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Original Research Article

Development and Validation of a Green HPLC Method for the Simultaneous Quantification of Major Alkaloids in Selected Herbal Formulations in Iraq

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Abstract: Cassia glauca Lam., a tree belonging to the Fabaceae circle of relatives and local to India, has been broadly used in traditional structures of medication such as Indian people medication and Brazilian ethnomedicine. It is known for its various pharmacological sports, including antidiabetic, antibacterial, antifungal, antioxidant, anti-hemolytic, anticancer, cardioprotective, and hepatoprotective outcomes. This examine aimed to isolate, discover, and quantify decided on phytochemical compounds from the aerial parts of Cassia glauca cultivated in Iraq, as no earlier phytochemical investigations were performed regionally in this plant. The aerial elements have been first defatted using n-hexane for forty-eight hours. Subsequently, extraction was completed with eighty-five % ethanol the use of the recent (Soxhlet) approach. The crude extract became then fractionated with extraordinary solvents: chloroform, ethyl acetate, and n-butanol. High-overall performance liquid chromatography (HPLC) was hired for the identification and quantification of compounds using authenticated reference standards. Preparative layer chromatography (PLC) became used for compound isolation, accompanied via structural affirmation through Liquid Chromatography–Mass Spectrometry (LC-MS/MS-Q-TOF). Results from chromatographic and spectrometric analyses showed the presence of luteolin within the ethyl acetate fraction and chlorogenic acid inside the n-butanol fraction. The predicted portions had been a hundred thirty.Seventy seven µg/g for luteolin and 0.0006% for chlorogenic acid, based on 50 g of plant material.

Keywords: Cassia glauca, luteolin, chlorogenic acid, HPLC, LC-MS/MS-Q-TOF.

INTRODUCTION

Cassia glauca is a leguminous tree characterised through its clean (glabrous) branches. It is native to East India and has a extensive geographical distribution that extends from the Himalayas via Sri Lanka and the Polynesian Islands to Australia (Hooker, 1879). This species flourishes in tropical and subtropical climates, inclusive of areas in Southeast Asia, Africa, and West India. It has also emerge as naturalized in numerous elements of the arena, along with Iraq, where it's miles now cultivated (Allen, Nelson, & Allen, 1981).

The Cassia genus in fashionable holds big economic fee, attributed to each its vibrant ornamental enchantment and ecological advantages; its plentiful floral display and the carpet of fallen petals make it a famous preference for planting alongside avenues in warm climates (Elsonbaty et al., 2020). Moreover, Cassia glauca is extensively utilized in conventional medicinal drug. It has been mentioned to behave as a valuable frightened system depressant, as well as owning purgative, antimalarial, and diuretic homes (Cook, 1911). The oil extracted from its seeds is utilized in indigenous medicinal drug for the treatment of pores and skin problems and leucoderma (Chopra, Nayar, & Chopra, 2002).

In Brazilian conventional medication, the plant is employed to relieve signs of influenza, fever, colds, and complications (Singh, 2018). Additionally, root decoctions are commonly used as a remedy for snake bites (Phuse & Khan, 2018). Phytochemical analyses of the plant have revealed the presence of several biologically active compounds, which includes polyphenols (consisting of flavonoids and tannins),

glycosides, carbohydrates, alkaloids, steroids, anthraquinones, and anthracenes (Veeraperumal et al., 2021). Specifically, the seeds incorporate alkaloids, sterols, carbohydrates, proteins, amino acids, and saponins.

Numerous scientific reports have confirmed that Cassia species showcase a wide variety of pharmacological activities, along with antidiabetic, antimicrobial, antimalarial, anticancer, and hepatoprotective outcomes (Srinivas, 2019). Polyphenols, specially, are a prominent group of herbal bioactive compounds defined via the presence of at the least two hydroxyl companies of their chemical systems. Their extensive presence in the plant country underscores their importance in each therapeutic applications and plant-primarily based bioactive studies.

MATERIALS AND METHODS

Plant Collection

The aerial parts of Cassia glauca had been accumulated from roadside regions positioned in Al-Karkh district, Baghdad, Iraq. The plant specimen turned into botanically recognized and authenticated at the Department of Biology, College of Science, University of Baghdad. After collection, the plant materials were very well washed with tap water to remove dirt and debris, air-dried at room temperature, after which floor into a first-rate powder the usage of a mechanical grinder.

Extraction Procedure

A total of 50 grams of the dried powdered aerial parts were subjected to defatting through maceration in n-hexane for 48 hours. The hexane extract was subsequently removed under reduced pressure using a rotary evaporator, and the resulting residue was weighed and labeled as **Fraction H1**.

The defatted plant material was then extracted using a Soxhlet apparatus with 85% ethanol until complete exhaustion was achieved. The obtained ethanolic extract was concentrated under reduced pressure using a rotary evaporator, yielding a dark green crude extract.

To obtain different fractions based on polarity, the crude extract was dissolved in distilled water and sequentially partitioned using a separatory funnel with three solvents: chloroform, ethyl acetate, and n-butanol. These yielded the following fractions:

- **H2** (chloroform fraction)
- **H3** (ethyl acetate fraction)
- **H4** (n-butanol fraction)

Each solvent fraction was evaporated to dryness under reduced pressure and weighed to prepare for further qualitative and quantitative analysis (Hafez, Ibrahim, & Ayoub, 2019).

Scientific Rationale

Polyphenols are recognized as essential bioactive compounds due to their potent antioxidant capabilities, which stem from their redox properties. These allow them to act as reducing agents, hydrogen donors, oxygen scavengers, metal ion chelators, and ferryl hemoglobin reducers. Recent findings suggest that polyphenols exhibit synergistic effects when present as a mixture, setting them apart from other natural bioactive substances (Kumar, 2013).

In light of their biological significance and pharmacological potential, numerous recent studies have focused on the extraction, identification, and quantification of polyphenolic compounds from medicinal plants, especially those long utilized in traditional medicine (Kittur, Srinivas, & Deshp, 2015). Accordingly, this study aimed to isolate and characterize specific polyphenolic constituents from Cassia glauca cultivated in Iraq.

Qualitative and Quantitative Analysis of Polyphenols in H3 and H4 Fractions

1. Preliminary Phytochemical Screening

Excerpts H3 and H4 have been subject to qualitative examinations to decide the presence of polyphenolic compounds. The screening concerned 5% ferric chloride and 10% lead acetate solutions using check tubes, indicating the formation of characteristic colored modifications or reflects the signal of polyphenol appearance. The results of these tests are supplied in the table (1) (wiciński et al., 2020):

Table 1. Qualitative Phytochemical Screening of H3 and H4 Fractions of Cassia glauca

Constituent	Test Procedure	Observation
Tannins and phenolic compounds	Feric Chloride Testing (5%): Each of each H3 and H4 degrees was mixed with 1 ml of 5% ferric chloride solution in a test tube.	The presence of a dark green or dark blue indicated the presence of tannins and polyphenols.
	Lead acetate test (10%): 1 mL of each of the H3 and H4 fractions was mixed with 1 mL of 10% lead acetate solution.	The formation of a white precipitation confirmed the presence of tannins and phenolic compounds.

2. Identification of Selected Compounds Using HPLC

High-demonstration liquid chromatography (HPLC) was analyzed to identify the active polyphenolic components

present in H3 and H4 fractions. The retention time received from analyzed samples was compared to certified standard compounds under the same chromatographic conditions.

For H3 fraction, luteolin was chosen as the standard reference compound. Chromatographic conditions were as follows:

- Flow Rate: 0.8 ml/min
- Detection Wavelling (λ): 278 Nm
- Mobile Phase:

- solvent a: 1% aquatic acetic acid solution
- solvent b: 100% methanol
- Lakshin technique: Grandient Election as mentioned in Table (2) (2) (Mitra et al., 2022).

Table 2. HPLC Gradient Elution Program for H3 Fraction and Luteolin Standard

Time (min)	Solvent A (%): 1% Aqueous Acetic Acid	Solvent B (%): Methanol
0 – 6	90%	10%
7 – 25	84%	16%
26 – 37	72%	28%
38 – 47	65%	35%
48 – 64	50%	50%
65 – 70	90%	10%

This shield alkaline program was used to separate the components of the H3 fraction and identify luteolin on the basis of retention time in controlled conditions. With detection at 278 Nm, the flow rate was maintained at 0.8 mL/min.

H4 fraction and chlorogenic acid analysis for standard

For H4 fraction, chlorogenic acid was chosen as a standard compound. The mobile phase system was included:

- Village A: 0.05% trifloroecetic acid (TFA) in delicked water
- solvent B: 0.05% TFA in methanol (adjusted to pH 2.5(

In a period of 15 minutes, the infection from 0% to 100% of the solvent B was employed, a shield alkaline was employed. The flow rate was set at 1 mL/min, and the detection was done on the wavelength of 280 nm (Sotiropolo, Megrame, and Tarantillis, 2020.(

To confirm the identification of chlorogenic acid peak in H4 chromatogram, an additional verification step was performed by spikeing the H4 fraction with a known amount of chlorogenic acid standard. The presence of a co-ingestion peak confirmed the identity on retention time (Mitra et al., 2022)

3. **Isolation of Polyphenolic Compounds Using Preparative Layer Chromatography (PLC)**

Preparative skinny-layer chromatography (PLC) became hired to isolate two goal polyphenolic compounds from Cassia glauca fractions. Silica gel GF254 plates (20 × 20 cm, zero.Five mm thickness) synthetic by using Taiyang, China, have been used. The plates were activated by using heating at one hundred ten °C for 30 minutes prior to application (Hussein & Kadum, 2020). For the isolation of luteolin from the H3 fraction, the cellular phase S1—a combination of ethyl acetate, formic acid, and hexane inside the ratio of seven.7:1.3:zero.Nine—became applied (Stoenescu, Trandafir, & Cosmulescu, 2022). For the isolation of chlorogenic acid from the H4 fraction, the cell section S2 consisted of formic acid, ethyl acetate, dichloromethane, acetic acid, and water in the ratio 0.6:6.Four:1.6:0.6:zero.7, respectively (Thangaraj, 2019) Detection of separated bands was executed below UV mild at 254 nm and 366 nm, as well as via visualization with five% ethanolic potassium hydroxide (KOH) spray (Jaafar et al., 2016) Identification become confirmed by means of comparing the Rf values and fluorescence characteristics of the remoted compounds to authenticated reference requirements.

4. **Quantification of Polyphenolic Compounds by HPLC**

Luteolin Quantification in H3 Fraction

The amount of luteolin was stagnated using the external standard calibration method. The analysis was organized in the Ministry of Science and Technology using Shimdzu liquid chromatograph LC-201010Aht. A calibration curve was installed by injecting standard luteoline solutions in concentrations of 5, 10, 15, and 20 μg/ml. The peak area (detector response) was plotted against concentration, and linear regression analysis was used to generate standard

curves. The correlation coefficient (R&) and regression equation was implemented to calculate luteoline concentration in H3 Excerpt (Dong, 2006)

Chlorogenic Acid Quantification in H4 Fraction

Chlorogenic acid quantification was performed at the **Iraqi National Center for Drug Control and Research** using a SHIMADZU LC-20AD system. Quantification was based on the comparison of the area under the curve (AUC) of the plant sample and the standard under identical chromatographic conditions. To confirm the identity of the peak, the H4 fraction was spiked with a small amount of authentic chlorogenic acid. The concentration was calculated using the following equation (Islam et al., 2020):

$$\% \text{ of compound in plant} = \left(\frac{\text{AUC of plant sample}}{\text{AUC of standard}} \times \frac{\text{Conc. of standard} \times \text{DF}}{\text{Weight of dried plant used in extraction}}\right) \times 100$$

Where:

- **AUC** = Area Under the Curve
- **Conc. of standard** = Concentration of authentic standard
- **DF** = Dilution factor

5. **Identification and Characterization by LC-MS/MS-QTOF**

To confirm the structure of the isolated polyphenolic compounds, advanced mass spectrometric analysis was carried out using **Liquid Chromatography – Tandem Mass Spectrometry with Quadrupole Time-of-Flight (LC-MS/MS-QTOF)**. The analysis was conducted at **Jordan University of Science and Technology**, Irbid, Jordan.

LC Conditions:

- Column: GL-Science C18 (250 mm × 4.6 mm, 5 µm particle size, Japan)
- Column oven temperature: 35 °C
- Injection volume: 10 µL
- Flow rate: 1.0 mL/min
- Run time: 25 minutes
- Solvent A: Formic acid in H_2O
- Solvent B: Acetonitrile

The elution followed a specific gradient system (see Table 3).

Mass Spectrometry Parameters:

- Instrument: X500 QTOF (AB Sciex)
- Software: AB-Sciex-OS
- Ionization mode: Electrospray Ionization (ESI) – Positive
- Scan range: 50–800 m/z
- Ion source voltage: 5500 V

Table 3. Gradient System for LC/MS/MS-Q-TOF Method

Time (min)	%A	%B
0.0	90	10
5.0	90	10
15.0	10	90
20.0	10	90
20.1	90	10
25.0	90	10

RESULTS

Fraction Yield and Weight Analysis The yield percentages and corresponding weights of each fraction obtained from 50 g of dried Cassia glauca plant material are summarized in Table 4. The defatted extract (H1) was obtained first, followed by fractions H2, H3, and H4 after the crude extract was subjected to fractionation.

Table 4. Weight and Yield Percentage of Each Fraction from Cassia glauca

Fraction	Weight of Fraction (g)	Percent Yield	Crude Extract Weight (g)	Initial Dried Plant Weight (g)
H1	1.63	23.26%	11.53	50
H2	1.62	3.24%	–	–
H3	5.01	10.02%	–	–
H4	4.82	9.64%	–	–

Preliminary Qualitative Analysis of Polyphenolic Compounds

The initial qualitative screening of polyphenolic compounds in fractions H3 and H4 revealed positive results, as shown in Table 5.

Table 5. Preliminary Qualitative Results for Polyphenols

Fraction	Test Applied	Result
H3	5% Ferric Chloride Test	Positive
H4	10% Lead Acetate Test	Positive

2. Identification of Two Compounds by HPLC – Results

The HPLC chromatographic analysis confirmed the presence of two target polyphenolic compounds in the tested fractions. In the H3 fraction, one of the peaks exhibited a retention time identical to that of the authenticated luteolin standard, as shown in **Figure 1**. Similarly, in the H4 fraction, one of the peaks matched the retention time of the chlorogenic acid standard, confirming its presence, as illustrated in **Figure 2**.

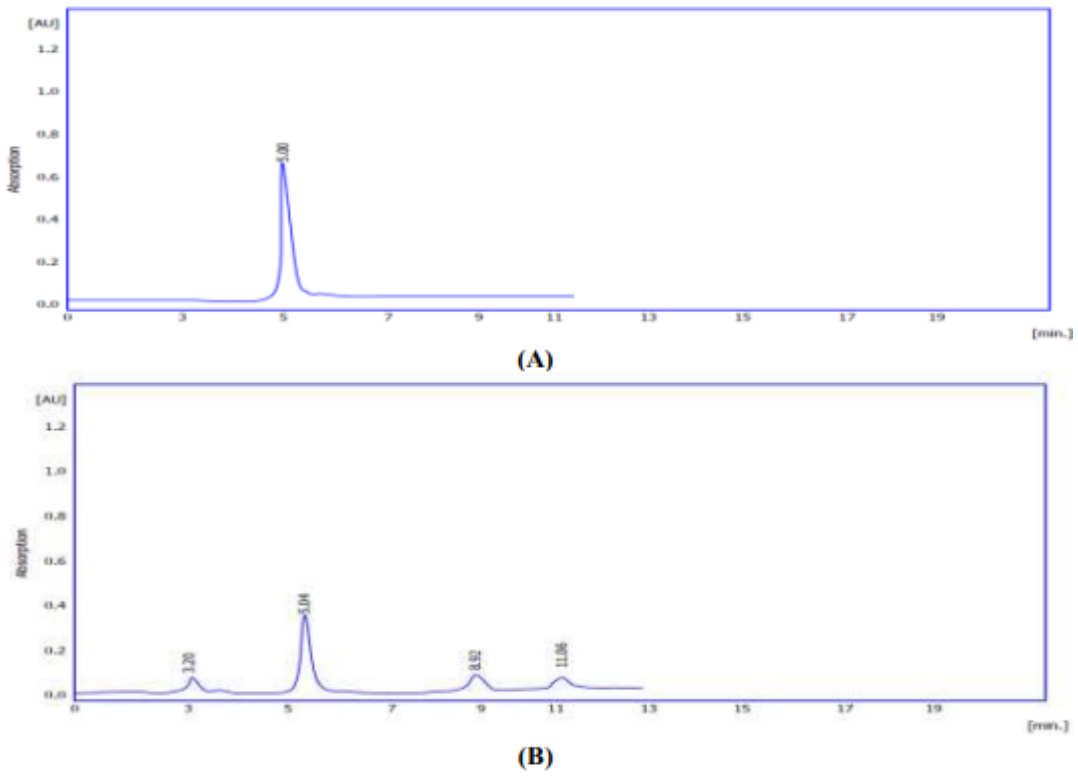


Figure 1. HPLC chromatogram of (A) luteolin standard and (B) H3 fraction.

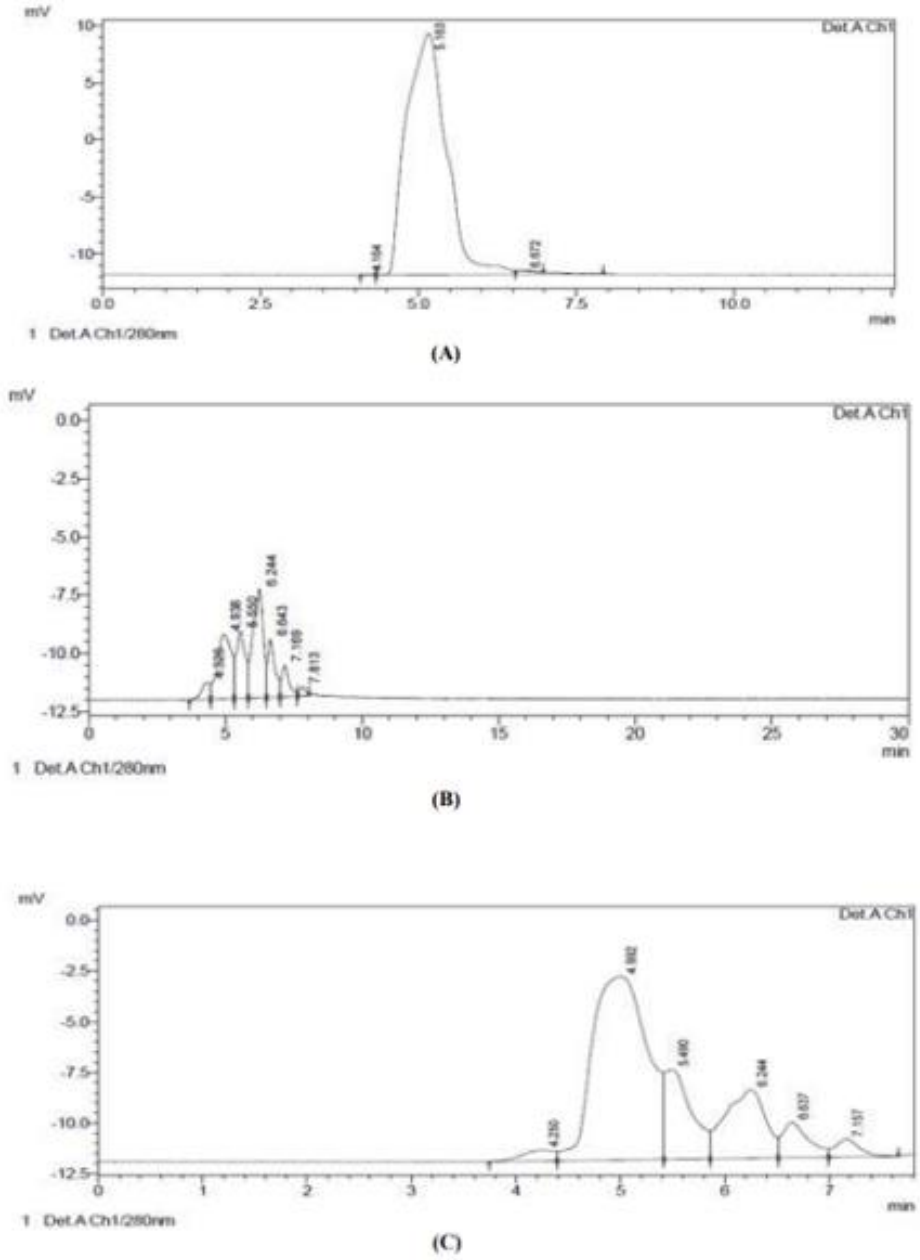


Figure 2. HPLC chromatograms of (A) chlorogenic acid standard, (B) H4 fraction, and (C) H4 fraction spiked with the chlorogenic acid standard.

3. Isolation of Two Polyphenolic Compounds by Preparative Layer Chromatography (PLC) – Results

The PLC technique, though simpler and less equipment-intensive than HPLC, allows for the parallel application of standards and test samples on a single plate and the use of visual spray reagents, which HPLC does not support. However, HPLC generally yields higher purity and quantity of isolated compounds. Isolated bands from H3 and H4 fractions were scraped from the plates, extracted with ethanol, filtered, and dried. Upon recrystallization, yellow and white crystalline solids were obtained from the H3 and H4 fractions, respectively, as seen in **Figures 3** and **4**.

Figure 3. PLC plate showing the detected luteolin band under UV at 254 nm. Developed in mobile phase S1 (Ethyl acetate: Formic acid: Hexane). H3: ethyl acetate fraction, L: luteolin standard.

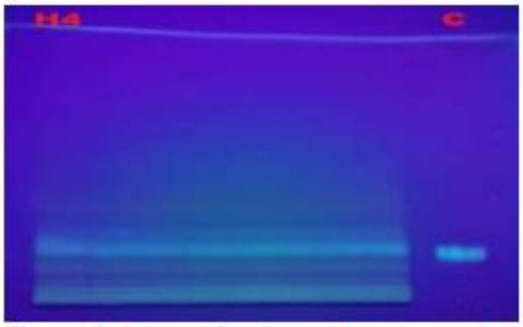


Figure 4. PLC plate showing the detected chlorogenic acid band under UV at 365 nm. Developed in mobile phase S2 (Formic acid: Ethyl acetate: Dichloromethane: Acetic acid: Water). H4: n-butanol fraction, C: chlorogenic acid standard.

4. Quantification of Polyphenolic Compounds Detected by HPLC – Results

The quantification of luteolin in the H3 fraction was performed using a calibration curve based on the external standard method. The regression equation obtained from the curve was:

Y = 15.63333 × X

Where **Y** is the detector response (peak area), and **X** is the concentration of luteolin. The calibration curve is displayed in **Figure 5**.

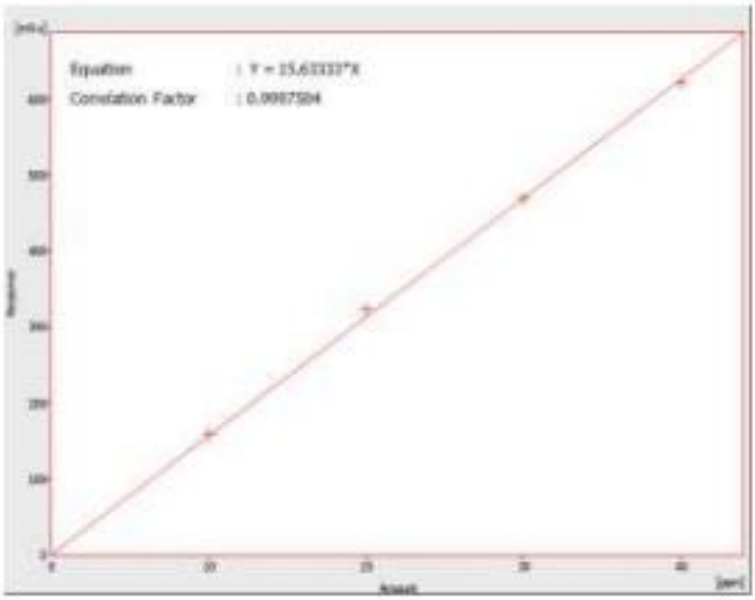


Figure 5. Calibration curve for quantifying luteolin in the H3 fraction using HPLC.

The final concentrations of the proposed luteolin and chlorogenic acid compounds isolated from the Cassia glauca

plant are presented in **Table 6**.

Table 6. Quantity of Luteolin and Chlorogenic Acid Isolated from Cassia glauca

Amount of Plant Material	Luteolin Content in H3 Fraction (µg/g)	Chlorogenic Acid Content in H4 Fraction (%)
50 g	130.77 µg/g	0.0006%

5. LC-MS/MS-Q-TOF Analysis – Results

Further identification and structural characterization of the compounds isolated by PLC were performed using LC–MS/MS-Q-TOF. The chromatographic profile and mass fragmentation spectra of the isolated luteolin are presented in **Figure 6**.

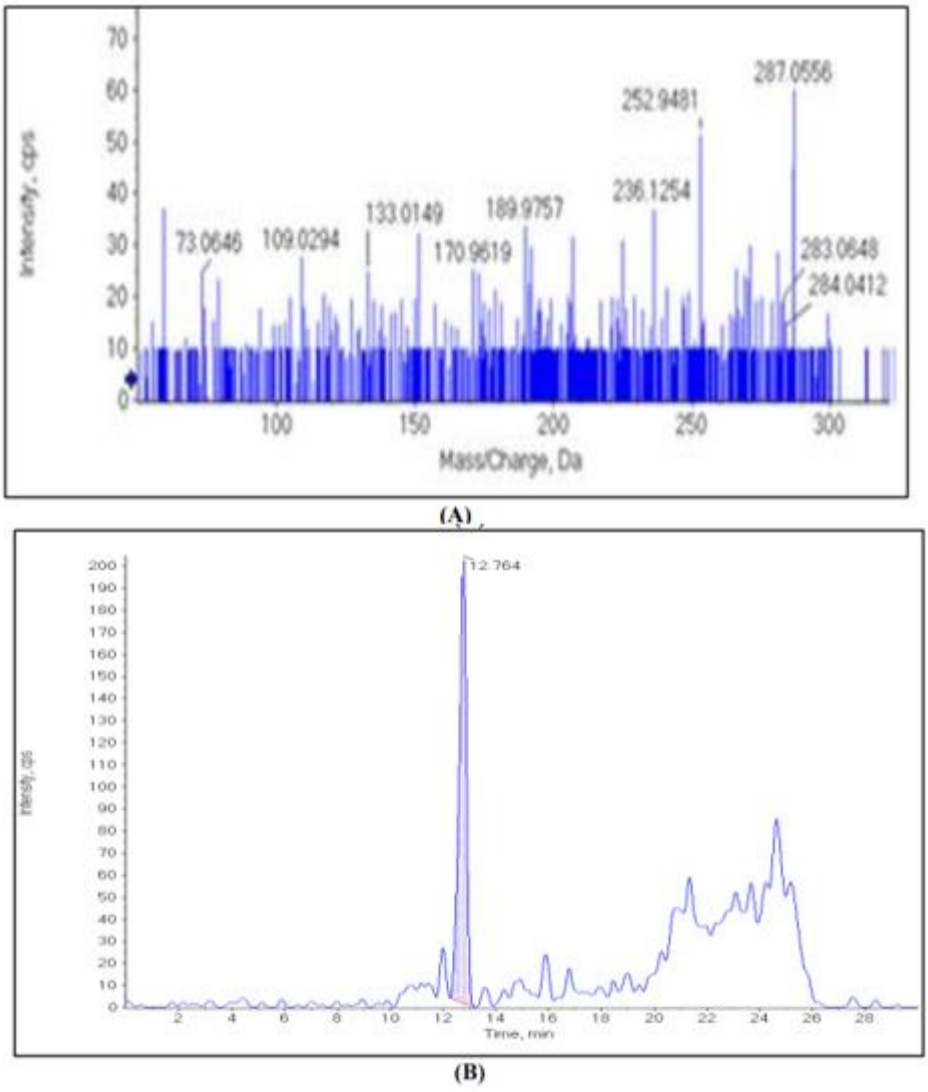
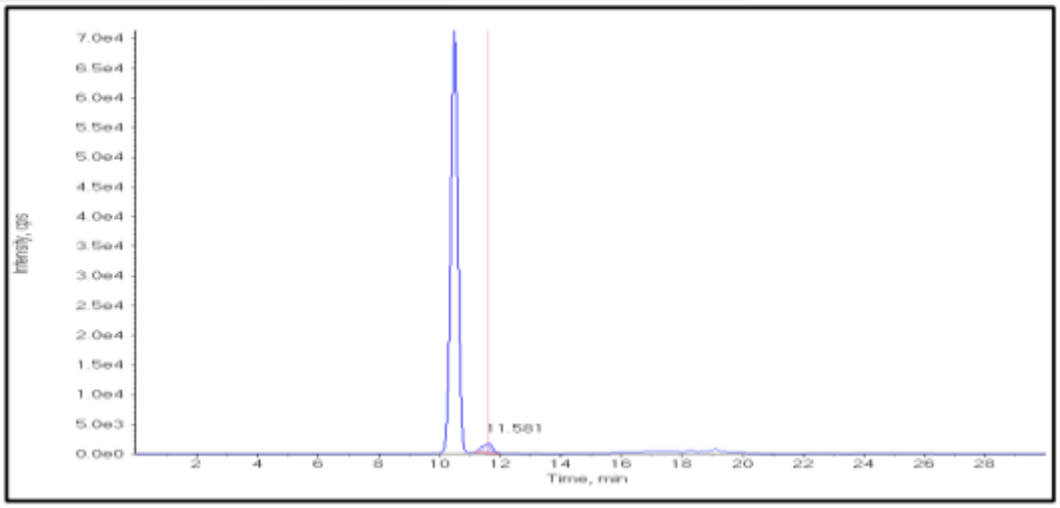


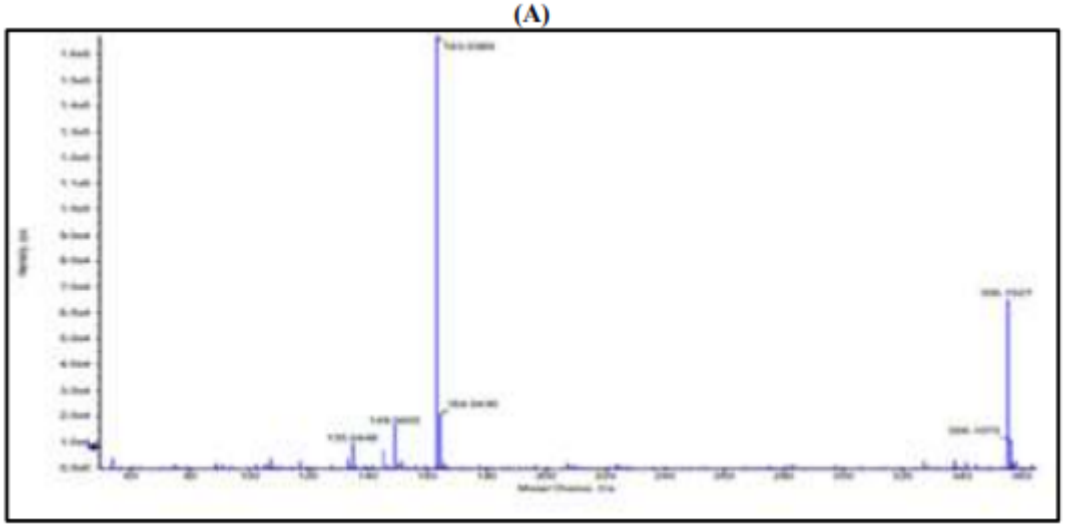
Figure 6. (A) Extracted ion chromatogram of luteolin, and (B) full scan product ion mass fragmentation spectra of the isolated luteolin.

The full-scan mass spectrum of the isolated luteolin showed a molecular ion peak at **m/z 287.05 [M+H]^+**, which was selected as the parent ion. The major fragmentation ions observed were **m/z 252 ($C_{15}H_8O_4^+$)**, **m/z 133 ($C_8H_5O_2^+$)**, and **m/z 109 ($C_6H_4O_2^+$)**. These mass spectral data are consistent with previously reported values for luteolin in the literature (Pitakpawasutthi et al., 2016)

Similarly, the chromatographic and mass fragmentation spectra for the isolated chlorogenic acid are shown in **Figure 7**.



(A)



(B)

Figure 7. (A) Extracted ion chromatogram of chlorogenic acid, and (B) full scan product ion mass fragmentation spectra of the isolated chlorogenic acid.

The full-scan mass spectrum of isolated chlorogenic acid revealed a molecular ion peak on M/Z 335.1027 [m+h]^+. The most prominent piece ions were considered M/z 163 ($c_9h_7o_3^+$), base peak, and M/Z 135 ($C_8h_7o_2^+$). These fragmentation patterns closely align with those reported for chlorogenic acid in pre -studies (Sarve, 2020(

DISCUSSION

Phytochemical screening and data presented in Table 2 confirm that the Cassia Glaka plant grown in Iraq is particularly rich in polyphenolic compounds. These compounds were mainly found in ethyl acetate (H3) and N-Butanel (H4) fractions.

Quantitative results showed that the ethyl acetate fraction contains polyphenols, especially luteolin, compared to an en-buttet fraction, which contained a small amount of chlorogenic acid. This difference can be attributed to the high solubility of some polyphenolic compounds in ethyl acetate or the order and efficiency of the calibration process.

The successful isolation of luteolin from Cassia Glaka provides scientific support for some traditional therapeutic uses of the plant, especially its antidiabic activity. Previous research has shown that luteolin fasting contributes to reducing blood sugar and HBA1C levels (Karakaya et al., 2020 (

On the other hand, the identity of chlorogenic acid helps explain the antioxidant properties of plant extracts, as chlorogenic acid is well documented to its powerful antioxidant effects (Sethuraman at al., 2021)

CONCLUSION

Based on the findings obtained, the following conclusions can be drawn:

1. The phytochemical analysis of the Cassia Glaka plant grown in Iraq, except for its seeds, discovered that air parts are rich in

polyphenolic compounds, especially polyphenolic compounds.

2. Both luteolin and chlorogenic acid were successfully detected through analytical TLC and HPLC, quantitatively measured using quantitative HPLC, and later separated using the preparation liquid chromatography (PLC). Their identity was confirmed compared with standard reference compounds and verified through LC-MS/MS Q-TOF analysis.
3. The results of this investigation closely align with the conclusions mentioned in the previous studies conducted on the species of this plant.

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