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Isolation and characterization of luteolin and ferulic acid from *Plumbago auriculata* cultivated in Iraq

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Abstract

Plumbago auriculata is a perennial plant belong to *Plumbaginaceae* family. It is an endemic genus of 18 species in South Africa. All plant parts have many phytochemical compounds appearing several pharmacological activities. The study was aimed to identify, isolate and structural elucidations of the bioactive poly-phenolic compounds (luteolin and ferulic acid) from *Plumbago auriculata* cultivated in Iraq by various chromatographic and spectroscopic techniques. The plant materials were defatted with n-hexane solvent by maceration for 48hr, and extracted by soxhlet apparatus using 85% methanol solvent, then fractionated with ethyl acetate solvent. High performance liquid chromatography (HPLC) was used to identify luteolin and ferulic acid in the fraction of ethyl acetate. The identified compounds were isolated using HPLC, then characterized and identified the isolated compounds using various detecting techniques including, HPLC by comparing with their standards and spiking, Fourier transform infrared spectroscopy (FTIR), in addition to the liquid chromatography mass spectrometry (LC-MS/MS). The results of chromatographic and spectroscopic techniques confirmed the existence of luteolin and ferulic acid in the fraction of ethyl acetate, as it concluded that the data for the isolated compounds were coincided with that reported for the compounds in literatures. These isolated polyphenolic compounds which have various pharmacological activities may demonstrate some of the therapeutic uses of *P.auriculata* plant.

Keywords: Ferulic acid, Flavonoids, FTIR, HPLC, LC-MS/MS, Luteolin, Phenols.

Introduction

The medicinal plants were recognized as an essential source of therapeutically active medicines due to the presence of secondary metabolites which considered a potential source of drugs. Natural products are safer healthier, and more dependable than synthetic products [(1)]. Medicinal plants had played an important role in folk remedies and herbal medicine around the world due to their accessibility, availability, potential for efficacy and affordability. Depending on the world health organization, 80% of people still used these natural products to decrease the side effects of using the synthetic drugs [(2-4)]. In addition, the increment using of medicinal plants in the developed countries was lead to detect many drugs and chemotherapeutic from these plants [(1)]. *Plumbago auriculata* is considered as a perennial, ornamental and medicinal plant belong to the family of *Plumbaginaceae* [(5)]. It is an endemic genus of 18 species in South Africa [(6)]. *P. auriculata* plant has sky blue flowers with trusses of pale and evergreen throughout the year [(7)]. The roots and aerial parts of *Plumbago auriculata* have an extensive range in pharmacological usages [(8,9)]. All plant parts have many phytochemical compounds appearing several pharmacological activities like antioxidant, antimalarial, antifungal, antimicrobial, anti-infertility, anti-inflammatory, anticancer, cardiotonic and hypoglycemic [(10,11)]. The phytochemical investigation was pointed that the alcoholic extract of *P. auriculata* showed the existence of naphthoquinones, tannins, saponins, flavonoids, carbohydrates, proteins and phenols which are the most abundant compounds [(12)]. Many studies were reported that the phenolic compounds have potential health benefits due to their strong antioxidant nature [(13-15)].

Luteolin compound (3′,4′,5,7-tetrahydroxyflavone) was described as a naturally flavonoids, that are the largest group of phenolic compounds present in numerous plant species [(16)]. It has many pharmacological activities like neuroprotective [(17)], anti-tumor [(18)], anti-tuberculosis immunity [(19)] anti-inflammatory [(20)] and antioxidant properties [(21)].

New studies have revealed that luteolin can inhibit the cancer development through intervening with the cell cycle course, inhibiting propagation, stimulate apoptosis, also prevents the migration of cancer cells and invasion [22,23], the structure of luteolin is demonstrated in Figure. 1.

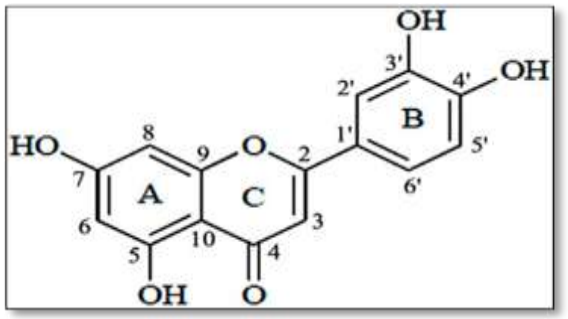

Figure 1. Luteolin chemical structure.

The phenolic compound, ferulic acid (4-hydroxy-3-methoxycinnamic acid) which present in the plants is committed as a phytochemical having potent antioxidant effect, and additional pharmacological effects such as anti-thrombosis, neuroprotective, antitumor, skin whitening, anti-inflammatory, antibacterial and UV absorptive effects [24,25], the structure of ferulic acid is shown in Figure. 2.

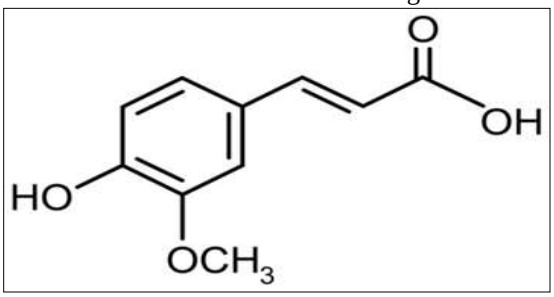

Figure 2. Ferulic acid chemical structure.

This study is the first phytochemical screening study of Iraqi Plumbago auriculata, aimed to identify, isolate and structural elucidations of the bioactive poly-phenolic compounds (luteolin and ferulic acid) by various chromatographic and spectroscopic techniques .

Materials and Methods

Preparation of plant materials

Whole plant of *P.auriculata* was obtained from farm in city of Baghdad in August 2023. The plant has been identified and authenticated by Assist. Prof. Dr. Israa Abdulrazaq Majeed at Biology Department, College of Science at the University of Baghdad. The plant was cleaned, dried in the shade, and grinded into coarse powder using mechanical grinder.

Extraction and fractionation

The powdered plant (about 100gm of whole plant) was weighted and defatted using 250ml of n-hexane solvent by maceration for 48hrs to get rid of wax and fatty materials [26]. Defatted plant materials were filtered, dried at room temperature and placed in a thimble for extraction by soxhlet apparatus for 16 hours using 750ml of 85% aqueous methanol as a solvent extractor. Then alcoholic extract was filtered, subjected to evaporation by rotary evaporator to get the crude extract [27]. Fractionation of the crude extract was performed through suspending in 250ml of distilled water, after that well partitioned in a separatory funnel using 250ml from ethyl acetate solvent, this procedure was repeated twice for the ethyl acetate fraction. Then the fraction has been dried using anhydrous sodium sulfate, filtered, subjected to evaporation by rotary evaporator and weighted to be assigned for the isolation of phenolic compounds [28].

Preliminary phytochemical screening

Test for flavonoids

Few milliliters of the fraction of ethyl acetate were mixed with little drops of 5% NaOH solution, when the yellow color appeared, a few drops of diluted HCL were added to get the colorless solution, this indicates the presence of flavonoids [27,29].

Test for phenols

Few milliliters of the fraction of ethyl acetate were mixed with little drops of 5% ferric chloride solution. The occurrence of a deep blue-green color suggests the existence of phenols [27,29,30].

Identification of luteolin and ferulic acid by high-performance liquid chromatography (HPLC)

The HPLC analysis was performed to select ferulic acid and luteolin present in the fraction of ethyl acetate using the instrument model SYKMAN (Germany), at the department of environmental and water research/Ministry of Sciences and Technology. The tested samples were compared with standard materials by their retention times under the same conditions [26].

The analysis of HPLC was prepared with a C18-ODS column (250 × 4.6 mm, 5 µm) using gradient mobile phase which consists of 95% acetonitrile + 0.01% Triflouroacetic acid as a solvent A, and 5% acetonitrile + 0.01% Triflouroacetic acid as a solvent B, the flow rate was at 1 mL/min, and the injection volume was 100 µL. Program of gradient was as follow: 10% A from 0–5 min; 25% A from 5-7 min; 40% A from 7–12 min; after that returning to primary conditions. A UV-visible detector was used to detect the phenolic compounds at 278 nm [31]. ***Isolation of luteolin and ferulic acid using high-performance liquid chromatography (HPLC)***

High performance liquid chromatography was carried out to isolate luteolin and ferulic acid from ethyl acetate fraction. The same chromatographic conditions and column used in the qualitative step were used for both the authenticated standards and fraction except the injection volume

was 300 µL and the flow rate was at 3 mL/min for separation process. By starting the collection of each of the two compounds from the beginning of the peak ending by the end of the peak that represent that compound [32].

Identification and characterization of the isolated compounds

The isolated luteolin and ferulic acid have been characterized by various chromatographic and spectroscopic techniques listed below:

1. Spiking analysis by analytical HPLC

This identification was done by taking10ppm/100 µL of the isolated samples which mixed with same amount of their standards to reanalyze by HPLC and compare the isolated compounds with their standards according to their retention times under the same conditions [33].

2. Fourier transform infrared spectroscopy (FTIR)

The characterization of the isolated constituents was further confirmed by FT-IR spectra using the instrument model Shimadzu (Japan) to identify the functional groups of the isolated ferulic acid and luteolin. It was carried out using KBr disc and the range of scanning was (4000-400 cm^{-1}).

3. Liquid chromatography mass spectrometry (LC-MS/MS)

LC-MS/MS analysis has been recorded in the Jordan University of Science and Technology, Jordan using the instrument model Shimadzu (Japan) according to the conditions as follow: column-GL-Science-C18-100mm x 4.6 (5um particle size), column oven was at 35 °C, injection volume was 5ul, flow rate was equal to 1 ml/min, run time was equal to 25 minute, Ionization mode was ESI Positive, Scan range (50-800 mz), and Ion source voltage 5500V, the used isocratic mobile phase was composed from water and 0.1% formic acid, in addition to acetonitrile: methanol (50: 50 v/v) and 0.1% formic acid [34].

Results

Quantity and percentage yield of fractions

Based on defatting, extraction, and fractionation by ethyl acetate solvent, the extracts of *P.auriculata* gave varying percentages. The amounts and percent yields of extracts in n-hexane, aqueous methanol, and ethyl acetate were determined and presented in Table 1.

Table 1. The quantities and percentage yield of the extracts with n-hexane, aqueous methanol, and ethyl acetate.

Fraction of plant extract	Quantity	Percentage yield
n-hexane	5 gram	5%
Aqueous methanol	16 gram	16%
Ethyl acetate	1 gram	1%

Preliminary phytochemical investigation

The existence of flavonoids and phenols was confirmed by the preliminary phytochemical analysis of ethyl acetate fraction, as demonstrated in Table 2.

Table 2. Preliminary screening of flavonoids and phenols in ethyl acetate fraction.

Test	Result
Flavonoids	+ (An intense yellow color which became colorless on addition of diluted acid)
Phenols	+ (Deep green color)

Identification of the two compounds using high-performance liquid chromatography (HPLC)

Results of the analyzed ethyl acetate fraction were revealed the existence of luteolin and ferulic acid compounds depending on their retention times as demonstrated in Table 3 as well as the chromatogram in Figure. 3 compared to that of luteolin and ferulic acid standards, as shown in Figure. 4 and 5 below:

Table 3. The retention time (R_t) of standards and the corresponding detected compounds in ethyl acetate fraction.

Standard used	R_t of standard peaks (min)	R_t of matched peaks (min)
Luteolin	5.92	5.92
Ferulic acid	4.05	4.00

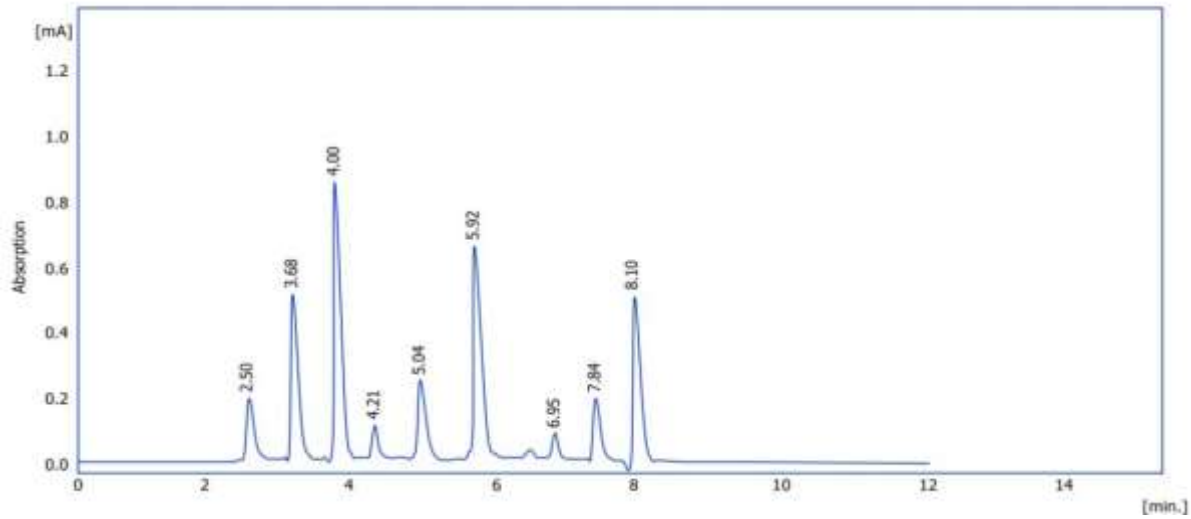

Figure 3. Chromatogram of HPLC for ethyl acetate fraction.

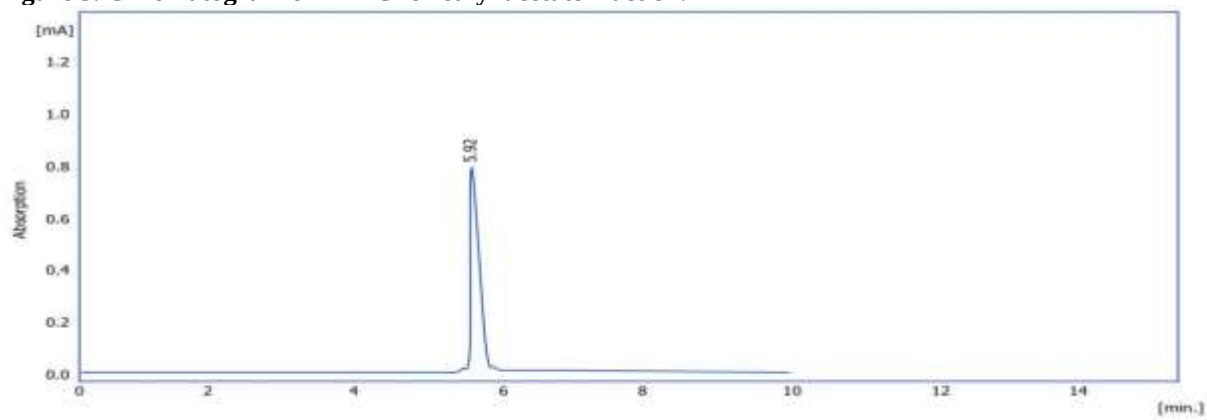

Figure 4. Chromatogram of HPLC for luteolin standard.

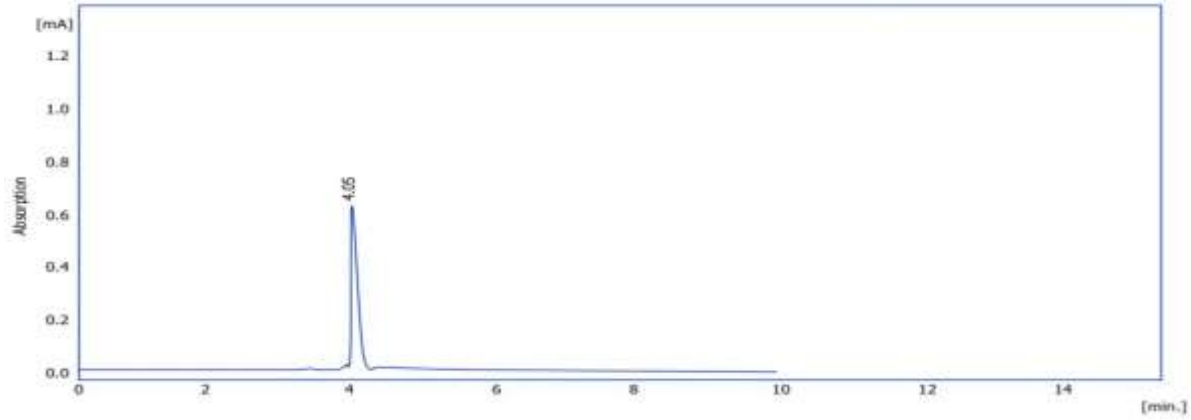

Figure 5. Chromatogram of HPLC for ferulic acid standard.

Isolation of luteolin and ferulic acid using high-performance liquid chromatography (HPLC)

Luteolin and ferulic acid compounds were isolated by HPLC using the chromatographic conditions that mentioned in the previous paragraphs, as shown in Figure. 6 and 7 below:

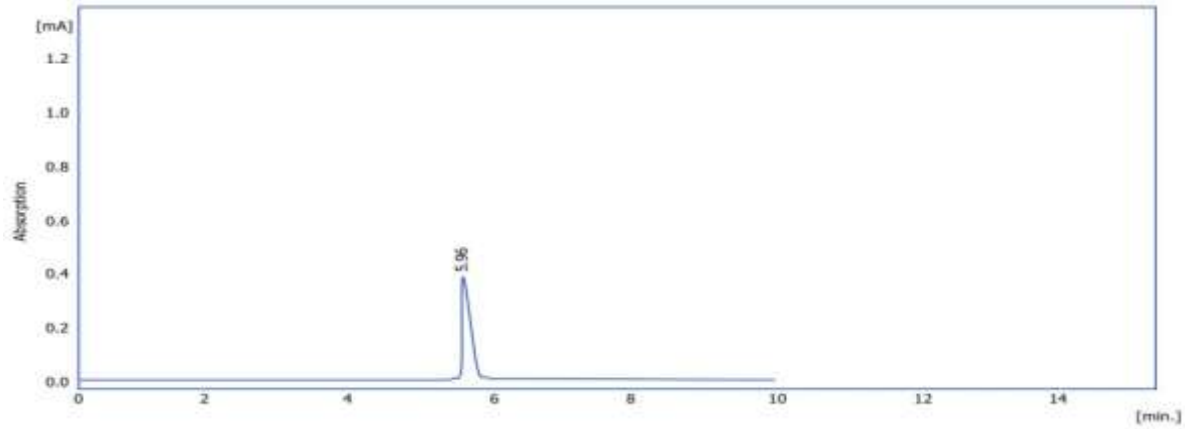

Figure 6. Chromatogram of HPLC for isolated luteolin.

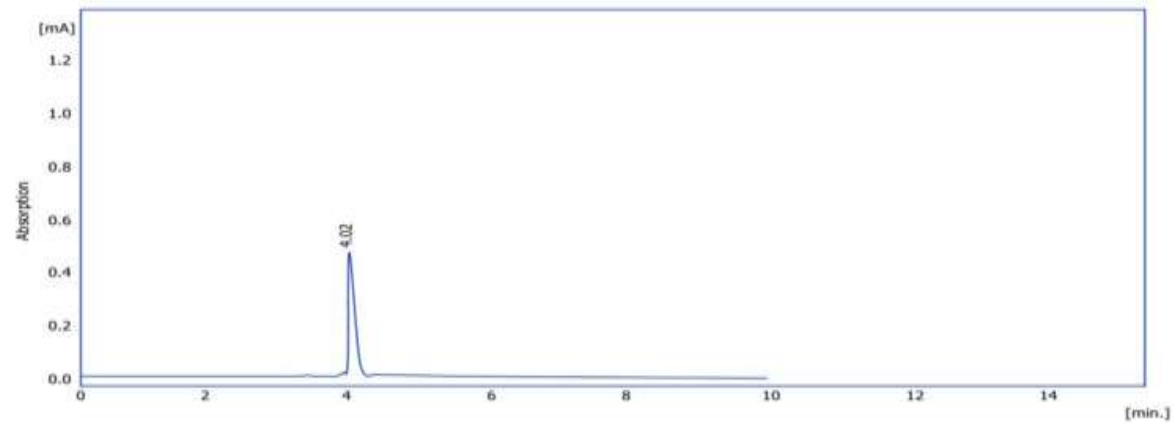

Figure 7. Chromatogram of HPLC for isolated ferulic acid.

Identification of the isolated luteolin and ferulic acid compounds by different spectroscopic and chromatographic techniques

1. Spiking analysis by analytical HPLC

Results of spiking analysis revealed an increase in the area of luteolin and ferulic acid as demonstrated in Figure. 8 and 9 respectively, the retention time of the isolated luteolin was (5.93min) which considered identical to that of the luteolin standard (5.92min). This was also performed to the isolated ferulic acid as its retention time (4.10min) was identical to that of the ferulic acid standard (4.05min).

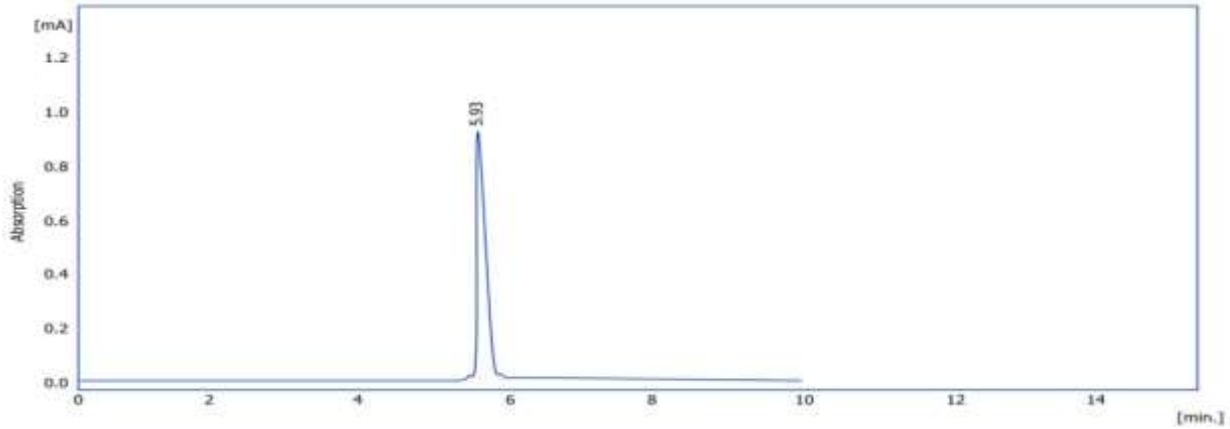

Figure 8. HPLC chromatogram of mixing standard luteolin and isolated compound.

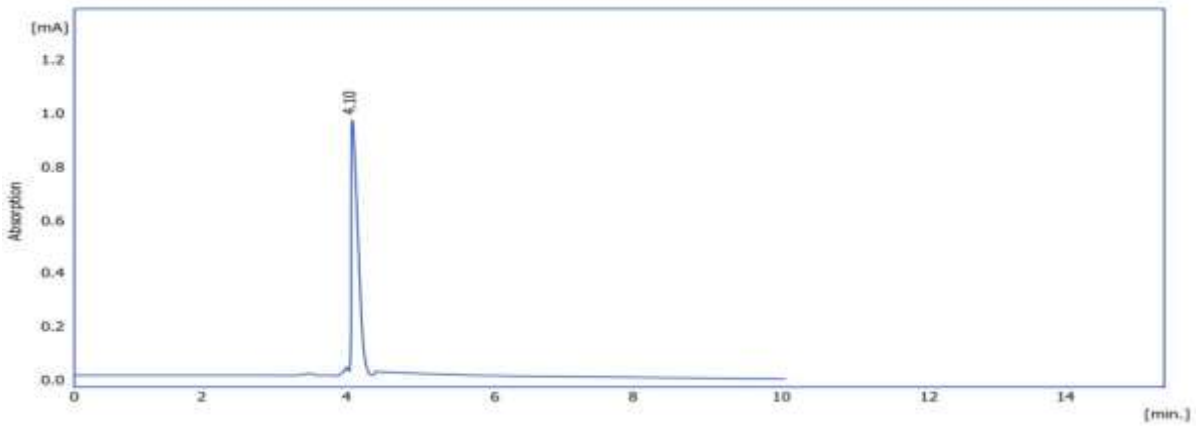

Figure 9. HPLC chromatogram of mixing standard ferulic acid and isolated compound.

2. Fourier transform infrared spectroscopy (FTIR)

The examination of FTIR was performed for isolated compounds , FTIR spectrum of isolated luteolin was presented in Figure. 10 and the interpretation of the band in Table 4.

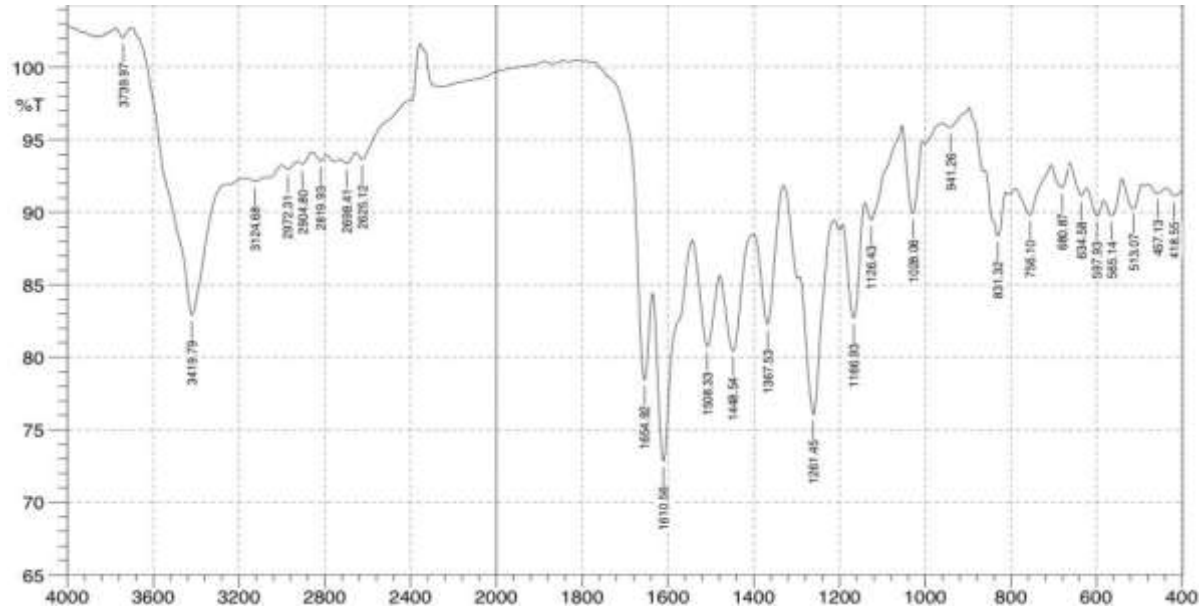

Figure 10. FTIR spectrum of isolated luteolin.

Table 4. Interpretation of the FTIR bands for the isolated luteolin [23,35].

FTIR bands of isolated luteolin	Interpretation
3419	OH stretching vibrations band
2972, 2904, 2819	C-H stretching
1654	C=O stretching
1610	C=C stretching of α,β- unsaturated system
1508	C=C stretching vibration of aromatic moiety
1261	C-O-C stretching

These data were coincided with that published for luteolin [23,35] . While the data of FTIR spectrum of isolated ferulic acid was demonstrated in Figure. 11 and the interpretation of the band in Table 5, as these data were coincided with that reported for ferulic acid [36].

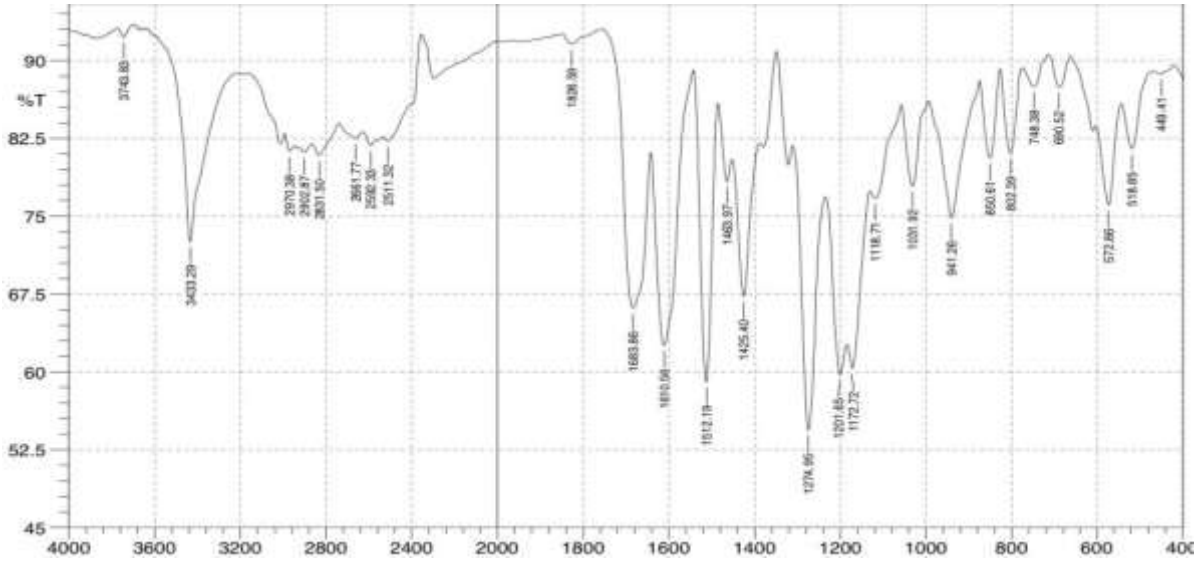

Figure 11. FTIR spectrum of isolated ferulic acid.

Table 5. Interpretation of the FTIR bands for the isolated ferulic acid [36].

FTIR bands of isolated ferulic acid	Interpretation
3433	OH stretching vibrations band
2970, 2902, 2831	C-H stretching
1683	C=O stretching
1610	C=C stretching of α,β- unsaturated system
1512	C=C stretching of aromatic moiety
1201, 1172	C-O-C stretching

3. Liquid chromatography mass spectrometry (LC-MS/MS)

For further identification and characterization of the isolated compounds, LC-MS/MS was done for the isolated compounds. The ion fragmentation spectra of isolated luteolin was demonstrated in Figure. 12.

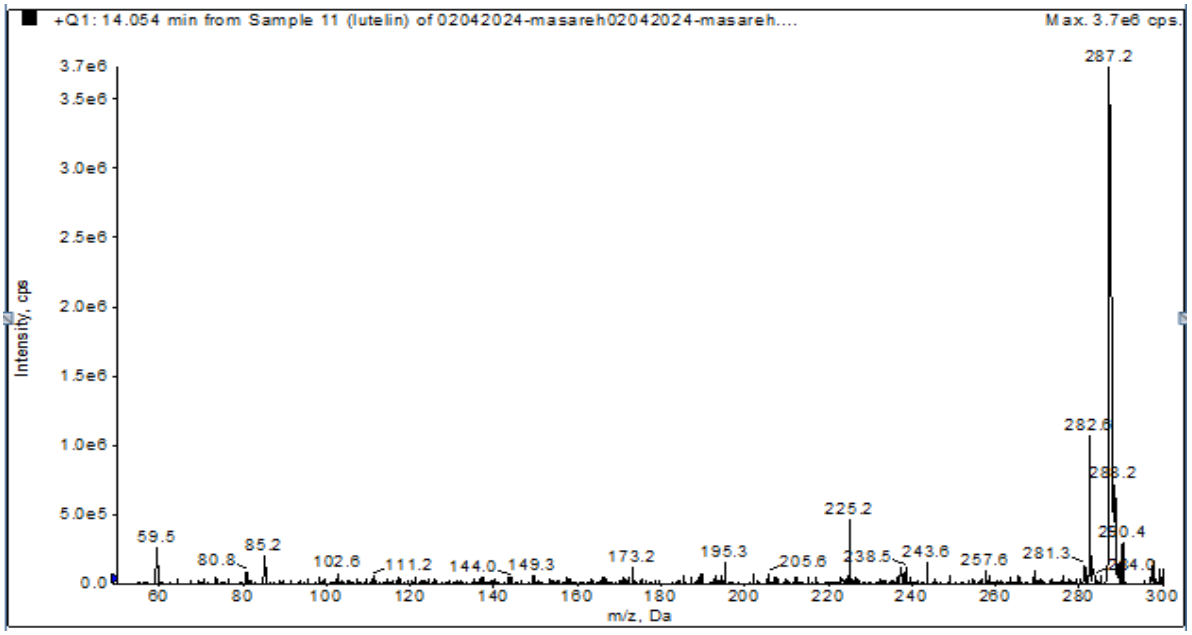

Figure 12. Representative ion mass fragmentation spectra of isolated luteolin.

According to data in Figure. 12, the $[M+H]^+$ ion with m/z 287.2, was considered as a molecular ion peak, the results showed that the most abundant fragments have been recorded at m/z 257 [$C_{14}H_9O_5^+$], 243 m/z [$C_{13}H_7O_5^+$], 144 m/z [$C_9H_4O_2^+$]. All these LC/MS data were coincided with that

published in literature for luteolin [37,38]. While the ion fragmentation spectra of isolated ferulic acid was shown in Figure. 13, where the molecular ion equal to m/z 194.2 and appeared as 195.3 $[M+H]^+$, the most abundant fragments were recorded at m/z 178 $[C_9H_6O_4^+]$, m/z 149 $[C_9H_9O_2^+]$, and m/z 134 $[C_8H_6O_2^+]$, these LC/MS data were coincided with that published in literatures for ferulic acid [39,40].

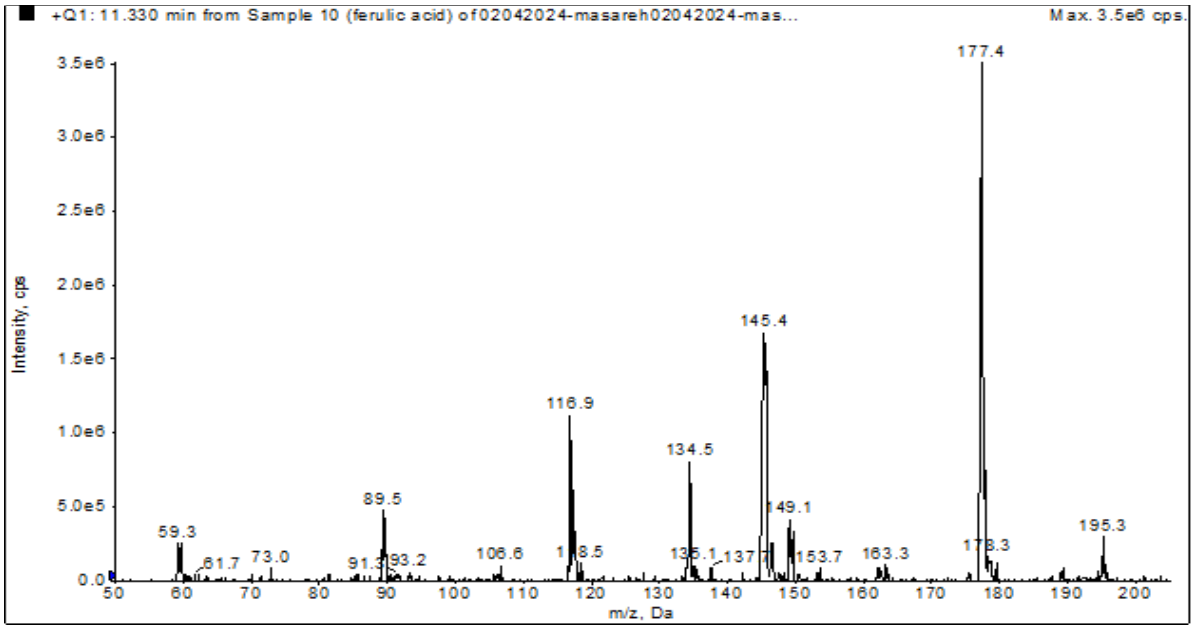

Figure 13. Representative ion mass fragmentation spectra of isolated ferulic acid.

Discussion

Natural products have always been a favored option due to their an important role in detecting new medicines. Among many phytochemicals, *Plumbago auriculata* was reported to have different types of polyphenolic constituents such as flavonoids and phenolic acids [12,41]. Polyphenolic compounds are secondary metabolites possess antioxidant activities which allow for surviving the plant under stressful environments [42,43]. From this study, positive results of preliminary screening for the occurrence of phenolic compounds in the fraction of ethyl acetate was accompanied by implying of spectroscopic methods to characterize the nature of these phenolic compounds. The use of these two phenolic compounds (luteolin and ferulic acid) as standards in the subsequent analytical methods, have a certain interest due to their powerful antioxidant effects. According to the results of analytical HPLC, the investigated luteolin and ferulic acid compounds were identified successfully in the fraction of ethyl acetate through comparing their retention times with the retention times of the standards using the same conditions. The mixture of luteolin and ferulic acid standard solutions with ethyl acetate fraction showed sharp peaks at 5.92min and 4.05min, respectively that confirmed the luteolin and ferulic acid peak positions as shown in Figure. 4 and 5. Such results encouraged subsequent isolation of these compounds by HPLC technique using the conditions mentioned previously.

Results of identification of the isolated luteolin and ferulic acid were identical to their standards when comparing by their retention times in HPLC (5.92min) and (4.05min) respectively. Furthermore, spiking by HPLC was performed to further confirm the isolated compounds as the area was increased by mixing the isolated compounds with their standards, Figure. 8 and 9. Results of FT-IR and LC-MS/MS spectrum for both compounds were further supporting the results of analytical HPLC as the data of interpretations and fragmentations were coincided with that published for luteolin and ferulic acid.

The potent antioxidant activity of these compounds was greatly examined and is associated with their polyphenolic nature [44.45], this led to use of these phenolic compounds like ferulic acid in some food manufacturers as a natural antioxidant [46] and they possess promising therapeutic effect in thrombosis, inflammations, oxidative damage, cancer, and microbial infections [47]. This study was the first to isolate luteolin and ferulic acid compounds from the *Plumbago auriculata* extract which may demonstrate some of its therapeutic uses such as antidiabetic as it was confirmed through researchers that luteolin possesses the ability to suppress fasting blood glucose and HbA1c [48,49]. Other researchers have proved that the antioxidant activity of ferulic acid may demonstrate that the plant has antioxidant effect [44,45].

Conclusion

The results of phytochemical analysis indicated that *Plumbago auriculata* plant has many polyphenol compounds. For the pharmacological activities of these compounds, many diseases were cured. Therefore, these polyphenols required to be isolated, and characterized to obtain the usefulness of *P.auriculata* cultivated in Iraq. Luteolin and ferulic acid are polyphenolic compounds with antioxidant, anti-inflammatory and anticancer activities were isolated from the plant by HPLC, and identified by different chromatographic and spectroscopic techniques included HPLC, FTIR and LC-MS/MS and the results of these techniques for the isolated compounds were coincided with that published for these compounds in literature.

Acknowledgment

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Conflicts of Interest

The authors declared that they have no conflict of interest.

Funding

For this research no financial support was received from any institution.

Ethics Statements

No need for ethical approval as the study was conducted on plant in vitro.

Author Contribution

Data gathering, analysis, practical work and writing of this study were performed by Massara Nazar Ahmed. The design of this study was performed by Amjed Haseeb Khamees and he provided the final agreement and approval of the study.

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عزل و تعريف اللوتولين وحامض الفريوليك لنبات الياسمين الازرق المزروع في العراق

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الخلاصة

الياسمين الازرق هو نبات معمر ينتمي الى عائلة (*Plumbaginaceae*). وهو يعد جنس مستوطن يضم ١٨ نوعا في جنوب افريقيا. تحتوي جميع أجزاء النبات على العديد من المركبات الكيميائية التي تظهر العديد من الانشطة الدوائية. هدفت هذه الدراسة الى تشخيص و عزل وتوضيحات هيكلية للمركبات متعددة الفينول النشطة بيولوجيا (اللوتولين وحامض الفريوليك) من نبات الياسمين الازرق المزروع في العراق باستخدام مختلف التقنيات الكروماتوغرافية والطيفية. تمت ازالة الدهون من المواد النباتية باستخدام مذيب الهكسان بطريقة التنقيع لمدة ٤٨ ساعة، واستخلاصها بواسطة جهاز السوكسليت باستخدام ٨٥ ٪ من مذيب الميثانول، ثم تجزئتها بمذيب أسيتات الايثيل. تم استخدام تحليل الكروماتوغرافي السائل عالي الاداء لتحديد اللوتولين وحامض الفريوليك في جزء اسيتات الايثيل. تم عزل المركبات التي تم التعرف عليها باستخدام تحليل الكروماتوغرافي السائل عالي الاداء، ومن ثم تم توصيف وتحديد المركبات المعزولة باستخدام تقنيات الكشف المختلفة بما في ذلك، تحليل الكروماتوغرافي السائل عالي الاداء من خلال المقارنة مع معاييرها وقياسها، والتحليل الطيفي للأشعة تحت الحمراء، بالإضافة الى مقياس الطيف الكتلي السائل. أكدت نتائج التحليل الكروماتوغرافي والطيفي وجود اللوتولين وحامض الفريوليك في جزء خلات الايثيل حيث خلصت الى أن البيانات الخاصة بالمركبات المعزولة تتطابق مع تلك الواردة للمركبات في البحوث. قد توضح هذه المركبات البوليفينولية المعزولة والتي لها أنشطة دوائية مختلفة بعض الاستخدامات العلاجية لنبات الياسمين الازرق.

الكلمات المفتاحية: حامض الفريولك، الفلافنويدات، التحليل الطيفي للأشعة تحت الحمراء، تحليل الكروماتوغرافي السائل عالي الاداء، مقياس الطيف الكتلي السائل، اللوتولين، الفينولات.