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Phytochemical investigation of some bioactive compounds from twigs and leaves of *Juniperus oxycedrus* L. plant grown in Iraq

Estabraq H. Naser*,[1] and Sarmed H. Kathem[2]

[1]Department of Pharmacognosy and Medicinal Plants, College of Pharmacy, University of Kerbala, Karbala, Iraq.
[2]Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad, Iraq.

Abstract

Herbal medicine has been used throughout history for the prevention and treatment of illnesses, and the promotion of health, and to enhance the quality and span of our lives. In this study *Juniperus oxycedrus* twigs and leaves are extracted with methanol after defatting with n-hexane using the soxhlet apparatus to separate the polar compounds according to the like dissolves-like rule. The flavonoids components are flavonol type, like rutin, quercetin; flavan-3-ol like catechin; as well as flavone, like apigetrin, were isolated by preparative high performance liquid chromatography (PHPLC), and examined using the ultraviolet spectrum (UV), Fourier-transform infrared spectroscopy (FTIR), mass spectrum by comparing the base peak mass m/z, and HPLC by comparing the retention times of the isolated and matching compounds. The isolated compounds have potent pharmacological activities like antioxidants, anticancer, antibacterial, and anti-inflammatory activities, and the plant needs further study to utilize it because this was the first, and the only research that focused on compounds isolation in Iraq and globally.

Keywords: Apigetrin, Catechin, *Juniperus oxycedrus*, LC MS QTOF, PHPLC, Rutin.

التشخيص الكيميائي النباتي لبعض المركبات النشطة بيولوجيا من اغصان واوراق نبات العرعر المزروع في العراق

استبرق حسين ناصر*،[١] و سرمد هاشم كاظم[٢]

[١]العقاقير والنباتات الطبية، كلية الصيدلة، جامعة كربلاء، كربلاء، العراق.
[٢]الادوية والسموم، كلية الصيدلة، جامعة بغداد، بغداد، العراق.

الخلاصة

استخدام طب الأعشاب على مر التاريخ للوقاية من الأمراض وعلاجها ، ولتعزيز الصحة ، ولتحسين نوعية حياتنا وامتدادها. في هذه الدراسة تم استخلاص أغصان وأوراق *Juniperus oxycedrus* بالميثانول بعد إزالة الدهن باستخدام n-hexane باستخدام جهاز soxhlet لفصل المركبات القطبية وفقًا لقاعدة الشبيه يذيب شبيهه. مركبات الفلافونويد من نوع الفلافونول ، مثل الروتين ، كيرسيتين. فلافان 3-ol مثل كاتشين ؛ بالإضافة إلى الفلافون ، مثل apigetrin ، تم عزلهما عن طريق تحليل كروماتوجرافي السائل عالي الأداء التحضيري (PHPLC) ، وفحصهما باستخدام الطيف فوق البنفسجي (UV) ، والتحليل الطيفي للأشعة تحت الحمراء (FTIR) ، والطيف الكتلي بمقارنة الكتلة الذروة الأساسية m / z ، و HPLC من خلال مقارنة أوقات الاحتفاظ بالمركبات المعزولة والمطابقة.
تم استنتاج ان للمركبات المعزولة أنشطة دوائية فعالة مثل مضادات الأكسدة ، ومضادات السرطان ، ومضادات الجراثيم ، ومضادة للالتهابات ، ويحتاج النبات إلى مزيد من الدراسة للاستفادة منها لأنه كان البحث الأول والوحيد الذي ركز على عزل المركبات في العراق والعالم. .

الكلمات المفتاحية: ابجترين ، كاتكين ، العرعر الشربيني ، جهاز استشراب السائل مع مطيافية الكتلة ، كروماتوغرافيا السائل عالي الاداء التحضيرية ، روتين.

Introduction

Herbal medicines have proven to be the primary treatment in the traditional medicine system and have been widely used since ancient times. This has led to the use of medicinal plants and their biological benefits in the production of drugs and medicines. Because of their availability, accessibility, affordability, and potential for efficacy, medicinal plants have played a significant role in traditional medicine and herbal remedies throughout the world. As a result, 80% of the global's population still depend on them to avoid the side effects associated with the use of synthetic drugs [(1-4)].

Many plants' therapeutic capabilities, effects on the human body, and methods of use were known up to the 18th century [(5,6)].

Under stress conditions, the secondary metabolites have long term effects on plant development and survival[(7)]. The plant kingdom contains over 100,000 secondary metabolites that are exclusive to particular taxonomic groupings. Based on their biochemical pathways, compounds containing nitrogen (alkaloids, and glucosinolates), phenolic compounds (flavonoids and phenylpropanoids), and terpenes (isoprenoids) are the three main families of secondary metabolites in plants [(8)].

*Corresponding author E-mail: estabraq.h@uokerbala.edu.iq.
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Juniperus oxycedrus contained flavones, flavonoids, terpenes, sesquiterpenes, monoterpenes, tannin, volatile oil, and resin. The plant contains cad oil with about 17-26% phenolic compounds such as guaiacol, and about 12% sesquiterpenes called cadinene besides the alcoholic compounds as cardinal and carburs. *Juniperus oxycedrus* Tar's constituents are cadinene, a sesquiterpene, but guaiacol and cresol were also recognized. The leaves contain terpenes, monoterpenes, and fatty acid: sabinic. The leaves oil was mainly composed of α-pinene (40-57)% and manoyl oxide (5-10) %. Fruits contain terpenes, diterpenes, monoterpenes, and sesquiterpenes. Unripe berry oil was predominately composed of pinene, which made up around 65% of the oil, with only minor levels of limonene, myrcene, gamma-murolene, or germacrene D. Additionally, terpinole, canfene, cadinene, and junene were said to be contained in them [9,10,11]. Figure (1) showed *Juniperus oxycedrus* plant morphology.

Figure 1.*Juniperus oxycedrus* plant

Rutin is a flavonoid glycoside present in plant, of flavonol type [12]. The primary flavonol found in fruits and vegetables is 3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside, which is a rhamnoglucoside of the flavonoid quercetin [13]. Its pharmacological properties include anti-oxidative, anti-allergic, anti-fungal, anti-inflammatory, and antibacterial effects. It is produced through the phenyl propanoid pathway [14]. It is a nontoxic flavonoid that is also utilized to treat numerous chronic disorders, including cancer, hypertension, hypercholesterolemia, and diabetes [15, 16], as shown in Figure(2).

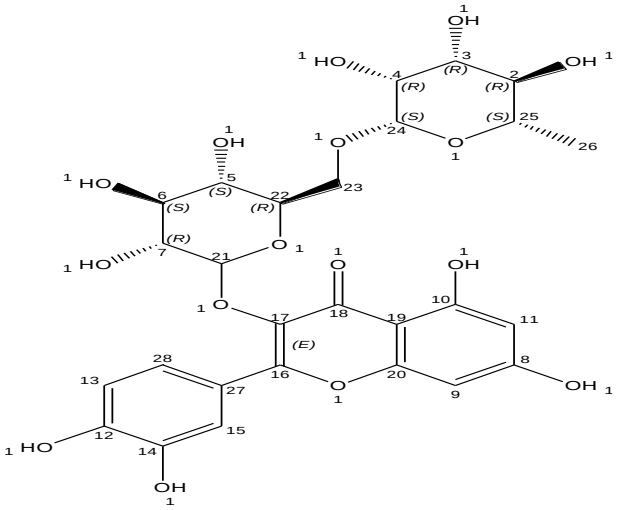

Figure 2. Rutin compound structure

Quercetin is a flavonoid aglycon of the flavonol class [17,18]. The versatile chemical quercetin has a wide range of therapeutic benefits, including antioxidant, neurologic, antiviral, anticancer, cardiovascular, antibacterial, anti-inflammatory, hepatoprotective, and anti-obesity activities. Also, it has other properties, including improving physical and mental performance [19, 20, 21] as shown in Figure (3).

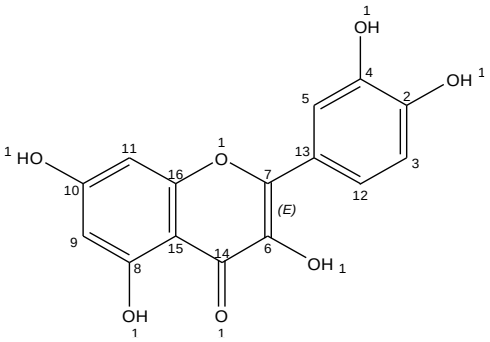

Figure 3. Quercetin compound structure

Apigetrin is 5,7,4-Trihydroxy flavones that belong to the subclass of flavones [22, 23]. The metabolic enzyme CYP2C9, which is responsible for the drug's metabolism, is one of the enzymes that apigetrin inhibits. Bioflavonoid apigetrin is referred to as a non-mutagenic compound [24]. Numerous *in vitro* investigations have shown that apigetrin has anti-proliferative, anti-inflammatory, and free radical scavenging effects. Apigetrin has many pharmacological activities like antispasmodic, antibacterial, and antiphlogistic impact [25, 26], as shown in Figure (4).

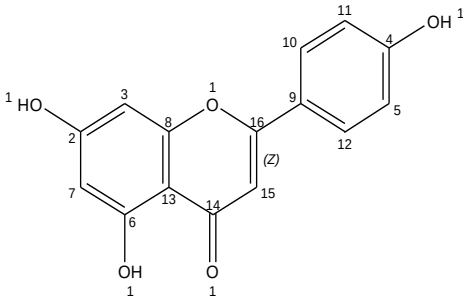

Figure 4. Apigetrin compound structure

Catechin is flavan-3-ol type that belongs to polyphenolic compounds. The condensation of catechin results in the formation of condensed or nonhydrolyzable tannins (epicatechin, and a catechin epimer) [27]. Catechins play a significant role in the defense against degenerative illnesses [28]. Other research has shown that catechin intake and the risk of cardiovascular illnesses are inversely related [29]. According to reports, catechins appear to have stronger antibacterial effects against gram-positive bacteria than Gram-negative ones [30, 31], as shown in Figure (5).

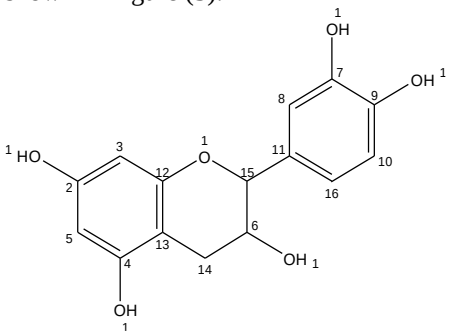

Figure 5. Catechin compound structure

This is the first study on the *Juniperus oxycedrus* plant in Iraq and globally to isolate, and investigate the flavonoid compounds rutin, apigetrin, quercetin, and catechin.

Materials and Methods

Plant collection

Dr. Haees Sayel Jarjes Mohammed Al-Jowary classified *Juniperus oxycedrus* plant at the college of agriculture and forestry, University of Mosul, Iraq, which was procured from the market in the north of country in Akra city in July 2020. The plant leaves and twigs were washed thoroughly, dried under shade, and ground in a mechanical grinder to a coarse powder.

Plant extraction

About 500 gram of plant parts (leaves, twigs) used together defatted by soxhlet with n-hexane then extracted sequentially by soxhlet with Ethylacetate and the remaining plant material re-extracted with more polar solvent methanol 98% using also soxhlet apparatus, all three crude extracts filtered and the solvents were evaporated by rotary evaporator [32-34]. The components in dried extracts from Ethylacetate and methanol are isolated by preparative HPLC, then identified by analytical HPLC, UV, LC MS QTOF and FT IR.

Preliminary phytochemical examination of crude extracts

Phytochemical analysis for the screening and identification of bioactive chemical constituents in the medicinal plants under study was carried out on crude extracts as well as powder specimens using the standard procedures as described.

Alkaloid test: 0.5 to 0.6 g of each plant extract were mixed in 8 ml of 1% HCl, warmed, and filtered. 2 ml of the filtrate was treated separately with both reagents (Mayer's and Dragendorff's), after which it was observed whether the alkaloids were present or absent in the turbidity or precipitate formation.

Test for saponins 0.5 g each plant extract were dissolved in boiling water in a test tube, cooled, and shaken vigorously to form a froth. 2.0 g of the powdered plant material was boiled in distilled water in a test tube in a boiling water bath and filtered. 10 ml of the filtrate mixed with 5 ml of distilled water and was shaken vigorously to the formation of stable, persistent froth. The frothing was mixed with three drops of olive oil and shaken vigorously for the formation of emulsion thus a characteristic of saponins.

Test for anthraquinones:1.0 g each plant extract were boiled in 6 ml of 1% HCl and filtered. The filtrate was shaken with 5 ml of benzene, and the benzene layer was removed. 10% NH_4OH was added, and the color in the alkaline phase was observed. The formation of pink/violet or red color indicated the presence of anthraquinones.

Test for sterols and terpenes: 0.5 g of each plant extract were shaken with petroleum ether to remove the coloring material. The residue was extracted with 10 ml chloroform, and the chloroform layer was dried over anhydrous sodium sulfate. 5 ml of chloroform layer was mixed with 0.25 ml of acetic anhydride, and then two drops of concentrated sulphuric acid were added. Different colors were observed to indicate the presence of sterol or terpenes. The green color indicated the presence of sterols while pink to purple terpenes and triterpenes.

Test for flavonoids: 0.5 g of each plant extract were shaken with petroleum ether to remove the fatty materials (lipid layer). The defatted residue was dissolved in 20 ml of 80% ethanol and filtered. 3 ml of the filtrate was mixed with 4 ml of 1% aluminum chloride in methanol in a test tube, and the color was observed. The formation of yellow color indicated

the presence of flavonoids [35-38].

Active ingredients are isolated and purified using preparative high performance liquid chromatography (PHPLC).

Crude natural product extracts and mixtures can sometimes consist of hundreds of compounds, and the isolation of particular components presents its own unique problems. Invariably, a fast and efficient technique is required to purify out the compounds of interest [39]. Separation and purification of natural materials by using Preparative HPLC systems can operate at pressures of up to 50 bar/725 psi and high flow rates of up to 4 ml/min on C18 (250x10) 5 m particle size from the USA Water Corporation [40]. Each crude extracts (Ethylacetate and methanol) were dissolved in 100µl of DMSO. The separation parameters for isolation of apigetrin and catechin from Ethylacetate fraction: Gradient of mobile A (0.1% TFA HPLC grade water) and mobile B (acetonitrile), and a gradient elution was performed by varying the proportion of solvent B to solvent A. The gradient elution was start with 35% to 65% A in a linear fashion, then gradient from B 80% to 20% for A for 3 min. duration, continue B 100% to 0% A for 30, and 40 min. The injection volume was 300µl, and total analysis time per sample was 40 min. The gradient elution was shown in Table 1.

Table 1. The elution program of PHPLC for Ethylacetate fraction.

Time (min.)	Mobile A concentration %	Mobile B concentration %	Flow rate ml/min
0	65	35	4
3	20	80	4
30	0	100	4
40	0	100	4

The separation parameters for isolation of quercetin, and rutin from methanol fraction: Gradient of mobile phase A (1% Phosphoric acid in HPLC grade water) and mobile phase B (acetonitrile). A gradient elution was performed by varying the proportion of solvent A to solvent B. The gradient elution was changed from100% A at the beginning and continue for 3min., then from 65% A to 35% B in a linear fashion for duration of 30 min, from 30% A to 70 % B in 40 min, finally continue only 100% B in 50 and 60 min. The injection volume was 300µl, and total analysis time per sample was 60 min. The gradient elution was shown in Table 2.

Table 2. The elution program of PHPLC for methanol fraction.

Time (min.)	Mobile A concentration %	Mobile B concentration %	Flow rate ml/min
0	100	0	4
3	100	0	4
30	65	35	4
40	30	70	4
50	0	100	4
60	0	100	

Detection of constituents by HPLC can be performed using several detection systems. Photodiode array detectors (DADs) are the most convenient for use because of their availability and easy maintenance, and mainly because of the valuable information for identifying compounds contained in their UV. The detection of each compound was confirmed by matching between the spectra of thesample with corresponding standards UV spectra [41, 42].The variable wavelength detector's operating principles are the same as those of the diode array detector (VWD). Instead of focusing on a single wavelength, the array of diodes allows for simultaneous acquisition across a number of them. The analyst can gather several spectra over a chromatographic peak using the spectral acquisition approach in conjunction with chromatographic separation. These spectra can be used to do a mathematical assessment of spectral peak purity and potentially identify substances after being gathered from an HPLC study [43], as in Table 3.

Table 3. The elution program of HPLC for Ethylacetate and methanol fraction

Time (min.)	Mobile A concentration %	Mobile B concentration %	Flow rate ml/min
0	100	0	1
3	100	0	1
30	65	35	1
40	30	70	1
50	0	100	1
60	0	100	

The FT-IR spectra (ATR type) of the isolated compounds were detected in the Department ofpharmaceutics, College of Pharmacy/ University of Kerbala by using a SHIMADZU device. The structural assignments have been correlated for characteristic bands of different chemical groups.
The m/z identification in Jordan by Bruker TOF MS is a Bruker Daltonik (Bremen, Germany) Impact II ESI-Q-TOF System equipped with Bruker Dalotonik Elute UPLC system (Bremen, Germany) with a high resolution made by using standards and the exact retention time of each analyst after chromatographic separation. This apparatus operates via Ion Source Apollo II ion Funnel electrospray source. The nebulizer gas was 2.0 bar, the capillary voltage was 2500 V, nitrogen flow (dry gas) was 8 L/min, and the dry temperature was 200 ºC. Less than one ppm was the mass accuracy; TOF repetition rate was up to 20 kHz, and the mass resolution was 50000 FSR (Full Sensitivity Resolution) [(44)]. LC MS QTOF instrumentation: Bruker Daltonik (Bremen, Germany) Impact II ESI-Q-TOF System equipped with Bruker Dalotonik Elute UPLC system (Bremen, Germany) was employed for screening components of potential. The m/z identification by Bruker TOF MS with high resolution made by using standards and the exact retention time of each analyte after chromatographic separation. The operation of this apparatus occurs via Ion Source Apollo II ion Funnel electrospray source. The nebulizer gas was 2.0 bar, capillary voltage was 2500 V, the flow of nitrogen (dry gas) was 8 L/min and the dry temperature was 200 ºC. Less than 1 ppm was the mass accuracy; TOF repetition rate was up to 20 kHz and the mass resolution was 50000 FSR (Full Sensitivity Resolution) [(45)]. Sample prepared as stock solutions, were prepared by dissolving of the appropriate amount of substance in Dimethyl sulfoxide-DMSO (analytical grade), then diluted with Acetonitrile then used for identification of exact MS and retention time. All the other reagents, Acetonitrile, methanol, water, and formic acid used were LC/MS grade. 2.0 ml DMSO was used to dissolve unknown sample, complete volume with Acetonitrile to 50 ml, centrifuge each sample for 2.0 min at 4000 rpm, then take 1.0 ml and transfer it to auto sampler and inject only 3.0 ul.

Results and Discussion

This phytochemical isolation and identification of secondary metabolites on *Juniperus oxycedrus* twigs and leaves is the first study achieved in Iraq. A preliminary study exhibited the result of flavonoids, alkaloids, saponins, anthraquinones, and terpenes, as shown in Table (4).

Table 4.preliminary phytochemical tests on *Juniperus oxycedrus* twigs and leaves powder

Test	Flavonoid	Alkaloid	Saponin	Anthraquinone	Terpene
Juniperus oxycedrus	+	+	+	+	+

Flavonoids are polyphenolic compounds; according to their chemical structure, flavonoids are divided into flavonol, flavanones, isoflavone, flavones, flavan-3-ol, anthocyanidins, etc. The soxhlet apparatus was used to extract the polar compounds from the crude extract of the *Juniperus oxycedrus* plant via methanol solvent. The results exhibit that 500 g of *Juniperus oxycedrus* twigs and leaves 98% give 25.78 g as crude extract after defatted by n-hexane, and 92.11 g as crude extract when extracted by methanol. The chromatogram of LC-MS QTOF and PHPLC chromatogram used for compounds isolation showed peaks of different compounds present in Ethylacetate and methanol extracts from *Juniperus oxycedrus* twigs and leaves as shown in Figure (6) and (7).

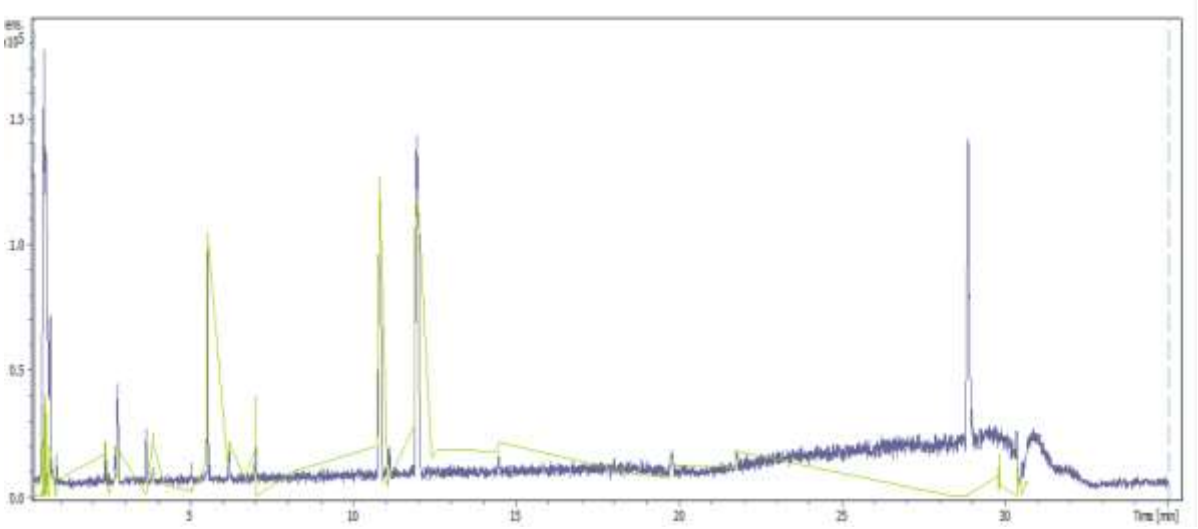

Figure 6. LC-MS QTOF peaks of compounds in methanolic crude extract of *Juniperus oxycedrus* plant

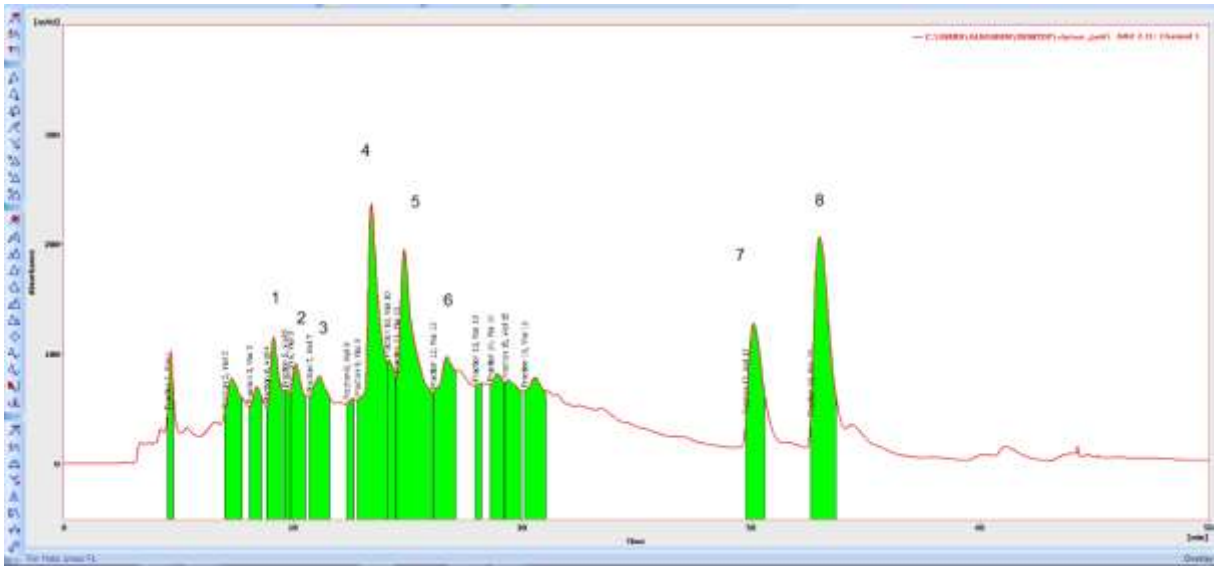

Figure 7. PHPLC chromatogram of *Juniperus oxycedrus* plant

For the separation and purification of natural materials, PHPLC is a versatile, reliable, and quick approach. Preparatory high-pressure chromatography is a superb purification method that was created to protect and identify significant compounds in the chemical, pharmaceutical, biotechnological, and biochemical sectors. The flavonoid compounds like flavonol class, including rutin, quercetin; flavan-3-ol like catechin; as well as flavones, like apigetrin were isolated by PHPLC using the assessment of standard compounds. Figures (8), (12), (16), (20) illustrate the UV spectrum used to identify the separated compounds quercetin, apigetrin, catechin, and rutin, respectively as it compared with reference standard; Figures (9), (13), (17), and (21) illustrate HPLC apparatus peaks that used to analyze the isolated compounds and standard ones by comparing the retention times of the isolated and their matching authentic standards for quercetin, apigetrin, catechin, and rutin respectively; Figures (11), (15), (19), and (23) illustrate FTIR spectrum achieved by comparing the characteristic IR bands of the isolated and standard components for quercetin, apigetrin, catechin, and rutin respectively; and Figures (10), (14), (18), and (22) illustrate Mass spectrum of each isolated compounds achieved by comparing its base peak mass m/z with the authenticated standard, for quercetin, apigetrin, catechin, and rutin respectively

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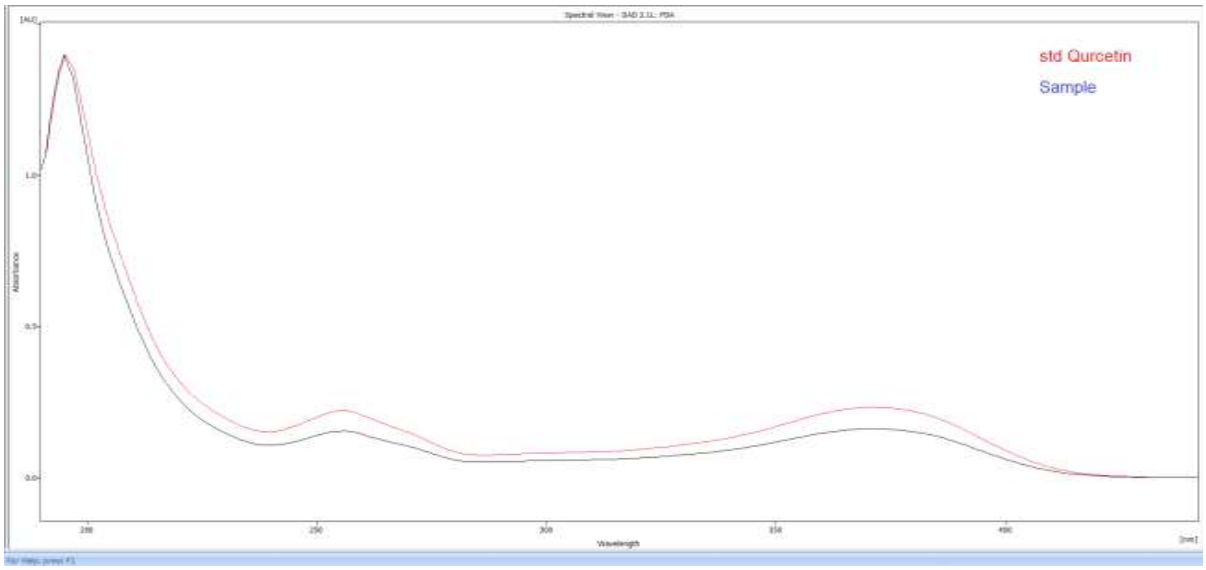


Figure 8. UV spectrum of isolated compounds quercetin from *Juniperus oxycedrus* plant.

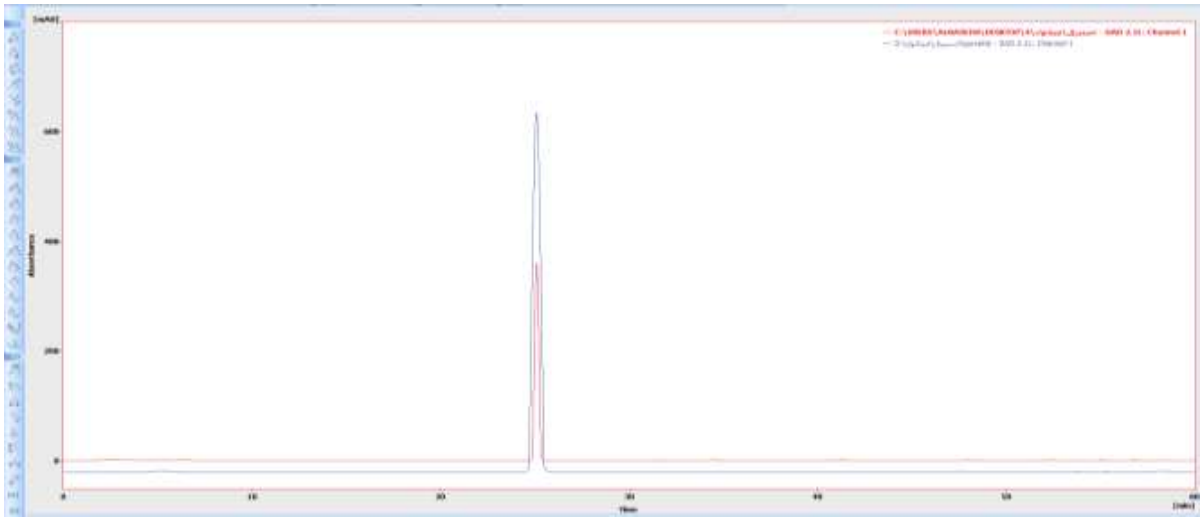


Figure 9. HPLC peak of isolated quercetin from *Juniperus oxycedrus* plant.

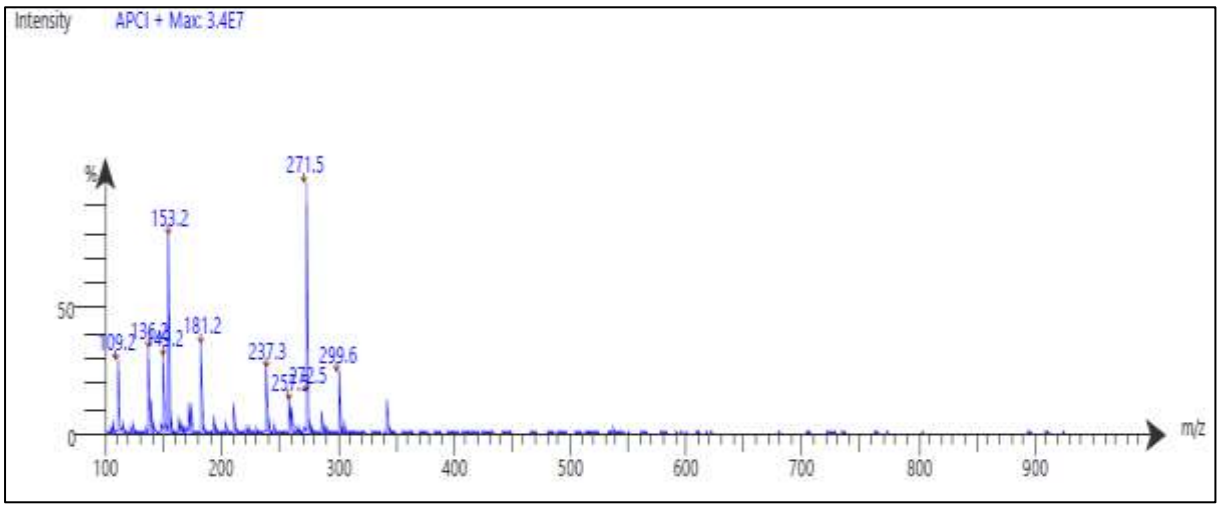


Figure 10. Mass spectrum of isolated quercetin from *Juniperus oxycedrus* plant.

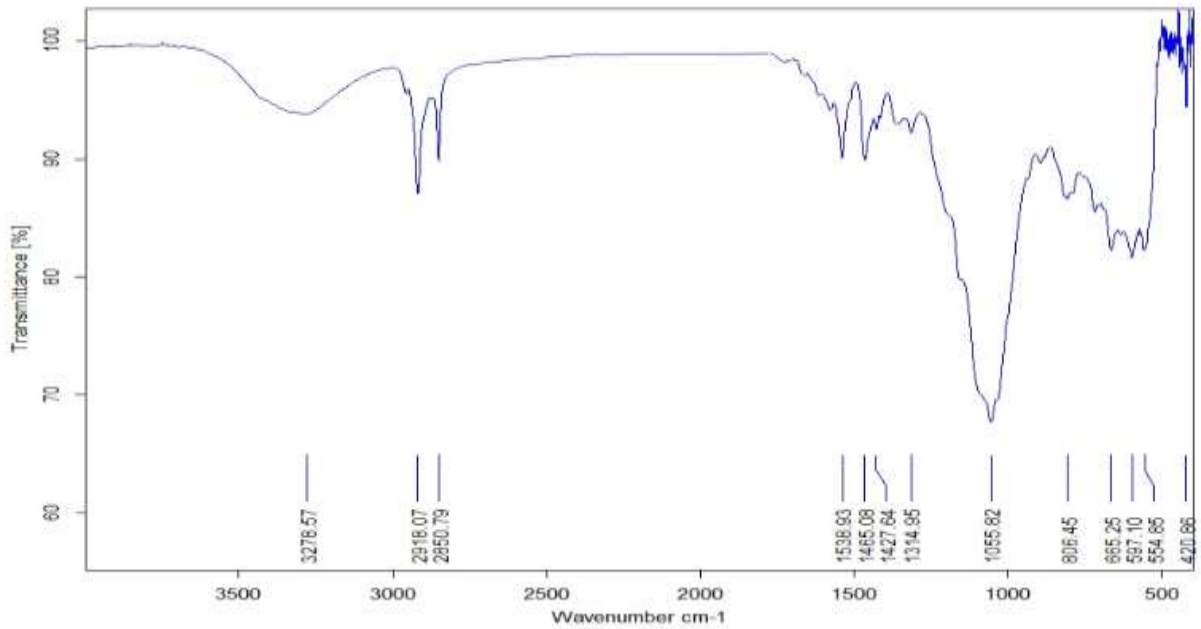


Figure 11. FTIR spectrum of isolated quercetin from *Juniperus oxycedrus* plant.

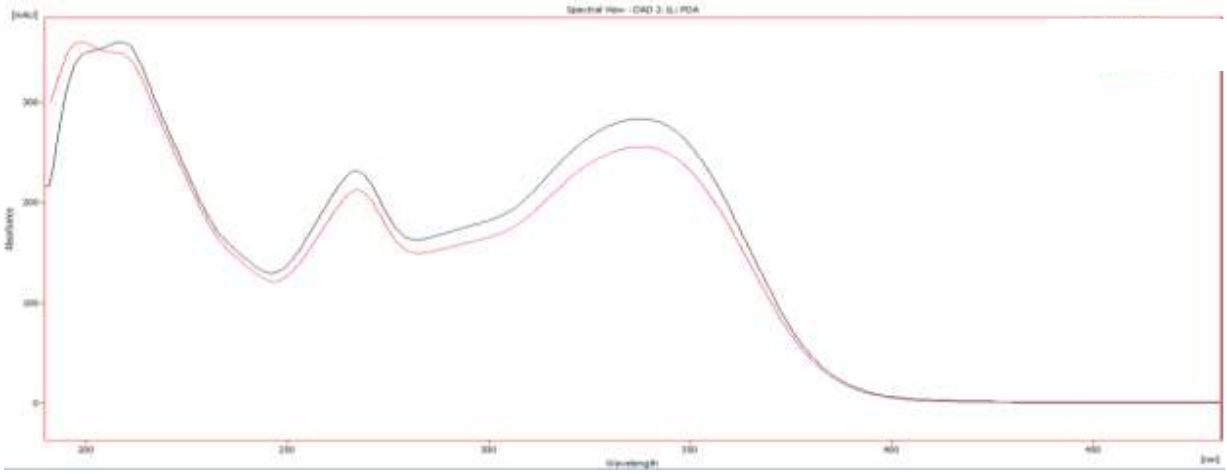


Figure 12. UV spectrum of isolated compounds apigetrin from *Juniperus oxycedrus* plant.

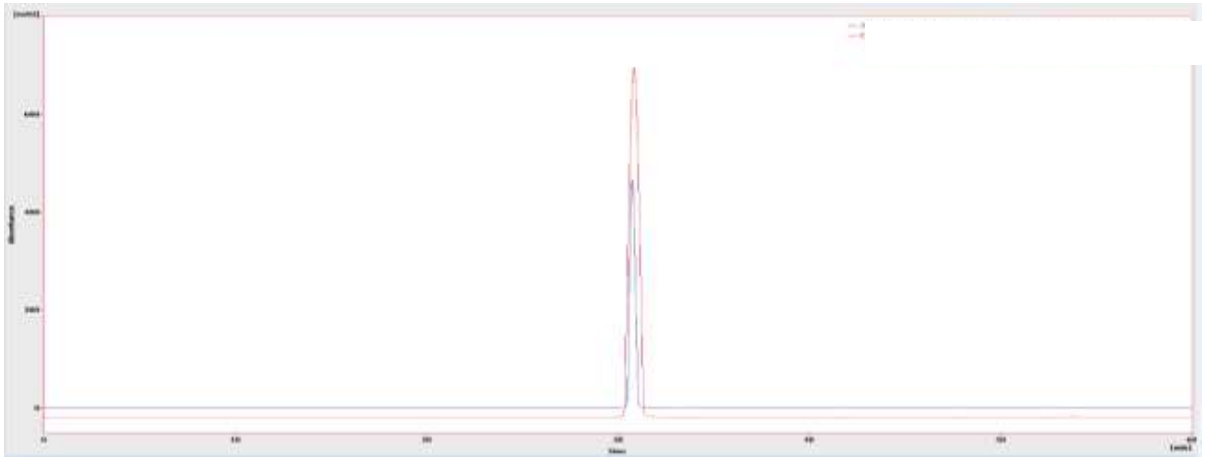
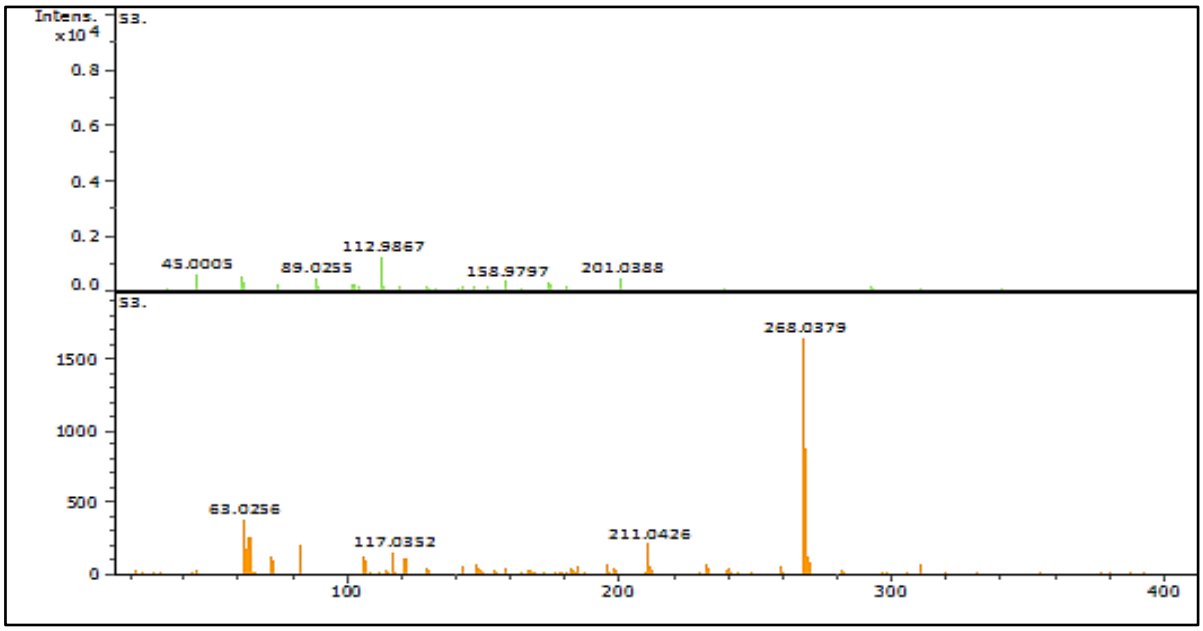


Figure 12. HPLC peak of isolated apigetrin from *Juniperus oxycedrus* plant.



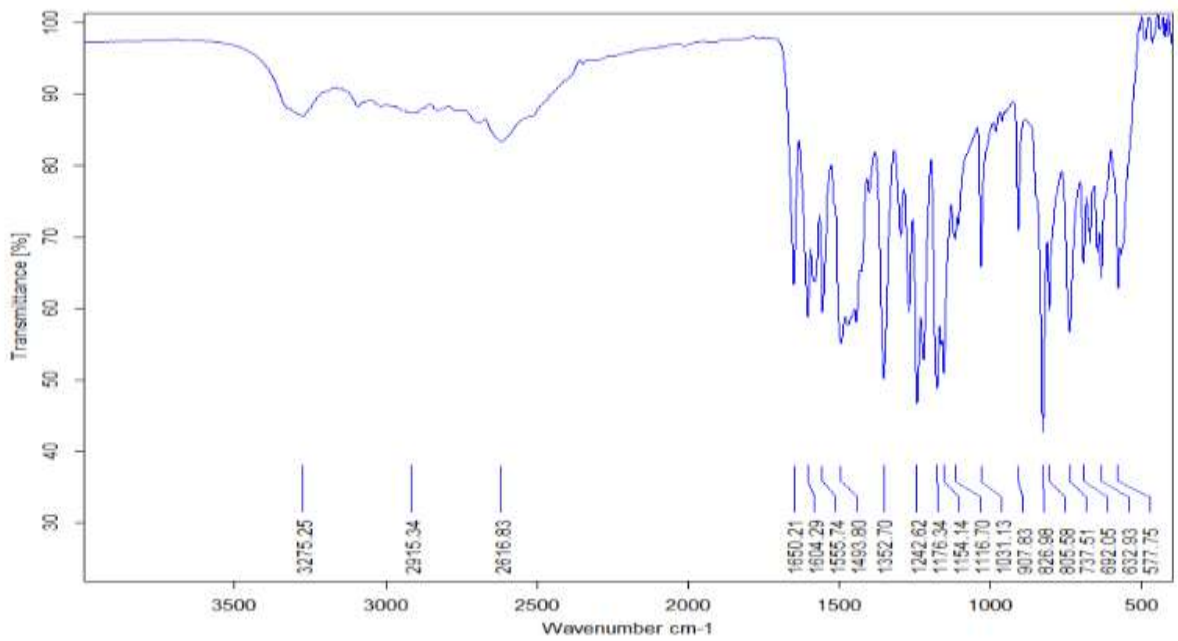


Figure 15. FTIR spectrum of isolated apigetrin from *Juniperus oxycedrus* plant.

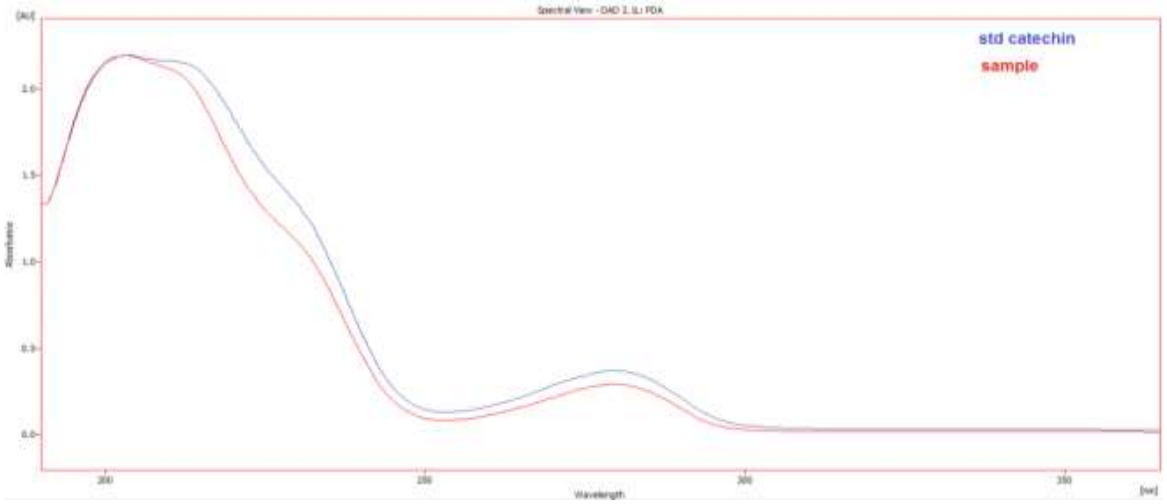


Figure 16. UV spectrum of isolated compounds catechin from *Juniperus oxycedrus* plant.

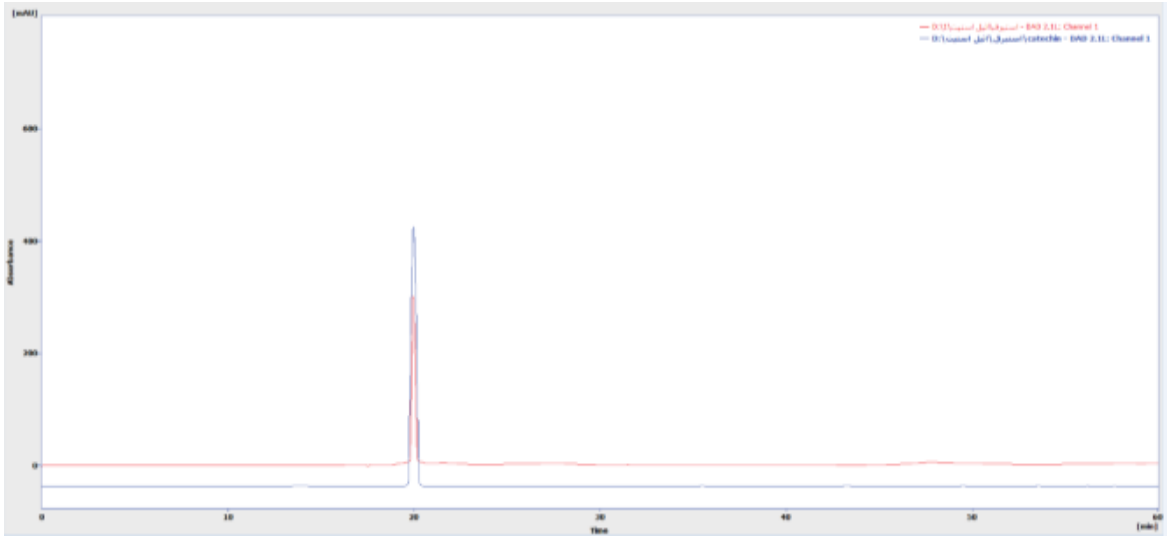


Figure 17. HPLC peak of isolated catechin from *Juniperus oxycedrus* plant.

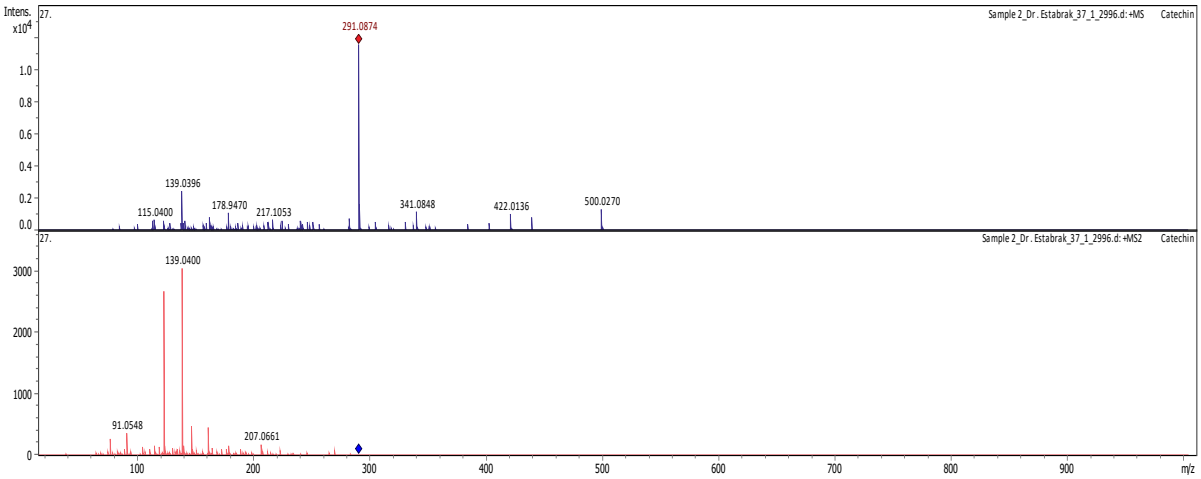


Figure 18. Mass spectrum of isolated catechin from *Juniperus oxycedrus* plant.

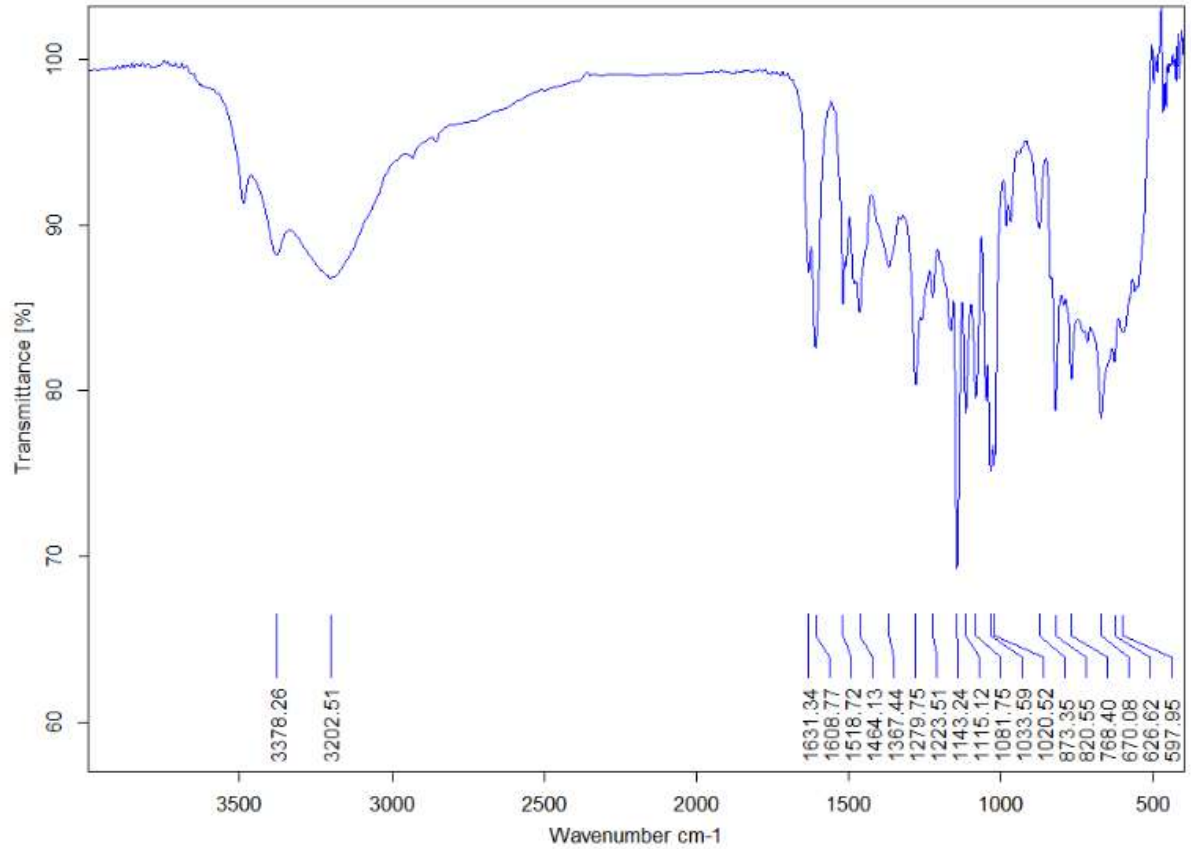


Figure 19. FTIR spectrum of isolated catechin from *Juniperus oxycedrus* plant.

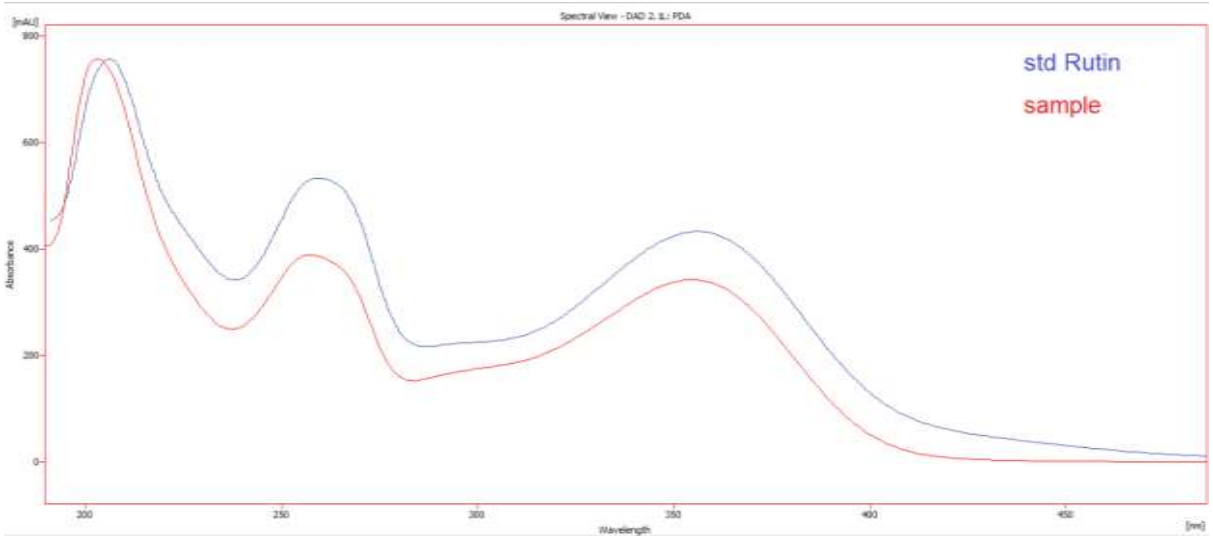


Figure 19. UV spectrum of isolated compounds rutin from *Juniperus oxycedrus* plant.

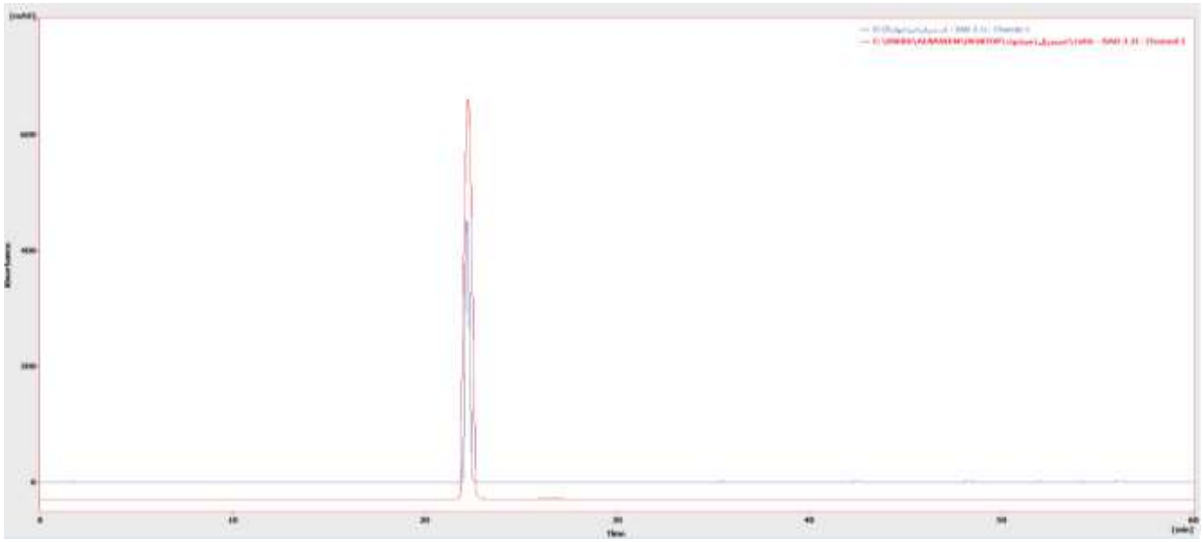


Figure 21. HPLC peak of isolated rutin from *Juniperus oxycedrus* plant.

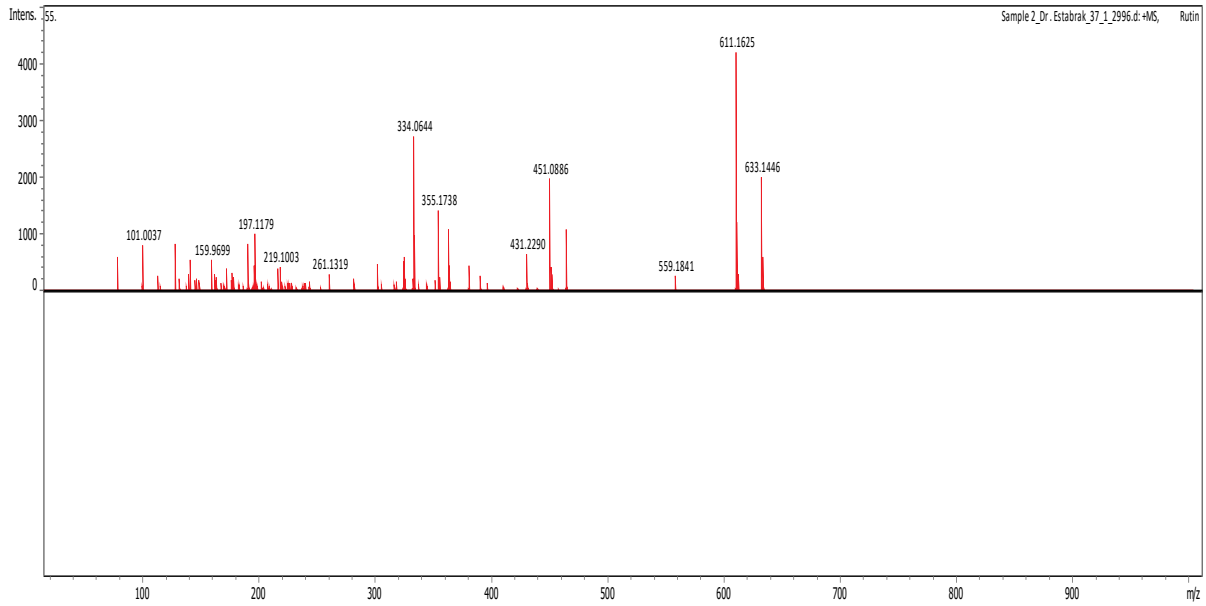


Figure 22. Mass spectrum of isolated rutin from *Juniperus oxycedrus* plant.

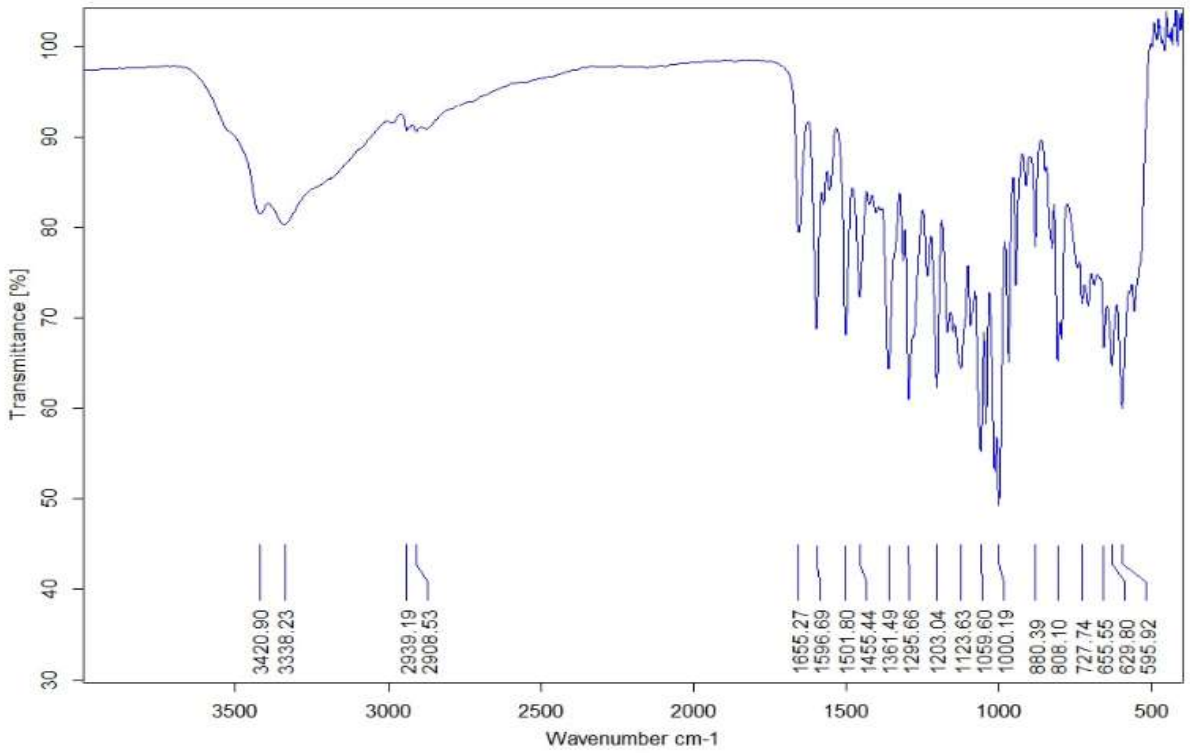


Figure 23. FTIR spectrum of isolated rutin from *Juniperus oxycedrus* plant.

Conclusion

Juniperus oxycedrus L. plant is rich in many secondary metabolites such as flavonoids, alkaloids, terpenes, anthraquinone, saponins and others. These compounds have potent pharmacological activities that cure many diseases, so these compounds needed to be isolated, and identified to get the whole benefits of this plant in Iraq. Rutin, quercetin, catechin, and apigetrin are flavonoids isolated from *Juniperus oxycedrus* plant that has antioxidant, anticancer, antibacterial, and anti-inflammatory activities.

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Conflicts of Interest

There is no conflict of interest regarding the publication of this manuscript.

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Ethics Statements

This article was approved by the scientific and ethical committees in College of Pharmacy/ University of Baghdad.

Author Contribution

The authors Estabraq H. Naser and Sarmed H. Kathem contribute together in plant selection, collection, investigation, and data analysis, revision and finally rearrangement.

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