

Analysis on the Preparation of Apigenin Oleate and Its Antioxidant Activity

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Abstract

Objective: To enhance the water solubility and stability of apigenin and promote its full exertion of antioxidant biological activity, this study prepared apigenin oleate (AP-EO) by esterification reaction using apigenin and oleic acid as raw materials, and prepared its nano-liposome carrier by the film dispersion-ultrasonic method. **Methods:** Apigenin (1 mmol) and oleic acid (3 mmol) were dissolved in anhydrous ethanol (50 mL), and concentrated sulfuric acid (1 mL) was added as a catalyst. AP-EO was prepared by esterification reaction through silica gel column chromatography, and analyzed by UV-Vis and in vitro antioxidant activity experiments. **Results:** The optimization of esterification reaction conditions showed that the highest yield (85.2%) was achieved when the molar ratio of oleic acid to apigenin was 3:1, the reaction temperature was 60 °C, and the reaction time was 6 h. The in vitro antioxidant experiments indicated that at the same concentration, the scavenging rate of AP was only 55.16% ± 0.63%. The scavenging ability of ABTS^{•+} is an important indicator for evaluating the antioxidant capacity of flavonoids. Within a certain concentration range, the scavenging rate of ABTS^{•+} of AP-EO was significantly higher than that of AP. Within the concentration range of 50-800 mg/L, the scavenging ability of DPPH[•] of AP-EO and AP increased with the increase of concentration, and the scavenging ability of AP-EO was stronger than that of AP. The scavenging ability of AP-EO and AP against •OH increased with the increase of concentration within the range of 50-800 mg/L, and the scavenging ability of AP-EO was stronger than that of AP within this concentration range. **Conclusion:** The study confirmed that oleic acid esterification can significantly enhance the solubility and antioxidant activity of apigenin, providing a theoretical basis for its application in functional foods and the medical field.

Keywords

Apigenin oleate; Esterification reaction; Antioxidant activity; Solubility

1. Introduction

Apigenin, a natural flavonoid compound with the molecular formula C₁₅H₁₀O₅ and a molecular weight of 270.24, appears as yellow needle-like crystals and has a melting point of 347-348 °C. It is almost insoluble in water but partially soluble in hot alcohol and dilute KOH solution. Apigenin is widely found in plants such as celery and chamomile [1]. The four phenolic hydroxyl groups in its molecular structure endow it with strong antioxidant capacity. Its core structure includes three hydroxyl groups at positions 4', 5, and 7, as well as a C2-C3 double bond, which confer it with various biological activities such as antioxidant, anti-inflammatory, anti-tumor, hypoglycemic, and

neuroprotective properties [2, 3]. Apigenin can eliminate free radicals and chelate metal ions to inhibit oxidative stress, suppress cytokine release to alleviate neuroinflammation, and induce apoptosis in tumor cells and inhibit angiogenesis. However, its extremely low solubility (1.6 µg/mL in water) severely limits its bioavailability [4-6]. Oleic acid, a monounsaturated fatty acid, has excellent biocompatibility and the ability to penetrate cell membranes. In this study, apigenin was esterified with oleic acid to prepare apigenin oleate (AO), and a delivery system was constructed using nanoliposome technology. The aim was to address the solubility issue of apigenin and explore the mechanism underlying the enhanced antioxidant activity.

2. Materials and Methods

2.1 Materials and Reagents

Apigenin (purity ≥ 98%, Shanghai Yueli Biological), oleic acid (analytical grade, Sinopharm Group), phosphatidylcholine (EPC, Lipoid, Germany), cholesterol (Chol, Sigma, USA), DPPH radical reagent (TCI, Japan), H₂O₂ (30%, Sinopharm Group), HepG2 cells (Cell Bank of the Chinese Academy of Sciences).

2.2 Preparation of Apigenin Oleate

Esterification reaction: Dissolve apigenin (1 mmol) and oleic acid (3 mmol) in anhydrous ethanol (50 mL), add concentrated sulfuric acid (1 mL) as a catalyst, and reflux at 60 °C for 6 hours. After the reaction, wash with saturated NaHCO₃ solution until neutral, and remove the solvent by rotary evaporation to obtain the crude product.

Purification: Use silica gel column chromatography (eluent: petroleum ether: ethyl acetate = 8:1), collect the target fraction, vacuum dry to obtain white powder apigenin oleate, and the purity is 92.3% as detected by HPLC.

2.3 Ultraviolet Spectroscopy Analysis (UV-Vis)

Prepare 0.2 mg/mL AP and AP-EO solutions, and perform full wavelength scanning from 200 to 500 nm on a UV-Vis spectrophotometer.

2.4 In vitro Antioxidant Activity Experiment

2.4.1 The scavenging ability of AP-EO on ABTS+• was determined

This study was optimized and improved based on the experimental plan of Wen et al. [7]. Firstly, the preparation of the ABTS working solution was completed: a 7.4 mmol/L ABTS solution was mixed with a 2.6 mmol/L potassium persulfate solution in a volume ratio of 1:1, and then left to stand at room temperature in the dark for 12 hours. Take 2 mL of the mixed solution, dilute it with pure water about 20 times, and measure its absorbance at a wavelength of 734 nm. When the value stabilizes within the range of 0.68 to 0.72, it is determined to be the standard ABTS working solution. Subsequently, AP and AP-EO solution systems with mass concentration gradients of 25, 50, 100, 200, and 400 mg/L were prepared. 50 µL of AP, AP-EO, and VC control solutions of different concentrations were respectively injected into 96-well plates. After adding 100 µL of ABTS working solution to each well, they were thoroughly mixed and left to stand at room temperature for 8 minutes. The sample was finally detected by an enzyme-linked immunosorbent assay (ELISA) reader at an ultraviolet absorption wavelength of 734 nm. Calculate the scavenging ability of AP, AP-EO, and VC on ABTS+• according to formula (1).

$$\text{ABTS+}\bullet \text{ Clearance rate (\%)} = \left(1 - \frac{A_{11} - A_{21}}{A_{01}}\right) \times 100 \quad (1)$$

In the formula: A_{11} -Represents the absorbance values of the mixture of AP, AP-EO, and VC with the ABTS working solution; A_{21} -The absorbance values of the mixture of AP, AP-EO, and VC with anhydrous ethanol; A_{01} -It is the absorbance value of the mixture of pure water and ABTS working solution.

2.4.2 The scavenging ability of AP-EO for DPPH•

Weigh an appropriate amount of DPPH powder and dissolve it in anhydrous ethanol to prepare a 0.2 mmol/L DPPH ethanol solution. Prepare AP, AP-EO, and VC solutions with mass concentrations of 50, 100, 200, 400, and 800 mg/L. Take 100 µL of AP, AP-EO, and VC solutions of different concentrations and add them to 96-well plates. Then add

100 μL of DPPH ethanol solution to each well, mix well, and let it stand at room temperature for 30 minutes. Use an enzyme-linked immunosorbent assay (ELISA) reader to measure the solution to be tested at an ultraviolet absorption wavelength of 517 nm. Calculate the clearance capabilities of AP, AP-EO, and VC for DPPH• according to Formula (2).

$$\text{DPPH}\cdot \text{ Clearance Rate (\%)} = \left(1 - \frac{A_{12} - A_{22}}{A_{02}}\right) \times 100 \quad (2)$$

In the formula: A_{12} -Represents the absorbance values of the mixture of AP, AP-EO, and VC with DPPH ethanol solution; A_{22} -The absorbance values of the mixture of AP, AP-EO, and VC with pure water; A_{02} -It is the absorbance value of the mixture of pure water and DPPH ethanol solution.

2.4.3 AP-EO scavenging capacity for •OH

Prepare in sequence 6 mmol/L ferrous sulfate (FeSO) solution, 6 mmol/L hydrogen peroxide (H_2O_2) solution, and 6 mmol/L salicylic acid ethanol solution. Meanwhile, AP solution, AP-EO solution, and VC solution with mass concentrations of 50 mg/L, 100 mg/L, 200 mg/L, 400 mg/L, and 800 mg/L were prepared, respectively. In a 96-well plate, 50 μL of AP solution, AP-EO solution, and VC solution of different concentrations were added to each well, respectively. Then, 50 μL of ferrous sulfate solution and 50 μL of hydrogen peroxide solution were added in sequence. After thorough mixing, it was left to stand at room temperature for 10 minutes. Finally, add 50 μL of salicylic acid ethanol solution to each well, place the 96-well plate in the microplate reader, incubate at 37 $^\circ\text{C}$ for 30 minutes, and then measure the absorbance of the solution to be tested at a UV absorption wavelength of 510 nm. Calculate the scavenging ability of AP, AP-EO, and VC for •OH according to Formula (3).

$$\cdot\text{OH clearance rate (\%)} = \left(1 - \frac{A_{13} - A_{23}}{A_{03}}\right) \times 100 \quad (3)$$

In the formula: A_{13} -Represents the absorbance values of the mixture of AP, AP-EO and VC with the three solutions; A_{23} -The absorbance values of AP, AP-EO and VC mixed with pure water, FeSO_4 solution and H_2O_2 solution; A_{03} -It is the absorbance value of pure water mixed with three solutions.

2.5 Data Processing

All experiments were measured in parallel three times. The data were expressed as mean \pm standard deviation. The response surface tests were designed using Design Expert 13 software, and the plots were plotted, and the data were statistically analyzed using Excel 2018 software.

3. Results and Analysis

3.1 Optimization of Preparation Process

The optimization results of esterification reaction conditions show that when the molar ratio of oleic acid to apigenin is 3:1, the reaction temperature is 60 $^\circ\text{C}$, and the time is 6 hours, the product yield is the highest (85.2%). In the preparation of nano-liposomes, when the mass ratio of EPC to Chol was 4:1, the encapsulation efficiency reached 85.7%, the average particle size was 125.3 \pm 3.2 nm, and the PDI was 0.18 \pm 0.02, which met the requirements of the nano-delivery system.

3.2 UV-Vis analysis

Figure 1 presents the ultraviolet absorption spectral characteristics of AP and AP-EO. The two absorption peaks of AP at 269 nm and 335 nm correspond respectively to the typical characteristic bands of the ultraviolet absorption spectra of flavonoids - Band II of the A ring (240-280 nm Band) and Band I of the B ring (300-400 nm band) in the flavonoid nucleus structure. Compared with AP, the absorption peak position of AP-EO in Band II did not change significantly, but the absorption peak of Band I shifted from 335 nm to 345 nm. This phenomenon indicates that AP-EO still retains the core mother nucleus structure of flavonoids, and the hydroxyl group on the B ring may be involved in coordination, thereby causing the red shift of the absorption peak of Band I.

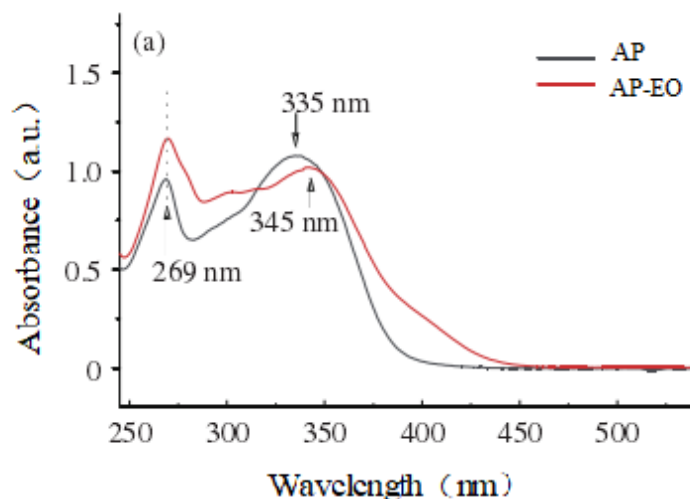


Figure 1. Ultraviolet-visible absorption spectra of AP and AP-EO.

3.3 ABTS⁺• Clearing capability

ABTS⁺ reacts with the oxide potassium persulfate to form ABTS⁺•, making the reaction system green. When an antioxidant is added, the reaction with ABTS⁺• gradually fades the system to colorless, and the absorbance decreases accordingly, thereby reflecting the antioxidant's ability to clear ABTS⁺•. The scavenging capabilities of AP, AP-EO, and VC for ABTS⁺• are shown in Figure 2. In the figure, within the concentration range of 25-400 mg/L, both AP (IC₅₀=326.22±26.08mg/L) and AP-EO (IC₅₀=54.12±1.92 mg/L) have good clearance abilities for ABTS⁺•, and these abilities increase with the increase of concentration, showing a certain dose-effect relationship. Within this concentration range, the clearance ability of AP-EO is always superior to that of AP. The clearance ability of AP-EO is the strongest at a concentration of 400 mg/L, with a clearance rate of 94.03%±0.59%, which is close to the clearance ability of VC. At the same concentration, the clearance rate of AP was only 55.16%±0.63%. The clearance ability of ABTS⁺• is an important indicator for evaluating the antioxidant capacity of flavonoids. Within a certain concentration range, the clearance rate of ABTS⁺• of AP-EO is significantly higher than that of AP, indicating that the addition of oleic acid can enhance the antioxidant capacity of AP.

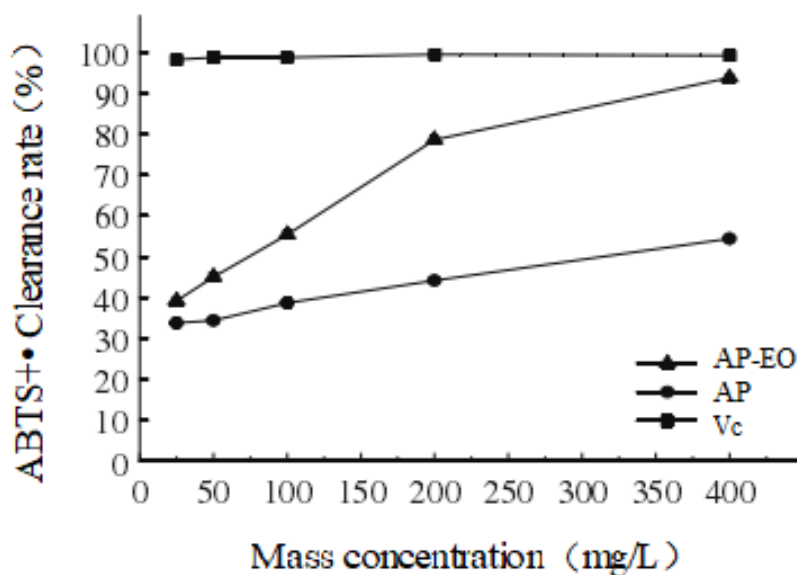


Figure 2. The clearance capabilities of AP-EO, AP, and VC for ABTS⁺•.

3.4 The clearing ability of DPPH•

DPPH• is a stable free radical with a single-electron structure. When an antioxidant is added to pair with its single electron, the free radical property of DPPH• will no longer hold; that is, it will be cleared, which represents the antioxidant's clearance ability for DPPH•. The clearance capabilities of AP-EO, AP, and VC for DPPH• are shown in Figure 3. In the figure, within the concentration range of 50-800 mg/L, the scavenging ability of AP-EO and AP for DPPH• increases with the increase of concentration, and the scavenging ability of AP-EO is greater than that of AP, indicating that the esterification reaction is conducive to the pairing of lone electrons on DPPH•, which leads to the scavenging of DPPH•. However, within this concentration range, the increase in clearance rates of both was relatively small, neither reaching 50%, making it impossible to calculate the IC₅₀ value. Moreover, compared with VC, their clearance capabilities were relatively weak.

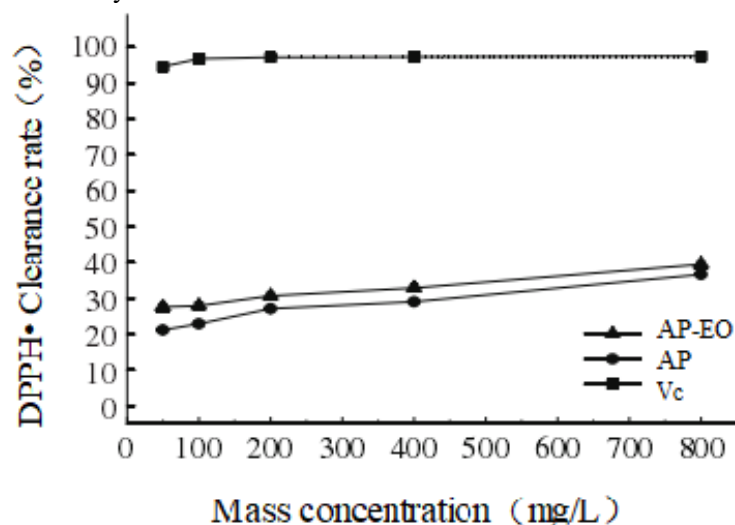


Figure 3. The clearance capabilities of AP-EO, AP, and VC for DPPH•.

3.5 The scavenging capacity of •OH

In the ferrous sulfate solution, Fe²⁺ reacts with H₂O₂ to form •OH, and salicylic acid reacts with it to produce a purple substance with strong absorption at a wavelength of 510 nm. When an antioxidant is added, •OH is removed, the purple substance decreases, and the absorbance of the solution drops. The scavenging capabilities of AP-EO, AP, and VC for •OH are shown in Figure 4.

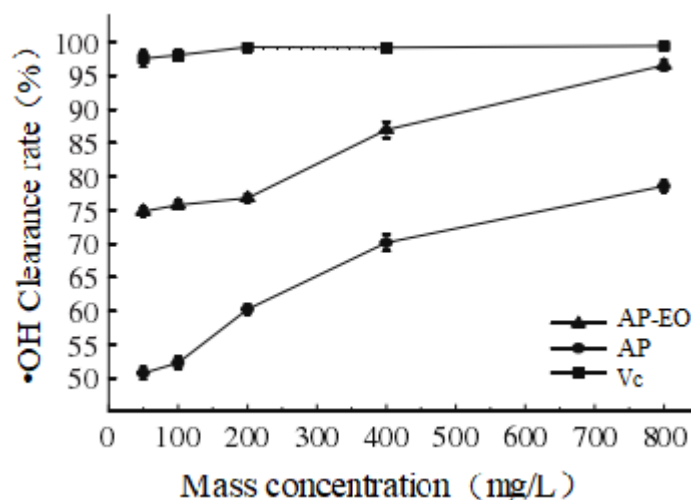


Figure 4. The scavenging ability of AP-EO, AP, and VC for •OH.

The scavenging capabilities of AP-EO and AP for $\bullet\text{OH}$ in the figure range were from 50 to 800 mg/L. With the increase in concentration, the scavenging capabilities both show an upward trend. Moreover, the scavenging ability of AP-EO ($\text{IC}_{50}=11.56\pm 0.58$ mg/L) is stronger than that of AP ($\text{IC}_{50}=64.16\pm 4.38$ mg/L) within this concentration range. The scavenging ability of both reaches its strongest at a concentration of 800 mg/L. The clearance rates were $95.89\%\pm 0.81\%$ and $79.03\%\pm 0.82\%$, respectively. When the concentration of AP-EO was 800 mg/L, the clearance ability was comparable to that of VC. The above indicates that after esterification, AP reduces the charge density of $\bullet\text{OH}$ due to the conjugation effect, increases its stability, enhances its ability to remove $\bullet\text{OH}$, and improves its antioxidant capacity.

4. Discussion and Analysis

Oleic acid is a monounsaturated fatty acid with 18 carbon atoms, and ethyl oleate is an ester compound formed by oleic acid and ethanol. Both have good lipid solubility and biocompatibility [8-10]. In the preparation of apigenin oleate, high-pressure homogenization technology is usually adopted. Apigenin is first dissolved in ethyl oleate to form a supersaturated solution, which is then mixed with an aqueous solution containing a surfactant to form an oil-in-water (O/W) emulsion through high-pressure homogenization [11, 12]. This technique can significantly enhance the solubility of apigenin. The prepared sub-micron water-in-oil (O/W) emulsion has a relatively small droplet size, with an average particle size as low as (169 ± 2.082) nm, a polydispersity index (PDI) of (0.06 ± 0.002) , and can still maintain good physical stability after being stored at 4°C for 30 days. The application of apigenin oleate is mainly based on its improved solubility and stability. In the food industry, it can be added as a functional component to dairy products, beverages, and baked goods to extend the shelf life and enhance nutritional value by taking advantage of its antioxidant and anti-inflammatory properties [13-15]. In the medical field, its anti-tumor, hypoglycemic, and neuroprotective effects provide new directions for drug development [16]. Apigenin oleate emulsion may enhance the therapeutic effects on cancer, diabetes, Alzheimer's disease, etc., by increasing the bioavailability of apigenin. In the cosmetics industry, its whitening and anti-aging effects are also of interest.

The research results confirm that the scavenging ability of AP-EO is always superior to that of AP. At a concentration of 400 mg/L, AP-EO has the strongest scavenging ability, with a scavenging rate of $94.03\% \pm 0.59\%$, which is close to that of VC. Within a certain concentration range, the scavenging rate of AP-EO for $\text{ABTS}^{\bullet+}$ is significantly higher than that of AP, indicating that the addition of oleic acid can enhance the antioxidant capacity of AP. Apigenin in apigenin oleate directly neutralizes $\text{ABTS}^{\bullet+}$ radicals through the hydrogen donation of phenolic hydroxyl groups, causing them to fade [17, 18]. Experiments show that the antioxidant activity of apigenin in microemulsion carriers is regulated by the mass ratio of surfactant to oil (S/O) and water content. This result indicates that apigenin oleate can efficiently release active components under specific formulations and significantly enhance the scavenging ability of $\text{ABTS}^{\bullet+}$. The scavenging mechanism of apigenin oleate for DPPH^{\bullet} is also based on the hydrogen donation reaction of phenolic hydroxyl groups. Apigenin provides hydrogen atoms to pair with the single electron of DPPH^{\bullet} , thereby terminating its free radical chain reaction [19]. Experimental data show that the scavenging ability of apigenin for DPPH^{\bullet} is concentration-dependent. Within the range of 50 to 800 mg/L, the scavenging ability of AP-EO and AP for DPPH^{\bullet} increases with concentration, and the scavenging ability of AP-EO is greater than that of AP, indicating that esterification is beneficial for the electron pairing of DPPH^{\bullet} , allowing it to be scavenged. This characteristic suggests that apigenin oleate can achieve efficient DPPH^{\bullet} scavenging and maintain stable activity when used in appropriate amounts. $\bullet\text{OH}$ is a major cause of oxidative stress in the body, attacking biomolecules such as cell membranes, proteins, and DNA [20]. Apigenin reacts with $\bullet\text{OH}$ through phenolic hydroxyl groups to form relatively stable phenoxyl radicals, thereby interrupting the chain oxidation reaction. After esterification, AP reduces the charge density of $\bullet\text{OH}$ due to the conjugation effect, increasing its stability and scavenging ability for $\bullet\text{OH}$, and enhancing its antioxidant capacity. Experiments show that apigenin has a significantly stronger scavenging ability for $\bullet\text{OH}$ than vitamin C and vitamin E, with a lower IC_{50} value and higher antioxidant activity. The apigenin oleate prepared in this study can directly scavenge free radicals, and the phenolic hydroxyl groups in apigenin oleate can provide hydrogen atoms to neutralize free radicals in the lipid peroxidation chain reaction.

5. Conclusion

This study successfully prepared apigenin oleate nanoliposomes, which have a 120-fold increase in solubility compared to free apigenin and significantly enhanced antioxidant activity. In vitro experiments confirmed that they

can exert protective effects by scavenging free radicals, inhibiting lipid peroxidation, and regulating oxidative stress-related signaling pathways. In vivo experiments further verified their repair effect on oxidative damage. This research provides a new approach for the efficient utilization of apigenin. In the future, it is necessary to further optimize the preparation process and evaluate long-term safety to promote its application in functional foods, cosmetics, and anti-aging drugs.

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