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# Isolation, Identification, and Quantification of Two Compounds from *Cassia glauca* Cultivated in Iraq

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## Abstract

The *Cassia glauca* Lam. is the tree that belongs to the Fabaceae family and is native to India has many uses in indigenous systems of medicine, folk medicine, and traditional Brazilian medicine. Has many pharmacological activities such as anti-diabetic, antibacterial, antifungal, antioxidant, anti-hemolytic, anticancer, cardio-protective, and Hepato-protection. The aim of study is to Isolation, identification, and quantification of some compounds from aerial parts of *Cassia glauca* since no phytochemical investigation had previously been done in Iraq for this plant. The aerial parts were defatted in n. hexane for 48 hours. The defatted materials were extracted in 85% ethanol using the hot method (soxhlet), then the extract was fractionated using different solvents (chloroform, ethyl acetate, and n-butanol). High-performance liquid chromatography (HPLC), was used for identification and quantification by using authenticated standards, and preparative layer chromatography (PLC) was used for the isolation of the identified compounds. The isolated compounds were identified after isolation by liquid chromatography Mass Spectrometry LC-MS/MS-Q-TOF method. The different chromatographic and spectroscopic methods results indicate the presence of luteolin and chlorogenic acid in the ethyl acetate fraction and n-butanol fraction respectively and estimated the quantity as (130.77µg/1g) for luteolin, and (0.0006%) for chlorogenic acid from 50g of plant material.

Keywords: ***Cassia glauca*, luteolin, Chlorogenic acid, HPLC, and LC-MS/MS-Q-TOF.**

# عزل، تعريف، و تحديد كمية لمركبين من نبات الكاسيا جلوكا المزروع في العراق

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**الخلاصه**

الكاسيا جلوكا هي شجره تنتمي الى عائله البقوليات وموطنها الاصلي الهند .لها استخدامات عديدة في أنظمة الطب الشعبي والطب البرازيلي التقليدي. لديها العديد من الأنشطة الدوائية مثل مضاد لكلا من للسكري , للبكتيريا ,الفطريات , للاكسده, لتكسر الدم و السرطان بلاضافه الى حماية القلب والكبد. الهدف من الدراسه هو عزل ,تعريف وتحديد كمية لبعض المركبات من الاجزاء الهوائية لنبات الكاسيا جلوكا حيث لم يتم اجراء اي دراسة تحقيق كيميائي سابقا لهذا النبات في العراق . تم ازاله الدهن من الاجزاء الهوائية للنبات بأستخدام الهكسان لمدة ٤٨ ساعه .ثم اجراء الاستخلاص بأستخدام ٨٥٪ من الايثانول بأستخدام الطريقة الساخنه , ثم تجزئه المستخلص بأستخدام مذيبات مختلفه (كلوروفورم, اسيتات أيثيل ,والبيوتانول). وبأستخدام الكروماتوغرافيا السائله عاليه الاداء ,كروماتوكرافيا الطبقة التحضيريه و طريقة قياس الطيف الكتلي تم تعريف وتحديد كمية المركبات المعزوله . تشير نتائج الطرق الكروماتوجرافية والطيفية المختلفة لوجود اللوتولين وحمض الكلوروجينك في جزء اسيتات الاثيل وجزء البيوتانول وقدرت كميتها بـ (١٣٠,٧٧ ميكروغرام / ١ جرام) من اللوتولين و(٠,٠٠٠٦٪) لحمض الكلوروجينيك على التوالي من ٥٠ جرام من المادة النباتية.

**الكلمات المفتاحية: كاسيا جلوكا ، لوتولين ، حمض الكلوروجينيك ، الكروماتوغرافيا السائله عاليه الاداء ، و قياس الطيف الكتلي.**

## Introduction

*Cassia glauca* is a leguminous tree with glabrous branches, native to the East India, distributed from the Himalayas, in India through Ceylon and the Polynesian island to Australia [1, 2]. The preferred climate zones are tropical and subtropical regions including Southeast Asia, Africa, and West India, and it is known to have escaped into the wild, naturalizing in many of these places and also being cultivated in Iraq [3]. All the *Cassia* species have diverse economic uses and because of their floriferous, ornamental beauty and the fallen petals carpet the ground beneath them which enhance their cultivation along avenues in all warm countries [2], besides that *Cassia glauca* has many traditional uses as central nervous system depressant, purgative, antimalarial, and diuretic [4] . The seeds oil is used in the indigenous system of medicine for the treatment of skin diseases and leucoderma [5]. In the case of traditional Brazilian medicine, it has been used for the treatment of flu, cold, fever, and headache [6].

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while the decoction of the roots is commonly used to treat snake bites [7]. In general, phytochemical verification performed on the plant revealed the presence of polyphenolic compounds such as flavonoids and tannins. Also contain glycosides, carbohydrates, alkaloids, steroids, anthraquinones, anthracenes, and their derivatives [8, 9] . While the seeds showed the presence of alkaloids, carbohydrates, sterols, proteins, amino acids, and saponins. Finally, many reports have shown that Cassia species possess anti-diabetic, antimicrobial, anti-malarial, anti-cancer, and hepato-protective activities [10-12] .

Polyphenols are natural bioactive compounds that form a diverse group in which at least two hydroxyl groups are present in their chemical structure. Due to their prevalence in the plant world, polyphenols constitute a significant food component and an essential class of antioxidant-containing chemicals because of their redox characteristics, which allow them to operate as reducing agents, hydrogen donors, oxygen quenchers, metal chelators, and ferryl hemoglobin reductants and recent study suggest that there is synergistic activity between polyphenol compounds, which distinguishes them from other naturally bioactive chemicals. [13-15] . In recent years, there has been an upsurge in the number of studies focused on identifying and quantifying these compounds, particularly in plants that have been utilized for centuries in traditional medicine [16] . So, due to the biological importance of these compounds, identification, isolation, and quantification for some polyphenol compounds from the *Cassia glauca* plant cultivated in Iraq were made.

## Materials and Methods

### *Plant collection*

The aerial parts mixture of *Cassia glauca* were taken from the side road of Baghdad city / Al-Karkh in April 2021. The plant was specified and authenticated by Prof. Dr. Sukaena Abass - Department of Biology-College of Sciences-University of Baghdad. The parts were washed, dried at room temperature, and crushed by using a mechanical grinder to powder.

### *Extraction method*

The dried powdered aerial plant material (50 g) were defatted by maceration in n-hexane solvent for 48hr. then evaporated to dryness under reduced pressure by rotary evaporator, weighted and designated as H1 fraction, then the defatted powder is extracted by soxhlet apparatus with 85% ethanol until complete exhaustion. This extract was evaporated to dryness by a rotary evaporator to get a dark green precipitate designated as a crude extract. The crude extract was dissolved in water and successively fractionated by using a separatory funnel with chloroform, ethyl acetate, and n-butanol solvents [17] designated as (H2, H3, and H4) respectively. Each fraction is dried by a rotary evaporator to dryness and weighed for further analysis.

**Qualification and quantification of polyphenol compounds in H3 and H4 fractions of *Cassia glauca* plant**

**1. Preliminary phytochemical screening of polyphenol*:*** The H3 and H4 fractions of the *Cassia glauca* plant were tested with (5% ferric chloride and 10% lead acetate) tests in test tubes [18] as listed in Table (1):

**Table 1. Qualitative phytochemical analysis of *Cassia glauca* H3 and H4 fraction.**

| Constituent | Test |
|---|---|
| Tannin and phenolic compounds | 5% ferric chloride test: 1ml of H3 and H4 fractions were mixed with 1ml of 5% ferric chloride solution in test tube. The formation of a deep green or deep blue color indicates the presence of tannins and polyphenol compounds. |
| | 10% lead acetate test: 1ml of H3 and H4 fractions were mixed with 1ml of a 10% lead acetate solution in test tube. The formation of white precipitate indicates the presence of tannins and polyphenol compounds. |

**2.Identification of two compounds by HPLC***:* HPLC was done for the identification of active constituents for H3, H4 fractions and, the retention times of analyzed samples were compared to retention times of standard materials under the same conditions as following :

For H3 fraction and Luteolin authenticated standard: the flow rate was 0.8 mL/min, and at $\lambda = 278$ nm, the mobile phases were 1% aqueous acetic acid solution (A) and 100% methanol(B), gradient elution [19] was used as in Table (2):

**Table 2. HPLC gradient elution program for H3 fraction and luteolin standard.**

| Solvent A% | Solvent B% | Strart from _ to _ |
|---|---|---|
| 90% | 10% | 0 to 6 min |
| 84% | 16% | 7 to 25min |
| 72% | 28% | 26 to 37 min |
| 65% | 35% | 38 to 47 min |
| 50% | 50% | 48 to 64 min |
| 90% | 10% | 65 to 70 min |

While for H4 fraction and Chlorogenic acid authenticates: the mobile phases were 0.05 % of tri-fluoroacetic acid in deionized water (A) with (B) 0.05 % of tri-fluoroacetic acid in methanol pH= 2.5, gradient program from gradient program from 0% B to 100% B for 15 min, the flow rate was1ml/min, and at λ = 280 nm [20]. In addition to performing HPLC analysis for chlorogenic acid standard and F4 fraction, a small amount of the standard was spiked into the F4 fraction sample to establish peak identity [21].

**3. Isolation of two polyphenolic compounds by preparative layer chromatography (PLC**): Preparative layer plates of thickness 0.5 mm of silica gel $GF_{254}$nm (20x20cm) manufactured in Taiyang, China, were utilized. The plates were activated for 30 minutes at 110 ° C [22]. For the isolation of proposed luteolin from H3 fraction, mobile phase S1 (Ethyl acetate: formic acid: hexane) (7.7:1.3:0.9) was used [23], whereas for the isolation of proposed chlorogenic acid from H4 fraction, mobile phase S2 (formic acid: Ethyl acetate: dichloromethane: acetic acid: water) (0.6:6.4:1.6:0.6:0.7) was used [24, 25]. The detection was conducted using UV light at 254 nm, 366 nm, and 5 % ethanolic KOH [26, 27]. The bands of proposed compounds were detected by matching with their respective authenticated reference standards.

4. **Quantification of the polyphenol compounds detected by HPLC:** for luteolin quantification by calibration (standardizing) of the HPLC method, the external type method was used in the (Ministry of Science and Technology) by SHIMADZU Liquid chromatograph LC-2010AHT, where a solution of known different concentrations of the luteolin authenticated standard (5, 10, 15, and 20) µg/ml vs. detector response (peak area) was plotted. Using linear regression analysis to construct the standard curve, the ($r^2$) correlation coefficient of the regression line is determined for proposed luteolin in H3 fraction utilizing the regression equation derived first from their standard curve[28,29].

While for the Chlorogenic acid quantification in the (Iraqi National Center for Drug Control and Research) by SHIMADZU Liquid LC-20AD, the area under the curve obtained under identical chromatographic conditions from the analyzed H4 fraction and authentic chlorogenic acid standard were used in the following equation [30]**.**

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

**AUC: Area under the curve Conc. St: Concentration of Standard DF: Dilution factor**

**5. Identification and characterization of isolated polyphenol compounds by liquid chromatography Quadruple Time-of-Flight Mass Spectrometry LC-MS/MS-Q-TOF method*:*** Analytical LC/MS/MS-Q-QTOF was done in Jordan University of Science and Technology, Irbid, Jordan. The following liquid chromatography conditions were used: column-GL-Science-C18-250mmx4.6 (5um particle size) –Japan, column oven at 35 ° C, injection volume of 10 um, flow rate equal to1 ml/min, run time equal to 25 min, solvent (A) was formic acid/H2O, and solvent (B) was acetonitrile according to the following gradient system in the Table (3). The mass parameter: LCMSMS-Q-TOF model X500 QTOF, Software AB-Siecx-OS, Ionization mode ESI Positive, Scan range (50-800 mz), and Ion source voltage 5500V.

**Table3. Gradient System for LC/MS/MS-Q-TOF Method.**

| TIME | %A | %B |
|---|---|---|
| 0 | 90 | 10 |
| 5 | 90 | 10 |
| 15 | 10 | 90 |
| 20 | 10 | 90 |
| 20.1 | 90 | 10 |
| 25 | 90 | 10 |

## Results

Weight and percent of the yield of each fraction resulted from 50g after the defatting process (H1 fraction) and from crude extract after fractionation (H2, H3, H4) fractions are shown in Table (4).

**Table 4. The weight and the percentage of yield of each fraction from *the Cassia glauca* plant.**

| The Percent yield of the plant of each fraction | The Weight of fraction | The fraction | The Crude extract weight from soxhlet | The Dried plant weight |
|---|---|---|---|---|
| 23.26% | 1.63g | H1 | 11.53g | 50g |
| 3.24% | 1.62g | H2 | | |
| 10.02% | 5.01g | H3 | | |
| 9.64% | 4.82g | H4 | | |

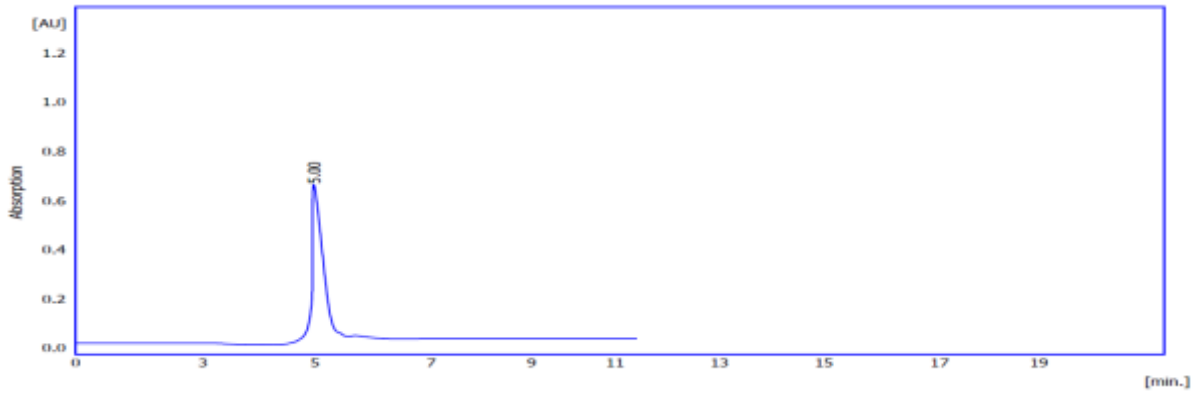
***Qualification and quantification of polyphenol compounds results***

**1. The preliminary qualitative result:** The results of the H3 and H4 fractions are shown in Table (5) below:

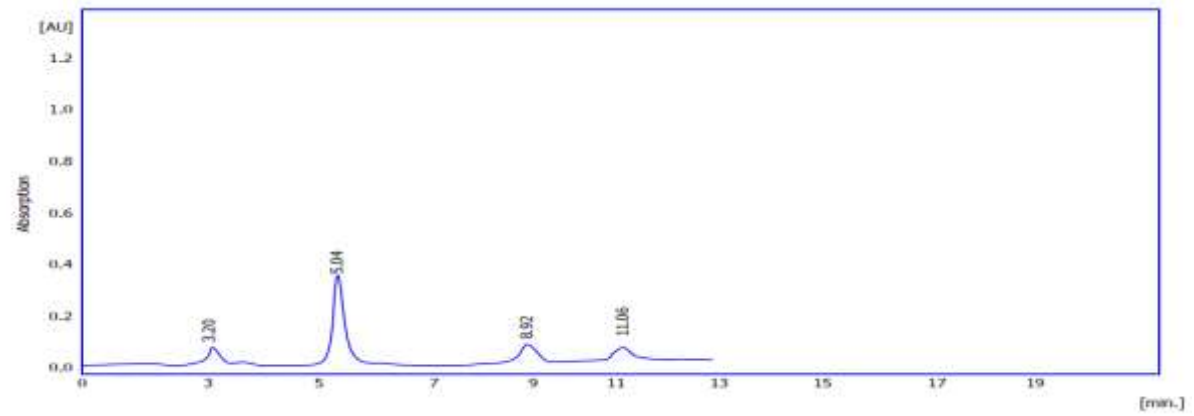
**Table 5. preliminary qualitative results**

| Fraction | Test name | Result |
|---|---|---|
| H3 | 5% ferric chloride test | Positive |
| H4 | 10% lead acetate | Positive |

**2. Identification of two compounds by *HPLC* results*:* the results** obtained from HPLC chromatogram showed that one of the expected retention times in H3 fraction was identical to the authenticated (Luteolin) standard retention time as illustrated in Figure (1), while in the H4 fraction the retention time of one expected compound was identical to the retention time of (Chlorogenic acid) authenticated standard retention time as illustrated in Figure (2).

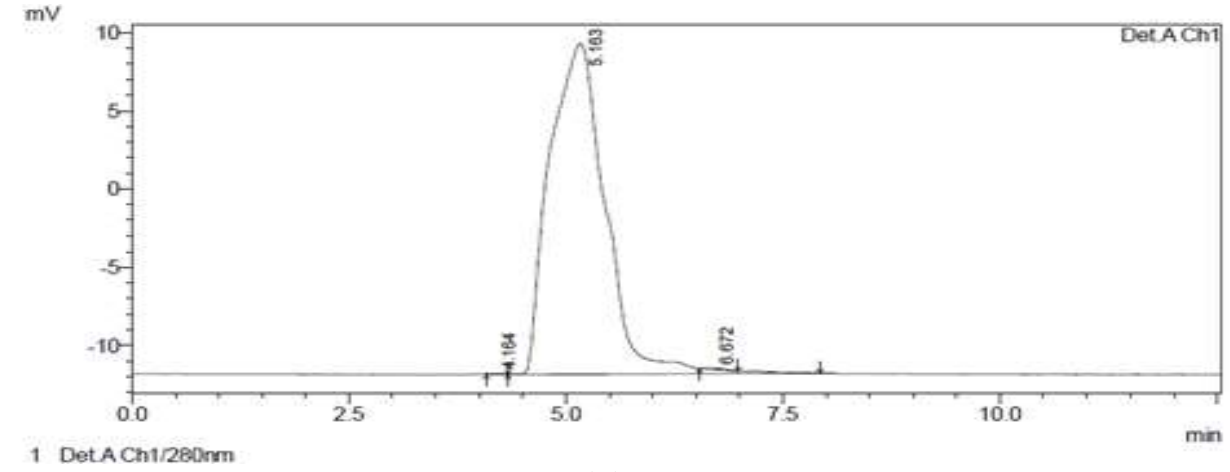



**(A)**

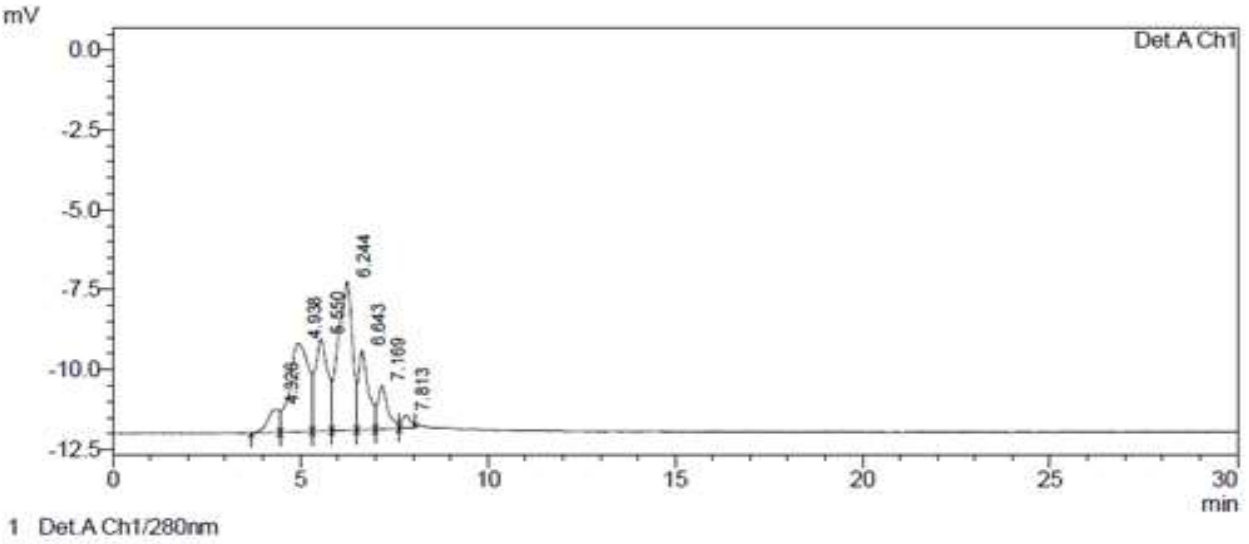



**(B)**

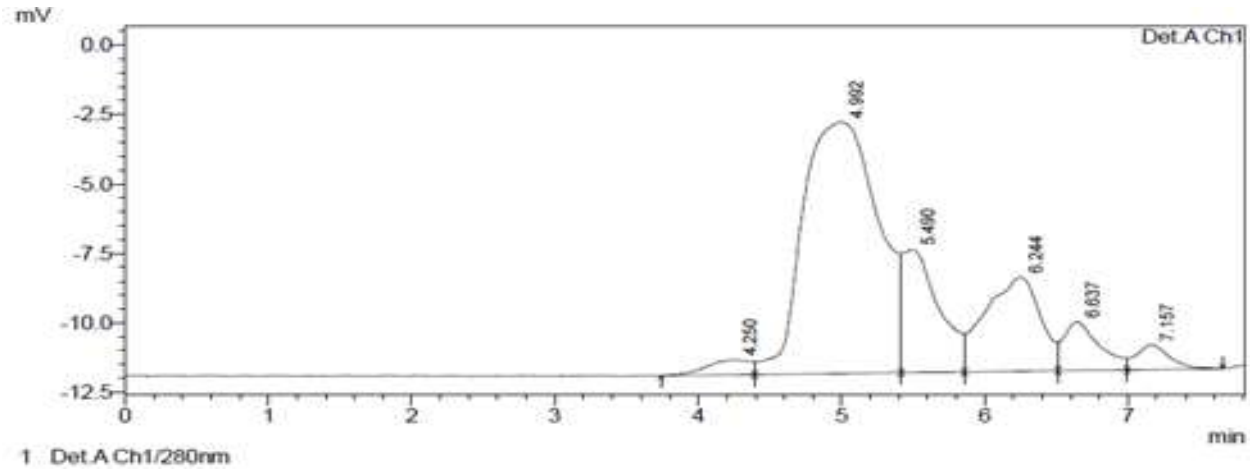
**Figure 1. HPLC chromatogram of luteolin standard (A), HPLC chromatogram of H3 fraction (B).**




**(A)**




**(B)**




**(C)**

**Figure 2.HPLC chromatogram of Chlorogenic acid standard (A), HPLC chromatogram of H4 fraction (B), HPLC chromatogram of H4 fraction spiked by Chlorogenic acid standard(C).**

**3. Isolation of two polyphenol compounds by *PLC results:*** the PLC technique is less complicated than the HPLC method, equipment used is simple and allows parallel runs of standards on one plate for detection. In addition to the use of spray reagent, which is not applicable to the HPLC technique [31,

[32], but still the quality (pure) and quantity of the isolated compounds by HPLC are higher than those obtained by PLC. The isolated bands from the H3 and H4 fractions from the plates are shown in Figures (3 and 4) which were extracted from the sorbent by ethanol, filtered, and evaporated to dryness. Upon re-crystallization, a yellow and white crystals were obtained from H3 and H4 fractions respectively.


**Figure 3. Preparative layer chromatography plate of detected luteolin, detected at 254nm. Developed in S1 (Ethyl acetate: formic acid: hexane) H3: ethyl acetate fraction, L: luteolin standard.**

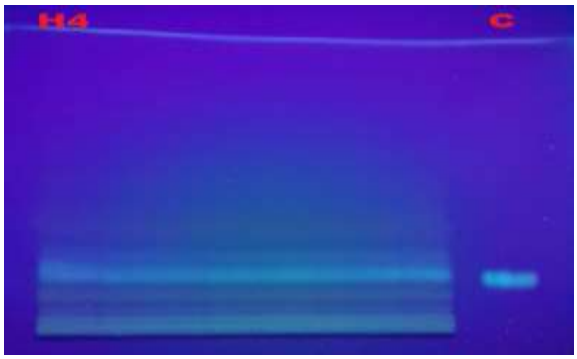



**Figure 4. Preparative layer chromatography plate of detected chlorogenic acid, developed in S2 (formic acid: Ethyl acetate: dichloromethane: acetic acid: water), Detected at 365nm. H4: n-butanol fraction, C: chlorogenic acid standard.**

**4. *Quantification of the polyphenol compounds detected by HPLC results:***

The amount of the proposed luteolin in the H3 fraction was determined by the regression equation (Y=15.63333*X) obtained from the calibration curve, as shown in Figure (5) below.

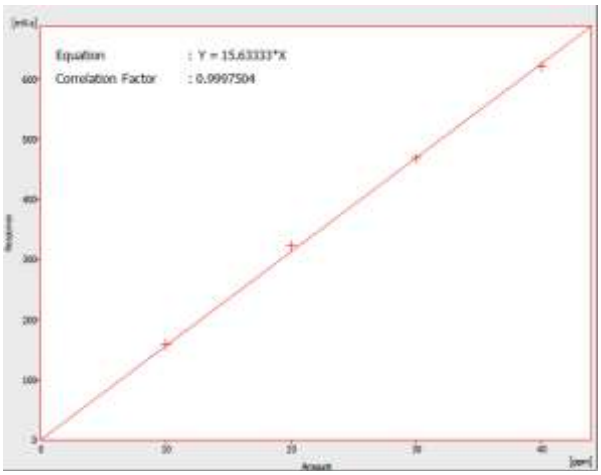



**Figure 5. Calibration curve of proposed Luteolin by HPLC**

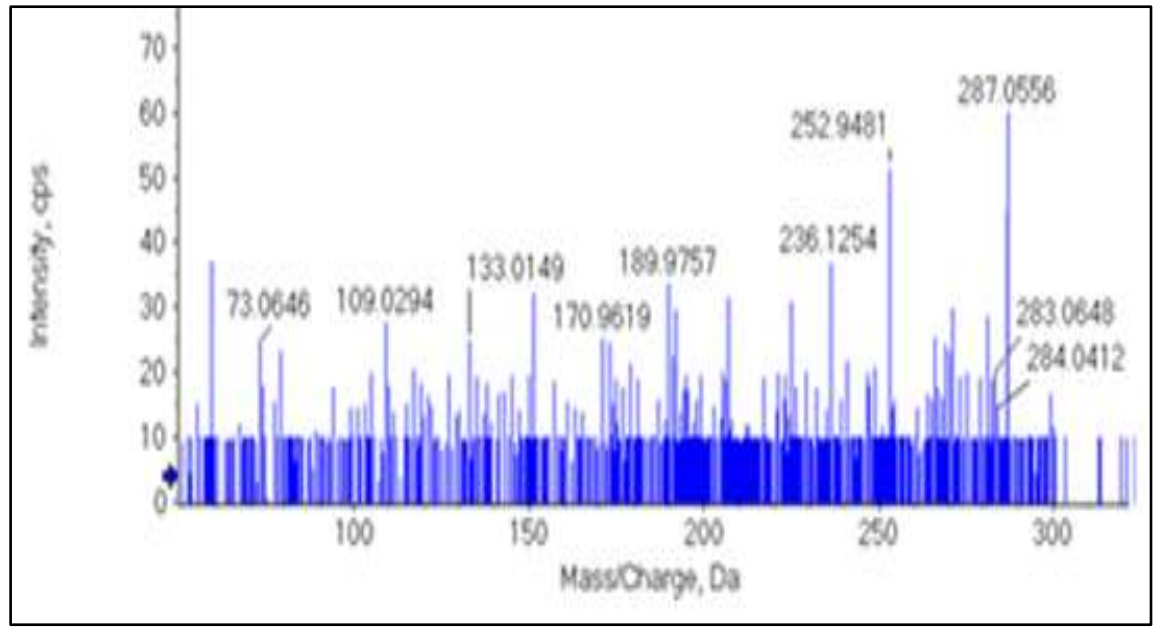
The amount of isolated proposed luteolin from H3 fraction and the percent of isolated proposed chlorogenic acid from H4 fraction are listed in Table (6).

**Table 6.The amount in (µg/g) of isolated proposed luteolin and the percent of the isolated proposed chlorogenic acid.**

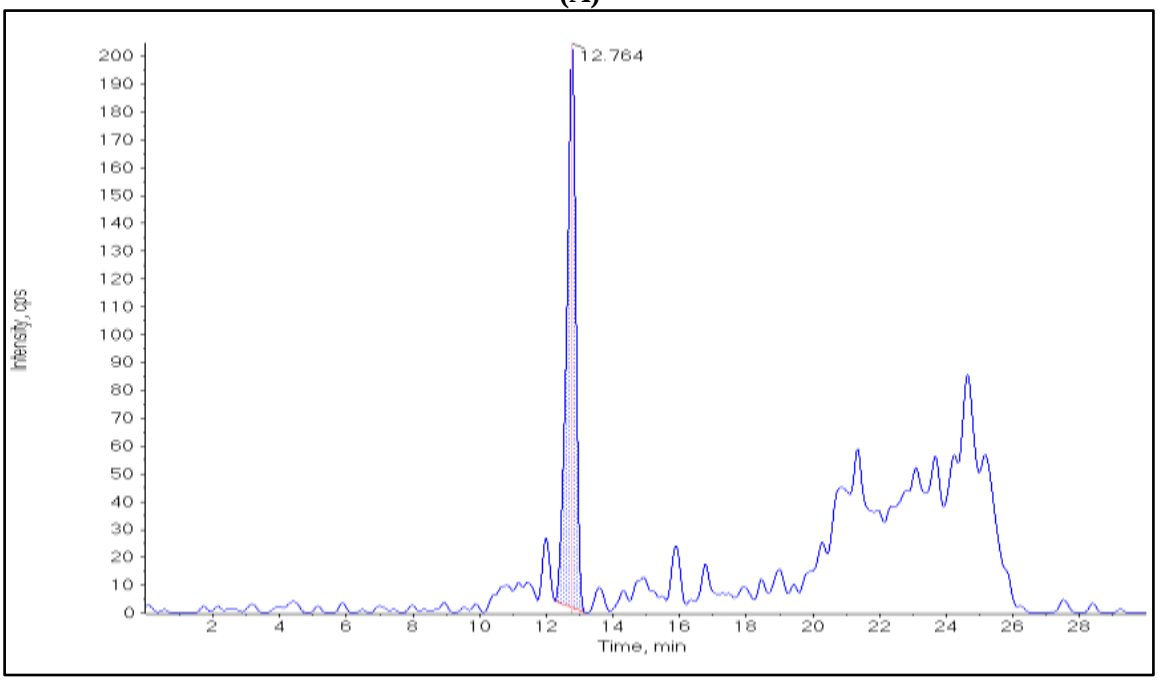
| Amount of plant material | Amount of luteolin in H3 fraction | Percent of chlorogenic acid in H4 fraction |
|---|---|---|
| 50g | 130.77 µg/g | 0.0006% |

**5. LC-MS/MS-Q-TOF results:**

The LC–TOF MS was carried out for further identification and characterization of isolated compounds by PLC and the isolated luteolin LC/MS chromatogram and ion mass fragmentation spectra are depicted in Figure (6).




**(A)**




**(B)**

**Figure 6. Luteolin extracted ion chromatogram (A), a full scan of isolated luteolin product ion mass fragmentation spectra (B).**

From the full scan mass spectra of the isolated luteolin, the $[M+H]^+$ ion with m/z was 287.05, was selected as a molecular ion peak, and the most abundant fragments were 252 m/z ($C_{15}H_8O_4^+$), 133 m/z($C_8H_5O_2^+$), 109 m/z($C_6H_4O_2^+$). All these data were closely similar to that reported in literature for luteolin[33].

While for the isolated Chlorogenic acid LC/MS chromatogram and ion mass fragmentation spectra are shown in Figure (7).

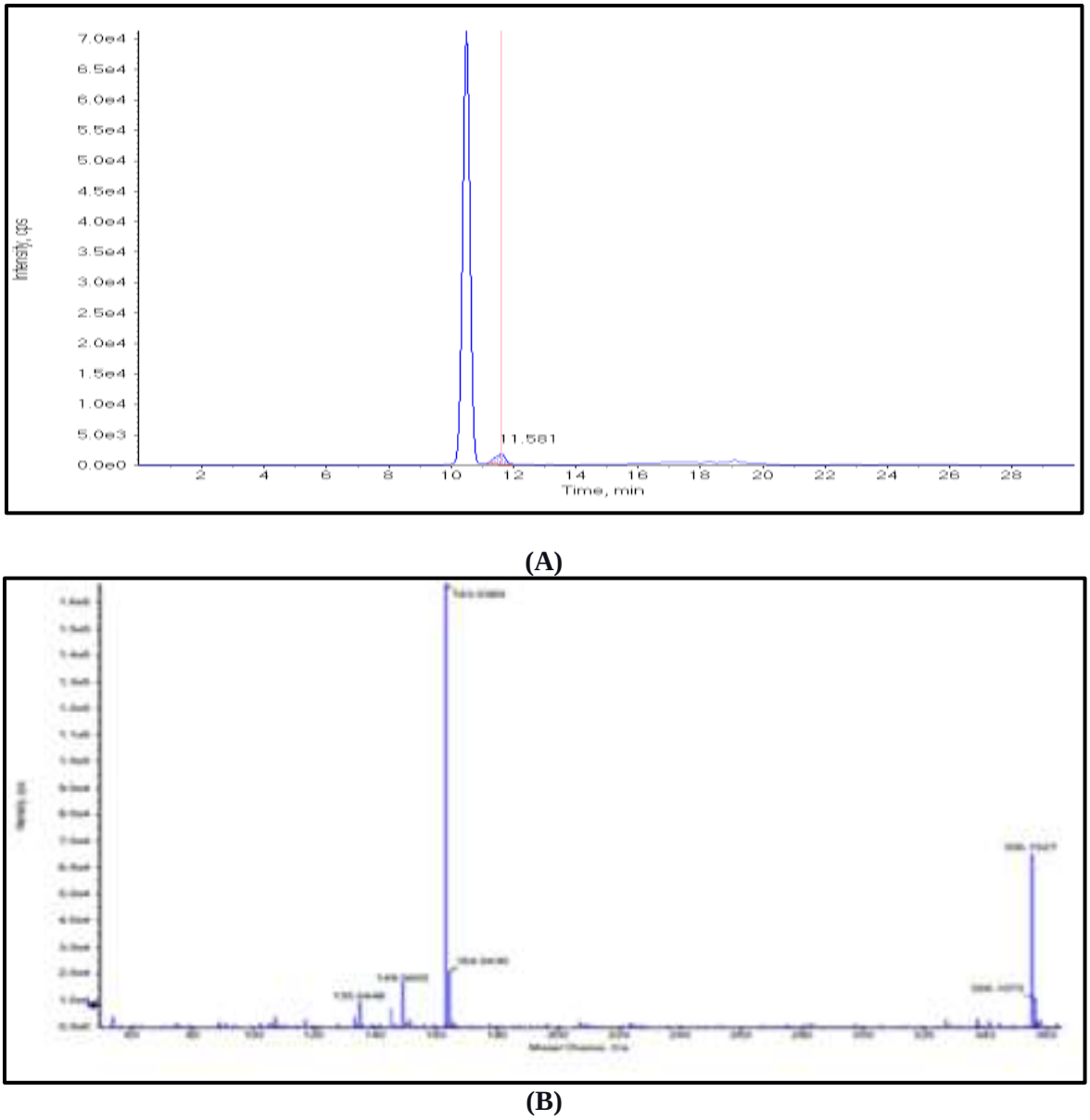



**Figure 7. chlorogenic acid extracted ion chromatogram (A), a full scan of isolated chlorogenic acid product ion mass fragmentation spectra (B).**

From the full scan mass spectra of the isolated Chlorogenic acid the $[M+H]^+$ ion with m/z was 335.1027, was selected as a molecular ion, the most abundant fragments are 163 m/z base peak($C_9H_7O_3^+$), and 135 m/z ($C_8H_7O_2^+$) .and All these data were closely similar to that reported in literature for Chlorogenic acid[34] .

## Discussion

Results from phytochemical screening and Table (2) indicate that the Iraqi *Cassia glauca* plant is extremely rich in polyphenolic compounds, which are found in the ethyl acetate fraction and also in the n-butanol fraction. From the concentration of the isolated two compounds, luteolin, and chlorogenic acid it is obvious that the ethyl acetate fraction has a much higher quantity of polyphenol compounds than the n-butanol fraction. This might be due to the solubility of the polyphenol compounds in the ethyl acetate or due to the order of fractionation.

The isolation of luteolin from the *Cassia glauca* plant explain some of the traditional uses of plant extract effect like antidiabetic as it has been proven by some studies that luteolin has suppression to fasting blood glucose and $HbA_{1c}$ [35, 36]. While the isolation of chlorogenic acid might explain the antioxidant effect of the plant extract since the studies had proved the antioxidant effect of the chlorogenic acid [37, 38].

## Conclusion

The following points were drawn based on the previous findings:

**1.** Phytochemical screening of the *Cassia glauca* plant cultivated in Iraq was done for aerial parts except for seeds and the results indicate that the plant is highly rich in polyphenol compounds.
**2.** The luteolin and chlorogenic acid were detected by analytical TLC, and analytical HPLC, quantified by analytical HPLC, and isolated by PLC compared with their corresponding authenticated standards, identified by *LC-MS/MS Q TOF*.
**3.** Most of the results of this study were harmonious with the results of research carried out on this plant.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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## Ethics Statements

This study was conducted in vitro on plant therefor no need for ethical approval.

## Author Contribution

Shamam Kanaan M. Abdulkareem: contributed to data gathering, analysis, practical (follow the procedure) and written parts of the study. Enas Jawad Kadhim gave final approval and agreement for all aspects of the study, supervision, revision, and rearrangement.

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