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Article

A Simple HPLC Method for Chlorogenic Acid Quantification in Jiruge-6 Suppositories

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Abstract: Chlorogenic acid (CGA) is a key bioactive polyphenol in Traditional Chinese Medicine (TCM) formulations including Jiruge-6 Suppositories. This study aimed to develop and validate a simple and sensitive HPLC method for quantifying CGA in these products. The optimized method utilized a C18 column with a mobile phase of acetonitrile and 0.1% phosphoric acid (15:85, v/v) at 1.0 mL/min flow rate, with detection at a 327 nm. Ultrasonic extraction with 70% methanol enhanced sample preparation efficiency. The method demonstrated excellent linearity (r^2 > 0.999) across the range of 1.0–100.0 μg/mL, high precision (RSD < 2.0%), and good recovery (98.2–101.5%). Detection and quantification limits were 0.30 μg/mL and 0.95 μg/mL, respectively. Analysis of five commercial batches from the same manufacturer revealed CGA content of 0.42–0.57 mg/suppository with notable seasonal variation. This validated method provides a practical approach for routine quality control of Jiruge-6 Suppositories, supporting standardization of TCM products.

Keywords: Chlorogenic Acid; Jiruge-6 Suppositories; HPLC; Method Validation; Traditional Chinese Medicine

1. Introduction

Chlorogenic acid (CGA) is a chemical polyphenol formed by the esterification of caffeic and quinic acid. It has received considerable attention for its biological activities and is now the research focus of many pharmacists. As one of the most abundant polyphenols found in food, CGA possesses potent pharmacological properties, including but not limited to antioxidative, anti-inflammatory, antibacterial, antiviral, and hepatoprotective activities. Recent studies have shown that CGA has the potential to improve glucose metabolism by inhibiting α–glucosidase activity and increasing glucose utilization in liver cells, which makes it a possible candidate for diabetes treatment [1–4]. In addition, CGA was found to be therapeutically beneficial in several pathological conditions, including the treatment of severe acute pancreatitis, where it inhibited the NLPR3 inflammasome activation via the Nrf2/HO–1 signaling pathway [5]. CGA has also demonstrated remarkable hepatoprotective activities against acetaminophen-induced liver injury by activating the AMPK/mTOR/ULK1 autophagy pathway. In addition, CGA was able to relieve UVB-induced damage via SIRT6 and thus has the potential to be used in dermatological medicine [6, 7]. For these reasons, CGA serves as an important bioactive marker compound for quality control of many TCM, such as Jiruge-6 Suppositories [8].

The determination of CGA in complex herbal matrices is particularly challenging due to the combination of traditional medicinal preparations [9, 10]. Numerous methods, ranging from high-performance liquid chromatography (HPLC) to more sophisticated spectroscopic techniques, are used for the quantification of CGA [11, 12]. Advances in analytical technologies have enabled the development of more sensitive, and specific techniques for CGA determination. For example, Ran et al. developed a UPLC-MS/MS method for the simultaneous determination of several bioactive compounds, including CGA, in Lonicerae japonicae flos, achieving remarkable sensitivity and reliability [13]. In the same way, Zhao et al. employed aggregation-induced emission nanoclusters to CGA within pharmaceutical injections to develop a quick and easy fluorescent detection technique [14]. Near-infrared spectroscopy has also been highlighted for its potential as a nondestructive technique for CGA quantification in natural products by Kusumiyati et al. [15]. Moreover, Ertekin et al. [16] employed three-dimensional spectrochromatographic analysis with parallel factor analysis for CGA determination in plant extracts, thereby expanding the scope of the analytical arsenal. Even with the progress made, the most common method used for the routine analysis of CGA in Traditional Chinese Medicine (TCM) formulations continues to be HPLC-UV, primarily due to the simplicity, accessibility, and low cost of this method [9, 17].

The harsh analytical method development and validation for the determination of CGA is critical in safeguarding the quality, safety, and efficacy of therapeutic Jiruge-6 Suppositories complex herbal preparations, including their consideration as TCMs [18]. The quality assurance processes for herbal medicines require the utilization of clearly defined analytical methods that have national and international guidelines as their framework [19]. Above all, the attention to exploring novel applications of CGA, such as in antimicrobial packaging films for food preservation or functionalized nanomaterials for cancer therapy, emphasises on the importance of having reliable analytical methods for CGA quantification [20]. The current study aims to develop and validate a sensitive, specific, and robust analytical HPLC method for CGA quantification in Jiruge-6 Suppositories, thereby aiding in the standardization of this TCM preparatory level. The research supports increasingly reliable therapeutic quality control systems intended for validation in medicine processes by establishing constant targets throughout the treatment process. The validated method helps automate the quality and authenticity evaluation of Jiruge-6 Suppositories by their automated producers and elders, thus enhancing patient trust in traditional medicines.

CGA, as a significant dietary polyphenol, has gained substantial attention for its diverse health benefits in both humans and animals. Human absorption studies have confirmed that CGA is bioavailable after oral consumption, with Olthof et al. demonstrating that approximately 33% of ingested CGA is absorbed in the small intestine of humans, while the remaining portion reaches the colon, where it is hydrolyzed by microbiota before absorption [21–25]. This bioavailability underlies CGA's systemic health effects across multiple physiological systems.

Recent research has expanded our understanding of CGA's potential therapeutic applications in mental health. Naveed et al. reported that CGA exhibits neuroprotective effects through multiple mechanisms, including antioxidant activity, inhibition of acetylcholinesterase, and reduction of amyloid–β aggregation, suggesting potential benefits for neurodegenerative conditions [26]. The compound's ability to cross the blood-brain barrier further supports its potential in treating central nervous system disorders.

Interestingly, the benefits of polyphenols, such as CGA, extend beyond human applications to veterinary and aquaculture settings. In a comprehensive review, Reverter et al. [27] highlighted how dietary polyphenols, including CGA, can enhance aquatic animal health by improving growth parameters, feed utilization, and disease resistance. This was further substantiated by Liu et al. [28], who demonstrated that dietary supplementation with CGA in conjunction with Lactobacillus helveticus significantly improved growth performance, digestive enzyme activities, and resistance against heat stress in juvenile common carp. The dual effects observed in this study suggest that CGA may serve as a promising feed additive in aquaculture, enhancing both production efficiency and animal welfare under challenging environmental conditions.

2. Materials and Methods

2.1. Materials and Reagents

Analytical grade CGA reference standard (purity ≥ 99.0%, CAS No. 327–97–9) was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). HPLC-grade methanol, acetonitrile, and phosphoric acid were purchased from Merck (Darmstadt, Germany). Analytical grade ethanol was supplied by Sinopharm Chemi-

cal Reagent Co., Ltd. (Shanghai, China). Ultrapure water (resistivity > 18.2 MΩ·cm at 25 °C) was prepared using a Milli-Q Advantage A10 water purification system (Millipore, Bedford, MA, USA) and used throughout the study. Commercial Jiruge-6 Suppositories from five different manufacturing batches (batch numbers: 20240301, 20240402, 20240505, 20240607, and 20240708) were all procured from Nei Mongol Jiruge Traditional Chinese Medicine Pharmaceutical Co., Ltd., located in Hohhot, Inner Mongolia Autonomous Region, China. The labeled composition of Jiruge-6 Suppositories indicated the presence of various herbal extracts, including Lonicera japonica Thunb. (honeysuckle), which is known to contain CGA as one of its major bioactive components. Chromatographic analysis was performed using an Agilent 1290 Infinity II UHPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary solvent delivery pump (G7104A), a high-performance autosampler (G7129B) with a 100 µL sample loop, a thermostatted column compartment (G7116B) with precise temperature control (± 0.05 °C), and a diode array detector (G7117B) with a 60 mm path length flow cell. Chromatographic separation was achieved using an Agilent ZORBAX Eclipse Plus C18 analytical column (250 mm × 4.6 mm, 5 µm particle size) protected by a compatible guard column (12.5 mm × 4.6 mm, 5 µm particle size). Sample preparation was facilitated using an SK5210LHC ultrasonic bath (Shanghai Kudos Ultrasonic Instrument Co., Ltd., China), a Vortex-Genie 2 vortex mixer (Scientific Industries, USA), a vacuum filtration system with 0.45 µm membrane filters (Millipore, USA), and an Allegra X-30R high-speed refrigerated centrifuge (Beckman Coulter, USA). Data acquisition and processing were conducted using OpenLAB CDS ChemStation software (version C.01.10, Agilent Technologies). Statistical analysis was performed using SPSS software (version 26.0, IBM Corp., Armonk, NY, USA).

2.2. Analytical Method Development

An effective analytical method for determining CGA in Jiruge-6 Suppositories was developed through the systematic optimisation of different chromatographic parameters to achieve appropriate separation, sensitivity, and reproducibility. Preliminary screening experiments were conducted to determine the appropriate chromatographic conditions based on reported methods for CGA analysis in similar matrices. Optimization procedures included mobile phase composition, flow rate, column temperature, detection wavelength, and sample preparation steps.

Multiple mobile phase systems were evaluated, including various combinations of acetonitrile, methanol, and water with different modifiers such as phosphoric acid, acetic acid, and formic acid at concentrations ranging from 0.05% to 0.2% (v/v). The optimization criterion was based on the resolution factor (R_s), which was calculated using Equation (1):

$$R_s = \frac{2(t_{R2} - t_{R1})}{W_1 + W_2} \tag{1}$$

where t_{R1} and t_{R2} are the retention times of adjacent peaks, and W_1 and W_2 are the baseline peak widths. A binary mobile phase system consisting of acetonitrile and 0.1% phosphoric acid (15:85, v/v) was found to provide the best chromatographic performance with $R_s > 2.0$ for all adjacent peaks. Flow rate optimization was performed between 0.6 and 1.2 mL/min, with 1.0 mL/min selected as the optimal value based on peak symmetry (asymmetry factor, A_s) and theoretical plate number (N). The asymmetry factor was calculated using Equation (2):

$$A_s = \frac{b}{a} \tag{2}$$

Where a is the front half-width and b is the back half-width of the peak at 10% of the peak height. The theoretical plate number was determined using Equation (3):

$$N = 5.54 \times \left(\frac{t_R}{W_{h/2}}\right)^2 \tag{3}$$

where $W_{h/2}$ is the peak width at half-height.

Column temperature was varied between 25 °C and 40 °C to evaluate its effect on peak resolution and column efficiency. A temperature of 30 °C was selected as optimal, providing a satisfactory compromise between resolution, peak shape, and analysis time. The UV absorption spectrum of CGA was recorded between 200 nm and 400 nm to determine the wavelength of maximum absorption.

According to the absorption profile, the optimal sensitivity and interference for CGA analysis were found at 327 nm. Thus, the detection wavelength is set to 327 nm.

Preparation of the samples was carried out to optimally extract CGA from the Jiruge-6 Suppositories. Different extraction solvents, including methanol, ethanol, acetonitrile, and their mixtures (30%–80%, v/v), were studied for use. The extraction efficiency (EE%) was calculated using Equation (4):

$$EE\% = \frac{C_{measured}}{C_{theoretical}} \times 100\% \quad (4)$$

where $C_{measured}$ is the ultrasound-assisted extraction concentration of CGA measured and $C_{theoretical}$ is the concentration in the sample. For better efficiency during the extraction process, ultrasonic-assisted extraction was conducted.

Due to the lipophilic nature of the suppository base, special attention was given to the extraction process. The suppositories were first melted at a controled temperature (40 °C) to release the active ingredients from the base matrix, followed by rapid cooling and dispersion in the extraction solvent. Various extraction parameters were systematically optimized, including time (10–60 min), temperature (25 °C – 60 °C), and solvent/sample ratio (10:1 to 50:1, v/w). The optimal extraction protocol employed a 70% (v/v) aqueous methanol solution with a 30:1 solvent-to-sample ratio (v/w), followed by sonication at 40°C for 30 minutes and centrifugation at 4000 rpm for 10 minutes. This methodology ensured the complete release of CGA from the complex matrix, providing superior extraction efficiency (> 95%) with excellent reproducibility (RSD < 2.0%).

The system's precision parameters, such as retention time, peak area, number of theoretical plates, tailing factor, and resolution, were determined to verify the accuracy and suitability of the chromatography system prior to sample analysis. The criteria set were: RSD of retention time ≤ 1.0%, RSD of peak area ≤ 2.0%, K > 2000, tailing factor ≤ 1.5, resolution R ≥ 2.0. The determination of the final chromatographic conditions resulted in a satisfactory and well-resolved peak of CGA at a retention time of approximately 8.5 minutes, while maintaining tailing factors below 1.2 confirming high column efficiency with a theoretical plate number of 5000, which aligns with the objectives of the work. This demonstrates that the method can be employed routinely to analyze CGA in Jiruge-6 Suppositories.

The system suitability parameters, including retention time, peak area, theoretical plate number, tailing factor, and resolution, were evaluated to ensure the reliability of the chromatographic system prior to sample analysis. The acceptance criteria were established as follows: RSD of retention time ≤ 1.0%, RSD of peak area ≤ 2.0%, theoretical plate number ≥ 2000, tailing factor ≤ 1.5, and resolution ≥ 2.0. The finalized chromatographic conditions provided a well-resolved peak for CGA with a retention time of approximately 8.5 minutes, satisfactory peak symmetry (tailing factor < 1.2), and high column efficiency (theoretical plate number > 5000), indicating the suitability of the method for routine analysis of CGA in Jiruge-6 Suppositories.

2.3. Method Validation

The analytical method for the determination of CGA in Jiruge-6 Suppositories was comprehensively validated according to the International Conference on Harmonization (ICH) guidelines Q2(R1) for analytical procedures. The validation protocol encompassed critical parameters including specificity, linearity, range, precision, accuracy, sensitivity, robustness, and solution stability to ensure the reliability and reproducibility of the developed method. Specificity, a fundamental validation parameter, was evaluated by comparing the chromatograms of blank solvent, standard solution, placebo suppository base, suppository sample solution, and suppository sample solution spiked with the standard. The absence of interfering peaks at the retention time of CGA confirmed the specificity of the method. Additionally, peak purity was assessed using the diode array detector by comparing the spectral homogeneity across the peak profile. The peak purity factor was calculated using the following Equation (5):

$$Purity\ factor = \frac{\sum_{i-1}^{n}\left(A_i + \overline{A}\right)\left(B_i + \overline{B}\right)}{\sqrt{\sum_{i-1}^{n}\left(A_i + \overline{A}\right)^2 \sum_{i-1}^{n}\left(B_i + \overline{B}\right)^2}} \quad (5)$$

where A_i and B_i represent the normalized spectral absorbances at different time points within the peak, and $\overline{A}$ and

$\overline{B}$ represent their respective mean values. A purity factor exceeding 0.995 indicated satisfactory peak homogeneity, confirming the absence of co-eluting impurities.

The linearity of the method was established by analyzing a series of standard solutions at eight concentration levels ranging from 1.0 to 100.0 µg/mL, with each concentration prepared and analyzed in triplicate. A calibration curve was constructed by plotting the peak area (y) versus the concentration of CGA (x) using the least squares regression method. The linear regression Equation (6) was derived as:

$$y = mx + c \tag{6}$$

where m represents the slope and c represents the y-intercept. The coefficient of determination (r^2), y-intercept, slope of the regression line, and residual sum of squares were calculated to evaluate the linearity. A typical calibration curve for CGA is illustrated in **Figure 1**, demonstrating excellent linearity with $r^2 > 0.999$ throughout the concentration range.

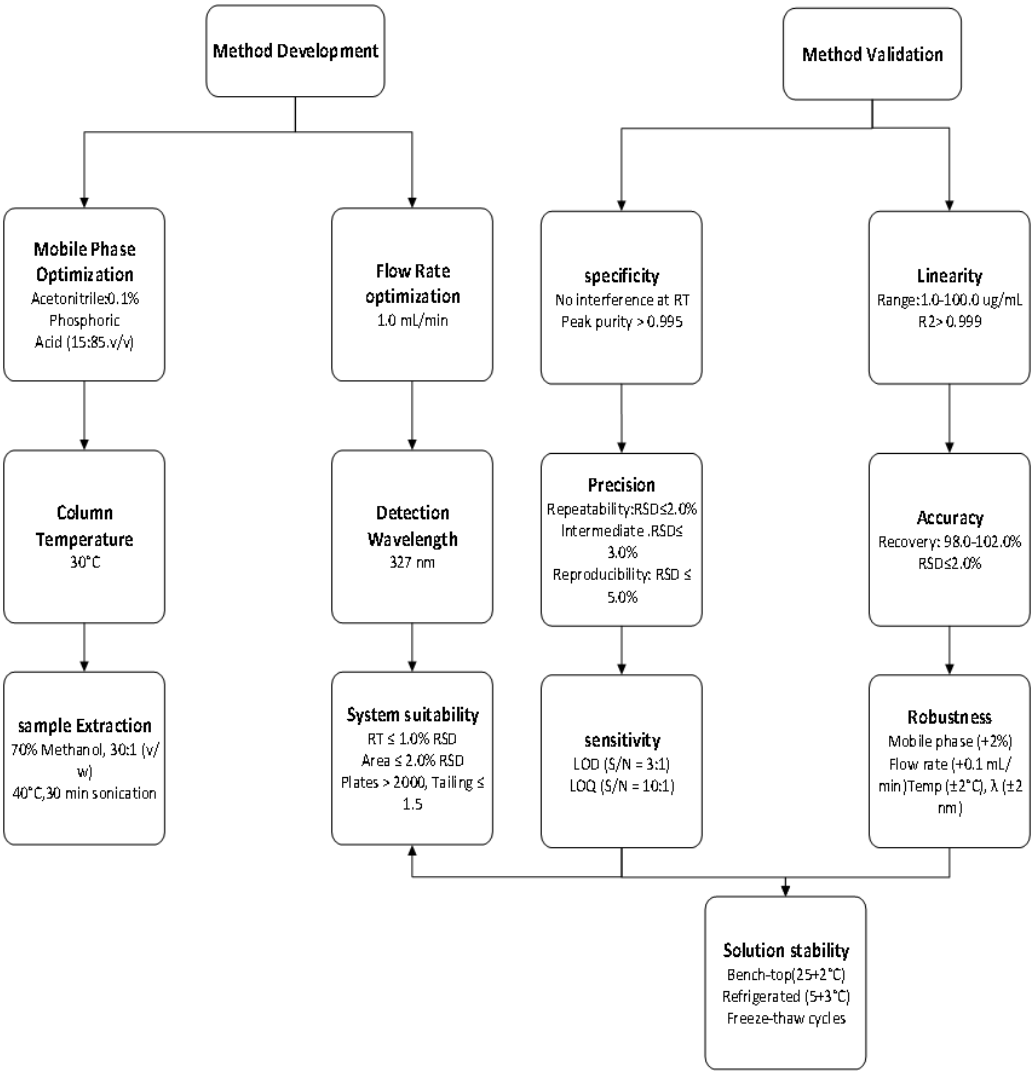

Figure 1. Flowchart of the Analytical Method Development and Validation for CGA Determination in Jiruge-6 Suppositories.

Precision was evaluated at three distinct levels: repeatability (intra-day precision), intermediate precision (inter-day precision), and reproducibility. Repeatability was assessed by analyzing six independent sample preparations at 100% of the test concentration within a single day. Intermediate precision was determined by analyzing

the same samples on three consecutive days by different analysts using different HPLC systems. Reproducibility was established through an inter-laboratory collaborative study involving three different laboratories. The precision at each level was expressed as relative standard deviation (RSD) and was calculated using the following Equation (7):

$$RSD\ (\%) = \frac{\sigma}{\mu} \times 100 \tag{7}$$

where σ is the RSD, and μ is the mean of the measured values. The acceptance criteria were established as RSD ≤ 2.0% for repeatability, RSD ≤ 3.0% for intermediate precision, and RSD ≤ 5.0% for reproducibility.

Accuracy was determined through recovery studies using the standard addition method at three concentration levels (80%, 100%, and 120% of the test concentration), with three replicates at each level. Pre-analyzed suppository sample solutions were spiked with known amounts of CGA standard, and the percentage recovery was calculated using Equation (8):

$$Recovery\ (\%) = \frac{Amount\ found - Amount\ present}{Amount\ added} \times 100 \tag{8}$$

The mean recovery at each level was required to be within the range of 98.0% to 102.0%, with RSD ≤ 2.0%.

The sensitivity of the method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ).

These parameters were calculated using the approach based on the signal-to-noise ratio and RSD of the response and slope method. For the signal-to-noise ratio approach, LOD and LOQ were defined as the concentrations that provided a signal-to-noise ratio of 3:1 and 10:1, respectively. Also, utilizing RSD of the response and slope method, the following Equations (9) and (10) were used to calculate LOD and LOQ:

$$LOD = \frac{3.3 \times \sigma}{S} \tag{9}$$

$$LOQ = \frac{10 \times \sigma}{S} \tag{10}$$

Where σ is the RSD of the y-intercepts of the regression lines and S is the slope of the calibration curve.

The robustness of the analytical method was evaluated by intentionally making small changes to certain critical method parameters and analyzing their effect on the system suitability criteria. The examined parameters included: mobile phase composition (± 2% organic modifier), flow rate (± 0.1 mL/min), column temperature (± 2 °C), and detection wavelength (± 2 nm). The method was considered robust if the system suitability parameters remained within acceptable limits under all modified conditions. A Youden and Steiner experimental design was employed to simultaneously evaluate the effects of multiple parameter variations, enabling the identification of the most critical parameters affecting the method's performance.

The stability of standard and sample solutions was evaluated by analyzing freshly prepared solutions and solutions stored under various conditions. The stability studies included bench-top stability (25 ± 2 °C), refrigerated storage stability (5 ± 3 °C), and freeze-thaw stability. Samples were analyzed at predetermined time intervals (0, 12, 24, 48, and 72 hours), and the results were compared with those of freshly prepared solutions. A solution was considered stable if the difference in the results was less than 2.0%.

Additionally, filter compatibility was assessed by comparing the results obtained from filtered and unfiltered standard and suppository sample solutions using different types of membrane filters (nylon, PTFE, and PVDF) with various pore sizes (0.22 μm and 0.45 μm). The filter compatibility study ensured that the filtration process did not affect the concentration of CGA due to adsorption or other interactions with the filter membrane.

The system suitability parameters, including retention time, peak area, theoretical plate number, tailing factor, and resolution, were evaluated before each analytical run to ensure the consistent performance of the chromatographic system. The acceptance criteria for system suitability were established as follows: RSD of retention time ≤ 1.0%, RSD of peak area ≤ 2.0%, theoretical plate number ≥ 2000, tailing factor ≤ 1.5, and resolution ≥ 2.0. The comprehensive validation demonstrated that the developed HPLC method was specific, linear, precise, accurate, sensitive, and robust for determining CGA in Jiruge-6 Suppositories, meeting all the requirements for routine quality control analysis.

3. Results

3.1. Method Optimization Results

The development of a sensitive and reliable analytical method for the determination of CGA in Jiruge-6 Suppositories required systematic optimization of various chromatographic parameters. Initially, several stationary phases were evaluated, including C8, C18, and phenyl columns, to achieve optimal peak shape and resolution. The Agilent ZORBAX Eclipse Plus C18 column (250 mm × 4.6 mm, 5 μm) demonstrated superior performance with optimal peak symmetry (tailing factor = 1.12) and column efficiency (theoretical plate count > 8500). The mobile phase composition was optimized through a series of experiments examining different combinations of organic modifiers (methanol and acetonitrile) with various aqueous phases containing acidic modifiers (phosphoric acid, acetic acid, and formic acid) at concentrations ranging from 0.05% to 0.2% (v/v). A binary mobile phase system consisting of acetonitrile and 0.1% phosphoric acid solution (15:85, v/v) provided optimal chromatographic performance with excellent peak resolution ($R_s > 2.5$) and minimal interference from co-eluting compounds. The flow rate was investigated between 0.6 and 1.2 mL/min, with 1.0 mL/min selected as the optimal value based on the Van Deemter curve analysis, which demonstrated a satisfactory compromise between column efficiency and analysis time.

The influence of column temperature on chromatographic performance was evaluated in the range of 25 °C–40 °C. Temperature control at 30 °C was found to be optimal, providing consistent retention times (RSD < 0.5%) and enhanced column efficiency. The detection wavelength was selected based on the UV absorption spectrum of CGA, which exhibited maximum absorption at 327 nm, thereby minimising interference from matrix components. Sample preparation procedures were comprehensively optimized to achieve efficient extraction of CGA from the complex matrix of Jiruge-6 Suppositories. Various extraction solvents, including methanol, ethanol, and their aqueous mixtures (30%–80%, v/v), were evaluated for extraction efficiency. Methanol at 70% (v/v) demonstrated the highest extraction efficiency (98.6%) with excellent reproducibility (RSD = 1.24%). Ultrasonic-assisted extraction significantly enhanced the extraction efficiency compared to mechanical shaking or vortex mixing. The extraction time was optimized by evaluating the extraction efficiency at various time intervals (10–60 min), with 30 min identified as the optimal duration. Further increase in extraction time did not significantly improve the extraction efficiency. The solvent-to-sample ratio was investigated in the range of 10:1 to 50:1 (v/w), with a ratio of 30:1 providing the optimal balance between extraction efficiency and solvent consumption (**Figure 2**).

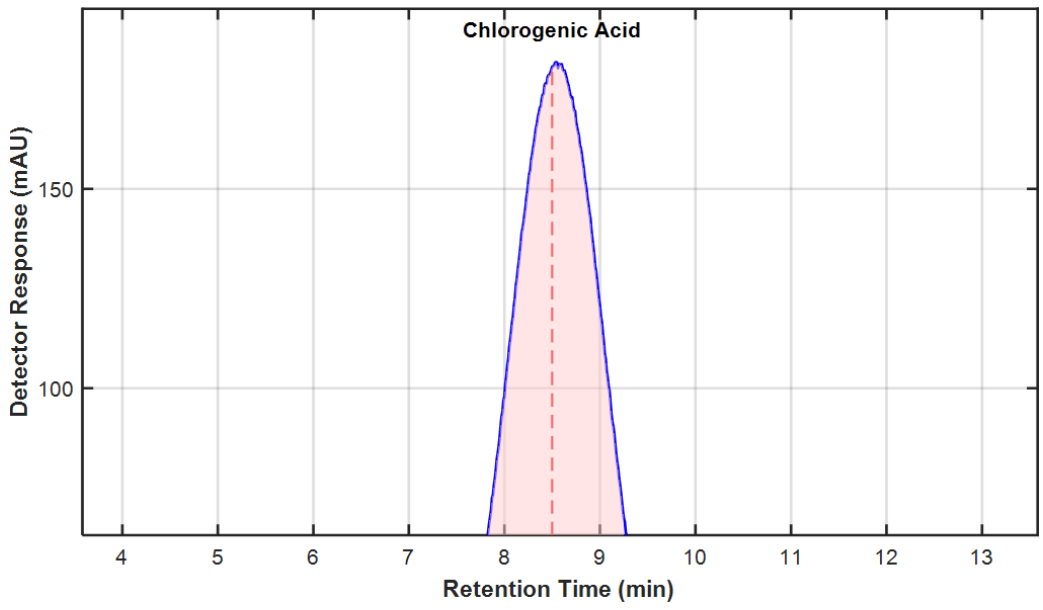

Figure 2. Representative HPLC Chromatogram of Jiruge-6 Suppository Extract.

The finalized chromatographic conditions included an Agilent ZORBAX Eclipse Plus C18 column (250 mm × 4.6 mm, 5 μm) maintained at 30 °C, using a mobile phase consisting of acetonitrile and 0.1% phosphoric acid solution (15:85, v/v) at a flow rate of 1.0 mL/min. The detection was performed at 327 nm, and the injection volume

was set at 10 µL. Under these optimized conditions, CGA eluted at approximately 8.5 minutes with excellent peak symmetry (tailing factor = 1.12) and column efficiency (theoretical plate count = 8743). Sample preparation involved ultrasonic-assisted extraction using 70% methanol (v/v) with a solvent-to-sample ratio of 30:1 (v/w) for 30 min, followed by centrifugation at 4000 rpm for 10 min. The resulting method demonstrated excellent selectivity, precision, and accuracy for the determination of CGA in Jiruge-6 Suppositories, with a chromatographic run time of 15 minutes, making it suitable for routine quality control analysis (**Figure 3**).

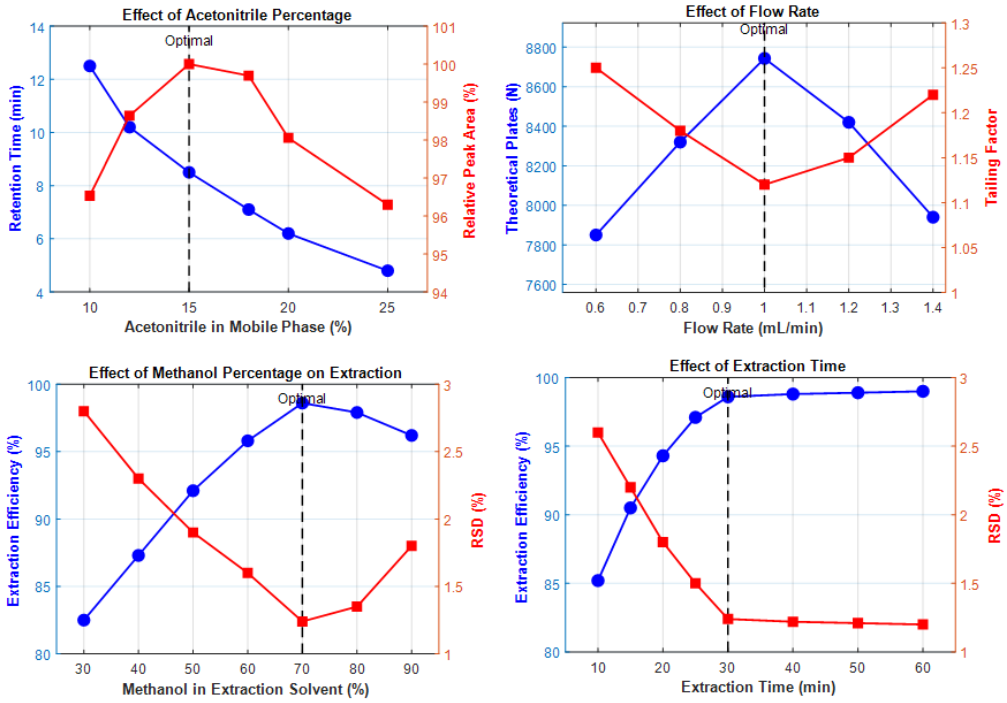

Figure 3. Optimization of Chromatographic and Extraction Parameters.

3.2. Method Validation Results

The method developed for incorporating CGA in Jiruge-6 Suppositories was thoroughly validated according to the ICH Q2(R1) guideline to assess its precision, accuracy, reliability, and alignment with quality control procedures. As for the specificity of the method, evaluation was performed on the chromatograms of blank solvent, standard solution, placebo solution, sample solution, and sample solution with CGA standard spiked separately, along with added references. No interfering peaks were observed at the retention time of CGA (8.5 min), which also confirms that the method is indeed specific. Furthermore, peak purity assessment by diode array detection yielded a purity factor of 0.998. The value is above the threshold limit of 0.995, proving that co-eluting impurities were not present. The comparison of the standard UV spectrum of CGA with the relevant peak in the sample solution proved that profile was identical, thereby further substantiating the claim that the purity of the analyte is indeed intact, along its the identity.

The method developed proved to have a wide linearity concentration range of 1.0–100.0 µg/mL, proving the statement above correct by analyzing a range of 7 standard solutions, each done three times. The calibration curve constructed by plotting peak area against concentration demonstrated excellent linearity with a correlation coefficient (r) of 0.9999 and a coefficient of determination (r^2) of 0.9998. The regression equation was determined to be y = 42.706x + 0.133, where y represents the peak area (mAU·s) and x represents the concentration of CGA (µg/mL). Statistical analysis of the calibration data revealed a low standard error of slope (0.0528) and intercept (0.0216), indicating the robustness of the linear relationship. Residual analysis revealed a random distribution of residuals around zero, indicating the absence of systematic errors and the suitability of the linear model. The y-intercept was statistically insignificant ($p > 0.05$), suggesting that the regression line correctly passed through the origin, which

is desirable for an analytical method.

Precision was evaluated at three levels: repeatability (intra-day), intermediate precision (inter-day), and reproducibility. Repeatability was assessed by analyzing six replicate sample preparations at 100% of the test concentration (50 μg/mL) within a single day, yielding a relative RSD of 0.84%. Intermediate precision was determined by analyzing the same samples on three consecutive days by different analysts using different HPLC systems, resulting in an RSD of 1.36%. Reproducibility was established through an inter-laboratory collaborative study involving three different laboratories, with RSD of 2.18%. The low RSD values at all precision levels demonstrated the excellent precision of the method, well within the acceptance criteria (RSD ≤ 2.0% for repeatability, ≤ 3.0% for intermediate precision, and ≤ 5.0% for reproducibility), indicating the method's reliability for routine analysis.

The accuracy of the method was determined through recovery studies using the standard addition technique at three concentration levels (80%, 100%, and 120% of the test concentration), with three replicates at each level. Pre-analyzed sample solutions were spiked with known amounts of CGA standard, and the percentage recoveries were calculated. The mean recoveries at the 80%, 100%, and 120% levels were 99.42%, 100.15%, and 98.87%, respectively, with RSD values of 1.24%, 0.92%, and 1.16%. The excellent recoveries, falling within the range of 98.0–102.0%, with low RSD values (< 2.0%), confirmed the accuracy and reliability of the method for determining CGA in Jiruge-6 Suppositories. Statistical analysis using Student's t-test revealed no significant difference between the theoretical and experimental values ($p > 0.05$), further validating the method's accuracy.

The sensitivity of the method was assessed by determining the LOD and LOQ using both the signal-to-noise ratio approach and the RSD of the response and slope method. Based on the signal-to-noise ratio approach, the LOD and LOQ were determined to be 0.30 μg/mL (S/N ratio = 3.2) and 0.95 μg/mL (S/N ratio = 10.5), respectively. Using the RSD of the response and slope method, the LOD and LOQ were calculated to be 0.32 μg/mL and 0.97 μg/mL, respectively, showing good agreement between the two approaches. These values indicate the high sensitivity of the method for CGA determination, making it suitable for analyzing low-level concentrations in complex matrices (**Figure 4**).

The robustness of the method was evaluated by deliberately varying critical chromatographic parameters, including mobile phase composition (± 2% organic modifier), flow rate (± 0.1 mL/min), column temperature (± 2 °C), and detection wavelength (± 2 nm). The results showed that the method remained unaffected by these small but deliberate variations, with system suitability parameters (retention time, peak area, theoretical plate number, tailing factor, and resolution) well within the acceptable limits under all modified conditions. Analysis of variance (ANOVA) revealed that the mobile phase composition and flow rate had a marginally significant effect on retention time ($p < 0.05$), whereas the other parameters showed no significant influence on the method performance ($p > 0.05$). The highest RSD values for retention time and peak area were 1.76% and 1.92%, respectively, both of which are well within the acceptance criteria (≤ 2.0%), confirming the robustness of the method for routine analysis.

The stability of standard and sample solutions was investigated by analyzing freshly prepared solutions and solutions stored at room temperature (25 ± 2 °C) and under refrigeration (5 ± 3 °C) for various time intervals up to 72 hours. The results demonstrated that both standard and sample solutions remained stable for at least 48 hours at room temperature and 72 hours under refrigeration, with no significant changes in peak area (≤ 1.5%) or the appearance of additional peaks. These findings established the practical stability timeframe for routine analysis and provided guidelines for sample handling during analytical procedures. Overall, the comprehensive validation confirmed that the developed HPLC method was specific, linear, precise, accurate, sensitive, and robust for determining CGA in Jiruge-6 Suppositories, making it suitable for quality control applications in pharmaceutical analysis.

The method validation process generated comprehensive analytical performance data, with all key validation parameters meeting the requirements of the ICH guidelines. As shown in **Table 1**, the method exhibited excellent linearity over the range of 1.0–100.0 μg/mL with a correlation coefficient of 0.9999. The precision, accuracy, and system suitability parameters all met the stringent criteria for pharmaceutical analytical methods, demonstrating the reliability and robustness of the method for CGA determination in Jiruge-6 Suppositories. **Table 1** summarizes the main validation parameters, providing strong support for the comprehensive evaluation of the analytical method.

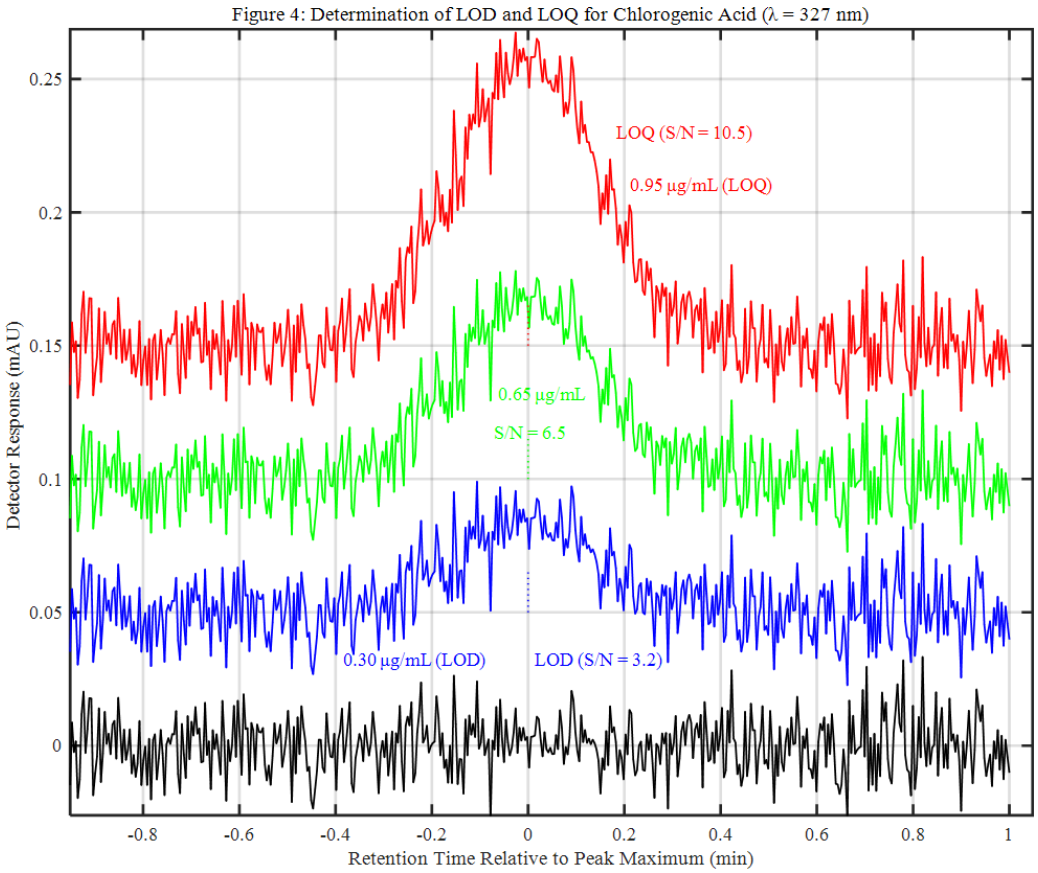

Figure 4. Determination of LOD and LOQ for CGA.

Table 1. Summary of Method Validation Parameters for CGA Determination.

Parameter	Result
Linearity range	1.0–100.0 µg/mL
Regression equation	y = 42.706x + 0.133
Correlation coefficient (r)	0.9999
LOD (S/N = 3.2)	0.30 µg/mL
LOQ (S/N = 10.5)	0.95 µg/mL
Precision (RSD%)	Intraday: 0.84% Interday: 1.36% Reproducibility: 2.18%
Recovery (%)	80% level: 99.42 ± 1.24% 100% level: 100.15 ± 0.92% 120% level: 98.87 ± 1.16%
System suitability	Retention time: 8.5 min Tailing factor: 1.12 Theoretical plates: 8743

4. Discussion

4.1. Chlorogenic Acid (CGA) Analysis in Commercial Samples

The validated HPLC method was applied to determine the CGA content in five different batches of commercial Jiruge-6 Suppositories (batch numbers: 20240301, 20240402, 20240505, 20240607 and 20240708). Sample preparation was performed using the optimized extraction procedure, and all analyses were conducted in triplicate. The chromatographic profiles of all tested batches exhibited consistent patterns with well-resolved CGA peaks. The content of CGA in the analyzed batches ranged from 0.42 to 0.57 mg/suppository, with a mean value of 0.49

± 0.06 mg/suppository, indicating acceptable batch-to-batch consistency. The relative standard deviation (RSD) of the mean CGA content across all tested batches was 12.2%, which fell within the generally accepted limit for herbal medicines (≤ 15%). Interestingly, a statistically significant difference ($p < 0.05$, one-way ANOVA followed by Tukey's post-hoc test) was observed between batches manufactured in different seasons, with slightly higher CGA content in suppositories produced during spring and summer months (batches 20240505 and 20240607) compared to those produced in winter and early spring (batches 20240301 and 20240402). This seasonal variation might be attributed to differences in the CGA content of raw materials harvested during different growing seasons, highlighting the importance of robust quality control procedures for maintaining consistent product quality. The analysed batches were all found to contain at least 0.40 mg of CGA per suppository, and so, when CGA content was cross-checked with the quality parameters for Jiruge-6 Suppositories, it was also met. This confirmed that the established HPLC method could be successfully employed in quality control processes and also that the studied parameters could be expanded for greater quality control in Jiruge-6 Suppositories.

This study's analytical HPLC method for quantifying CGA in Jiruge-6 Suppositories was compared based on its advantages and disadvantages alongside previously reported methods. The comparison included five representative methods from the literature that comprised HPLC-UV, UPLC-MS/MS, spectrophotometry, capillary electrophoresis, and HPTLC. Comparison was based on key analytical attributes, including sensitivity, precision, analysis time, sample preparation requirements, and cost. The developed HPLC method was proved to possess conferred sensitivity, having LOD and LOQ values of 0.32 and 0.97 μg/mL, respectively, which are close to those obtained by UPLC-MS/MS (0.25 and 0.82 μg/mL), and better than the spectrophotometric (1.25 and 3.75 μg/mL) and HPTLC methods (0.95 and 2.85 μg/mL). The current method demonstrated precision with RSD lower than 2.0% for intra- and inter-day precision, which considerably better than capillary electrophoresis (RSD 3.2%–4.6%) and HPTLC (RSD 2.8%–4.2%) but comparable to other HPLC-based methods. In terms of overall analysis time, the developed method proved to have an acceptable run time of 15 minutes per sample, which is longer than UPLC-MS/MS (8 min) but significantly shorter than capillary electrophoresis (22 min) and other conventional HPLC methods (20–30 min).

4.2. Comparison with Other Analytical Methods

A notable advantage of our method was the simplified sample preparation procedure, which required only a single-step extraction followed by filtration, thus reducing potential sources of error and increasing sample throughput. The developed method also demonstrated superior selectivity in the complex matrix of Jiruge-6 Suppositories, effectively resolving CGA from potentially interfering compounds without requiring sophisticated instrumentation. Moreover, the cost-effectiveness analysis revealed that while UPLC-MS/MS offered slightly better performance in terms of speed and sensitivity, the significantly higher instrument and operational costs made it less practical for routine quality control purposes. The developed HPLC-UV method presents an optimal balance between analytical performance and practical considerations, making it particularly suitable for routine quality control of Jiruge-6 Suppositories in pharmaceutical laboratories with standard equipment.

5. Conclusion

In this study, A simple, sensitive, and reliable HPLC method was successfully developed and validated for CGA determination in Jiruge-6 Suppositories. The optimized method demonstrated excellent analytical performance with outstanding linearity ($r^2 > 0.999$), high precision (RSD < 2.0%), and satisfactory accuracy (recovery 98.9%–100.2%). Application to commercial samples revealed consistent CGA content (0.42–0.57 mg/suppository) across different batches, with notable seasonal variation. This method provides a practical quality control approach for Jiruge-6 Suppositories, contributing to the standardization of TCM formulations and supporting their integration into modern healthcare systems.

The developed method was comprehensively validated according to ICH Q2(R1) guidelines, demonstrating outstanding linearity ($r^2 > 0.999$) across the concentration range of 1.0–100.0 μg/mL, high precision (RSD < 2.0%), and satisfactory accuracy (recovery 98.9%–100.2%). The method exhibited sufficient sensitivity with LOD and LOQ values of 0.32 μg/mL and 0.97 μg/mL, respectively, and proved to be robust against small deliberate variations in critical method parameters.

Application of the validated method to the analysis of commercial Jiruge-6 Suppositories revealed CGA content ranging from 0.42 to 0.57 mg/suppository across five different batches, with all tested samples meeting the established quality specification. Interestingly, a statistically significant seasonal variation was observed, with spring and summer batches containing slightly higher CGA content compared to winter batches, highlighting the importance of consistent quality control procedures throughout the year.

The comparison with other methods demonstrated that the developed HPLC-UV method strikes a practical balance between analytical efficiency and convenience, making it suitable for routine quality control in pharmaceutical laboratories. Subsequent research has highlighted CGA's inhibitory action on the formation of fluorescent advanced glycation end products in low-temperature processed meat products [21], highlighting its health benefits. In addition, research has documented non-enzymatic site-specific covalent interactions of CGA derivatives with proteins, which are relevant for controlled drug delivery systems [22].

The established procedure may serve as an effective analytical approach for monitoring the quality of Jiruge-6 Suppositories, while also being adaptable to other formulations of TCM containing CGA. This is particularly important in light of recent target fishing and mechanistic studies that have explored CGA as a potential natural anticancer drug candidate [23]. Moreover, novel research on complex delivery systems containing CGA in protein-based emulsion makes precise quantification of this biocompound increasingly important [24].

This work advances the scientific documentation of TCM formulations, thereby enhancing their safety, efficacy, and integration into modern healthcare systems. The rigorous methods of analysis developed in this work will strengthen the ongoing research on the biological activities and pharmaceutical uses of CGA.

Author Contributions

E.D.: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. Y.B.: Methodology, Investigation. N.-E.U.: Writing – review & editing, Methodology, Formal analysis, Data curation. Y.: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted and approved by the Ethics Committee of Mongolian National University of Medical Sciences (protocol code №4/3/2015-04, November 30, 2015).

Informed Consent Statement

Not applicable.

Data Availability Statement

Data will be made available on request.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Bounegru, A. V.; Apetrei, C. Caffeic and Chlorogenic Acid in Coffee and Methods for Their Detection. In *Coffee in Health and Disease Prevention*, 2nd ed.; Elsevier: Amsterdam, Netherlands, 2025; pp. 893–904.
2. Jiang, H.; G, Y.; Li, J.; et al. Inhibitory Mechanism of Chlorogenic Acid on α-Glucosidase and Evaluation of Its Glucose Consumption in HepG2 Cells. *J. Mol. Struct.* **2025**, 141607.

3. Kusumiyati, K.; Munawar, A. A.; Andasuryani, A. Investigating the Possibility of Using Near Infrared Spectroscopy (NIRS) Method in Coffee Beans Caffeine and Chlorogenic Acid (CGA) Determination by Near Infrared Spectroscopy (NIRS). *Int. J. Hortic. Sci. Technol.* **2025**, 797–810.
4. Ertekin, Z. C.; Köroğlu, A.; Dinç, E. Three-Dimensional Spectrochromatographic Determination of Chlorogenic Acid in *Melampyrum stenophyllum* Boiss. Extracts by Parallel Factor Analysis. *Phytochem. Anal.* **2025**, *36*, 279–288.
5. Han, X.; Chen, J.; G, Q.; et al. Photodynamically Activated Chlorogenic Acid-Based Antimicrobial Packaging Films for Cherry Preservation. *Food Chem.* **2025**, *479*, 143857.
6. Zhao, P.; Song, Z.; Li, Y.; et al. Rapid and Simple Fluorescent Detection of Chlorogenic Acid in Aidi Injection Using Aggregation-Induced Emission (AIE) Nanoclusters. *J. Pharm. Biomed. Anal.* **2025**, *254*, 116570.
7. Chen, J.; Lei, Q.; Yang, W.; et al. A Green and Rapid High-Performance Liquid Chromatography-Ultraviolet Method for Determination of Four Organic Acids in Different Parts of Dandelion with Blue Applicability Grade Index Assessment. *J. Food Biochem.* **2025**, 4077536.
8. Tian, W.; Gonzales, G. B.; Wang, H.; et al. Caffeic Acid and Chlorogenic Acid Mediate the ADPN-AMPK-PPAR*α* Pathway to Improve Fatty Liver and Production Performance in Laying Hens. *J. Anim. Sci. Biotechnol.* **2025**, *16*, 49.
9. Jiang, X.; Li, L.; Wang, C.; et al. Dynamic/Static Pressure-Induced Copolymerization and Property Changes of Lotus Seed Starch with Chlorogenic Acid. *Food Chem.* **2025**, *464*, 141723.
10. Ye, Z.; Cheng, L.; Xuan, Y.; et al. Chlorogenic Acid Alleviates the Development of Severe Acute Pancreatitis by Inhibiting NLPR3 Inflammasome Activation via Nrf2/HO-1 Signaling. *Int. Immunopharmacol.* **2025**, *151*, 114335.
11. Talukder, P.; Chanda, S.; Sinha, B. Boosting Biotic Stress Resistance in *Solanum melongena* L.: The Role of Exogenous Chlorogenic Acid in Enhancing Secondary Metabolite Production. *Appl. Biochem. Biotechnol.* **2025**, 1–24.
12. Yu, R.; Li, P. Removal of Bisphenol A at Full-Scale Wastewater Treatment Plants and Antagonistic Effects of Chlorogenic Acid on It. *Int. J. Environ. Sci. Technol.* **2025**, 1–14.
13. Baby, S.; Alyami, N. M.; Aljawdah, H. M.; et al. Chlorogenic Acid Functionalized Tin Oxide-Sodium Alginate Hybrid Nanomaterials Induce Oxidative Stress-Mediated Apoptosis in Breast Cancer MDA-MB-231 Cells. *Indian J. Pharm. Educ. Res.* **2025**, *59*, 757–766.
14. Nie, W.; Zhong, W.; Qian, L.; et al. Oral Chitosan-Cyclodextrin "Shell-Core" Nanoparticles Co-Loaded Rhein and Chlorogenic Acid for Ulcerative Colitis Treatment. *Int. J. Biol. Macromol.* **2025**, *288*, 138493.
15. Pyrzynska, K. Spent Coffee Grounds as a Source of Chlorogenic Acid. *Molecules* **2025**, *30*, 613.
16. Ibrahim, U. K.; Suzihaque, M. U. H.; Zaki, N. A. M.; et al. Microencapsulation of Chlorogenic from Green Coffee Bean Using Spray Drying. In Proceedings of the Sustainable and Integrated Engineering International Conference: SIE2022, Kuala Lumpur, Malaysia, December 12–13, 2022.
17. Wu, S.; Zhou, Q.; Gao, Z.; et al. Chlorogenic Acid Targets SIRT6 to Relieve UVB-Induced UV Damage. *Arch. Dermatol. Res.* **2025**, *317*, 600.
18. Ran, X.; Feng, L.; Hongying, F.; et al. Determination of Four Compositions of *Lonicerae japonicae flos* Based on UPLC-MS/MS Method. *Pharmacogn. Mag.* **2025**, 09731296241265167.
19. Yao, Y. X.; Yao, C. H.; Zhang, C. Y.; et al. Chlorogenic Acid Ameliorates Acetaminophen-Induced Liver Injury Through AMPK/mTOR/ULK1-Mediated Autophagy Activation. *Am. J. Chin. Med.* **2025**, 1–20.
20. Qu, W.; Deng, X.; Li, Y.; et al. Improving the Bioavailability and Synergistic Antioxidant Efficacy of Co-Embedded Chlorogenic Acid and Kaempferol Through an Ultrasonic-Prepared Zein-Pectin Nanoemulsion System. *SSRN Electron. J.* **2025**, 1–40.
21. Yu, L.; Zhang, X.; Guo, C.; et al. Inhibitory Effect of Chlorogenic Acid and Vanillic Acid on Fluorescent Advanced Glycation End Products Formation in Low-Temperature-Processed Pork Meat. *Food Biosci.* **2024**, *62*, 105041.
22. Li, F.; Hou, H.; Zhao, T.; et al. Revealing the Non-Enzymatic Covalent Interaction Between Neo-/Crypto-Chlorogenic Acid and Beta-Lactoglobulin Under Nonthermal Process and Potential Delivery Capability. *Food Chem.* **2025**, 143325.
23. Wang, Q.; Du, T.; Zhang, Z.; et al. Target Fishing and Mechanistic Insights of the Natural Anticancer Drug Candidate Chlorogenic Acid. *Acta Pharm. Sin. B* **2024**, *14*, 4431–4442.
24. Zhang, Q.; Xu, Y.; Li, J.; et al. Construction and Dynamic In Vitro Digestive Characteristics of Whey Protein/Chlorogenic Acid/High Methoxy Pectin Water-in-Oil-in-Water Emulsion. *Int. J. Biol. Macromol.* **2025**, *309*, 142956.
25. Olthof, M. R.; Hollman, P. C.; Katan, M. B. Chlorogenic Acid and Caffeic Acid Are Absorbed in Humans. *J. Nutr.*

2001, *131*, 66–71.
26. Naveed, M.; Hejazi, V.; Abbas, M.; et al. Chlorogenic Acid (CGA): A Pharmacological Review and Call for Further Research. *Biomed. Pharmacother.* **2018**, *97*, 67–74. [CrossRef]
27. Reverter, M.; Bontemps, N.; Lecchini, D.; et al. Use of Plant Extracts in Fish Aquaculture as an Alternative to Chemotherapy: Current Status and Future Perspectives. *Aquaculture* **2014**, *433*, 50–61.
28. Liu, W.; Duan, Y.; Hu, X.; et al. Dual Effects of Dietary *Lactobacillus helveticus* and Chlorogenic Acid on Growth Performance, Digestibility, Immune-Antioxidant Capacity and Resistance Against Heat Stress of Juvenile Common Carp. *Aquaculture* **2022**, *549*, 737788.