

Article

Purification, Ionic-Liquid-Associated Elemental Marker Reduction, Stability and Bioactivity Evaluation of Monoterpene Glycoside-Enriched Extracts from *Radix Paeoniae Alba*

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Abstract

Ionic liquid-assisted extraction can improve the recovery of monoterpene glycosides from *Radix Paeoniae Alba*, whereas post-extraction purification and ionic-liquid-associated marker-level quality control remain necessary before further bioactivity evaluation. In this study, a macroporous resin-based purification process was developed for monoterpene glycoside-enriched extracts obtained by conventional reflux extraction and ionic liquid-assisted microwave-ultrasound extraction. Among the five tested resins, AB-8 resin showed the best adsorption performance and was selected for further process optimization. The optimized purification conditions were a loading concentration of 13 mg/mL, loading pH of 7, elution with 70% ethanol, loading volume of 6 BV and elution volume of 9 BV. After purification, the total quantified monoterpene glycoside content increased from 6.59% to 51.68% in the conventional extract and from 15.30% to 75.30% in the ionic liquid-assisted extract. The ionic-liquid-associated elemental marker content in the ionic liquid-assisted extract decreased from 5.593% to 0.072% after purification, corresponding to an approximately 98.7% reduction at the elemental-marker level. Stability evaluation indicated that Na₂SO₃, Fe³⁺, ascorbic acid, sucrose and light exposure affected the detected monoterpene glycoside content to different extents, whereas short-term heating within 20–80 °C caused only slight fluctuations. The purified extracts showed stronger DPPH and ABTS radical scavenging activities and reducing power than the crude extracts. In LPS-stimulated RAW264.7 cells, the purified extracts reduced NO production and downregulated the mRNA expression of IL-6, IL-1 β , TNF- α , iNOS and COX-2, suggesting anti-inflammatory potential at the NO and gene-expression levels. AB-8 resin purification enriched the monitored monoterpene glycosides, reduced the selected ionic-liquid-associated elemental marker, and generated purified fractions suitable for subsequent stability and bioactivity evaluation.

Keywords: *Radix Paeoniae Alba*; monoterpene glycosides; AB-8 resin; macroporous resin purification; ionic-liquid-associated elemental marker; stability; antioxidant activity; anti-inflammatory potential



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1. Introduction

Radix Paeoniae Alba, the dried root of *Paeonia lactiflora* Pall., is an important medicinal plant material widely used in traditional Chinese medicine, and its chemical constituents and pharmacological actions have been systematically reviewed [1]. Monoterpene glycosides are among the characteristic bioactive constituents of *Paeonia* species and are

associated with multiple pharmacological activities [2]. Paeoniflorin, albiflorin and oxy-paeoniflorin are representative monoterpene glycosides in *Radix Paeoniae Alba* and have been used as characteristic constituents in pharmacokinetic and quality-related studies [3]. Therefore, the enrichment and purification of monoterpene glycosides are important for improving extract quality, supporting biological evaluation and facilitating further development of *Radix Paeoniae Alba*-derived products.

Ionic liquid-assisted extraction has attracted increasing attention in natural product extraction because ionic liquids can interact with plant metabolites through hydrogen bonding, electrostatic interactions and other noncovalent forces [4]. Ionic liquids and deep eutectic solvents have also been discussed as alternative extraction media for natural products, owing to their tunable physicochemical properties and extraction efficiency [5]. In addition, ionic liquid solutions have been reviewed as extractive solvents for recovering value-added compounds from biomass and natural resources [6]. However, the use of ionic liquids also introduces a post-extraction challenge: residual ionic liquids may remain in crude extracts and affect downstream purification, bioactivity evaluation and potential application. Therefore, a suitable post-extraction purification strategy is required not only to enrich target compounds but also to reduce selected ionic-liquid-associated elemental marker levels. Green-solvent-based extraction strategies have also highlighted the importance of connecting extraction efficiency with downstream quality and applicability [7].

Macroporous resin chromatography is widely used for the enrichment and purification of natural products because of its adsorption capacity, operational simplicity, solvent compatibility and potential for scale-up. For monoterpene glycosides from Paeonia resources, resin-based purification has been reported as an effective approach to increase purity. For example, monoterpene glycosides from Paeonia suffruticosa seed meal were successfully enriched using macroporous resin purification [8]. Subcritical water extraction combined with macroporous resin enrichment has also been applied to obtain total glucosides from Paeonia lactiflora Pall. 'Zhongjiang' [9]. In addition, large-scale extraction, enrichment and separation of paeoniflorin and albiflorin from oil tree peony seed cake further supports the feasibility of resin-based preparation of Paeonia-derived monoterpene glycoside fractions [10]. These findings provide a methodological basis for applying AB-8 resin to post-extraction purification of *Radix Paeoniae Alba* extracts.

Although AB-8 macroporous resin has been widely used to enrich plant-derived glycosides, its use as a post-extraction quality-improvement step for ionic liquid-assisted botanical extracts remains less clearly defined. In this study, a resin-based purification workflow was developed for conventional reflux extract and ionic liquid-assisted microwave-ultrasound extract of *Radix Paeoniae Alba*. Five macroporous resins were screened, and AB-8 resin was further optimized. The resulting purified fractions were evaluated not only by their total quantified monoterpene glycoside content, but also by a selected ionic-liquid-associated elemental marker, stability profiles, antioxidant activity and anti-inflammatory potential in LPS-stimulated RAW264.7 cells. This integrated evaluation was intended to determine whether resin purification could simultaneously enrich marker glycosides, reduce the P-marker-level and provide purified fractions more suitable for downstream stability and bioactivity assays.

2. Materials and Methods

2.1. Materials, Reagents and Instruments

Dried roots of Paeonia lactiflora Pall. (*Radix Paeoniae Alba*) were purchased from Songyaotang medicinal materials supplier (Bozhou, China) and authenticated by Prof. Xiaoming Peng (Beijing Institute of Petrochemical Technology, Beijing, China). The material was pulverized and passed through a 45-mesh sieve before extraction, and the powder

passing through the sieve was used for all experiments. 1-Propyl-3-methylimidazolium dihydrogen phosphate ($[\text{C}_3\text{mim}][\text{H}_2\text{PO}_4]$) was used as the ionic liquid in the ionic liquid-assisted extraction route and was prepared in 70% ethanol-water solution at 0.12 mol/L. D101, S-8 and AB-8 macroporous resins were obtained from Rhawn Reagent Co., Ltd. (Shanghai, China). HPD100B and H103 resins were obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Sodium hydroxide was purchased from Beijing Chemical Works (Beijing, China). Ferric chloride, sodium sulfite, sucrose, ethanol, acetonitrile, phosphoric acid and hydrochloric acid were of analytical or chromatographic grade, as required.

Reference standards of paeoniflorin, oxypaeoniflorin and albiflorin were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China), with HPLC purity $\geq 98\%$. Stock solutions were prepared in methanol. Briefly, 5.10 mg of paeoniflorin, 5.02 mg of oxypaeoniflorin and 5.17 mg of albiflorin were accurately weighed, dissolved in methanol and diluted to volume. Equal volumes of the three standard solutions were mixed to obtain a mixed standard stock solution containing paeoniflorin, oxypaeoniflorin and albiflorin at concentrations of 0.255, 0.251 and 0.259 mg/mL, respectively.

DPPH, ABTS, potassium persulfate, trichloroacetic acid, ferric chloride, acetylacetone, p-dimethylaminobenzaldehyde, hyaluronidase, sodium hyaluronate, calcium chloride, sodium hydroxide, ascorbic acid, lipopolysaccharide, CCK-8 reagent, NO assay kit, PBS, DMEM medium, fetal bovine serum, penicillin–streptomycin and other cell culture reagents were used for antioxidant and anti-inflammatory assays.

The main instruments used in the purification experiments included an SL-100 shaker (Yongkang Songqing Hardware Factory, Yongkang, China), a PH818 pH meter (SMART-SENSOR, Dongguan, China) and a W-T-600EAS peristaltic pump (Jieheng Technology Co., Ltd., China). HPLC analysis was performed using a Waters e2695 HPLC system (Waters Corporation, Milford, MA, USA) equipped with an InertSustain C18 column (4.6×250 mm, $5 \mu\text{m}$; GL Sciences Inc., Tokyo, Japan). Ionic-liquid-associated elemental marker analysis was performed by inductively coupled plasma atomic emission spectroscopy using an iCAP 7400 Radial ICP-OES instrument equipped with a CID detector and a high-salt nebulizer (Thermo Fisher Scientific, Waltham, MA, USA). Antioxidant, hyaluronidase inhibition, CCK-8 and NO assays were measured using a Synergy LX microplate reader, model SLXFATS (BioTek Instruments, Inc., Winooski, VT, USA). Cell experiments were performed using a 3111 CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA), a YT-CJ-1ND clean bench (Beijing Yatai Kelong Experimental Technology Development Center, Beijing, China), an IX71 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan), a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), an MX-S vortex mixer (DLAB Scientific Co., Ltd., Beijing, China), a 3K15 refrigerated centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), a TC20 automated cell counter (Bio-Rad Laboratories, Hercules, CA, USA) and a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA).

2.2. Preparation of Crude and Purified Extracts

Four types of *Radix Paeoniae Alba* extracts were prepared and used in this study: conventional crude extract (CCE), conventional purified extract (CPE), ionic liquid-assisted crude extract (ICE) and ionic liquid-assisted purified extract (IPE). The abbreviations are summarized in Table 1.

As shown in Table 1, the samples were divided into crude and purified fractions from conventional and ionic liquid-assisted extraction routes. These abbreviations were used consistently throughout the manuscript to clarify comparisons between the extraction and purification processes.

Table 1. Sample abbreviations used in this study.

Abbreviation	Full Name	Description
CCE	Conventional crude extract	Reflux extraction with 70% ethanol
CPE	Conventional purified extract	CCE purified using AB-8 resin
ICE	Ionic liquid-assisted crude extract	Ionic liquid-assisted microwave–ultrasound extract
IPE	Ionic liquid-assisted purified extract	ICE purified using AB-8 resin
PF	Paeoniflorin	Reference compound
Vc	Ascorbic acid	Positive antioxidant control

For the conventional crude extract, *Radix Paeoniae Alba* powder was passed through a 45-mesh sieve. A total of 100 g of powder was extracted with 70% ethanol at a solid-to-liquid ratio of 1:26 g/mL by reflux extraction for 60 min. The extract solution was collected, concentrated and freeze-dried.

Both crude extracts were prepared using a single extraction cycle. Repeated extraction was not applied in the present purification study, because the objective was to obtain reproducible crude extract feed solutions for AB-8 resin purification rather than to establish an exhaustive isolation process for the individual glycosides. If exhaustive recovery is required for another application, repeated extraction should be separately validated.

For purified extracts, the CCE and ICE powders were separately redissolved in 70% ethanol to prepare the resin-loading solutions at 13 mg/mL. Thus, the same ethanol–water system was used for sample dissolution and later elution; during loading, 70% ethanol was used to maintain extract dissolution, while its elution suitability was confirmed in the ethanol-concentration optimization experiment. The loading pH was adjusted to 7 using diluted HCl or NaOH and confirmed with a pH meter before column loading. The optimized purification process was as follows: loading concentration, 13 mg/mL; loading pH, 7; loading volume, 6 BV; washing with 2 BV water to remove impurities; elution with 70% ethanol; and elution collection volume, 9 BV. The collected eluent was concentrated under reduced pressure and freeze-dried to obtain CPE and IPE.

2.3. HPLC Determination and Method Validation of Monoterpene Glycosides

Paeoniflorin, oxypaeoniflorin and albiflorin were quantified using an established HPLC method. In this manuscript, the comparative purification index is the total quantified monoterpene glycoside content, defined as the sum of these three marker compounds. The individual analytes were used for HPLC quantification and calculation of the summed value, whereas the Results Section focuses on the total marker content because the objective was to evaluate extract-level enrichment rather than the separation behavior of each individual glycoside.

The chromatographic conditions were as follows: InertSustain C18 column, 4.6 × 250 mm, 5 µm; flow rate, 1.0 mL/min; mobile phase A, acetonitrile; and mobile phase B, 0.05% phosphoric acid in water. The gradient program was 0–15 min, 14% A; 15–16 min, 14–17% A; 16–20 min, 17% A; and 20–25 min, 14% A. Oxypaeoniflorin was detected at 258 nm, while albiflorin and paeoniflorin were detected at 230 nm. The injection volume was 10 µL.

The mixed standard stock solution was filtered through a 0.45 µm membrane before HPLC analysis. The source, purity and working concentrations of the reference standards are also summarized in Supplementary Table S10.

To support the reliability of quantitative comparison in this purification study, the HPLC method was verified in terms of system suitability, linearity, precision, instrumental repeatability, limits of detection, limits of quantification and recovery. The verification re-

sults are summarized in Tables S1–S5. These verification results supported the quantitative comparison of the three marker compounds in the purification experiments.

2.4. Pretreatment, Screening and Optimization of Macroporous Resins

AB-8, HPD100B, D101, H103 and S-8 macroporous resins were pretreated before use. The resins were washed with deionized water to remove floating impurities and then soaked in 95% ethanol with continuous stirring to remove air bubbles and residual impurities. After sufficient soaking, the resins were washed with deionized water until no ethanol odor remained and were stored in water before use.

The technical parameters of the five resins are shown in Table 2.

Table 2. Technical parameters of the tested macroporous resins.

Resin	Polarity	Matrix	Average Pore Diameter
AB-8	Weakly polar	Styrene type	130–140 Å
HPD100B	Non-polar	Styrene type	90–100 Å
D101	Non-polar	Styrene type	500–550 Å
H103	Non-polar	Styrene type	8.4–9.4 Å
S-8	Polar	Styrene type	28–30 Å

The polarity and pore characteristics listed in Table 2 were used as the basis for comparing resin adsorption behavior. For resin screening, 4.0 g of each pretreated resin was accurately weighed into a 50 mL conical flask. Then, 50 mL of crude extract solution with an initial concentration of 10 mg/mL was added. The mixture was shaken at room temperature to reach adsorption equilibrium. The samples were filtered through a 0.45 µm membrane and analyzed by HPLC.

For adsorption kinetic analysis, 4.0 g of each resin was mixed with 50 mL of 10 mg/mL crude extract solution and shaken at 300 rpm. Samples were collected at 10, 20, 35, 75, 135, 255 and 435 min and analyzed by HPLC. For desorption kinetic analysis, the saturated resin was mixed with 50 mL of 60% ethanol and shaken at 300 rpm. Samples were collected at the same time points and analyzed by HPLC.

The adsorption capacity and adsorption rate were calculated as follows:

$$Q_e = ((C_0 - C_e) \times V)/W \quad (1)$$

$$E_1 = ((C_0 - C_e)/C_0) \times 100\% \quad (2)$$

where Q_e is the adsorption capacity, C_0 is the initial concentration, C_e is the equilibrium concentration, (V) is the sample solution volume, (W) is the resin weight and E_1 is the adsorption rate.

The desorption rate was calculated as follows:

$$E_0 = (C_a \times V_1)/((C_0 - C_a) \times V_2) \quad (3)$$

where E_0 is the desorption rate, C_a is the concentration in the desorption solution, V_1 is the desorption solution volume and V_2 is the adsorption solution volume.

Based on resin screening, AB-8 resin was selected for further optimization. The loading concentrations were 1.25, 2.5, 5, 7, 10, 13 and 15 mg/mL. The pH values of the loading solutions were adjusted to 1, 3, 5, 6, 7, 9, 10 and 11. Ethanol solutions of 40%, 50%, 60%, 70%, 80% and 90% were used to investigate the desorption efficiency. For breakthrough curve analysis, AB-8 resin was packed into a 100 mL column, and the sample solution was loaded at a flow rate of 0.5 BV/h. For elution endpoint investigation, the resin column was

eluted at a flow rate of 2 BV/h. Based on the optimization results, the final purification process used 70% ethanol as the eluent and 9 BV as the elution volume.

2.5. Determination of Ionic-Liquid-Associated Elemental Marker Content

The ionic-liquid-associated elemental marker content in ICE and IPE was determined using an inductively coupled plasma-based elemental analysis method. Because the ionic liquid used in this study was 1-propyl-3-methylimidazolium dihydrogen phosphate, the monitored element was used as a selected elemental marker associated with the phosphate-containing ionic liquid-assisted extraction system. This ICP-based method was used to monitor the selected ionic-liquid-associated elemental marker rather than to directly quantify the intact ionic liquid molecule. Therefore, the result should be interpreted as marker-level evidence of residue-associated reduction.

The samples were prepared at suitable concentrations before analysis. The instrumental parameters were as follows: RF power, 1.20 kW; plasma flow, 15.0 L/min; auxiliary flow, 1.50 L/min; nebulizer flow, 0.75 L/min; and sample uptake delay, 10 s. Blank samples and standard working solutions were analyzed after instrument stabilization to establish the working curve.

The ionic-liquid-associated elemental marker content was calculated using the following equation:

$$C_x = (C_0 \times f \times V_0 \times 10^{-3}) / (m \times 10^{-3}) \quad (4)$$

where C_x is the elemental marker content in the sample, C_0 is the measured elemental concentration, f is the dilution factor, V_0 is the sample volume and m is the sample mass.

2.6. Stability Evaluation

The stability of CPE, IPE and PF was evaluated under different environmental and formulation-related conditions. Sodium sulfite was used to evaluate the effect of reducing substances; ferric chloride was used to evaluate the effect of Fe^{3+} ; ascorbic acid and sucrose were used to evaluate the effect of additive-related components; light exposure and dark storage were compared to evaluate light stability; and heating at 20, 40, 60 and 80 °C was used to evaluate short-term thermal stability. Samples were analyzed by HPLC after treatment, and the detected content of monoterpene glycosides or paeoniflorin was recorded. This evaluation was designed to provide preliminary handling, storage and compatibility information for the purified fractions rather than to identify degradation products or establish a complete stability mechanism.

2.7. Antioxidant Assays

The DPPH radical scavenging activity was determined using a modified microplate method with reference to the free radical method described by Brand-Williams et al. [11]. DPPH and the four extract samples were dissolved in methanol. A 1 mM DPPH solution and 2 mg/mL sample solutions were prepared, and the sample solutions were serially diluted to 0.125 mg/mL. In a 96-well plate, 40 μL of sample solution was mixed with 120 μL of DPPH solution. The blank group contained methanol and DPPH solution, and the sample control contained sample solution and methanol. After reaction in the dark at room temperature for 30 min, absorbance was measured at 540 nm according to the final experimental protocol. The scavenging rate was calculated as follows:

$$\text{Scavenging rate(\%)} = (1 - (A_{\text{sample}} - A_{\text{control}})) / A_{\text{blank}} \times 100 \quad (5)$$

The ABTS radical scavenging activity was determined using a modified microplate assay based on the ABTS radical cation decolorization method [12]. ABTS solution at

3.75 mg/mL and potassium persulfate solution at 0.67 mg/mL were mixed at a ratio of 1:1 and diluted 35-fold with PBS to prepare the ABTS working solution. In a 96-well plate, 200 µL of ABTS working solution was mixed with 10 µL of sample solution. The absorbance was measured at 540 nm according to the final experimental protocol. The scavenging rate of samples was calculated as (5).

The reducing power assay was performed using the ferric reducing method. Extracts and Vc were prepared at 0.0625, 0.125, 0.25, 0.5, 1 and 2 mg/mL. The sample solution was mixed with PBS and potassium ferricyanide and incubated at 50 °C for 20 min. After cooling, 10% trichloroacetic acid was added, and the mixture was centrifuged. The supernatant was mixed with ethanol and ferric chloride solution in a 96-well plate. After shaking for 20 s, absorbance was measured at 700 nm.

2.8. Hyaluronidase Inhibition Assay

Hyaluronidase inhibitory activity was evaluated using a modified Morgan–Elson colorimetric method [13]. PF, CCE, CPE, ICE and IPE were prepared at 1.25, 2.5, 5 and 10 mg/mL. The reaction system contained sample solution, hyaluronidase, calcium chloride and sodium hyaluronate. After incubation, acetylacetone solution, sodium hydroxide solution and p-dimethylaminobenzaldehyde color reagent were added according to the established procedure. Absorbance was measured at 530 nm.

The inhibition rate was calculated as follows:

$$\text{Inhibition rate} = ((C - D) - (A - B)) / (C - D) \times 100\% \quad (6)$$

where A represents the complete reaction system containing sample and enzyme, B represents the sample background control, C represents the enzyme control without sample and D represents the reagent blank.

2.9. Cell Culture, CCK-8 Assay and NO Assay

RAW264.7 murine macrophages were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

For the CCK-8 assay, RAW264.7 cells were seeded into 96-well plates at 1.6×10^4 cells per well in 160 µL of medium. After the cell density reached 70–80%, the cells were treated with LPS at 1 µg/mL, ibuprofen at 0.05 mg/mL and extract samples at 10, 25, 50, 75, 100, 150 and 200 µg/mL. After 24 h of incubation, 50 µL of supernatant was collected for NO analysis. Then, 10 µL of CCK-8 reagent was added to each well, and the plate was incubated for 2 h. Absorbance was measured at 450 nm. Cell viability was calculated according to the absorbance values of the treatment, control and blank wells following the CCK-8 kit instructions and the final experimental data-processing protocol.

NO production was determined using the Griess method [14]. Sodium nitrite standards were prepared at 1.25, 2.5, 5, 10, 20, 40, 80 and 160 µmol/L. Cell culture supernatants were mixed with Griess reagent I and Griess reagent II, reacted in the dark and measured at 540 nm. NO concentration was calculated from the sodium nitrite standard curve.

2.10. RNA Extraction, cDNA Synthesis and qRT-PCR

RAW264.7 cells were seeded into 6-well plates and treated with CPE and IPE at 50, 75 and 100 µg/mL in the presence of LPS according to the final experimental design. The total RNA was extracted from treated cells, and cDNA was synthesized according to the manufacturer's instructions. qRT-PCR was performed to determine the mRNA expression levels of IL-6, IL-1β, TNF-α, iNOS and COX-2, with GAPDH used as the internal reference gene. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$

method [15,16]. This analysis was used to evaluate anti-inflammatory potential at the gene-expression level. It was not designed to provide protein-level validation or pathway-specific mechanistic confirmation.

The primer sequences are listed in Table 3.

Table 3. Primer sequences used for qRT-PCR analysis.

Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')
GAPDH	GTCATTGAGAGCAATGCCAG	GTGTTCTACCCCAATGTG
IL-6	CTGACAATATGAATGTTGGG	TCCAAGAAACCATCTGGCTAGG
IL-1 β	GAGCCTGTGTTTCCTCCTTG	CAAGTGCAAGGCTATGACCA
TNF- α	GGGAGCAAAGGTTCAAGTAT	CCTGGCCTCTCTACCTTGT
iNOS	AAGCAGCTGGCCAATGAG	CCCCATAGGAAAAGACTGCA
COX-2	TCCTCACATCCCTGAGAACC	GTGCGCACTCTGTTGTGCT

2.11. Statistical Analysis

Data are expressed as mean $\pm$ standard deviation (SD). Unless otherwise specified, chemical assays were performed using three replicate measurements. For cell-based assays, each treatment group was analyzed using three replicate wells under the present experimental conditions, and qRT-PCR reactions were performed in technical triplicate. Statistical differences among groups were analyzed by one-way analysis of variance (ANOVA). Dunnett's multiple comparison test was used when treatment groups were compared with a single control group, such as the blank control or the LPS model group. Tukey's multiple comparison test was used when multiple groups were compared with each other. A value of $p < 0.05$ was considered statistically significant. For cell experiments, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ indicate significant differences versus the blank control group; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate significant differences versus the LPS model group. The cell-based results were interpreted as preliminary bioactivity evidence under the tested conditions. Statistical analysis was performed using GraphPad Prism 10.6.1 (GraphPad Software, Boston, MA, USA), and figure preparation was performed using OriginPro 2018 (OriginLab Corporation, Northampton, MA, USA) and GraphPad Prism 10.6.1.

3. Results and Discussion

3.1. Screening and Optimization of AB-8 Resin Purification

The overall post-extraction purification and evaluation workflow is shown in Figure 1. Conventional crude extract (CCE) and ionic liquid-assisted crude extract (ICE) were first prepared from *Radix Paeoniae Alba*. The two crude extracts were then subjected to macroporous resin purification to obtain conventional purified extract (CPE) and ionic liquid-assisted purified extract (IPE). After purification, the extracts were evaluated in terms of total quantified monoterpene glycoside content, ionic-liquid-associated elemental marker content, stability and bioactivity-related responses.

Figure 1 summarizes the experimental sequence from crude extract preparation to resin purification and subsequent quality evaluation. Conventional crude extract (CCE) and ionic liquid-assisted crude extract (ICE) were purified using AB-8 resin to obtain CPE and IPE, respectively. The crude and purified fractions were then compared for total quantified monoterpene glycoside content, selected ionic-liquid-associated elemental-marker content, stability and bioactivity-related responses.

The screening and optimization results of macroporous resin purification are shown in Figure 2. Five macroporous resins, namely AB-8, HPD100B, D101, H103 and S-8, were compared. The adsorption performance differed among the tested resins, and AB-8 showed

the best overall adsorption performance under the experimental conditions. AB-8 is a weakly polar styrene-type macroporous resin with an average pore diameter of 130–140 Å. Its adsorption behavior may be related to a suitable balance of weak polarity, hydrophobic interaction and pore accessibility for the representative monoterpene glycosides. In comparison, the non-polar resins and the polar S-8 resin showed lower overall suitability under the tested conditions, which may be associated with differences in polarity matching, pore size and mass-transfer behavior.

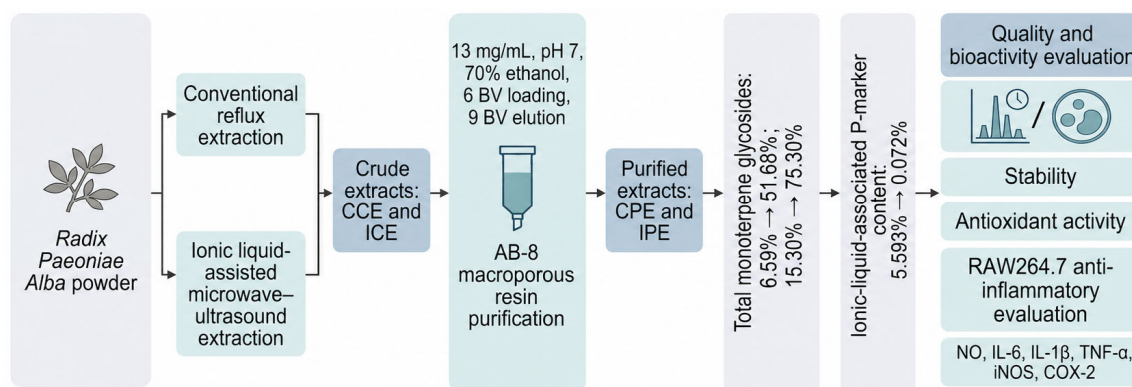


Figure 1. Overall workflow for purification, ionic-liquid-associated elemental marker reduction, stability evaluation and bioactivity assessment of monoterpene glycoside-enriched extracts from *Radix Paeoniae Alba*. Colors are used only to distinguish different workflow modules and do not indicate quantitative categories.

Although AB-8 showed the highest adsorption performance among the tested resins, the adsorption rate was not extremely high. This may be partly related to the use of a hydroethanolic loading solution. The crude extract showed limited solubility in pure water, and ethanol was required to maintain sample dissolution. However, ethanol can also weaken adsorption because it acts as an eluting solvent for relatively polar natural products. Thus, AB-8 was selected as the most suitable resin under the hydroethanolic loading conditions required for maintaining extract solubility.

The loading concentration affected the adsorption capacity of AB-8 resin. The adsorption capacity increased as the loading concentration increased and reached the highest level at 13 mg/mL. When the loading concentration was too low, the resin adsorption sites may not have been fully utilized. When the concentration was further increased to 15 mg/mL, decreased solubility and increased viscosity may have hindered mass transfer and resin adsorption. Therefore, 13 mg/mL was selected as the loading concentration for subsequent purification.

The effect of loading pH suggested that neutral conditions were more favorable for the purification of monoterpene glycosides. The adsorption capacity gradually increased as the pH approached neutrality and reached its maximum at pH 7. Strongly acidic or alkaline conditions may affect the stability, ionization state or HPLC-detectable content of extract components and were therefore unfavorable for purification. Thus, pH 7 was selected for subsequent experiments.

The ethanol concentration affected the desorption efficiency. The content of monoterpene glycosides in the eluent first increased and then decreased with the increasing ethanol concentration, and 70% ethanol provided the most suitable elution strength. Lower ethanol concentrations may be insufficient to disrupt analyte–resin interactions, whereas excessively high ethanol concentrations may not favor the recovery of relatively polar glycosides. Therefore, 70% ethanol was selected as the eluent.

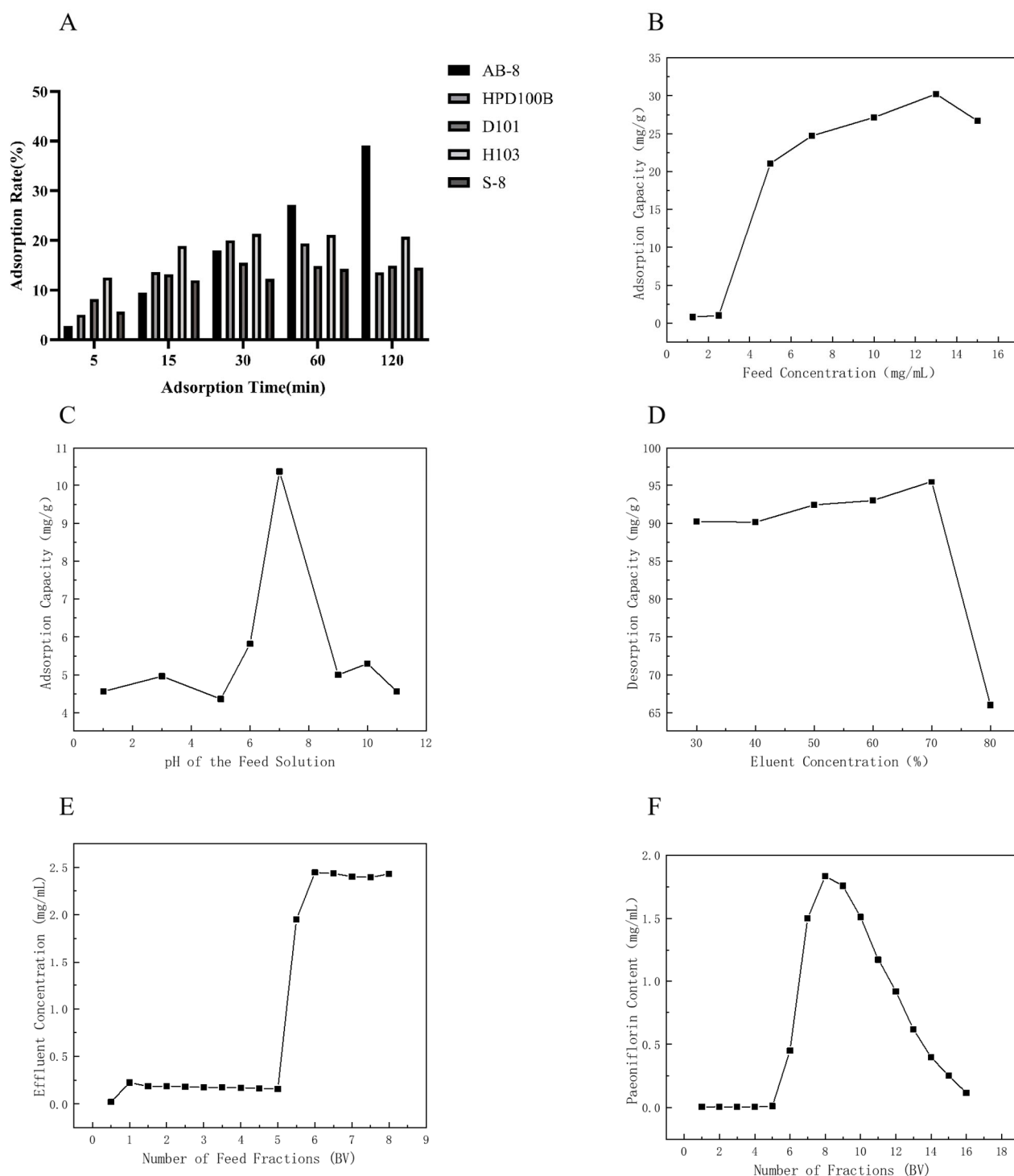


Figure 2. Screening and optimization of AB-8 resin purification for monoterpene glycosides from *Radix Paeoniae Alba*. (A) Adsorption rates of five macroporous resins; (B) effect of loading concentration; (C) effect of loading pH; (D) effect of ethanol concentration; (E) breakthrough curve; (F) elution profile.

Breakthrough and elution profiles were further investigated to determine practical column operation parameters. When the loading volume reached 5 BV, the effluent concentration increased rapidly, indicating that the resin was approaching saturation. At 6 BV, the effluent concentration approached the loading concentration, suggesting adsorption saturation; therefore, 6 BV was selected as the loading volume. During elution, the highest monoterpene glycoside content was observed around 9 BV, and the elution peak was rela-

tively sharp without obvious tailing under the selected flow rate. Thus, 9 BV was selected as the elution volume.

Based on the above optimization, the AB-8 resin purification conditions were set as follows: loading concentration, 13 mg/mL; loading pH, 7; elution with 70% ethanol; loading volume, 6 BV; and elution volume, 9 BV. These parameters were used for the preparation of CPE and IPE in the following experiments.

3.2. Enrichment of Monoterpene Glycosides After AB-8 Resin Purification

The optimized AB-8 resin process was applied to both conventional and ionic liquid-assisted crude extracts. The changes in the total quantified monoterpene glycoside content are shown in Figure 3. The total quantified monoterpene glycoside content was calculated as the sum of paeoniflorin, oxypaeoniflorin and albiflorin. Individual analyte concentrations were used to calculate this sum but were not used as separate response endpoints in Figure 3, because the purpose was to compare extract-level enrichment before and after purification.

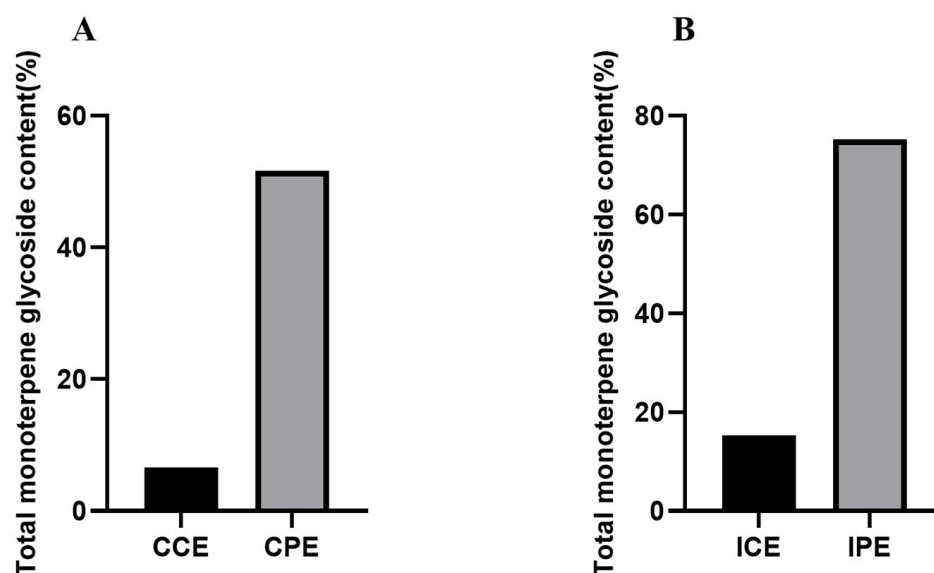


Figure 3. Improvement of total quantified monoterpene glycoside content after AB-8 resin purification. (A) CCE and CPE and (B) ICE and IPE.

After AB-8 resin purification, both crude extracts showed marked increases in total quantified monoterpene glycoside content. In the conventional extraction group, the content increased from 6.59% in CCE to 51.68% in CPE, corresponding to an approximately 7.84-fold increase. In the ionic liquid-assisted extraction group, the content increased from 15.30% in ICE to 75.30% in IPE, corresponding to an approximately 4.92-fold increase. These changes support the enrichment function of the AB-8 resin process for monoterpene glycosides in both extract systems.

The enrichment fold of the conventional extract was higher because of its lower initial marker content. However, IPE showed the highest final total quantified monoterpene glycoside content among the tested fractions. This result suggests that ionic liquid-assisted extraction provided a crude extract with higher initial marker abundance, while AB-8 resin purification further concentrated the quantified monoterpene glycosides. Therefore, the combination of ionic liquid-assisted extraction and AB-8 resin purification may provide a practical route for preparing monoterpene glycoside-enriched fractions from *Radix Paeoniae Alba*.

These results demonstrate that, beyond conventional enrichment, AB-8 resin can serve as a post-extraction quality-improvement step for ionic liquid-assisted botanical extracts. In this context, purification performance should be evaluated together with P-marker-level reduction, stability and bioactivity-related responses, rather than by enrichment fold alone.

3.3. Reduction in Ionic-Liquid-Associated Elemental Marker Content

The ionic-liquid-associated elemental marker content in ICE and IPE was evaluated by ICP-based elemental analysis. The results are shown in Table 4. Because the ionic liquid used in the extraction process was 1-propyl-3-methylimidazolium dihydrogen phosphate, the selected elemental marker was used to monitor residue-associated changes related to the phosphate-containing ionic liquid-assisted extraction system. This analysis was not intended to directly quantify the intact ionic liquid molecule.

Table 4. Ionic-liquid-associated P-marker content in ionic liquid-assisted crude and purified extracts.

Sample	P Concentration C0 (mg/L)	P-Marker Cx (mg/kg)	Ionic-Liquid-Associated P-Marker Content W (%)
ICE	431.20	55,927.37	5.593
IPE	1.38	717.19	0.072

3.4. Stability Evaluation of Purified Extracts and Paeoniflorin

The stability of CPE, IPE and paeoniflorin (PF) was evaluated under different environmental and formulation-related conditions, including a reducing agent, metal ion, additive-related components, light exposure and short-term heating. The summarized results are shown in Figure 4, and the complete stability profiles are provided in Figure S1.

When the Na₂SO₃ concentration was 0.00–0.04%, the total quantified monoterpene glycoside content in CPE and IPE showed no obvious change. When the Na₂SO₃ concentration reached 0.05%, a slight decreasing trend was observed in purified extracts, and a more obvious decrease was observed in PF. This result suggests that high concentrations of reducing agents should be avoided during storage or formulation development.

With an increasing Fe³⁺ concentration, the detected monoterpene glycoside content in CPE, IPE and PF decreased to different extents. Although degradation products were not identified in the present study, the decrease in detected content indicates that Fe³⁺ may negatively affect the compound stability or HPLC-detectable response. Possible explanations include metal ion-associated complexation, oxidation-related changes or matrix effects. Because no structural characterization of degradation products was performed, these explanations should be regarded as cautious interpretations rather than confirmed degradation pathways.

Ascorbic acid and sucrose also affected the detected contents of monoterpene glycosides. The influence of ascorbic acid was more evident, whereas sucrose produced a milder effect. These results suggest that compatibility with additive-related components should be considered when purified extracts are further developed into product forms. The observed changes may reflect chemical instability, changes in the matrix environment or analytical response variation under the tested conditions.

Light exposure produced a greater decrease in detected monoterpene glycoside content than dark storage. CPE and IPE showed decreases under light exposure, whereas changes under dark storage were relatively small. PF was generally more stable than the extract fractions, although the detected content under dark conditions was still slightly higher than that under light exposure. These results support storage protected from prolonged light exposure.

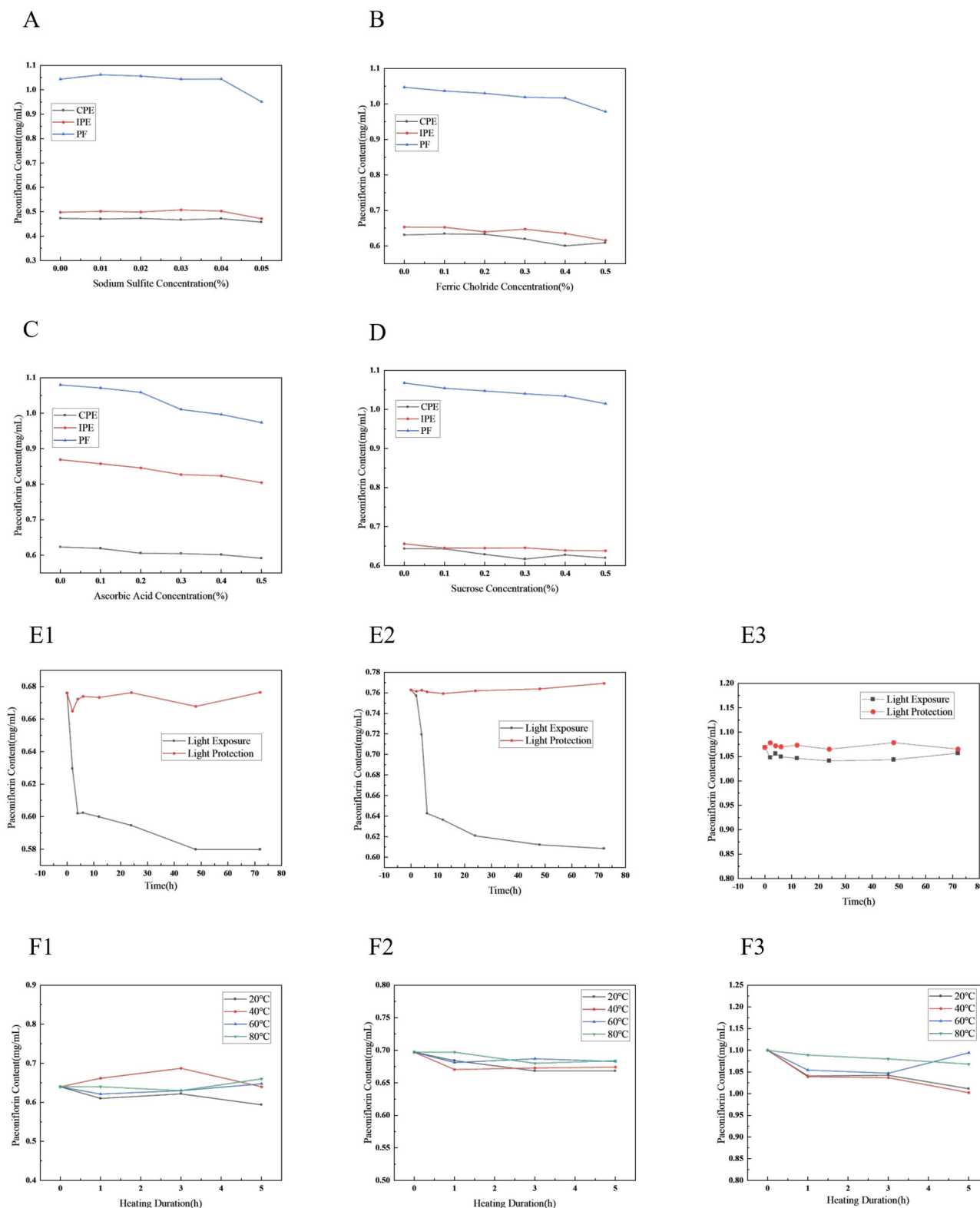


Figure 4. Stability evaluation of purified extracts and paeoniflorin under different environmental and formulation-related conditions. (A) Na_2SO_3 ; (B) Fe^{3+} ; (C) ascorbic acid; (D) sucrose; (E1–E3) light exposure and light-protection profiles of CPE, IPE and PF, respectively; (F1–F3) short-term heating profiles of CPE, IPE and PF, respectively. Different symbols and line styles correspond to the sample or condition shown in each panel.

For thermal stability, CPE, IPE and PF showed only slight fluctuations within 20–80 °C under the tested short-term heating conditions. The maximum change was approximately

0.1 mg/mL. Therefore, short-term heating within this temperature range had limited influence on the detected monoterpene glycoside content. Overall, the purified extracts should be stored at room temperature, protected from light and kept away from Fe^{3+} and incompatible additive-related components.

These stability tests provide preliminary formulation- and handling-related information for the purified fractions. Because degradation products were not characterized, further forced-degradation studies are needed to clarify the degradation pathways.

3.5. Antioxidant Activities of Crude and Purified Extracts

The antioxidant activities of CCE, CPE, ICE and IPE were evaluated using DPPH radical scavenging, ABTS radical scavenging and reducing power assays. Vc was used as the positive control. The results are shown in Figure 5.

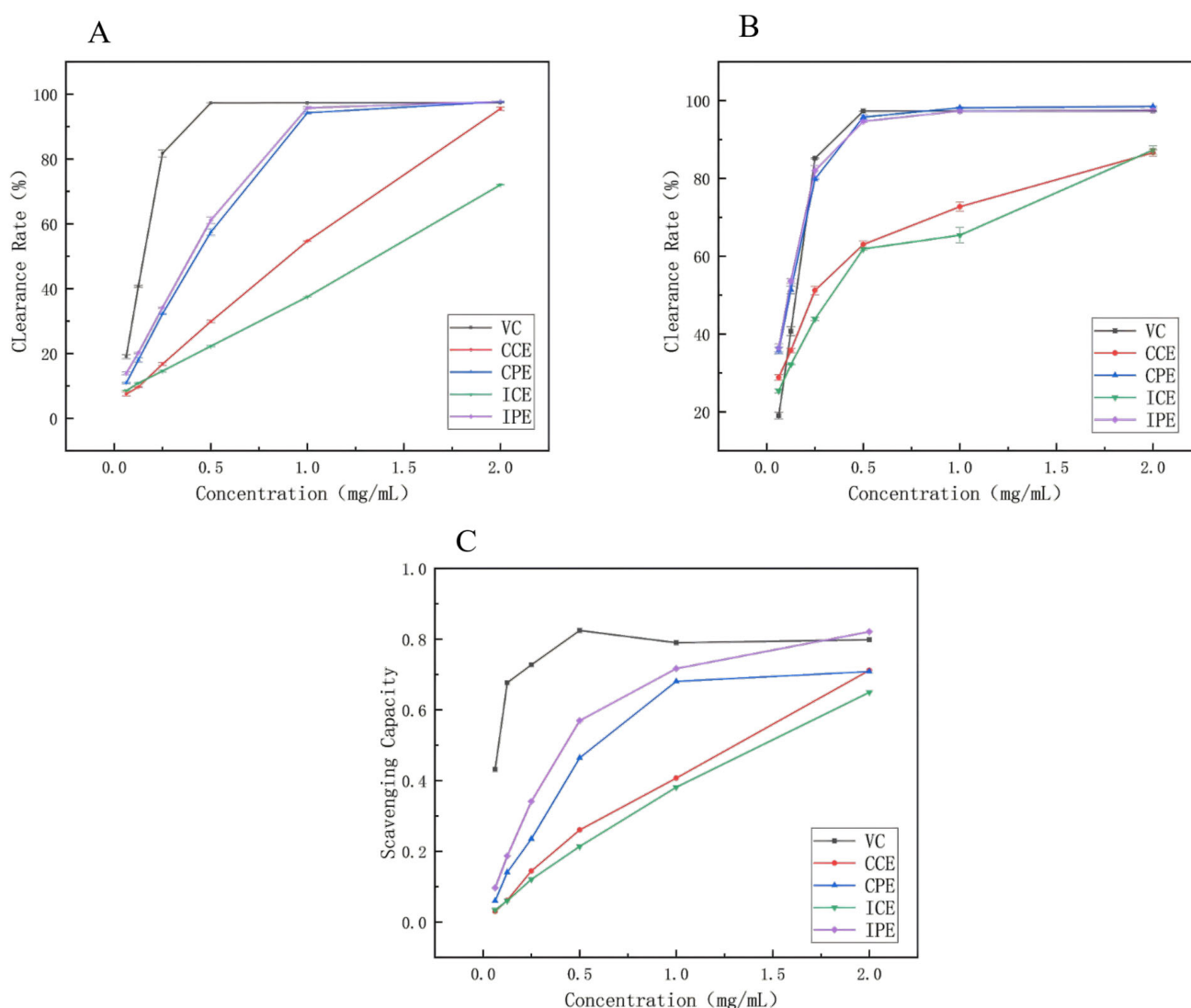


Figure 5. Antioxidant activities of crude and purified extracts from *Radix Paeoniae Alba*. (A) DPPH radical scavenging activity; (B) ABTS radical scavenging activity; (C) reducing power.

All four extracts showed concentration-dependent antioxidant responses in the tested concentration range. In the DPPH assay, the radical scavenging rates of the crude extracts increased with increasing concentration, whereas the purified extracts reached a high scavenging level at relatively lower concentrations and then tended to plateau. Overall, the purified extracts showed stronger DPPH radical scavenging activity than the corresponding

crude extracts. At 1 mg/mL, the DPPH radical scavenging activities of CPE and IPE approached that of Vc, with scavenging rates of approximately 97% according to the experimental results.

In the ABTS assay, CPE and IPE also showed stronger radical scavenging capacity than the crude extracts. The curves of CPE and IPE were close to that of Vc within the tested concentration range. At 0.0625 mg/mL, the scavenging rates of the two purified extracts were approximately 38%, and at 0.5 mg/mL, the scavenging rates approached 100%. The two crude extracts showed weaker ABTS radical scavenging activity, and the overall order described by the experimental results was Vc > IPE > CPE > CCE > ICE.

The reducing power assay showed a similar trend. The reducing power of the purified extracts was higher than that of the crude extracts. At 1 mg/mL, CPE and IPE showed reducing power close to that of Vc, whereas the crude extracts remained lower. These results indicate that AB-8 resin purification improved the antioxidant performance of *Radix Paeoniae Alba* extracts under the tested in vitro conditions.

The enhanced antioxidant response of the purified extracts may be related to the enrichment of quantified monoterpene glycosides and other co-enriched phenolic or antioxidant-related constituents during resin purification. However, the present study did not isolate individual antioxidant contributors or determine the antioxidant contribution of each compound. Therefore, the antioxidant results should be interpreted as extract-level activity rather than direct evidence that paeoniflorin, oxypaeoniflorin or albiflorin alone accounted for the observed activity.

3.6. Preliminary Anti-Inflammatory Screening, Cell Viability and NO Production

The anti-inflammatory potential of crude and purified extracts was first evaluated using a hyaluronidase inhibition assay as a preliminary screening method. Hyaluronidase inhibition has been used as an in vitro indicator related to inflammatory processes, but the assay can be affected by sample matrix, color interference and reaction-system variability. Therefore, in the present study, the hyaluronidase assay was used only as a preliminary screening assay and not as the main basis for ranking the anti-inflammatory activity of the different extracts.

The hyaluronidase inhibition results are provided in Figure S2. All tested samples, including CCE, CPE, ICE, IPE and PF, showed hyaluronidase inhibitory activity within the tested concentration range of 1.25–10 mg/mL. However, relatively high variability was observed in this assay system. Therefore, the hyaluronidase result was used as supportive preliminary evidence, while the subsequent RAW264.7 cell-based assays were used as the main in vitro evaluation of anti-inflammatory potential.

Before interpreting NO inhibition [17,18], the cytotoxicity of the samples was evaluated using the CCK-8 assay in LPS-stimulated RAW264.7 cells [19]. The summarized cell viability results are shown in Figure 6A, and the complete results are provided in Figure S3. RAW264.7 cells were treated with CCE, CPE, ICE, IPE and PF at concentrations of 10, 25, 50, 75, 100, 150 and 200 µg/mL in the presence of LPS.

The CCK-8 results showed that CCE, IPE and PF did not cause obvious cytotoxicity compared with the blank group within the tested concentration range of 10–200 µg/mL. In contrast, CPE showed decreased cell viability at higher concentrations, and ICE reduced cell viability to approximately 68.6% at 200 µg/mL. These results indicate that the apparent bioactivity of ICE at high concentrations should be interpreted cautiously, because reduced NO production at cytotoxic or near-cytotoxic concentrations may partly result from decreased cell viability rather than a specific anti-inflammatory response.

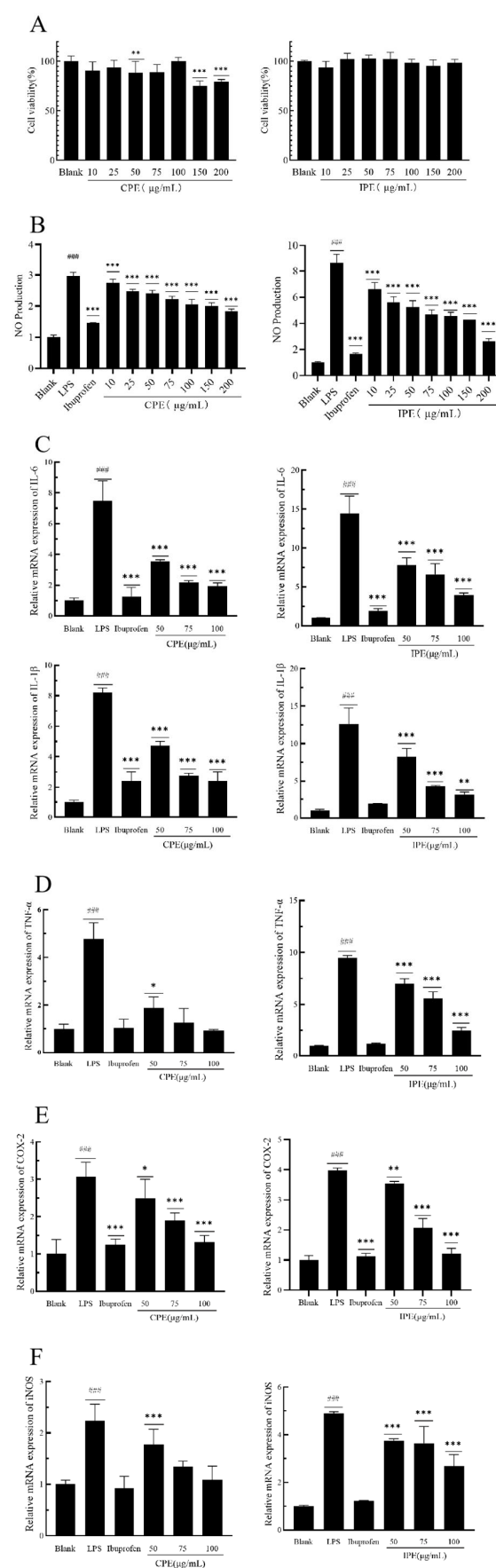


Figure 6. Cell-based evaluation of anti-inflammatory potential of crude and purified extracts in LPS-stimulated RAW264.7 cells. (A) Cell viability results from the CCK-8 assay; (B) NO production;

(C) mRNA expression of IL-6 and IL-1 β ; (D) mRNA expression of TNF- α ; (E) mRNA expression of COX-2; (F) mRNA expression of iNOS. CCE, conventional crude extract; CPE, conventional purified extract; ICE, ionic liquid-assisted crude extract; IPE, ionic liquid-assisted purified extract; PF, paeoniflorin. Data are expressed as mean $\pm$ SD. ### $p < 0.001$ versus the blank control group; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus the LPS model group.

Based on the CCK-8 results, concentrations of 50, 75 and 100 $\mu\text{g/mL}$ were selected for subsequent qRT-PCR analysis of purified extracts. This concentration range avoided high-dose cytotoxicity and was therefore more suitable for evaluating anti-inflammatory potential at the gene-expression level.

NO production was then measured using the Griess method. The summarized NO results are shown in Figure 6, and the NO standard curve and complete sample results are provided in Figure S4. The sodium nitrite standard curve was $y = 0.0053x + 0.0395$, with $R^2 = 0.9996$, indicating good linearity for NO quantification.

LPS stimulation significantly increased NO production compared with the blank control group, indicating successful establishment of the inflammatory cell model. Ibuprofen significantly reduced NO production, and the NO level was close to that of the blank control group. For the conventional extraction group, CPE showed stronger NO-inhibitory activity than CCE. At 100 $\mu\text{g/mL}$, the NO-inhibitory effect of CPE approached that of ibuprofen under the present experimental conditions. For the ionic liquid-assisted extraction group, both ICE and IPE reduced NO production. However, because ICE showed reduced cell viability at high concentration, its NO-inhibitory effect should be interpreted cautiously. IPE showed NO-inhibitory activity with better cell viability than ICE, suggesting that purification improved the suitability of the ionic liquid-assisted extract for subsequent anti-inflammatory evaluation.

Overall, the NO results suggest that AB-8 resin purification improved the in vitro anti-inflammatory evaluation profile of the extracts. However, NO production alone cannot establish a complete anti-inflammatory mechanism. Therefore, the NO assay was interpreted as evidence of anti-inflammatory potential at the inflammatory mediator level rather than as definitive mechanistic proof.

3.7. Regulation of Inflammatory Cytokine and Inflammatory Enzyme mRNA Expression

To further evaluate the anti-inflammatory potential of the purified extracts at the gene-expression level, qRT-PCR was used to determine the mRNA expression levels of IL-6, IL-1 β , TNF- α , COX-2 and iNOS in LPS-stimulated RAW264.7 cells. Based on the cell viability and NO production results, CPE and IPE were selected for qRT-PCR analysis at 50, 75 and 100 $\mu\text{g/mL}$. The summarized qRT-PCR results are shown in Figure 6C–F, and the complete qRT-PCR profiles are provided in Figure S5.

LPS stimulation markedly increased the mRNA expression levels of IL-6, IL-1 β , TNF- α , COX-2 and iNOS compared with the blank control group, further supporting successful establishment of the LPS-induced inflammatory response model. Treatment with ibuprofen and purified extracts reduced the expression of these inflammatory markers to different extents.

For the pro-inflammatory cytokines IL-6 and IL-1 β , both CPE and IPE showed significant downregulation, with $p < 0.001$ versus the LPS model group. For TNF- α , IPE showed a stronger inhibitory trend than CPE under the present experimental conditions. These results suggest that the purified extracts, especially IPE, were associated with reduced pro-inflammatory cytokine mRNA expression in LPS-stimulated RAW264.7 cells.

For the inflammatory enzymes, both CPE and IPE reduced the mRNA expression of COX-2 and iNOS. The decrease in iNOS expression was consistent with the NO inhibition results, because iNOS is closely related to NO production in activated macrophages. Within

the tested concentration range of 50–100 µg/mL, IPE showed a stronger inhibitory trend than CPE for inflammatory enzyme mRNA expression.

Taken together, the cell-based results indicate that AB-8 resin-purified fractions, particularly IPE, showed anti-inflammatory potential in LPS-stimulated RAW264.7 cells at the NO and mRNA-expression levels. These findings are consistent with the increased total quantified monoterpene glycoside content after purification and suggest that resin purification improved the suitability of the extracts for downstream bioactivity evaluation.

Together with the NO results, the qRT-PCR data suggest that CPE and IPE attenuated LPS-induced inflammatory responses at the mediator and mRNA-expression levels. Further protein-level and pathway-specific analyses are still needed to define the underlying mechanism.

4. Conclusions

In this study, an AB-8 macroporous resin-based post-extraction purification process was developed for monoterpene glycoside-enriched extracts from *Radix Paeoniae Alba* prepared by conventional reflux extraction and ionic liquid-assisted microwave-ultrasound extraction. Among the five tested resins, AB-8 showed the best overall adsorption performance under the experimental conditions. The optimized purification parameters were a loading concentration of 13 mg/mL, loading pH of 7, elution with 70% ethanol, loading volume of 6 BV and elution volume of 9 BV. Under these conditions, the total quantified monoterpene glycoside content increased from 6.59% to 51.68% in the conventional extract and from 15.30% to 75.30% in the ionic liquid-assisted extract.

In the ionic liquid-assisted group, the ionic-liquid-associated elemental marker content decreased from 5.593% to 0.072%, indicating a substantial reduction at the elemental-marker level. This finding supports the potential of AB-8 resin purification as a post-extraction quality-improvement step for ionic liquid-assisted botanical extracts. However, the ICP-based analysis used in this study measured an elemental marker and did not directly quantify the intact ionic liquid molecule. Therefore, direct molecular-level residue characterization by LC-MS, NMR or other targeted analytical methods would still be required to clarify the chemical forms and safety relevance of residual ionic liquid-derived components.

Stability evaluation indicated that Na₂SO₃, Fe³⁺, ascorbic acid, sucrose and light exposure affected the detected monoterpene glycoside content to different extents, whereas short-term heating within 20–80 °C caused only slight fluctuations. These results suggest that the purified extracts should be stored so that they are protected from light and away from potentially incompatible formulation-related components. Because degradation products were not identified, the stability results should be regarded as preliminary compatibility evidence based on HPLC-detectable marker contents.

The purified extracts showed improved antioxidant activity compared with the crude extracts and reduced NO production as well as the mRNA expression of IL-6, IL-1β, TNF-α, iNOS and COX-2 in LPS-stimulated RAW264.7 cells. These results suggest antioxidant activity and anti-inflammatory potential at the NO and gene-expression levels. However, the present cell-based assays did not include protein-level validation, pathway-specific assays or direct target identification. Therefore, the anti-inflammatory mechanism cannot be considered fully elucidated.

Overall, this work provides an integrated post-extraction evaluation workflow linking monoterpene glycoside enrichment, ionic-liquid-associated elemental marker reduction, stability evaluation and preliminary bioactivity assessment. These findings support the use of AB-8 resin as a practical post-extraction purification step for ionic liquid-assisted

Radix Paeoniae Alba extracts. Further studies should include direct molecular-level ionic liquid residue analysis, toxicological safety assessment, degradation-product identification, characterization of co-enriched active constituents and protein-level validation of inflammation-related pathways.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/separations13070189/s1>. Figure S1: Complete stability profiles of purified extracts and paeoniflorin under Na₂SO₃, Fe³⁺, ascorbic acid, sucrose, light exposure and heating conditions; Figure S2: Hyaluronidase inhibitory activity of CCE, CPE, ICE, IPE and PF; Figure S3: Complete CCK-8 cell viability results for crude extracts, purified extracts and paeoniflorin in RAW264.7 cells; Figure S4: NO standard curve and NO production results in LPS-stimulated RAW264.7 cells; Figure S5: Complete qRT-PCR results of IL-6, IL-1 β , TNF- α , iNOS and COX-2 expression in LPS-stimulated RAW264.7 cells; Table S1: System suitability of the HPLC method; Table S2: Linearity of the HPLC method; Table S3: Precision and instrumental repeatability of the HPLC method; Table S4: Limits of detection and quantification; Table S5: Recovery of the HPLC method; Table S6: HPLC conditions for the determination of paeoniflorin, oxypaeoniflorin and albiflorin; Table S7: Optimized AB-8 resin purification parameters for monoterpene glycosides from *Radix Paeoniae Alba*; Table S8: Experimental conditions for antioxidant assays; Table S9: Cell experiment grouping and treatment concentrations; Table S10: Reference standards and mixed standard stock solution used for HPLC analysis.

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Abbreviations

The following abbreviations are used in this manuscript:

CCE	Conventional crude extract
CPE	Conventional purified extract
ICE	Ionic liquid-assisted crude extract
IPE	Ionic liquid-assisted purified extract
PF	Paeoniflorin
Vc	Ascorbic acid

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