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Development and Evaluation of Artemisia annua L. Soft Capsules: Formulation Optimization, Quality Characterization, and Preliminary Safety Assessment

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Abstract

Objective

To develop and evaluate a soft capsule formulation of *Artemisia annua* L. extract, with the aim of improving formulation performance of artemisinin, particularly in relation to its poor aqueous solubility and limited stability.

Methods

The extract was prepared using supercritical CO_2 extraction followed by ethyl acetate purification. Chemical constituents were identified by UPLC-MS/MS. The soft capsule formulation was optimized through single-factor experiments. Artemisinin content and dissolution behavior were determined by HPLC. Quality evaluation (fill weight variation, disintegration time, microbial limits, and stability) was conducted according to the Chinese Pharmacopoeia (2025 Edition). Safety was assessed through acute oral toxicity and 28-day repeated-dose toxicity studies in rats.

Results

The optimal extraction conditions were 25 MPa, 50°C, with 50% ethanol as co-solvent, yielding 2.5% extract after purification. A total of 78 components were identified, with artemisinin and representative sesquiterpene lactones as major constituents. The prepared soft capsules met pharmacopoeial quality standards, showing consistent artemisinin content (~ 6.4 mg/capsule), favorable dissolution (> 85% within 45 min), and good stability. The acute oral LD_{50} was greater than 2000 mg/kg, and the NOAEL in the 28-day study was 250 mg/kg, with only reversible liver changes observed at high doses.

Conclusion

This study establishes a reproducible preparation process and provides initial evidence regarding the quality attributes and short-term safety of *Artemisia annua* soft capsules. The findings suggest improved formulation performance in terms of dissolution and stability; however, further studies, including long-term toxicity, pharmacokinetics, and clinical evaluation, are required.

1. Introduction

Artemisia annua L., *A.annua* is an important medicinal plant. Its dried aerial parts are used as the traditional Chinese medicine "Qinghao" and have the effects of clearing deficiency heat, eliminating bone steaming, relieving summer heat, stopping malaria, and reducing jaundice [1]. Among them, the discovery of artemisinin has had a revolutionary impact on the treatment of malaria worldwide. Artemisinin-based combination therapy has been recommended by the World Health Organization (WHO) as a first-line antimalarial treatment regimen [2–3]. In addition, studies have shown that *A.annua* is also rich in a variety

of active ingredients such as flavonoids, coumarins, and volatile oils, which exhibit a wide range of pharmacological activities such as antioxidation, immunomodulation, anti-inflammation, and antiviral, suggesting that its medicinal potential is far more than just antimalarial [4–5]. However, artemisinin has poor water solubility and poor photostability [6], which seriously limits the development of its formulations and clinical applications. At the same time, how to extract the active ingredients from *A.annua* efficiently and environmentally, and to preserve its pharmacological activity to the maximum extent, has become a key issue for industrialization. Supercritical CO_2 extraction technology is particularly suitable for extracting heat-sensitive natural products such as *A.annua* due to its advantages, such as high extraction efficiency, low operating temperature, and no organic solvent residue [7–9]. In terms of dosage form, soft capsules can effectively mask the unpleasant odor of drugs, improve drug stability, and promote bioavailability. Therefore, soft capsules are an ideal carrier for oily extracts or light-labile drugs [10–11]. In addition, with the help of modern analytical techniques such as UPLC-MS/MS, which have high resolution and high sensitivity, the material basis of complex systems of traditional Chinese medicine can be thoroughly analyzed, and effective support can be provided for quality control. At present, systematic research on *A.annua* soft capsules is still insufficient, especially the lack of comprehensive exploration that organically combines preparation process, component analysis, and toxicological safety evaluation. Given that the development of new traditional Chinese medicine preparations must attach importance to safety assessment, standardized animal toxicology tests and related technical guidelines are the key basis for ensuring their safe clinical application [12]. The present study aims to develop a rational pharmaceutical formulation (soft capsules) of *A.annua* extract, with a focus on addressing key formulation-related challenges of artemisinin, particularly its poor aqueous solubility and limited photostability [6]. To achieve this, an integrated approach was employed, encompassing extraction optimization, formulation development, comprehensive quality evaluation, and preliminary toxicological assessment. The performance of the developed soft capsules was systematically evaluated in terms of dissolution behavior, stability, and content uniformity, providing evidence of improved formulation characteristics.

2. Materials and Methods

2.1 Instruments and Materials

HA221-50-06-C Supercritical CO_2 Extraction Apparatus (Nantong Huaan Supercritical Extraction Co., Ltd.), AB204-S Analytical Balance (Mettler Toledo), KQ-500DE Ultrasonic Cleaner (Kunshan Shumei), Rotary Evaporator, SCIEX X500R QTOF Liquid Chromatography-Mass Spectrometry System (AB SCIEX), LC-40 Nexera Series (Shimadzu), 2.7 μm C18 Column (Dikmatech), 0.22 μm Filter (Millex-GP), YWJ-100 Fully Automatic Soft Capsule Machine, HJG-100 Temperature-Controlled Capsule Gel Dispensing Tank (Beijing Xinhangcheng Technology Development Co., Ltd.), DHG-9140(A) Constant Temperature Drying Oven (Shanghai Yiheng Scientific Instruments Co., Ltd.), ACQUITY PREMIER (Waters Corporation), Distek 2100C Dissolution Tester, QT-BJ-001 Disintegration Tester (Tianda Tianfa Technology Co., Ltd.), DSX-30L-I High-Pressure Steam Sterilizer (Shanghai Shenan Medical Instrument Factory).

Artemisinin reference standard (purity ≥ 98%, batch number: G2003140), ethanol (batch number: 20231107), ethyl acetate (batch number: L2402693), di-tert-butyl-p-cresol (batch number: H2408072, GC),

ethylparaben (batch number: H2413063) were all analytical grade. Methanol, acetonitrile, formic acid, and ammonium acetate were all mass spectrometry grade. Beeswax (batch number: B2304609), soybean oil (grade 1), gelatin (batch number: C17170085), glycerol (batch number: C17051006), titanium dioxide (batch number: G2417016), caramel color (batch number: C304687), pepsin (batch number: F2509419), tryptic soybean agar medium, and Sabouraud dextrose agar medium were used.

A.annua was collected in August 2024 from the Wuling Mountains in Chongqing. It was identified by Professor Lü Hongfei of Zhejiang University of Technology as the dried aerial part of *A.annua*, a plant of the genus Artemisia in the family Asteraceae. The sample was placed in the International Joint Laboratory for the Development of Traditional Medicine and Big Health Products in Zhejiang Province.

2.2 Preparation and active ingredient analysis of *A.annua* extract

2.2.1 Preparation of *A.annua* extract

1 kg of *A.annua* powder (10 mesh) was placed in a 5 L extraction vessel, and 50% ethanol was added as an entrainer. Based on single-factor experiments, the optimal extraction process was determined by examining extraction pressure, temperature, and entrainer concentration, with artemisinin extraction rate as the indicator. Specifically, supercritical CO_2 extraction was performed at 25 MPa and 50 ℃. The extract was collected in separation vessel I (55 ℃, 7.5 MPa) and separation vessel II (25 ℃, 5 MPa), combined, and ethyl acetate was added at a ratio of 1:10 (w/v). Ultrasonic extraction was then performed (30 min × 3 times), followed by filtration. The filtrates were combined and concentrated to dryness by rotary evaporation under reduced pressure at 45 ℃, yielding a dark green extract for later use. The extraction parameters (25 MPa, 50°C, and 50% ethanol as co-solvent) were selected based on preliminary single-factor experiments aimed at maximizing artemisinin yield while minimizing co-extraction of undesired components. Higher pressures improved extraction efficiency but increased the co-extraction of lipophilic impurities, whereas lower pressures resulted in reduced yield. A temperature of 50°C was selected to balance extraction efficiency and thermal stability of sesquiterpene lactones. The addition of ethanol as a co-solvent enhanced the polarity of supercritical CO_2, thereby improving the extraction of moderately polar compounds, consistent with previous studies on supercritical CO_2 extraction [7–9].

2.2.2 UPLC-MS/MS system for identifying active ingredients in extracts

Accurately weigh 10 mg each of extracts I, II, and the extract paste, and dissolve them in 1 mL of methanol to prepare a stock solution with a concentration of 10.0 mg/mL. Subsequently, dilute the stock solution to 100.0 μg/mL, filter through a 0.22 μm microporous membrane, and collect the filtrate for later use. Analysis was performed using a Shimadzu Nexera Series liquid chromatography system and an AB SCIEX X500R QTOF mass spectrometer. Chromatographic separation was performed using a Dikmatech Leapsil C18 column (2.1 mm × 150 mm, 2.7 μm), with the column temperature set at 40 ℃ and the injection volume at 3 μL. Mobile phase: in positive ion mode, 0.1% formic acid aqueous solution (A)-acetonitrile (B); in negative ion mode, 5 mM ammonium acetate aqueous solution (A)-acetonitrile (B). The flow rate was 0.3 mL/min,

and the gradient elution program was as follows: 0–5 min, 5% B; 5–18 min, 5%–55% B; 18–25 min, 55%–80% B; 25–31 min, 80%–90% B; 31–38 min, 90%–95% B; 38–42 min, 95%–5% B. Mass spectrometry was performed using an electrospray ionization (ESI) source, scanning in both positive and negative ion modes, with information-dependent acquisition (IDA) mode. The mass scan range was m/z 80–1300. The spray voltage was 5500 V in positive ion mode and − 4200 V in negative ion mode; the collision energy was set to 30 V; and the ion source temperature was 550 ℃. The qualitative identification of the components was based on the following criteria: First, the error between the precise mass number of the quasi-molecular ion peak in the primary mass spectrometry and the theoretical value must be less than 5 × 10^-6; second, the fragment information from the secondary mass spectrometry was matched with the mzCloud and MassBank databases, and the matching score must be greater than 70%; simultaneously, isotope distribution was examined, and the data were compared with those from the PubChem and ChemSpider databases and relevant literature to tentatively identified based on MS/MS fragmentation and databases. For key target components such as artemisinin, the retention time and secondary mass spectra were compared with those of its reference standards under the same conditions for further confirmation.

2.3 Preparation process of *A.annua* soft capsules

Based on preliminary experiments, single-factor studies were conducted on factors such as the drug loading of *A.annua* extract, the proportion of suspending agent (beeswax), and the capsule shell formulation, using the viscosity of the contents, dispersion uniformity, and oil leakage rate during soft capsule preparation as evaluation indicators. The final formulation and process were determined. Three batches of soft capsules were prepared according to the determined formulation. The specific process is as follows: First, weigh 20 g of beeswax, 730 g of soybean oil, 250 g of *A.annua* extract, and 0.1 g of BHT. Add the beeswax to the soybean oil and heat to 80 ℃ using a magnetic stirrer, continuing stirring until the beeswax is completely melted. Then stop heating and allow the system to cool naturally to 60 ℃. At this temperature, add the *A.annua* extract and BHT, and continue mechanical stirring for 15–20 min until a uniform suspension is formed. Finally, allow to cool naturally to room temperature and stand to obtain the soft capsule contents. The formulation composition was optimized through preliminary experiments evaluating viscosity, dispersion uniformity, and leakage rate during encapsulation. Beeswax was selected as a suspending agent to improve physical stability, while soybean oil was used as a lipid carrier to ensure compatibility and homogeneity. The final formulation represents a balance between manufacturability and formulation stability.

Separately prepare 10 kg of gelatin, 10 kg of purified water, 5 kg of glycerin, 75 g of titanium dioxide, 100 g of caramel coloring, and 75 g of ethyl paraben. Add all excipients except gelatin to a mixing tank, start stirring, and slowly heat to 70°C until the excipients are completely dissolved. Then add the gelatin, maintain the temperature and stirring rate, and continue processing for 45 min until a uniform and transparent gel solution is formed. Afterward, perform vacuum degassing for 1 h to completely remove air bubbles, and the resulting capsule material is ready for use.

The soft capsules were produced using a fully automated soft capsule press. The capsule material temperature was set at 40°C, the contents temperature at 35°C, the rolling mill speed at 3–5 r/min, and the capsule shell thickness at 0.8 mm. Three batches of soft capsules were pressed according to these

parameters. The resulting soft capsules were placed in a setting tray and allowed to stand at room temperature (40%–50% relative humidity) for 2 hours to complete the setting process. Subsequently, the surface of the soft capsules was wiped with a 75% (v/v) ethanol solution to remove residual oil phase, and then placed in a constant temperature drying oven at 30°C and 30%–40% relative humidity for 8 hours. Finally, the three batches of soft capsules underwent quality inspection.

2.4 Quality testing of *A.annua* soft capsules

2.4.1 Inspection of fill weight variation of *A.annua* soft capsules

Following the method described in Section 0103, Capsules, of the 2025 edition of the Chinese Pharmacopoeia, three batches of soft capsules were tested for fill weight variation. Ten capsules were randomly selected from each of the three batches, and the weight of each capsule was accurately measured. The contents were poured out (without damaging the capsule shell), and the shells were washed with 75% (v/v) ethanol and placed in a ventilated area to allow the solvent to evaporate completely. The weight of the shell for each capsule was then accurately measured again. The fill weight per capsule and the average fill weight for each batch were calculated by subtracting the weight of the shell from the total weight of each capsule. According to the pharmacopoeia, in each batch of 10 soft capsules, no more than two capsules should exceed the fill weight variation limit (± 10%) compared to the average fill weight, and no single capsule should exceed the fill weight variation limit by more than 20%.

2.4.2 *A.annua* soft capsule disintegration time test

According to the Disintegration Time Test Method in General Chap. 0921 of Part IV of the 2025 Edition of the Chinese Pharmacopoeia, three batches of soft capsules were tested for disintegration time. A basket was suspended from a support via a stainless-steel shaft at the top and immersed in a 1000 ml beaker. The basket was adjusted so that the screen was 25 mm from the bottom of the beaker when it descended. The beaker contained artificial gastric fluid at (37 ± 1) ℃ as a medium. The liquid level was adjusted so that the screen was 15mm below the liquid surface when the basket rose, and the top of the basket was not submerged in the solution. Six capsules were randomly selected from each of the three batches and placed in the glass tube of the basket. The disintegration apparatus was started, and baffles were added as required. The test was conducted in artificial gastric fluid at (37 ± 1) ℃. All soft capsules from each batch should disintegrate completely within 60 minutes. If one capsule failed to disintegrate completely, six more capsules from the same batch were taken for retesting. The results of each retest should meet the requirements.

2.4.3 Microbial limit test of *A.annua* soft capsules

The tests were conducted according to General Chaps. 1105, 1106, and 1107 of Part IV of the 2025 edition of the Chinese Pharmacopoeia. Three batches of soft capsules were tested. 10 g of the test sample was taken, and the total aerobic bacteria count, mold count, and yeast count were performed according to the plate count method under General Chap. 1105, "Microbial Limit Tests for Non-Sterile Products: Microbial Count Method." The tests for Escherichia coli and Salmonella were performed according to General

Chap. 1106, "Control Bacteria Test Method." In each batch of test samples, the total aerobic bacteria count should not exceed 1.0×10^3 CFU/g, the total mold and yeast count should not exceed 1.0×10^2 CFU/g, and Escherichia coli should not be detected.

2.4.4 Determination of Artemisinin Content and Dissolution Rate in *A.annua* Soft Capsules by HPLC

Chromatographic conditions: The column was a Waters ACQUITY™ Premier CSH C18 (1.7 μm, 2.1 mm × 100 mm, VanGuard™ FIT), the mobile phase was methanol-0.1% formic acid aqueous solution (50:50), the detection wavelength was 260 nm, the flow rate was 0.3 mL/min, the column temperature was 30 ℃, and the injection volume was 10 μL.

Preparation of reference solutions: Accurately weigh 10.0 mg of artemisinin reference standard (≥ 98%), dissolve in ethanol and dilute to 10 mL to obtain a 1 mg/mL stock solution, which should be stored at 4 ℃ protected from light. Take an appropriate amount of the stock solution, dilute with 0.2% NaOH solution, and after alkaline hydrolysis at 50 ℃ for 30 min, dilute to volume with 0.08 mol/L acetic acid to prepare a series of standard solutions of 5, 10, 20, 40, and 50 μg/mL.

Preparation of test solution: Weigh about 1 g of the contents of the soft capsule, dissolve it in ethanol and make up to 10 mL; take 1 mL and follow the same steps as the "reference solution" for alkaline digestion and make up to 10 mL, and dilute it tenfold to obtain a 1 mg/mL test solution. Filter the solution through a 0.45 μm filter membrane and then test.

Dissolution and release determination (Method II, Paddle Method) was performed according to Chap. 0931, General Chapter, Part IV, Chinese Pharmacopoeia 2025 Edition. Three batches of soft capsules were tested. 900 mL of phosphate buffer (pH 6.8) containing 0.5% sodium dodecyl sulfate (SDS) was used as the dissolution medium at 37 ℃ and a rotation speed of 50 r/min. Six capsules from each batch were placed in dissolution vessels. Appropriate amounts of solution were taken at 10, 20, 30, 45, and 60 min, filtered through a 0.45 μm microporous membrane, and the filtrate was used as the test solution. The concentration of artemisinin was determined under the chromatographic conditions described above. The cumulative dissolution amount of artemisinin per soft capsule was calculated using the external standard method. The dissolution amount of each batch of 6 capsules should meet the following requirements: the dissolution amount per capsule, calculated based on the labeled amount, should not be less than the specified limit (Q); unless otherwise specified, Q is 80%. If 1–2 particles have a dissolution rate below Q but not below 70%, and their average dissolution rate is not lower than Q, they can also be judged to meet the requirements.

2.4.5 Stability study of *A.annua* soft capsules

Quality standards for each inspection item: see Table 1.

Accelerated Testing: Three batches of *A.annua* soft capsule samples were stored at a temperature of (40 ± 2) ℃ and a relative humidity of (75 ± 5) %, and samples were taken at 0, 3, and 6 months, respectively, and tested according to the quality standard for *A.annua* soft capsules.

Long-Term Testing: Three batches of *A.annua* soft capsule samples were stored at a temperature of (25 ± 2) ℃ and a relative humidity of (60 ± 5) %, and samples were taken at 0, 3, 6, 9, and 12 months, respectively, and tested according to the quality standard for *A.annua* soft capsules.

Table 1
Quality specifications and acceptance criteria for *A. annua* soft capsules

Inspection items	Quality standards stipulate
Appearance and shape	This product is a soft capsule containing a dark green paste; it has an aromatic odor and a bitter taste.
Filling volume difference	No more than two units may exceed the limit (± 10%), and no single unit may exceed the limit by more than 20%.
Disintegration time limit(min)	It should completely disintegrate within 1 hour.
Microbial limits	Bacterial count must not exceed 1.0×10^3 CFU/g, mold count must not exceed 1.0×10^2 CFU/g, and Escherichia coli must not be detected.
Content (mg/capsule)	Each capsule must contain at least 5 mg of artemisinin.

2.5 Toxicological safety evaluation

2.5.1 Laboratory animals and grouping

130 SPF-grade SD rats, half male and half female, weighing 170-270g, were purchased from Zhejiang Vital River Laboratory Animal Technology Co., Ltd., license number SCXK(浙)2024-0001. The animals were housed at the Zhejiang Provincial Institute for Food and Drug Control at a temperature of 20–24℃, relative humidity of 50%-70%, with 12 hours of light per day, and free access to food and water. The animal experiments were approved by the Laboratory Animal Ethics Committee of the Zhejiang Provincial Institute for Food and Drug Control, approval number 20250716Aazz06190620154-7. According to the dosage specified in the 2025 edition of the Chinese Pharmacopoeia, after 7 days of acclimatization, rats were randomly divided into a control group, a high-dose (2000 mg/kg) group, and a low-dose (1000 mg/kg) group based on body weight, with half being male and half female. For the 28-day oral toxicity test, rats were randomly divided into three dose groups based on body weight: high (1000 mg/kg, 30 rats), medium (500 mg/kg, 20 rats), and low (250 mg/kg, 20 rats), as well as a control group (30 rats), with half being male and half female. To reduce animal use and focus on potential toxic reactions, the number of animals in the medium and low-dose groups was set at 20.

2.5.2 Acute oral toxicity test

Following the "Technical Guidelines for Pharmacological and Toxicological Research of New Traditional Chinese Medicines" (2021) and OECD Trial Guideline 420 (Fixed-Dose Method), an acute oral toxicity test was conducted using the limit method. Thirty healthy SPF-grade SD rats (half male, half female) weighing (238.45 ± 30.75) g were used. The animals were fasted for approximately 16 hours before the experiment (water was allowed). A suspension of the test substance at a concentration of 200 mg/mL was prepared

using soybean oil as the solvent. The rats were randomly divided into three groups: a high-dose group (2000 mg/kg, administration volume 10 ml/kg), a low-dose group (1000 mg/kg, administration volume 10 ml/kg), and a blank control group (equal volume of soybean oil, 10 mL/kg). The high and low doses were equivalent to 57 times and 28.5 times the intended adult clinical dose, respectively. The test substance was administered orally as a single dose, and animals were observed for 14 days. The symptoms and deaths of the animals were carefully observed and recorded. After 14 days, the rats were anesthetized with Tiletamine-Zolazepam (Virbac, France), weighed, and then grossly dissected and pathologically examined.

2.5.3 28-day repeated-dose toxicity test

Following the OECD 407 guidelines for repeated-dose oral toxicity testing over 28 days, and with laboratory animal use license number SCXK (□) 2024-0001, 100 SPF-grade SD rats (half male, half female) were selected. After acclimatization for 7 days, they were randomly divided into a negative control group and three dose groups (low, medium, and high doses, 250, 500, and 1000 mg/kg body weight, equivalent to 7.2, 14.3, and 28.6 times the recommended human dose, respectively). The high-dose and control groups each contained 30 rats (half male, half female), while the medium-dose and low-dose groups each contained 20 rats (half male, half female). All rats were fed continuously for 28 days. During the experiment, rats had free access to water and food. General clinical manifestations were observed daily, and the signs, severity, duration, and mortality of poisoning were recorded. Body weight and food intake were recorded weekly, and weight gain was calculated at the end of the experiment. Before, at the end of, and at the end of the recovery period, all animals underwent ocular examinations (cornea, conjunctiva, iris). Fresh urine was collected from all animals within 4 hours using a rat metabolic cage before the end of the sampling and recovery period for routine urinalysis. Animals were fasted overnight before necropsy, and blood was drawn from the abdominal aorta for hematological and blood biochemical examinations. Rats scheduled for necropsy at the end of the sampling and recovery periods underwent gross anatomical examination of their organs after blood collection. The heart, thymus, adrenal glands, liver, kidneys, spleen, and testes were harvested, weighed, and their organ-to-body ratio was calculated. The brain, thyroid gland, thymus, heart, liver, spleen, kidneys, adrenal glands, stomach, duodenum, colon, pancreas, mesenteric lymph nodes, ovary, testes, and bladder were also harvested. The harvested tissues and organs were fixed in 10% neutral formalin, routinely embedded in paraffin, sectioned (4–5 μm thick), and stained with hematoxylin and eosin (H-E). Two pathologists, unaware of the rat group assignments, observed and evaluated the samples under an optical microscope to investigate the toxic effects of administering *A.annua* soft capsules to SPF-grade SD rats for 28 days and 14 days after recovery. The test substance was administered via gavage at doses of 1000, 500, and 250 mg/kg.

2.6 Statistical analysis

Data are expressed as mean ± SD. Group differences were analyzed by one-way ANOVA followed by Dunnett's test versus the control group, using SPSS. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 UPLC-MS/MS Identification Results of Active Ingredients in *A.annua* Extract

The chemical composition of two intermediate extracts and the final refined extract from the supercritical CO_2 extraction process was systematically identified and compared using UPLC-MS/MS. The results showed that all three extracts were rich in flavonoids and terpenoids, but their chemical components differed significantly in quantity and composition. Specifically, 69, 56, and 78 components were identified in extracts I, II, and the final extract, respectively. Further analysis revealed that the three extracts shared 29 core components, which constituted the main material basis for enrichment during the supercritical CO_2 extraction process. **Table 2** lists nine of the most representative common active components, including the recognized main active component artemisinin, as well as various terpenoid lactones with anti-inflammatory and anticancer activities such as ternolactone and costone lactone, as shown in **Figure 1**. It also covers other important active components such as coumarins and alkaloids. Typical UPLC-MS/MS extraction ion chromatograms are shown in **Figure 2.** It is noteworthy that the final refined extract not only contains all the core components but also boasts the richest number of unique components, reaching 22, including molecules known to possess significant pharmacological activity such as gemmaconone and thymequinone. This result indicates that the extract obtained through a complete extraction and purification process possesses a more complex chemical composition profile, providing a more solid material basis for its multi-target and synergistic pharmacological effects.

Table 2. Major shared bioactive compounds identified in the three extracts by UPLC–MS/MS

Serial Number	Test results	Retention time RT (min)	Molecular formula	Molecular Weight	Category
1	Artemisinin	21.05	$C_{15}H_{22}O_5$	283.15	Terpenes
2	Parthenolide	18.46	$C_{15}H_{20}O_3$	249.15	Terpenes
3	Costunolide	23.45	$C_{15}H_{20}O_2$	232.32	Terpenes
4	Dehydrocostus lactone	16.97	$C_{15}H_{18}O_2$	231.14	Terpenes
5	Scopoletin	12.00	$C_{10}H_8O_4$	192.17	Coumarin
6	Coumarin	14.19	$C_9H_6O_2$	147.04	Coumarin
7	Chrysosplenetin B	19.70	$C_{19}H_{18}O_8$	373.09	Flavonoid
8	Atractylenolide I	19.60	$C_{15}H_{18}O_2$	231.14	Terpenes
9	Resveratrol 3-O-glucuronide	30.32	$C_{20}H_{20}O_9$	424.37	Polyphenol

Note: Retention time and molecular weight may vary depending on the extract or detection mode.

3.2 Quality evaluation results of soft capsules

3.2.1 The content variation of soft capsules complies with regulations

The average total weight of the three batches of soft capsules was 0.508 g, 0.507 g, and 0.508 g, respectively; the average weight of the capsule shell was 0.168 g, 0.169 g, and 0.169 g, respectively; and the average content was 0.340 g, 0.338 g, and 0.339 g, respectively. The deviation range of the content of each capsule from the average content was -3.24% to 4.12%, -2.37% to 4.15%, and -2.66% to 4.44%, respectively, all within ±10% of the limit, and none of the capsules exceeded the limit by more than 10%, which complies with the pharmacopoeia regulations.

3.2.2 The disintegration time of the soft capsules complies with regulations

All six *A.annua* soft capsules from the three batches tested completely disintegrated within 60 minutes, and the disintegration times of each batch were relatively similar, ranging from 18 to 25 minutes, 16 to 28 minutes, and 17 to 26 minutes, respectively, with no abnormal delays. According to General Chapter 0921 of Part IV of the 2025 edition of the Chinese Pharmacopoeia, the disintegration time limits of the three batches of samples all meet the requirements.

3.2.3 The soft capsules passed the microbial limit test

As shown in **Table 3**, the microbial limit test results of the three batches of *A.annua* soft capsules all met the requirements for non-sterile oral preparations in General Chapter 1107 of Part IV of the 2025 edition of the Chinese Pharmacopoeia. Specifically, the total aerobic bacteria count, as well as the total mold and yeast counts, were far below the standard limits, and Escherichia coli was not detected.

Table 3. Microbial limit test results of *A. annua* soft capsules

Inspection methods based on	Inspection items	Measurement results	Limits and Standards (General Rule 1107)	Eligible conditions
Microbial Counting Method (General Rule 1105)	Total bacterial count (cfu/g)	1.8×10^2	$\leq10^3$ cfu/g	conform to
	Total number of mold and yeast cells (cfu/g)	23	$\leq10^2$ cfu/g	conform to
Control the bacteria testing method (General rule 1106)	Escherichia coli (/g)	Not detected	Not detectable	conform to

3.2.4 Artemisinin content is stable, and dissolution behavior is good

The artemisinin contents of the three batches of soft capsules were 1.900%, 1.883%, and 1.842%, respectively, with an average content of 1.875%, meaning each capsule contained 6.375 mg of artemisinin. The consistent artemisinin content across three batches is shown in **Figure 3 (B)**. A standard curve was

plotted against concentration using peak area, and quantification was performed using the external standard method. Artemisinin showed good linearity in the concentration range of 5-50 μg/mL, with a regression equation of Y = 7530X - 15327.5 (r = 0.9975).

The results showed that the cumulative dissolution rate of artemisinin at 45 min was greater than 86%, meeting the conventional requirements for the dissolution rate of oral solid dosage forms. Therefore, 45 min was determined as the sampling time point for dissolution testing. Three batches of *A.annua* soft capsules (numbers: 20250520, 20250525, and 20250530) were taken, and 6 capsules were accurately weighed. The dissolution rate of the samples was determined according to the above method, and the results were 86.56%, 87.63%, and 87.12%, respectively. This indicates that there was no significant difference in the dissolution rate between different batches of samples. The dissolution curves are shown in **Figure 3 (A)**.

3.2.5 Stability test

Three batches of samples underwent accelerated testing for six months, and all test indicators, including appearance, content variation, disintegration time, and microbial limits, met the requirements. The artemisinin content in the three batches was 6.34, 6.13, and 5.92 mg/capsule (batch number 20250520); 6.37, 6.18, and 5.96 mg/capsule (batch number 20250525); and 6.39, 6.15, and 5.91 mg/capsule (batch number 20250530), respectively. There was no significant change in the artemisinin content of the formulation, which meets the quality standard requirements.

After a 12-month long-term trial, the samples met all the required standards for appearance, content variation, disintegration time, and microbial limits. The artemisinin content in the three batches of samples was 6.34, 6.23, 6.12, 6.25, and 6.31 mg/capsule (batch number 20250520); 6.37, 6.21, 6.27, 6.30, and 6.15 mg/capsule (batch number 20250525); and 6.39, 6.37, 6.13, 6.24, and 6.16 mg/capsule (batch number 20250530), respectively. There was no significant change in the artemisinin content of the formulation, which meets the quality standard requirements **Figure 4**.

This figure illustrates the distribution of artemisinin content across three batches of *A.annua* soft capsules after 12 months of stability testing, showing minimal variation and confirming that all batches remain within the target specification, demonstrating the stability and consistency of the formulation over time.

3.3 Acute oral toxicity test showed no obvious toxicity.

During the experiment, none of the rats showed signs of death or poisoning, their weight increased normally, and no abnormalities were found during gross necropsy. The results indicate that, under the experimental conditions, the acute toxicity test of *A.annua* soft capsules in rats did not show any significant toxic reactions related to the test substance, and the acute oral LD50 for both male and female rats was greater than 2000 mg/kg.

3.4 A 28-day repeated-dose toxicity study revealed reversible liver adaptation changes.

3.4.1 General physiological state observation

During the administration and recovery periods, no abnormalities were observed in the general behavior or clinical manifestations of male and female rats in any of the dosage groups, and no animals died. There was no statistically significant difference in the average daily food intake between the dosage groups and the control group (P>0.05). Systemic ophthalmological examinations before and after administration also revealed no abnormalities.

3.4.2 Weight measurement

The results are shown in **Table 4**. During the sampling period, the body weight of both male and female rats in each sampling group increased. Compared with the control group, there was no significant difference in body weight among the groups (P>0.05), indicating that the test substance had no significant effect on the animal body weight.

Table 4. Effects of *A. annua* soft capsules on body weight changes in rats (mean ± SD)

Gender	Group	Control Group (n=15)	Low-Dose Group (n=10)	Medium-Dose Group (n=10)	High-Dose Group (n=15)
Female	First Sample Period	181.0 ± 8.3	183.2 ± 7.4	185.9 ± 7.0	184.9 ± 8.2
	Weight Gain	70.5 ± 15.9	62.0 ± 17.5	74.7 ± 9.1	57.3 ± 13.9
Male	First Sample Period	257.8 ± 7.5	254.0 ± 4.4	259.0 ± 9.1	260.4 ± 8.8
	Weight Gain	182.8 ± 21.7	178.9 ± 33.6	184.7 ± 28.7	160.0 ± 29.1

3.4.3 hematological examination

The results are shown in **Table 5**. At the end of the sampling period, compared with the control group, female rats showed increased NEUT% in the low-dose group, decreased PT in the medium-dose group, and decreased BASO% in the low, medium, and high-dose groups (P < 0.01 or P < 0.05). Male rats showed decreased HGB and MCH in the medium and high-dose groups, increased MPV and NEUT% in the high-dose group, decreased LYMPH%, increased MONO% and decreased PT in the medium-dose group, and decreased BASO% in the low, medium, and high-dose groups (P < 0.01 or P < 0.05). No significant differences were observed in the remaining hematological parameters among the groups. However, these changes were all within the range of our laboratory's historical normal background data, and there was no clear dose-response relationship, nor were there any corresponding histopathological changes. Therefore, these differences are considered to be without toxicological significance and may be due to physiological fluctuations or adaptive responses to the test substance.

Table 5. Effects of *A. annua* soft capsules on hematological parameters in rats (mean ± SD)

Gender	Parameter	Control Group (n = 10)	Low-Dose Group (n = 10)	Medium-Dose Group (n = 10)	High-Dose Group (n = 15)
Female	NEUT (%)	8.76 ± 2.32	11.5 ± 3.4*	9.9 ± 3.9	12.0 ± 6.2
	BASO (%)	0.2 ± 0.1	0.1 ± 0.1*	0.1 ± 0.1*	0.1 ± 0.1*
	PT (s)	7.3 ± 0.3	7.1 ± 0.3	6.8 ± 0.3**	7.1 ± 0.4
	HGB (g/dL)	148 ± 4	146 ± 6	143 ± 4*	143 ± 5*
	MCH (pg)	19.0 ± 0.5	18.7 ± 0.7	18.4 ± 0.4*	18.2 ± 0.8*
	MPV (fL)	7.0 ± 0.2	6.9 ± 0.3	7.1 ± 0.3	7.2 ± 0.2*
Male	NEUT (%)	11.37 ± 2.95	13.1 ± 6.1	12.7 ± 1.7	17.6 ± 8.1*
	LYMPH (%)	84.1 ± 3.7	82.1 ± 6.5	81.5 ± 2.0	77.3 ± 9.0*
	MONO (%)	3.7 ± 1.2	3.9 ± 0.7	5.1 ± 1.3*	4.4 ± 1.1
	BASO (%)	0.2 ± 0.1	0.1 ± 0.1*	0.1 ± 0.0*	0.1 ± 0.1*
	PT (s)	7.8 ± 0.3	7.8 ± 0.3	7.5 ± 0.3*	7.6 ± 0.5

*Note: Compared with the control group, *P < 0.05, **P < 0.01; NEUT%: Neutrophil percentage; BASO%: Basophil percentage; PT: Prothrombin time; HGB: Hemoglobin; MCH: Mean corpuscular hemoglobin; MPV: Mean platelet volume; LYMPH%: Lymphocyte percentage; MONO%: Monocyte percentage.*

3.4.4 Serum biochemistry test

The results are shown in **Table 6**. At the end of the sampling period, compared with the control group, female animals showed decreased A/G, TBIL, and CL in the low, medium, and high dose groups, increased CHOL, increased TG in the high dose group, and decreased P in the low dose group (P < 0.05 or P < 0.01). Male animals showed decreased ALP and CL and increased TP in the medium and high dose groups, decreased TBIL in the low, medium, and high dose groups, and decreased GLU and A/G and increased CHOL and K in the high dose group (P < 0.05 or P < 0.01). No significant differences were observed in serum biochemical indicators among the remaining groups. However, these changes were all within the normal background data range of our laboratory, and there was no clear dose-response relationship, nor were there corresponding histopathological changes. Therefore, these differences are considered to be without toxicological significance and may be due to physiological fluctuations or adaptive responses to the test substance.

Table 6. Effects of *A. annua* soft capsules on serum biochemical parameters in rats (mean ± SD)

Gender	Parameter	Control Group (n = 10)	Low-Dose Group (n = 10)	Medium-Dose Group (n = 10)	High-Dose Group (n = 10)
Female	A/G	1.5 ± 0.1	1.4 ± 0.0**	1.4 ± 0.1**	1.3 ± 0.1**
	TBIL	1.8 ± 0.4	1.3 ± 0.2**	1.1 ± 0.2**	1.2 ± 0.2**
	CHOL	1.41 ± 0.29	1.79 ± 0.41*	1.93 ± 0.48**	2.23 ± 0.35**
	TG	0.35 ± 0.05	0.41 ± 0.09	0.39 ± 0.06	0.52 ± 0.09*
	P	2.43 ± 0.26	2.20 ± 0.14*	2.44 ± 0.17	2.32 ± 0.11
	Cl	105.3 ± 0.7	104.1 ± 1.2*	104.2 ± 1.2*	103.0 ± 0.9*
	TP	58.9 ± 1.4	59.2 ± 1.6	60.9 ± 2.4*	62.9 ± 3.8*
Male	A/G	1.3 ± 0.0	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1**
	TBIL	1.5 ± 0.3	1.1 ± 0.2**	1.0 ± 0.2**	1.0 ± 0.2**
	ALP	179 ± 38	148 ± 27	142 ± 29*	136 ± 39*
	GLU	7.73 ± 1.12	7.24 ± 0.62	6.88 ± 0.97	6.06 ± 0.98*
	CHOL	1.64 ± 0.25	1.42 ± 0.26	1.64 ± 0.29	1.86 ± 0.18*
	K	5.03 ± 0.20	5.19 ± 0.25	5.04 ± 0.20	5.39 ± 0.20**
	Cl	105.3 ± 1.2	104.8 ± 0.9	103.7 ± 1.1**	103.0 ± 1.3**

*Note: Compared with the control group, *P < 0.05, **P < 0.01; A/G: Albumin-to-globulin ratio; TBIL: Total bilirubin; CHOL: Total cholesterol; TG: Triglyceride; P: Phosphorus; CL: Chloride; TP: Total protein; ALP: Alkaline phosphatase; GLU: Glucose; K: Potassium.*

3.4.5 Urine test

The results are shown in **Table 7**. After the sampling and recovery periods, the urine was clear and without turbidity. During the sampling period, some dose groups showed statistically significant changes in urine specific gravity and pH, and positive results for urinary protein and ketones. After the recovery period, all of these changes returned to normal and were not different from the control group, indicating that the changes were reversible.

Table 7. Effects of *A. annua* soft capsules on urinalysis parameters in rats (mean ± SD)

Gender	Group	Ketone Bodies				Urine Protein				White Blood Cells				proportion	PH
		□	±	+	++	□	±	+	++	□	±	+	++		
Female	Control	4	6			2	2	5	1	8	1	1		1.019 ± 0.006	8.0 ± 1.0
	Low	4	6			5	4	1		4	6			1.018 ± 0.006	7.4 ± 0.8
	Medium	4	6			8		2		3	6	1		1.027 ± 0.005*	7.0 ± 1.0*
	High	10				10				7	3			1.023 ± 0.006	6.4 ± 0.7**
Male	Control	1	4	5			1	9		2	8			1.015 ± 0.002	8.6 ± 0.3
	Low	4	5	1		10				1	8	1		1.016 ± 0.002	8.4 ± 0.2
	Medium	1	5	4		2	1	4	3		3	5	2	1.017 ± 0.003*	8.6 ± 0.4
	High	3	5	2			7	3			2	7	1	1.019 ± 0.004**	8.0 ± 0.7*

*Note: Negative (-), Equivocal (±), Positive (+), Strongly positive (≥++). Compared with the control group, *P < 0.05, **P < 0.01.*

3.4.6 Histopathological examination confirmed that high-dose hepatic vacuolar degeneration is reversible

High-dose group (1000 mg/kg): Clear pathological changes related to the test substance were observed in the liver. As shown in **Figures 5D and 6H**, mild cytoplasmic vacuolar degeneration was observed in hepatocytes of some female (3/10) and male (4/10) rats. No pathological changes related to the test substance were observed in other organs examined, including the heart, spleen, kidneys, brain, testes, and ovaries. Medium- and low-dose groups (500 and 250 mg/kg): No abnormalities were observed in any tissues (including the liver) **(Figures 5B, 5C, 5F, 5G)**. The liver tissue structure of the control group **(Figures 5A and 5E)** showed no significant difference. After 14 days of drug withdrawal, the vacuolar degeneration observed in the livers of the high-dose group animals completely disappeared, confirming that the lesion was a reversible adaptive response. In this study, the mild hepatocellular vacuolar changes observed at 1000 mg/kg were considered an adaptive and reversible response rather than progressive or severe liver injury, as they were limited to the high-dose group, were not observed at 250 or 500 mg/kg, and completely resolved after the 14-day recovery period. Although no treatment-related histopathological liver changes were detected at 500 mg/kg, some clinical pathology parameters at this dose showed slight, treatment-related alterations that remained within the historical background range and were not associated with structural liver damage. Taking these findings into account, together with the clear, dose-dependent liver changes observed at 1000 mg/kg, a conservative approach was adopted, and 250 mg/kg was selected as the NOAEL in this 28-day study.

4. Discussion

This study focuses on the development and evaluation of a soft capsule formulation of *A.annua*, with particular emphasis on formulation performance and quality optimization. The work integrates extraction optimization, formulation design, quality evaluation, and preliminary safety assessment within a systematic framework. The results showed that the established preparation process exhibited good stability and reproducibility, the overall quality of the formulation was controllable, the main chemical components were clearly identified, and no significant safety risks were observed within the experimental dosage range set in this study. This provides experimental evidence for further research and potential clinical translation of this formulation. Supercritical CO_2 extraction technology has become an ideal method for extracting heat-sensitive active ingredients from *A.annua* due to its advantages such as high efficiency, low operating temperature and no organic solvent residue. Through process optimization, the optimal extraction conditions were determined to be an extraction pressure of 25 MPa, a temperature of 50 ℃, and an entrainer of 50% ethanol. This ensured the efficient extraction of artemisinin while preserving other unstable active ingredients to the greatest extent [13]. UPLC-MS/MS analysis showed that 78 chemical components were identified in the final purified extract [14]. In addition to artemisinin, it also included terpene lactones with clear anti-inflammatory and anti-tumor activities, such as valerin lactone and costone lactone, as well as various coumarins and flavonoids [15]. It is worth noting that compared with traditional *A.annua* preparations with complex components and large batch-to-batch variations [16], the soft capsules prepared in this study showed significant advantages in terms of component clarity and quality control. Through standardized supercritical fluid extraction, efficient and targeted enrichment of multiple active ingredients, with artemisinin as the core, was achieved, and the stable content of artemisinin in the formulation (1.875%) was determined [17]. This multi-component system provides a material basis for the formulation to exert multi-target and synergistic pharmacological effects, suggesting that artemisinin, parthenolide, and other components may produce synergistic effects through similar or complementary mechanisms of action. The specific mechanisms of action still require further in-depth research. To address the poor water solubility and low photostability of artemisinin, this study selected soft capsules as the dosage form to improve its taste masking properties, stability, and bioavailability. Through systematic optimization of the contents and capsule shell formulations, three batches of soft capsule samples were successfully prepared, all exhibiting good formability and appearance quality. Quality evaluation results showed that the artemisinin content was uniformly distributed across the three batches, with an average content of 1.875%. Key quality indicators such as fill weight variation, disintegration time, microbial limits, and dissolution rate all met the relevant requirements of the Chinese Pharmacopoeia (2025 edition), indicating that the established preparation process has good reproducibility and the overall formulation quality is stable and controllable. Compared with conventional crude extract forms, the developed soft capsules demonstrated improved physicochemical performance, including consistent artemisinin content, favorable dissolution (> 85% within 45 min), and stability under accelerated and long-term conditions. These findings suggest that the formulation may partially address known limitations of artemisinin, particularly in terms of stability and dissolution behavior [10–11]. Toxicological safety evaluation is an important part of preclinical research on new traditional Chinese medicine drugs. Acute oral toxicity tests showed that the LD_{50} of the *A.annua* soft capsules in rats was greater than 2000

mg/kg, which, according to relevant evaluation standards, classifies it as practically non-toxic[18]. In a 28-day repeated oral toxicity test, some animals in the high-dose group (1000 mg/kg) exhibited hepatocellular vacuolar degeneration. This phenomenon needs to be comprehensively analyzed in conjunction with the research background of the "dual effects" of artemisinin-based drugs on the liver. Previous studies have shown that while artemisinin and its derivatives exert potential hepatoprotective effects, such as anti-inflammatory and antioxidant properties, they may also induce a certain degree of hepatic response under high-dose conditions, and this effect is significantly dose-dependent [19–20]. The liver histological changes observed in this study were reversible and only occurred in the high-dose group. All changes were completely recovered after the drug withdrawal recovery period, suggesting that they are more likely an adaptive response to high-dose exposure rather than progressive or irreversible liver damage. Furthermore, while some hematological, serum biochemical, and urological indicators showed statistically significant differences, no clear dose-response relationship was observed [21]. All relevant indicators remained within the laboratory's historical normal reference range and were not accompanied by corresponding histopathological abnormalities. These changes also resolved spontaneously during the recovery period. Therefore, a comprehensive assessment suggests that these changes do not have clear toxicological significance and are more likely to reflect physiological adaptation processes or individual differences. While acknowledging the findings of this study, its limitations should also be fully recognized. First, the toxicological evaluations in this study were conducted using healthy SD rat models, which differ somewhat from the pathophysiological state of patients in clinical applications. Second, the 28-day repeated-dose toxicity test cannot fully reflect the potential safety risks under long-term or chronic administration conditions. Furthermore, the molecular mechanism of reversible hepatocyte vacuolar degeneration observed in the high-dose group remains unclear. Additionally, the pharmacokinetic characteristics of the soft capsule in vivo and its bioavailability in humans require further systematic investigation. Clearly identifying these limitations aims to provide a clearer research direction and focus for in-depth research and subsequent development of *A.annua* soft capsules. In conclusion, this study established a preliminary and reproducible preparation process for *A.annua* soft capsules and provided initial data on their quality attributes and short-term toxicological safety. While the formulation demonstrated acceptable stability, dissolution behavior, and no significant toxicity under the tested conditions, these findings remain preliminary. Further studies, including long-term toxicity, pharmacokinetics, and clinical validation, are required to confirm the safety and efficacy of this formulation [22].

Declarations

FUNDING INFORMATION

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Author Contribution

QIU K.; Farukh-S S.; Adam J.; Gadallah M.; SUN Y.; Methodology: Farukh-S S.; Adam J.; Validation: QIU K.; Gadallah M.; Formal analysis: QIU K.; Adam J.; Data curation: QIU K.; Farukh-S S.; Writing – original draft preparation: QIU K.; Writing – review & editing: QIU K.; Farukh-S S.; Adam J.; Gadallah M.; SUN Y.; Funding acquisition: SUN Y. All authors have read and agreed to the published version of the manuscript.

Data Availability

All data generated or analyzed during this study are included in this article and its supplementary information files.

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Figures

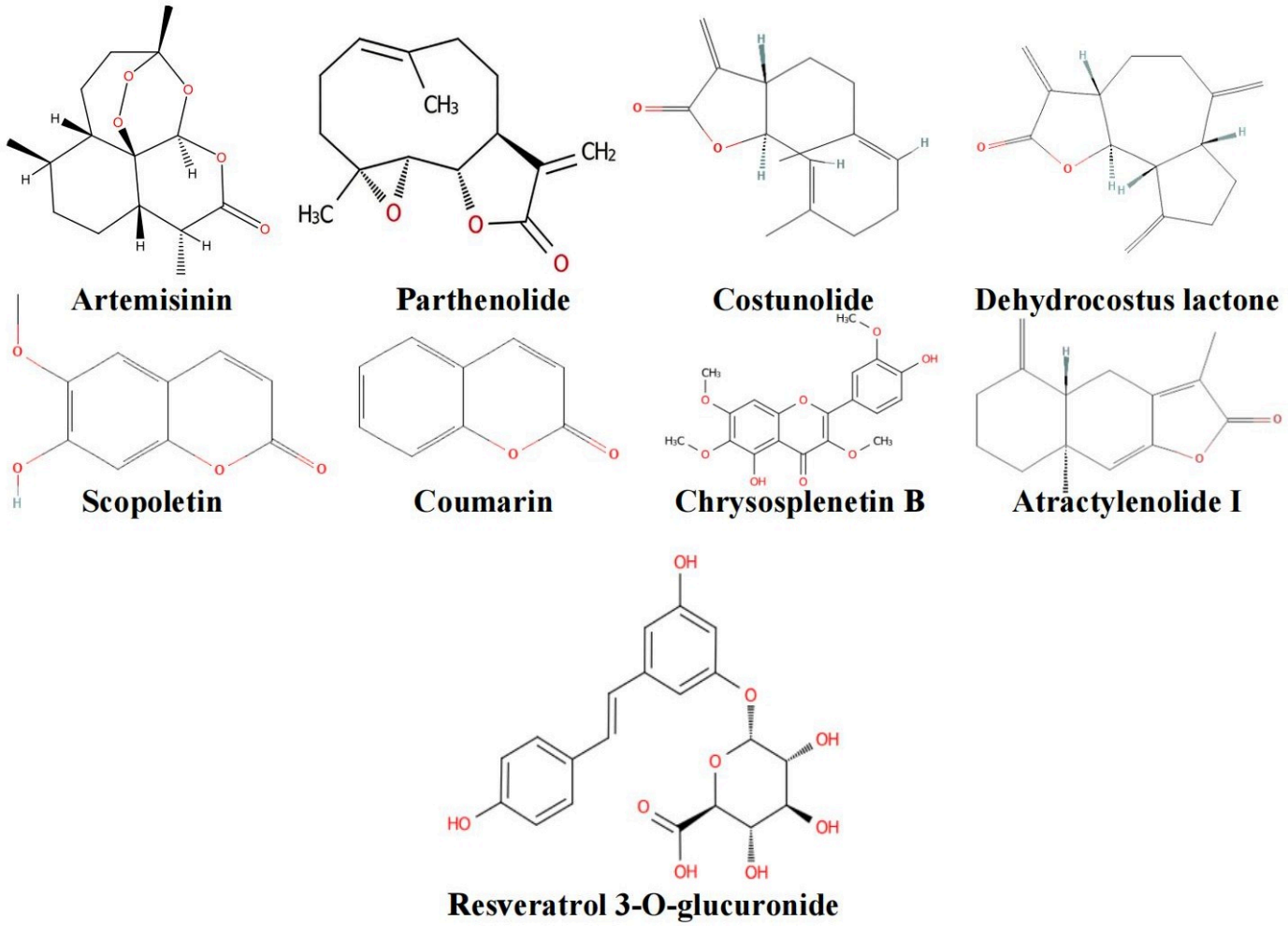


Figure 1

Chemical structures of the main common active ingredients identified in the three extracts

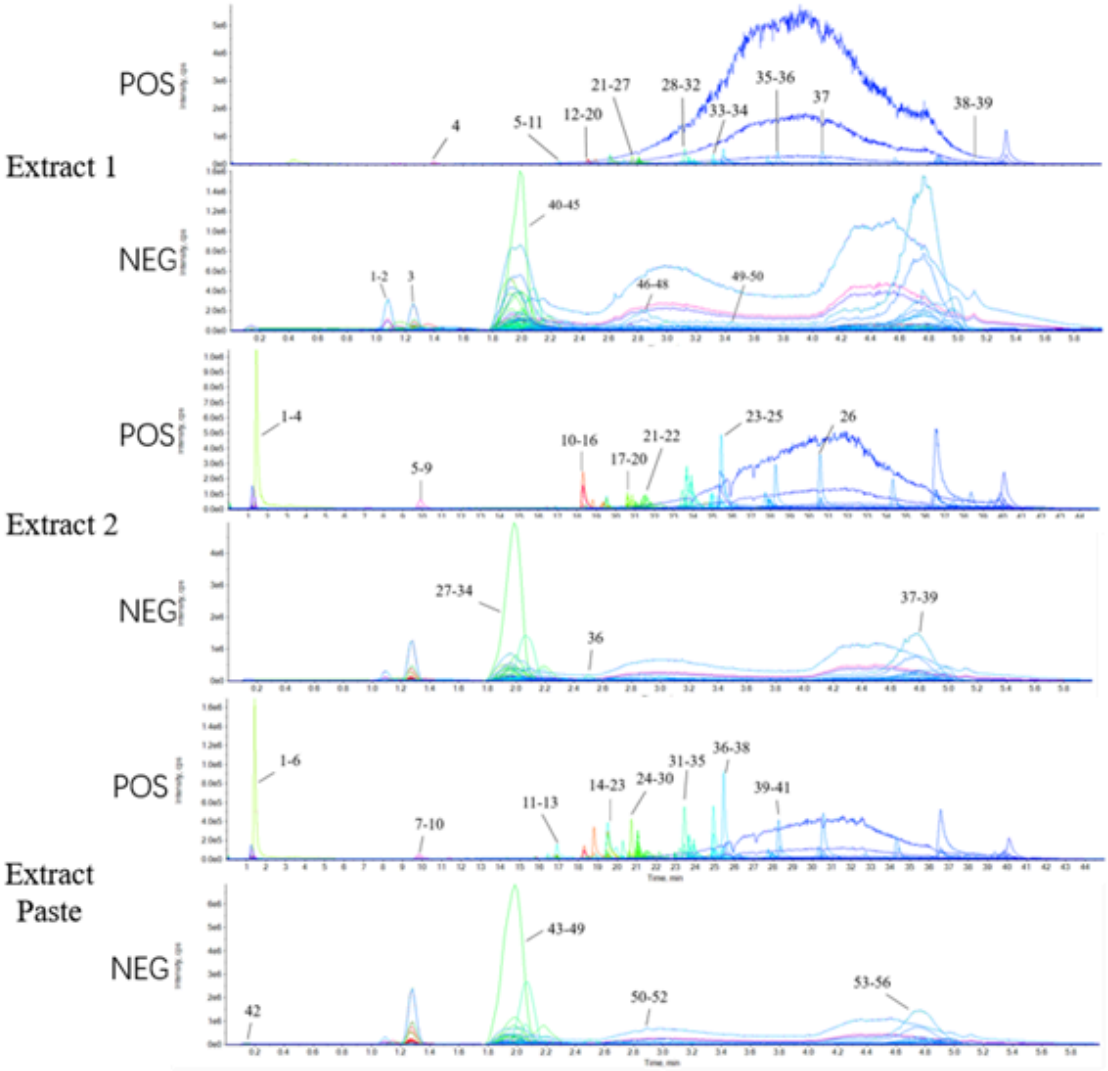


Figure 2

Typical extracted ion chromatograms of *A. annua* extracts

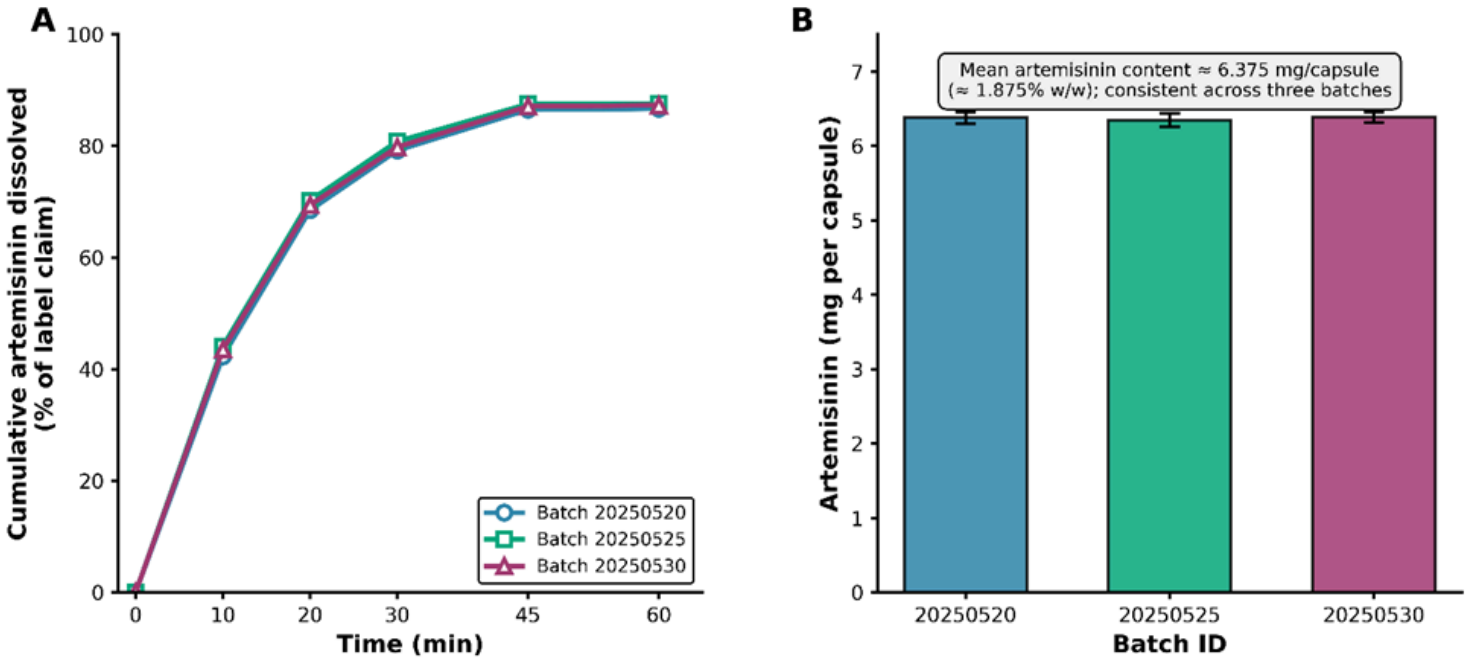


Figure 3

Artemisinin content and *in vitro*dissolution profiles of *A.annua* soft capsules across three manufacturing batches (20250520, 20250525, 20250530).

(A) Cumulative dissolution curves showing >86% release at 45 min for all batches.

(B) Consistent artemisinin content averaging 6.375 mg/capsule (1.875% w/w).

These results demonstrate batch-to-batch consistency in both content uniformity and dissolution performance.

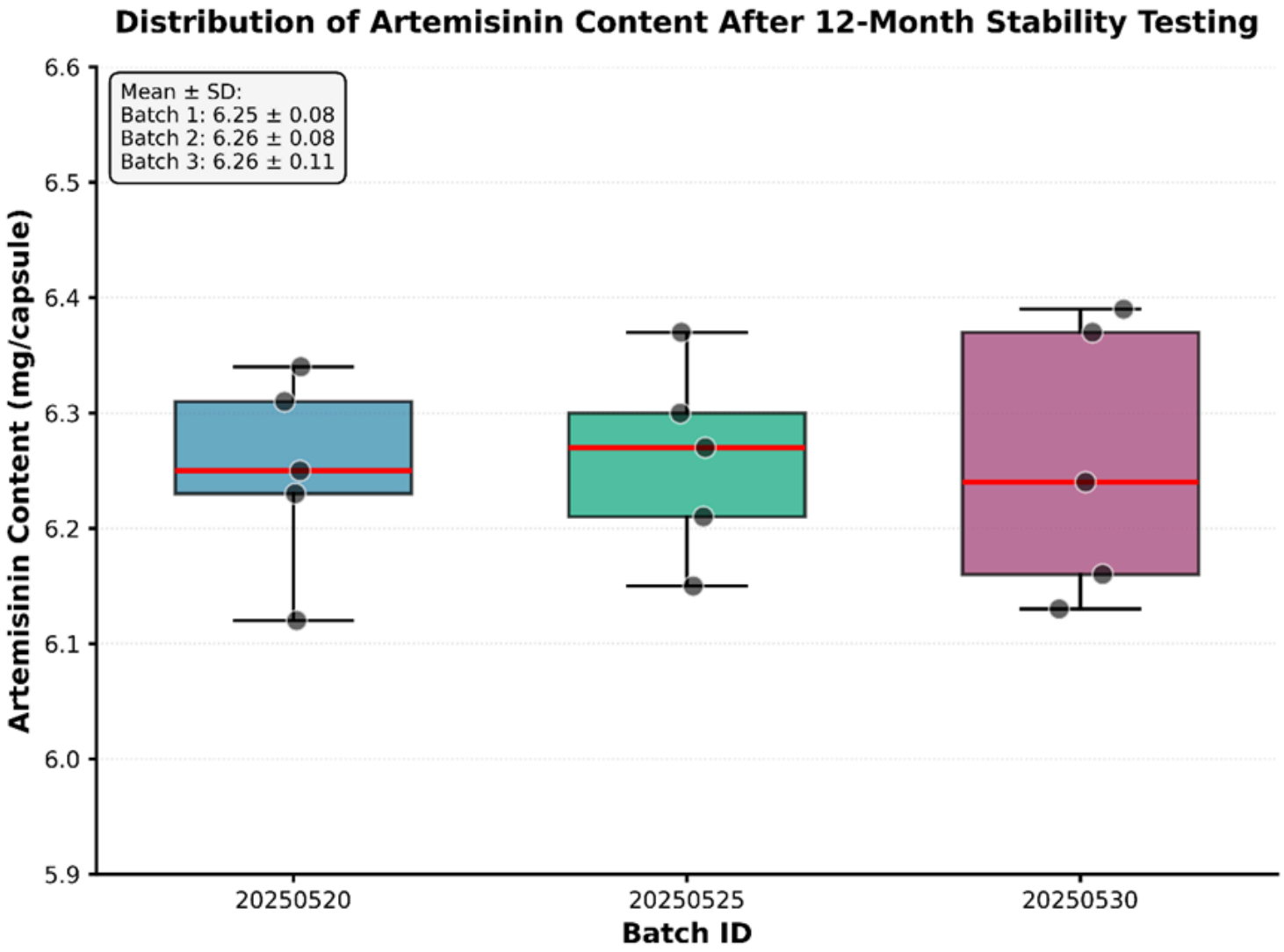


Figure 4

Distribution of artemisinin content after 12-month stability testing of *A. annua* soft capsules

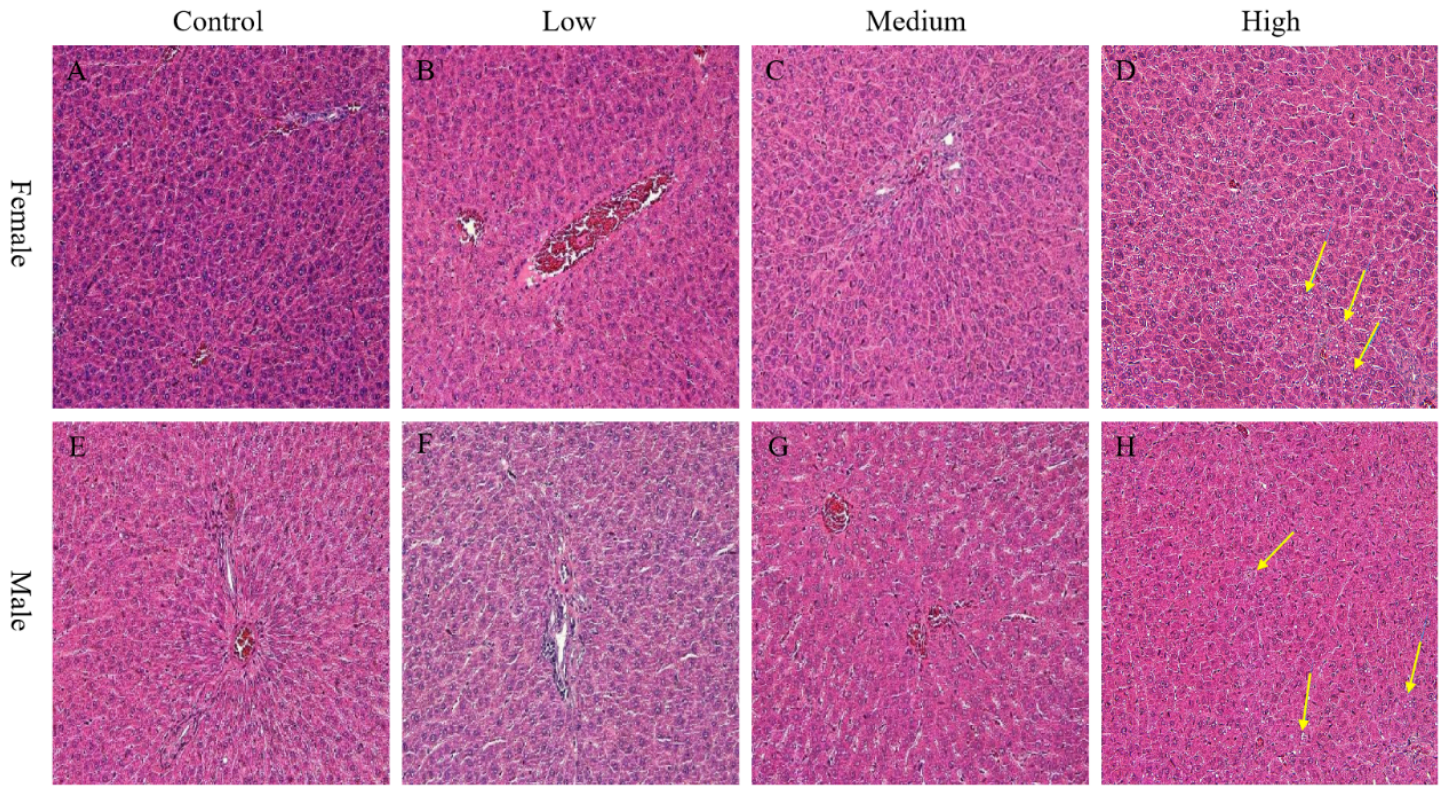


Figure 5

Histopathological evaluation of rat liver tissues following oral administration *of A. annua* soft capsules (H&E staining, 200×)

A: Control group, female, no abnormal changes observed; B: Low-dose group (250 mg/kg), female, no abnormal changes observed; C: Middle-dose group (500 mg/kg), female, no abnormal changes observed; D: High-dose group (1000 mg/kg), female, showing cytoplasmic vacuolation in hepatocytes (indicated by arrows); E: Control group, male, no abnormal changes observed; F: Low-dose group (250 mg/kg), male, no abnormal changes observed; G: Middle-dose group (500 mg/kg), male, no abnormal changes observed; H: High-dose group (1000 mg/kg), male, showing cytoplasmic vacuolation in hepatocytes (indicated by arrows).

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