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Study on the isolation、 purification and bioactivities of triterpenoids from the endangered ethnic medicine *Euchresta tubulosa* Dunn

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Abstract: *Euchresta tubulosa* Dunn is an endangered ethnic medicinal plant belonging to the family Fabaceae. This study systematically investigated the isolation and purification of triterpenoids from this plant and evaluated their in vitro bioactivities. Four monomeric compounds, namely trifolirhizin, oleanolic acid, lupeol and quercetin, were isolated using column chromatography, recrystallization, and thin-layer chromatography. Among these, trifolirhizin, oleanolic acid, and lupeol are isolated from this species for the first time. The antioxidant and hypoglycemic activities of the four monomers were assessed through DPPH• and ABTS⁺ radical scavenging assays, as well as α -amylase and α -glucosidase inhibition tests. Furthermore, the antibacterial potential of two pentacyclic triterpenoids (oleanolic acid and lupeol) was evaluated against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*. The underlying mechanisms were analyzed by determining bacterial growth curves, time-kill curves, nucleotide leakage rates, and transmission electron microscopy (TEM) observations. The results showed that all four monomers possessed moderate antioxidant and hypoglycemic activities. Among them, quercetin exhibited the strongest antioxidant effect ($IC_{50} = 8 \mu\text{g/mL}$), while lupeol demonstrated superior inhibitory capacity in the hypoglycemic activity test ($IC_{50} = 5 \mu\text{g/mL}$). The two pentacyclic triterpenoids acted primarily by disrupting bacterial cell wall and cell membrane structures, altering membrane permeability, and causing leakage of intracellular contents, thereby inhibiting bacterial growth and exerting bactericidal effects, which indicates considerable antibacterial potential. This study confirms that the traditional use of *Euchresta tubulosa* Dunn for antioxidant, anti-inflammatory, antibacterial, and hypoglycemic purposes is supported by a definite pharmacodynamic material basis and scientific rationale.

Keyword: *Euchresta tubulosa* Dunn; Triterpenoids; Isolation and identification; Antioxidant activity; Antibacterial; Hypoglycemic activity

1. Introduction

Euchresta tubulosa Dunn (Fabaceae), a plant species under Grade II national key protection in China, is regarded as a “highly reputable ethnomedicine” in the Tujia ethnic inhabited areas and is commonly used for the treatment of laryngeal cancer and esophageal cancer [1-2]. Due to its strict habitat requirements and scarcity of wild resources, currently, most domestic and international research on *Euchresta tubulosa* Dunn, focuses on its ecological characteristics, morphological features, as well as the isolation and identification of alkaloids and flavonoids. However, systematic studies on its triterpenoid compounds remain scarce. Regarding its chemical

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constituents, Matsuura et al. (1993)^[3], based on wild samples collected from Enshi, Hubei Province, China, identified 13 flavonoids and one novel benzofuran; our research group, following successful in situ conservation of this species, has preliminarily isolated and identified six alkaloids, two flavonoids, and two other types of compounds^[4-6]. Pharmacological studies have been mostly conducted in the 1980s–1990s, covering burn healing^[7], central nervous system inhibition^[8], and the anti-inflammatory, analgesic, and antitumor activities of alkaloids such as matrine^[9]. More recently, our group has investigated the antioxidant and antibacterial activities of flavonoid and alkaloid constituents^[10-11]. Nevertheless, to date, no studies have reported the isolation, purification, or biological evaluation of triterpenoids from *Euchresta tubulosa* Dunn.

Although more than 30 triterpenoids have been documented in other species of the genus *Euchresta*^[12-15], the extraction, isolation, structural identification, and bioactivity assessment of triterpenoids from *Euchresta tubulosa* Dunn remain entirely unexplored. Therefore, this study aims to preliminarily investigate the triterpenoids of *Euchresta tubulosa* Dunn, covering extraction, isolation, purification, structural elucidation, and biological evaluation. The antioxidant and hypoglycemic activities of four isolated monomeric compounds will be assessed, and the antibacterial activity of two triterpenoid monomers will be evaluated against three common bacterial strains (*Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*).

This work will help fill the knowledge gap regarding triterpenoids in *Euchresta tubulosa* Dunn, provide a scientific basis for exploring its medicinal potential and rational utilization, facilitate the transition from folk medicine to modern drug development, and support the conservation of this rare and endangered plant resource.

2. Material and Reagent

2.1 Plant collection

The *Euchresta tubulosa* Dunn samples were collected in June 2023 from the Tianping Mountain Reserve, which is under the jurisdiction of Badagongshan National Forest Park, Hunan Province, China. After collection, the roots, stems, and leaves were separated, thoroughly cleaned individually, and dried at 50 °C. Subsequently, the dried plant material was pulverized and passed through a standard sieve with a mesh size of 250 μm. The resulting powder was stored in a dry environment for further use.

2.2 Reagents

Methanol, absolute ethanol, petroleum ether, dichloromethane, ethyl acetate, n-butanol, column chromatography silica gel (100–200 mesh), column chromatography silica gel (200–300 mesh), vanillin, perchloric acid, glacial acetic acid, α-Amylase (100 U/mg), α-Glucosidase, acarbose, 4-nitrophenyl-α-D-glucopyranoside (PNPG), 3,5-dinitrosalicylic acid (DNS, analytical grade), soluble starch, PBS buffer, nutrient agar and MH broth were purchased from Shanghai Titan Technology Co., Ltd. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and potassium persulfate were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was supplied by Fujian Feijing Biotechnology Co., Ltd. Chloroform was purchased from Maoming Runjing Chemical Co., Ltd. Thin-layer chromatography silica gel G was provided by Suzhou Ruoxiang Biotechnology Co., Ltd. SiLiaSphere PC C₁₈ Monomeric was acquired from SiLicycLe Inc., Canada.

2.3 Test bacterial strains

Escherichia coli and *Staphylococcus aureus* were provided by the research group of Associate Professor Yao Shufeng, College of Pharmacy, Jishou University. *Bacillus subtilis* and

Pseudomonas aeruginosa were donated by the Laboratory of Forest Chemical Engineering, Jishou University.

3. Extraction and Isolation

3.1 Preparation of crude triterpenoid extracts from *Euchresta tubulosa* Dunn

A total of 3.00 kg of dried powder of *Euchresta tubulosa* Dunn was accurately weighed and extracted three times according to the optimized process. The extracts were combined, and the solvent was recovered. The crude triterpenoid extract was obtained by vacuum concentration at 50 °C. Ultra-pure water was added at a ratio of 1:5 (w/v), and the mixture was centrifuged at 8000 r/min for 10 min. The water-soluble fraction was concentrated again under vacuum at 50 °C to obtain an extract, which was sequentially extracted with petroleum ether, dichloromethane, ethyl acetate, and n-butanol (each at least three times). The extracts were collected and dried for further use.

3.2 Isolation and Purification

Four grams of the ethyl acetate fraction extract was dissolved in an appropriate solvent, mixed with 8.000 g of 200–300 mesh silica gel, stirred evenly, and the solvent was evaporated by heating. A column was packed with 100–200 mesh silica gel using petroleum ether by wet method, equilibrated, and left overnight before dry loading. Gradient elution was performed with chloroform-methanol (100:1, 50:1, 20:1, 10:1, 8:2, 1:1, 0:100, V/V). Fractions with similar R_f values were combined by thin-layer chromatography (TLC) detection to obtain 7 fractions (Fr.1–Fr.7), which were concentrated by rotary evaporation, dried at 50 °C, and stored for use.

Fr.4 was loaded onto a C₁₈ reversed-phase column (extract:silica gel = 1:2, column packing ratio = 1:30) and eluted with methanol-water (0:100→100:0, V/V), yielding 14 subfractions (Fr.4-1–Fr.4-14) after TLC combination. Compound 1 (22.88 mg) was obtained from Fr.4-7 by repeated recrystallization and elution with solvents of different polarities.

Fr.2 was eluted with methanol-water on a C₁₈ reversed-phase column to obtain 14 subfractions (Fr.2-1–Fr.2-14). Fr.2-9 and Fr.2-10 were combined according to TLC results, further purified by silica gel column chromatography with methanol-chloroform (3:7, V/V) elution, and separated by high-performance preparative thin-layer plates. Compound 3 (9.12 mg) was obtained by repeated collection, recrystallization and elution with different polar solvents.

Similarly, Fr.6 was eluted with methanol-water on a C₁₈ reversed-phase column to yield 14 subfractions. Fr.6-7 was separated by silica gel column chromatography with ethyl acetate-methanol (10:1→5:5, V/V) elution, obtaining 6 subfractions (Fr.6-7-1–Fr.6-7-6). Compound 2 (11.03 mg) was obtained from Fr.6-7-3 by high-performance preparative thin-layer plate separation, repeated recrystallization and elution with different polar solvents.

Four grams of the n-butanol fraction extract was dissolved in an appropriate solvent, mixed with 8.000 g of 200–300 mesh silica gel, and the solvent was evaporated by heating. A column was packed with 100–200 mesh silica gel using petroleum ether by wet method, equilibrated, and left overnight before dry loading. Gradient elution was performed with ethyl acetate-methanol-water (100:5:1→5:5:1, V/V/V), and 7 fractions (Fr.1–Fr.7) were obtained by combining fractions with similar R_f values via TLC detection, followed by concentration and drying to enrich target components.

Fr.3 was subjected to C₁₈ reversed-phase column chromatography as described above with methanol-water (0:100→100:0, V/V) elution, yielding 14 subfractions (Fr.3-1–Fr.3-14). Fr.3-6 was separated by silica gel column chromatography (chloroform-methanol, 10:1→3:7, V/V) to

obtain 10 subfractions (Fr.3-6-1–Fr.3-6-10). Compound 4 (8.61 mg) was obtained from Fr.3-6-6 by purification with high-performance preparative thin-layer plates and repeated recrystallization.

3.3 Structural identification

The four isolated monomeric compounds were structurally identified by nuclear magnetic resonance (NMR) and electron impact mass spectrometry (EI-MS). The obtained spectral data were systematically sorted and compared with the corresponding data reported in the literature to confirm the chemical structures of the four monomeric components.

4. In Vitro Activity Studies

4.1 DPPH· Scavenging rate assay

The DPPH· scavenging rate assay was modified according to the method described in Reference^[16] to determine the antioxidant activity of the four isolated monomers from *Euchresta tubulosa*. The four monomers were dissolved in ethanol to prepare solutions with mass concentrations of 0.005, 0.2, 0.5, 1.0, 1.5, and 2.0 mg/mL. Precisely, 3.00 mL of each sample solution was mixed with 3.00 mL of DPPH· solution in a test tube, reacted in the dark at room temperature for 30 min, and the absorbance (A_1) was measured at 517 nm in triplicate. Vitamin C (Vc) was used as the positive control, and the same operation was performed. The DPPH· scavenging rate was calculated by Formula (1):

$$\eta_{DPPH\cdot}(\%) = \left[1 - \frac{A_1 - A_2}{A_3} \right] \times 100\% \quad (1)$$

Where: A_1 = sample + DPPH·; A_2 = sample + absolute ethanol (replacing DPPH·); A_3 = absolute ethanol + DPPH· (replacing sample solution).

4.2 ABTS· Scavenging rate assay

The ABTS· scavenging rate assay was modified according to Reference^[17]. A total of 100 mL of 7 mmol/L ABTS· solution and 100 mL of 2.5 mmol/L potassium persulfate solution were mixed and incubated in the dark at room temperature for 12 h to prepare the ABTS· stock solution. The four monomers were dissolved in ethanol to prepare solutions with mass concentrations of 0.005, 0.2, 0.4, 0.8, and 1.0 mg/mL. Precisely, 2.00 mL of each sample solution was mixed with 4.00 mL of diluted ABTS· stock solution, reacted in the dark at room temperature for 10 min, and the absorbance (A_1) was measured at 734 nm in triplicate. Vc was used as the positive control, and the ABTS· scavenging rate was calculated by Formula (1).

4.3 Hypoglycemic activity assay

4.3.1 α -Glucosidase inhibitory activity assay

The α -Glucosidase inhibitory activity assay was modified according to Reference^[18]. The four monomers were dissolved in ethanol to prepare solutions with mass concentrations of 0.005, 0.1, 0.3, 0.5, 0.7, and 0.9 mg/mL. A total of 200 μ L of sample solution and 300 μ L of α -Glucosidase solution (4 U/mL) were incubated at 37 °C for 10 min, then 500 μ L of pNPG (2 mmol/L) was added and incubated at 37 °C for 1 h. The reaction was terminated by adding 500 μ L of Na_2CO_3 (0.1 mol/L), and the absorbance was measured at 405 nm. Acarbose was used as the positive control, and the α -Glucosidase inhibitory rate was calculated by Formula (2):

$$\text{Inhibition rate}(\%) = \left[1 - \frac{(A_1 - A_2)}{A_3} \right] \times 100\% \quad (2)$$

Where: A₁ = enzyme + sample; A₂ = distilled water (replacing enzyme) + sample; A₃ = distilled water (replacing sample solution) + enzyme.

4.3.2 α -Amylase inhibitory activity assay

The α -Amylase inhibitory activity assay was modified according to Reference^[19]. The monomers were dissolved in ethanol to prepare solutions with mass concentrations of 0.005, 0.1, 0.3, 0.5, 0.7, and 0.9 mg/mL. A total of 500 μ L of each sample solution was mixed with 500 μ L of α -Amylase solution (1 U/mL, prepared with 0.1 mol/L pH 6.8 phosphate buffer (PBS), incubated in a water bath at 37 °C for 5 min. Then 500 μ L of 1% soluble starch was added to initiate the reaction. After 5 min, 500 μ L of DNS was added for color development, reacted in a boiling water bath for 5 min, cooled in a water bath, and fixed to 5 mL with PBS. The absorbance was measured at 540 nm. Acarbose was used as the positive control, and the α -Amylase inhibitory rate was calculated by Formula (2).

4.4 Antibacterial activity assay

4.4.1 Bacterial strain activation

Pseudomonas aeruginosa, *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus* were inoculated into LB broth for activation. The bacterial suspension was collected, centrifuged and washed twice with sterile normal saline, adjusted to 10⁹ CFU/mL (OD_{600nm} = 1), and then diluted to 1 $\times$ 10⁶ CFU/mL by 10-fold dilution for subsequent experiments^[20].

4.4.2 Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of triterpenoid monomers against the four bacteria were determined with reference to Reference^[21] by the micro-dilution method. In a 96-well plate, 100 μ L of bacterial suspension was added to each well, followed by triterpenoid monomer solutions diluted with MH broth to final concentrations of 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9, and 0 μ g/mL. After incubation at 37 °C for 24 h, the OD_{600nm} was measured by a microplate reader.

4.4.3 Growth curves of three bacteria treated with two triterpenoid monomers

The growth curves of three bacteria were determined with modification according to Reference^[22]. A total of 50 μ L of triterpenoid monomer solutions with different concentrations (0, 1/8, 1/4, 1/2, 1, and 2 $\times$ MIC) were added to a 96-well plate, followed by 150 μ L of bacterial solution mixed with MH broth. After incubation in a constant temperature incubator at 37 °C, the OD_{600nm} was measured every 1 h within the first 12 h by a microplate reader.

4.4.4 Bactericidal curves of three bacteria treated with two triterpenoid monomers

Sterile test tubes were added with 0.5 mL of bacterial suspension, 1 mL of MH broth, and 0.5 mL of triterpenoid monomer solutions with different concentrations (0, 1, 2, and 4 $\times$ MIC), mixed well and incubated at 37 °C. Samples were taken at 0, 4, 8, 12, 20, and 24 h, spread on MH agar plates, and colony counting was performed after incubation at 37 °C for 24 h.

4.4.5 Effect of two triterpenoid monomers on cellular nucleic acid leakage of three bacteria

The effect on nucleic acid leakage was determined with modification according to Reference^[23]. Four sterile test tubes were added with 0.5 mL of bacterial suspension, triterpenoid monomer solutions, and 1 mL of MH broth to adjust the final concentrations (0, 1, 2, 4 $\times$ MIC). After incubation at 37 °C for 4, 8, 12, 16, 20, and 24 h, the mixture was centrifuged at 6000 r/min for 5 min, and the absorbance of the supernatant was measured at 260 nm. The nucleic acid leakage rate was calculated by Formula (3):

$$\text{Leakage rate (\%)} = \frac{E_1}{E_2} * 100\% \quad (3)$$

Where: E_1 = OD value of the sample; E_2 = OD value of the control.

4.4.6 TEM detection of three bacteria treated with two triterpenoid monomers

Triterpenoid monomer solutions were added to the bacterial suspensions of *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis* to a final concentration of $3 \times \text{MIC}$, and cultured in a shaker at 37°C and 200 r/min. Distilled water was used instead of triterpenoid monomers as the control. After 24 h of culture, the mixture was centrifuged at 10000 r/min for 5 min, the supernatant was discarded, and the precipitated bacteria were washed twice with PBS. A $5\ \mu\text{L}$ aliquot of the bacterial suspension was dropped on a support film, observe after freeze-drying..

5. Results and Analysis

5.1 Structural elucidation of compounds

Compound 1: White powder. Its molecular weight was determined to be 447 by EI-MS (m/z). ^1H NMR (600 MHz, Methanol- d_4) δ : 7.38 (d, $J = 8.4$ Hz, 1H, H-1), 6.81 (s, 1H, H-7), 6.79 (dd, $J = 8.7, 2.5$ Hz, 1H, H-2), 6.64 (d, $J = 2.4$ Hz, 1H, H-4), 6.38 (s, 1H, H-10), 5.88 (s, 1H, H-12), 5.86 (s, 1H, H-12), 5.49 (d, $J = 7.0$ Hz, 1H, H-11a), 4.90 (d, $J = 6.8$ Hz, 1H, H-1'), 4.26 (dd, $J = 11.0, 4.8$ Hz, 1H, H-6), 3.89 (dd, $J = 12.0, 2.5$ Hz, 1H, H-6'), 3.69 (dd, $J = 12.4, 5.7$ Hz, 1H, H-6a), 3.60 (t, $J = 10.7$ Hz, 1H, H-4'), 3.56–3.49 (m, 1H, H-5'), 3.48–3.42 (m, 3H, H-6, 2', 3'), 3.39 (d, $J = 8.6$ Hz, 1H, H-6'). ^{13}C NMR (151 MHz, Methanol- d_4) δ : 160.24 (C-3), 157.89 (C-4a), 155.53 (C-10a), 149.51 (C-9), 143.21 (C-8), 133.03 (C-1), 119.63 (C-7a), 115.87 (C-11b), 111.70 (C-2), 105.97 (C-7), 105.74 (C-4), 102.54 (C-12), 102.07 (C-1'), 94.25 (C-10), 79.70 (C-5'), 78.18 (C-11a), 77.94 (C-3'), 74.85 (C-2'), 71.31 (C-4'), 67.53 (C-6), 62.45 (C-6'), 41.6 (C-6a). By comparing its ^1H NMR and ^{13}C NMR data with those reported in Reference^[24], Compound 1 was identified as trifolirhizin (Figure 1A).

Compound 2: Yellow powder. Its molecular weight was 301 as determined by EI-MS (m/z). ^1H NMR (600 MHz, Methanol- d_4) δ : 7.73 (d, $J = 2.2$ Hz, 1H, H-2'), 7.63 (dd, $J = 8.5, 2.2$ Hz, 1H, H-6'), 6.88 (d, $J = 8.5$ Hz, 1H, H-5'), 6.39 (d, $J = 2.1$ Hz, 1H, H-8), 6.18 (d, $J = 2.1$ Hz, 1H, H-5). ^{13}C NMR (151 MHz, Methanol- d_4) δ : 177.33 (C-4), 165.57 (C-7), 162.50 (C-5), 158.23 (C-9), 148.77 (C-2), 148.01 (C-4'), 146.22 (C-3'), 137.22 (C-3), 124.15 (C-1'), 121.68 (C-6'), 116.23 (C-5'), 116.00 (C-2'), 104.52 (C-10), 99.24 (C-6), 94.42 (C-8). Comparison of its spectral data with those in Reference^[5] indicated that Compound 2 was quercetin (Figure 1B).

Compound 3: White powder. Its molecular weight was 427 as determined by EI-MS (m/z). ^1H NMR (600 MHz, Methanol- d_4) δ : 4.69 (d, $J = 2.5$ Hz, 1H, H-29), 4.57 (d, $J = 2.6$ Hz, 1H, H-29), 3.13 (dd, $J = 11.7, 4.7$ Hz, 1H, H-3), 2.41 (td, $J = 11.1, 5.8$ Hz, 1H, H-19), 1.99–1.89 (m, 1H, H-21), 1.79–1.70 (m, 4H, H-2, 6, 11), 1.70–1.68 (m, 1H, H-7), 1.69 (s, 3H, H-30), 1.67–1.57 (m, 1H, H-2), 1.57–1.52 (m, 1H, H-15), 1.49 (ddd, $J = 13.0, 4.6, 2.5$ Hz, 1H, H-16), 1.47–1.38 (m, 6H, H-12, 15, 18, 21, 22), 1.39–1.29 (m, 3H, H-1, 12), 1.28–1.18 (m, 2H, H-6, 7), 1.12 (td, $J = 13.4, 4.7$ Hz, 1H, H-13), 1.08 (s, 3H, H-26), 1.07–1.01 (m, 1H, H-16), 0.99 (s, 3H, H-23), 0.96 (s, 3H, H-27), 0.92 (dd, $J = 13.1, 4.2$ Hz, 1H, H-9), 0.87 (s, 3H, H-28), 0.83 (s, 3H, H-25), 0.76 (s, 3H, H-24), 0.71 (dd, $J = 8.9, 2.8$ Hz, 1H, H-5). ^{13}C NMR (151 MHz, Methanol- d_4) δ : 151.95 (C-30), 110.13 (C-29), 79.68 (C-3), 56.84 (C-5), 51.89 (C-9), 49.55 (C-18), 44.11 (C-19), 43.96 (C-17), 42.07 (C-14), 41.05 (C-8), 40.08 (C-4), 39.96 (C-1), 39.53 (C-13), 38.31 (C-10), 36.71 (C-16), 35.54 (C-7), 30.89 (C-21), 28.62 (C-23), 28.62 (C-2), 28.05 (C-15), 26.51 (C-12), 22.07 (C-11), 19.56 (C-30), 19.46 (C-6), 18.41 (C-28), 16.72 (C-26), 16.59 (C-24), 16.12 (C-27), 15.02 (C-25). Based on the comparison of its NMR data with those in Reference^[25], Compound 3 was identified as lupeol (Figure 1C).

Compound 4: White amorphous powder. Its molecular weight was 455 as determined by EI-MS (m/z). ^1H NMR (600 MHz, Methanol- d_4) δ : 5.24 (t, $J = 3.7$ Hz, 1H, H-12), 3.15 (dd, $J = 11.5, 4.7$ Hz, 1H, H-3), 2.85 (dd, $J = 14.1, 4.7$ Hz, 1H, H-18), 2.01 (td, $J = 13.5, 4.0$ Hz, 1H, H-11), 1.90 (tt, $J = 10.0, 5.3$ Hz, 2H, H-22), 1.82–1.64 (m, 3H, H-2, 11), 1.67–1.52 (m, 5H, H-1, 6, 7), 1.55–1.47 (m, 1H, H-16), 1.47–1.35 (m, 2H, H-21), 1.35–1.28 (m, 2H, H-1, 19), 1.24–1.17 (m, 1H, H-19), 1.16 (s, 3H, H-23), 1.15–1.05 (m, 2H, H-15), 1.02–0.98 (m, 1H, H-16), 0.97 (s, 3H, H-27), 0.95 (s, 3H, H-30), 0.94 (s, 3H, H-24), 0.91 (s, 3H, H-26), 0.90–0.84 (m, 1H, H-9), 0.82 (s, 3H, H-29), 0.78 (s, 3H, H-25), 0.81–0.74 (m, 1H, H-5). ^{13}C NMR (151 MHz, Methanol- d_4) δ : 181.86 (C-28), 145.21 (C-13), 123.66 (C-12), 79.72 (C-3), 56.77 (C-5), 49.07 (C-9), 47.64 (C-17), 47.26 (C-19), 42.90 (C-14), 42.74 (C-18), 40.56 (C-8), 39.84 (C-4), 39.84 (C-1), 38.18 (C-10), 34.90 (C-21), 34.03 (C-29), 33.82 (C-7), 33.57 (C-22), 31.61 (C-20), 28.84 (C-23), 28.75 (C-15), 27.87 (C-2), 26.40 (C-27), 24.52 (C-30), 24.07 (C-16), 23.98 (C-11), 19.51 (C-6), 17.73 (C-26), 16.31 (C-24), 15.89 (C-25). By comparing its spectral data with those reported in Reference [26], Compound 4 was identified as oleanolic acid (Figure 1D).

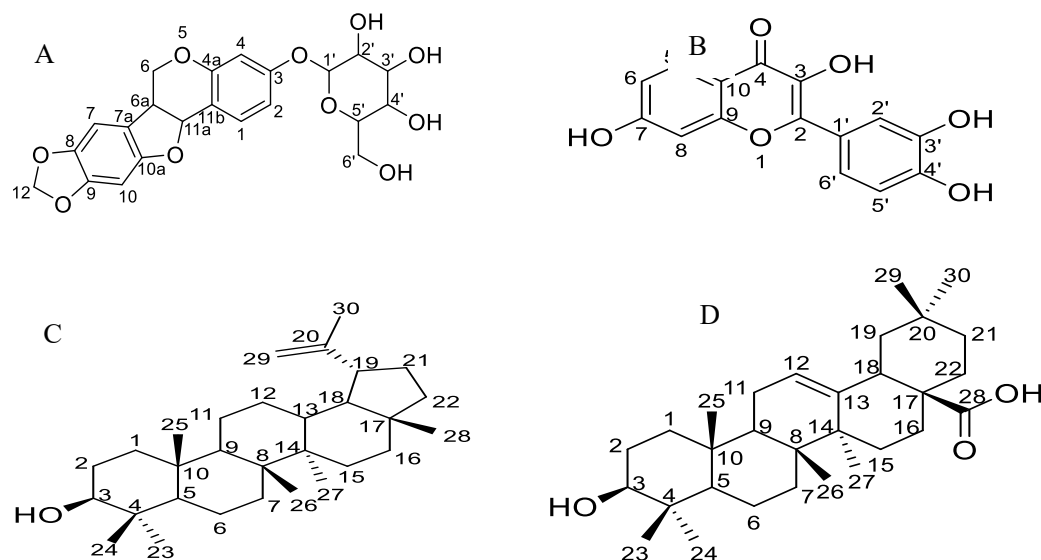


Fig 1 Structural diagrams of the four monomer compounds

5.2 Antioxidant activity

5.2.1 DPPH $\cdot$ and ABTS $\cdot$ scavenging rate assays

The DPPH $\cdot$ scavenging effects of the four monomers are shown in Figure 2 (A), with IC_{50} values of 0.932, 0.746, 0.065 and 0.153 mg/mL for oleanolic acid, lupeol, quercetin, and trifolirhizin, respectively. Their ABTS $\cdot$ scavenging effects are presented in Figure 2 (B), with corresponding IC_{50} values of 0.153, 0.057, 0.008 and 0.027 mg/mL. Within the tested concentration range, all four monomers exhibited certain DPPH $\cdot$ and ABTS $\cdot$ scavenging capacities, which increased with increasing monomer concentration. The results indicated that the flavonoid monomers had higher antioxidant activity than the triterpenoid monomers, with quercetin showing the best performance. The antioxidant capacity of quercetin is attributed to two antioxidant pharmacophores in its molecular structure with optimal free radical scavenging configurations: the catechol group on the B ring and the hydroxyl group at the 3-position of the AC ring, which can stabilize intermediates generated in free radical reactions and thus achieve efficient scavenging^[27].

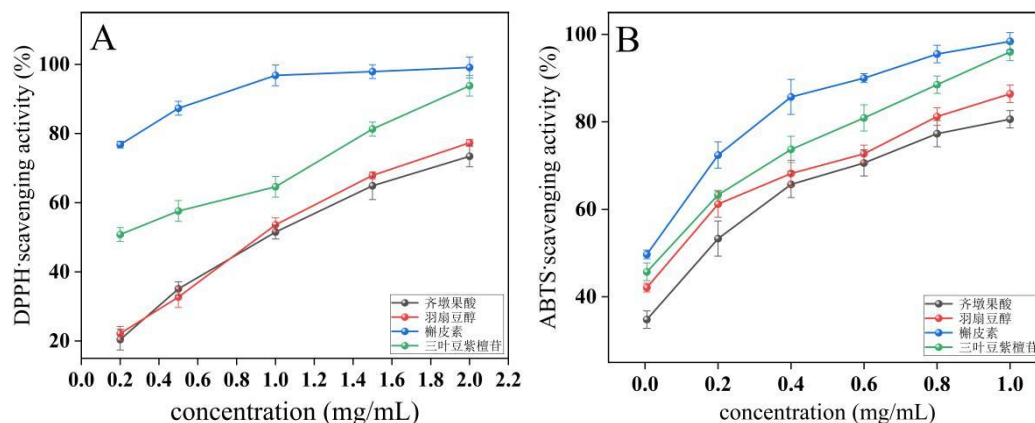


Fig 2 Elimination effects of four monomers on DPPH (A) and ABTS (B)

5.3 Hypoglycemic activity

5.3.1 α -Glucosidase and α -Amylase inhibition assays

Inhibiting α -Glucosidase can significantly delay the conversion of carbohydrates to monosaccharides and reduce postprandial blood glucose peaks^[28]. The α -Glucosidase inhibitory effects of the four monomers are shown in Figure 3 (A), with IC_{50} values of 0.033, 0.005, 0.036 and 0.309 mg/mL for oleanolic acid, lupeol, quercetin, and trifolirhizin, respectively. α -Amylase is an active enzyme capable of hydrolyzing starch, and its inhibition can reduce starch metabolism to achieve hypoglycemic effects^[28]. The α -Amylase inhibitory effects of the four monomers are presented in Figure 3 (B), with corresponding IC_{50} values of 0.368, 0.324, 0.352 and 0.496 mg/mL. Within the tested concentration range, lupeol exhibited excellent inhibitory activity against both enzymes, while oleanolic acid and quercetin showed comparable inhibitory effects, and trifolirhizin had slightly weaker activity. Notably, the inhibitory effects of all four monomers significantly improved with increasing concentration. These results are consistent with the findings of Zhang et al and Suvro Biswas et al^[29-30], who reported that lupeol can inhibit carbohydrate-degrading enzymes in the digestive tract, restrict the decomposition and absorption of carbohydrates in the gastrointestinal tract, which is beneficial for controlling postprandial hyperglycemia, indicating its potential positive role in preventing diabetes.

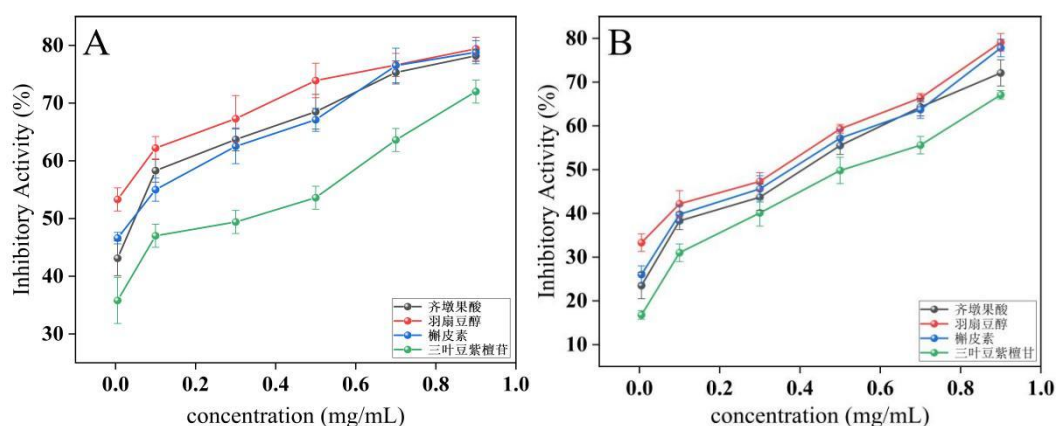


Fig 3 Inhibition rates of four monomers on α -Amylase (A) and α -Glucosidase (B) activity

5.4 Antibacterial activity

5.4.1 MIC and MBC of triterpenoid monomers

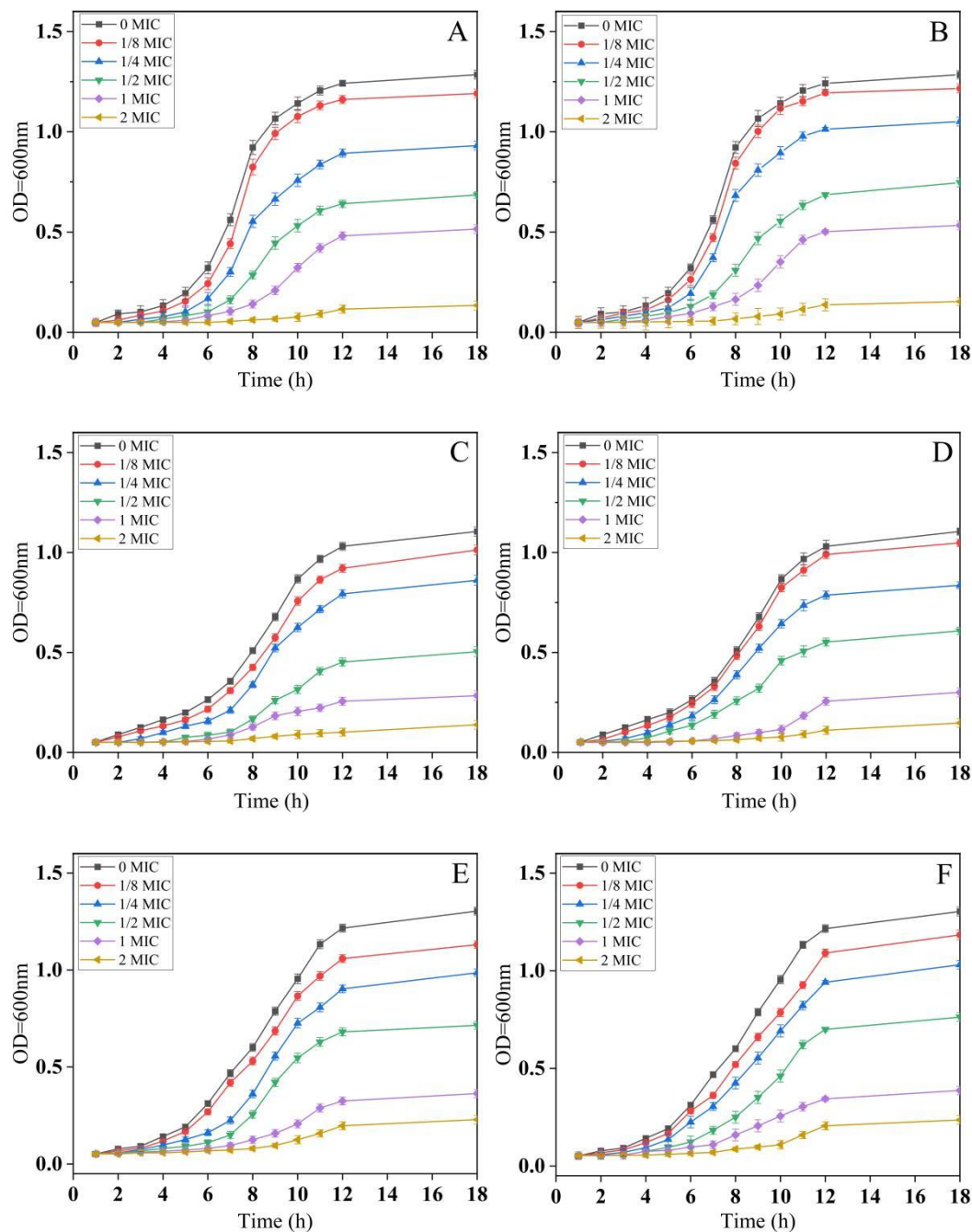
As shown in Table 1, the two triterpenoid monomers exhibited certain inhibitory effects against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. For oleanolic acid, the MIC values against the four bacteria were 31.25, 31.25, 15.6, and 31.25 $\mu\text{g/mL}$, respectively, with corresponding MBC values of 62.5, 31.25, 31.25, and 62.5 $\mu\text{g/mL}$. For lupeol, the MIC values were 62.5, 31.25, 15.6, and 31.25 $\mu\text{g/mL}$, and the MBC values were 125, 62.5, 31.25, and 62.5 $\mu\text{g/mL}$, respectively. The MIC and MBC values of both lupeol and oleanolic acid were basically consistent or slightly different, indicating that both monomers possessed bactericidal activity. Comprehensive comparison of the antibacterial performance of the two monomers showed the best inhibitory effect against *Bacillus subtilis*. Since *Staphylococcus aureus* and *Escherichia coli* are the two most common bacteria in the digestive and respiratory systems, these three bacteria were used for subsequent experiments to further analyze the antibacterial activity and mechanism of action of the two monomers.

Table 1 MIC and MBC results of two triterpenoid monomers

Test strain	MIC ($\mu\text{g/mL}$)		MBC ($\mu\text{g/mL}$)	
	oleanolic acid	lupeol	oleanolic acid	lupeol
<i>Staphylococcus aureus</i> (positive)	31.25	62.5	62.5	125
<i>Pseudomonas aeruginosa</i>	31.25	31.25	31.25	62.5
<i>Bacillus subtilis</i> (positive)	15.6	15.6	31.25	31.25
<i>Escherichia coli</i>	31.25	31.25	62.5	62.5

5.4.2 Growth curves of bacteria treated with two triterpenoid monomers

As shown in the growth curves (Figure 4), all bacterial groups grew normally under the 0 MIC condition: they were in the lag phase within 0–4 h, entered the logarithmic growth phase within 5–8 h with a linear increase in bacterial concentration, the $\text{OD}_{600\text{nm}}$ value increased slowly after 8 h, and gradually stabilized after 12 h with a significantly reduced growth rate. For the three bacterial strains treated with oleanolic acid and lupeol, a notable inhibitory effect was observed starting from 1/4 MIC. When the concentration exceeded 1/2 MIC, the growth of the two triterpenoid monomers was significantly inhibited; at 2 MIC, the growth of *Bacillus subtilis* and *Escherichia coli* was almost completely inhibited. Although the growth of *Staphylococcus aureus* was significantly inhibited, the inhibitory effect was weaker than that on *Bacillus subtilis* and *Escherichia coli*. The experiments indicated that oleanolic acid had overall better antibacterial activity than lupeol. This difference is mainly attributed to the stronger amphiphilic characteristics of oleanolic acid: its molecular structure contains a hydrophilic carboxyl group and a hydrophobic triterpenoid skeleton, which can more effectively insert into and interfere with the phospholipid bilayer of bacterial cell membranes, thereby increasing membrane permeability and leading to leakage of intracellular substances. In contrast, lupeol has weak polarity; although it can embed into membrane lipids through hydrophobic interactions, it lacks specific strong binding with the membrane surface, resulting in relatively low membrane damage efficiency^[31].

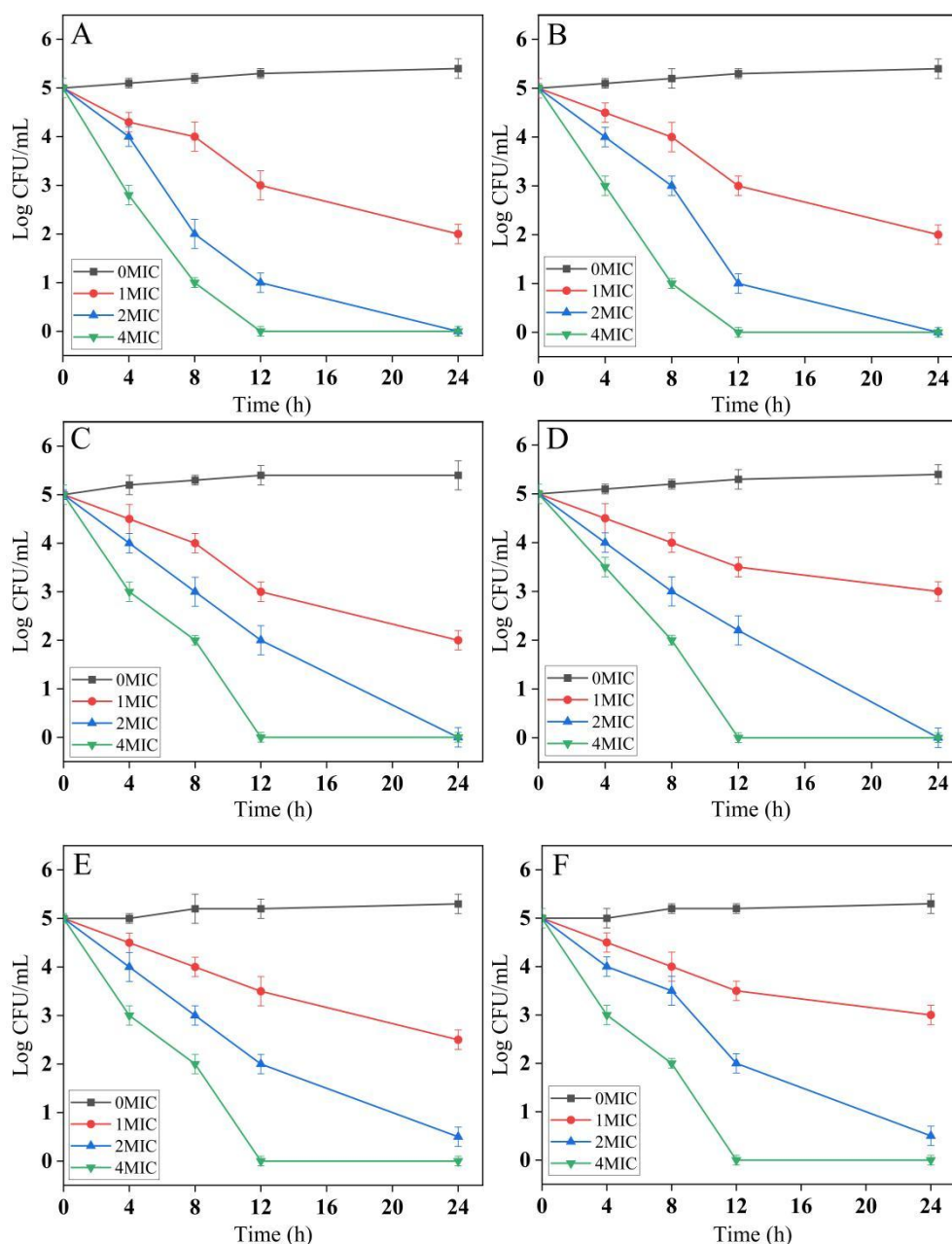


(A: *Bacillus subtilis*-Oleanolic acid; B: *Bacillus subtilis*-lupeol; C: *Escherichia coli*-Oleanolic acid; D: *Escherichia coli*-lupeol; E: *Staphylococcus aureus*-Oleanolic acid; F: *Staphylococcus aureus*-lupeol)

Fig 4 Effect of triterpenoid monomers at different concentrations on the growth curves of three bacterial strains
5.4.3 Bactericidal curves of bacteria treated with two triterpenoid monomers

The time-kill curves intuitively reflected the bactericidal effects of the two monomers on the three bacterial strains. As shown in Figure 5, the viable counts of the three bacteria treated with oleanolic acid and lupeol decreased significantly within 4 h, and no viable bacteria were detected after 12 h of treatment with 4 MIC. These results indicated that both triterpenoid monomers had strong bactericidal effects, which enhanced with increasing concentration. The hydroxyl group on the A ring and the carboxyl group on the E ring of oleanolic acid are not only key active groups

for inhibiting bacterial growth but also can affect bacterial morphology and induce macromolecular degradation and denaturation^[32].



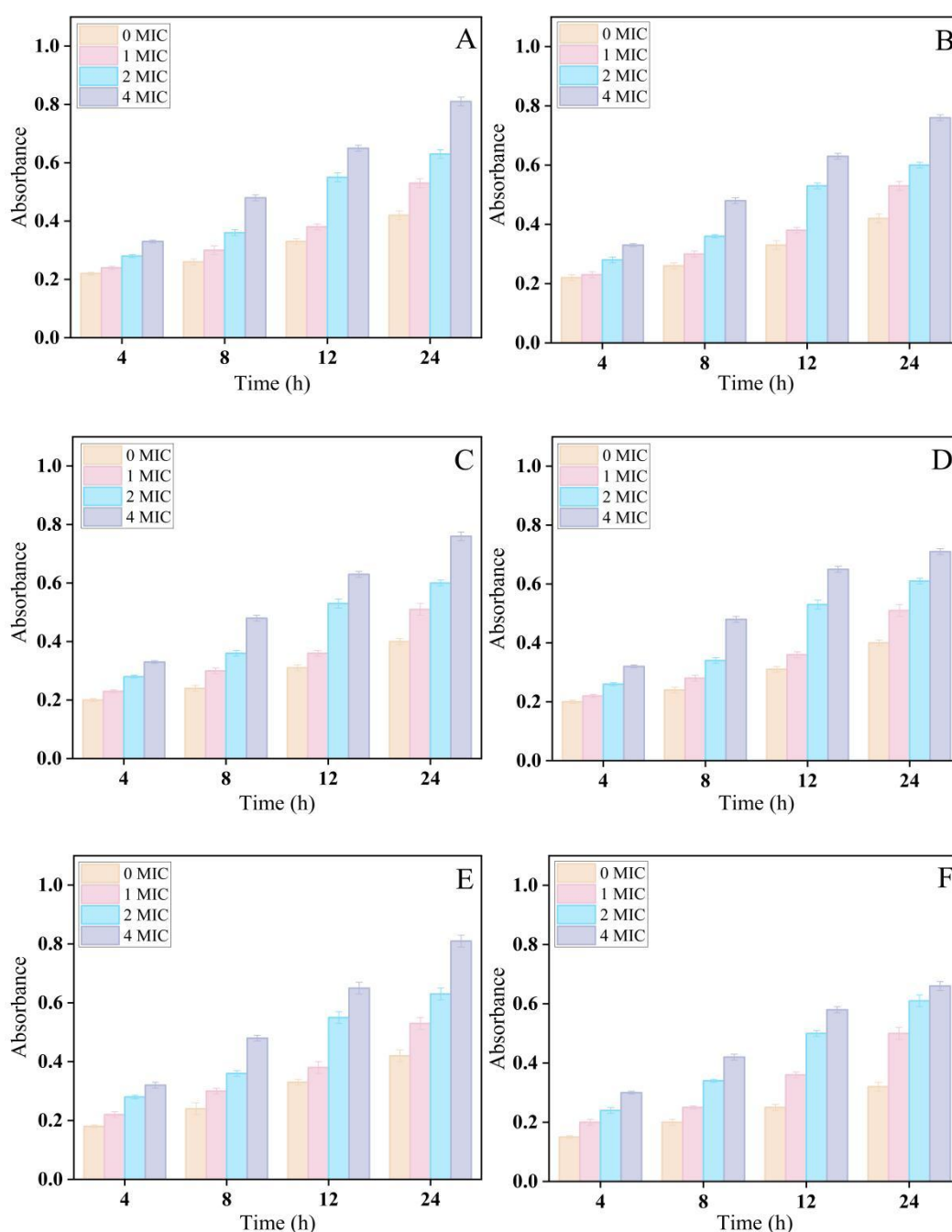
(A: *Bacillus subtilis*-Oleanolic acid; B: *Bacillus subtilis*-lupeol; C: *Escherichia coli*-Oleanolic acid; D: *Escherichia coli*-lupeol; E: *Staphylococcus aureus*-Oleanolic acid; F: *Staphylococcus aureus*-lupeol)

Fig 5 Effect of triterpenoid monomers at different concentrations on the bactericidal curves of three bacterial strains

5.4.4 Nucleic acid leakage rate of bacteria treated with two triterpenoid monomers

To further explore whether the two triterpenoid monomers exert antibacterial effects by acting on bacterial cell membranes, the nucleic acid leakage method was used to observe the degree of cell membrane damage caused by the two monomers on the three bacterial strains. As shown in Figure 6, the nucleic acid leakage in the 0 MIC group did not change significantly with time, while that in the experimental groups treated with oleanolic acid and lupeol increased

significantly. In addition, the nucleic acid leakage of the three bacteria gradually increased with the increase of monomer concentration. The determination of nucleic acid leakage rate further confirmed the damaging effect of the two compounds on bacterial cell membranes. The leakage of intracellular substances such as nucleic acids into the extracellular environment is an important indicator of severe and irreversible damage to the cell membrane^[33]. The experiments showed that both oleanolic acid and lupeol could significantly increase the cell membrane permeability of the tested bacteria, even leading to membrane structure loss and ultimately disrupting membrane integrity. This is consistent with the findings of Wang^[34], who reported that oleanolic acid can efficiently increase membrane permeability and damage membrane integrity. Meanwhile, Broniatowski et al.^[35] found that lupeol can insert into the bacterial membrane phospholipid monolayer and interact specifically with phosphatidylglycerol, thus also possessing membrane-damaging ability.



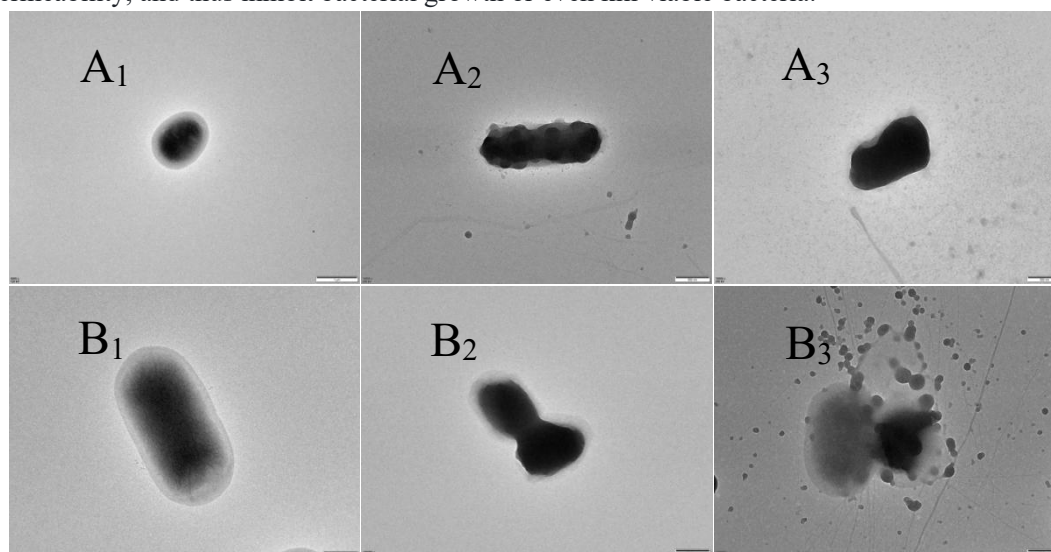
(A: *Bacillus subtilis*-Oleanolic acid; B: *Bacillus subtilis*-lupeol; C: *Escherichia coli*-Oleanolic acid; D: *Escherichia coli*-lupeol; E: *Staphylococcus aureus*-Oleanolic acid; F: *Staphylococcus aureus*-lupeol)

Fig 6 Impact of triterpenoid monomers at different concentrations on leakage assessment of three bacterial strains

5.4.5 TEM observation of three bacteria treated with two triterpenoid monomers

After the antibacterial experiment, to more intuitively demonstrate the action mode of the two triterpenoid monomers on the three bacteria, TEM was used to observe the bacteria treated with 3 MIC. As shown in Figure 4.8, *Bacillus subtilis* (A₁), *Escherichia coli* (B₁), and *Staphylococcus aureus* (C₁) before treatment had regular cell shapes, smooth surfaces, no obvious debris, and intact and clear intracellular substances. In contrast, the three bacterial cells treated with oleanolic acid (A₂, B₂, C₂) and lupeol (A₃, B₃, C₃) showed different states on their surfaces.

TEM observation results showed that there were differences in the morphological changes of different bacterial strains treated with oleanolic acid and lupeol, reflecting the similarities and differences in their mechanisms of action. For *Bacillus subtilis* (Gram-positive bacteria): oleanolic acid caused significant bacterial swelling and blurred cell outline, which is related to the compound damaging the cell wall and membrane structure of Gram-positive bacteria, leading to osmotic imbalance and content leakage^[36]; lupeol mainly caused bacterial surface shrinkage and morphological collapse, whose mechanism involves membrane depolarization and apoptosis-like death induced by lipid peroxidation^[37]. For *Escherichia coli* (Gram-negative bacteria): both compounds caused significant morphological changes, with blurred edges in individual bacteria. Oleanolic acid exerts its inhibitory effect by inhibiting bacterial motility, interfering with the two-component signal system and iron absorption pathway^[38]; lupeol exerts an auxiliary antibacterial function by inhibiting the efflux pump system^[39]. For *Staphylococcus aureus* (Gram-positive bacteria): both oleanolic acid and lupeol caused significant shrinkage and even fragmentation of bacterial cells. Among them, oleanolic acid mainly acts by damaging membrane integrity and inhibiting biofilm formation^[37]; lupeol can enter the interior of bacteria and directly inhibit protein synthesis, thereby interfering with bacterial growth and reproduction^[39]. Collectively, these results indicate that the two pentacyclic triterpenoid monomers can affect the structure of bacterial cell walls and membranes within a certain concentration range, alter cell permeability, and thus inhibit bacterial growth or even kill viable bacteria.



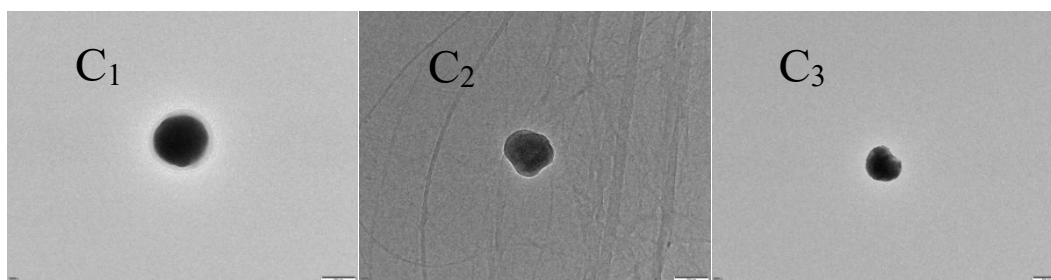


Fig 7 Electron microscopic observation of triterpenoid monomers on three bacterial species

6. Results and Discussion

Four compounds were purified and isolated from the triterpenoid extract of *Euchresta tubulosa* Dunn, and their structures were identified by nuclear magnetic resonance (NMR) and mass spectrometry (MS) as trifolirhizin (Compound 1), quercetin (Compound 2), lupeol (Compound 3), and oleanolic acid (Compound 4). Among them, two are pentacyclic triterpenoids, one is a flavonoid glycoside, and one is a flavonoid. Notably, trifolirhizin, oleanolic acid, and lupeol were isolated from this plant for the first time. The antioxidant and hypoglycemic capacities of the four isolated monomers were determined, and the results showed that quercetin exhibited the best antioxidant activity, while lupeol showed excellent enzyme inhibitory activity in the hypoglycemic activity test.

The antibacterial activity and mechanism of action of the two triterpenoid monomers against *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus* were systematically compared. By observing the bacterial growth curves, time-kill curves, nucleic acid leakage rates, and TEM images after inhibition, both compounds showed significant antibacterial effects, but their intensity and mechanisms differed.

Preliminary investigations on the antioxidant, hypoglycemic, and antibacterial activities of the isolated monomers confirmed that the folk use of *Euchresta tubulosa* Dunn in antioxidation, anti-inflammation, antibacterial, and hypoglycemic aspects has a certain pharmacodynamic material basis and scientific basis.

Declaration of Interest Statement

There is no conflict of interest in this study

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