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Phytoscreening and Isolation of Some Phenolic Compounds from the Iraqi *Gundelia tournefortii* and Detecting the Antioxidant Activity of its Crude Extract

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ABSTRACT

The *Gundelia tournefortii* is considered one of the plants that harbor the most bioactive components. They can potentially be used to treat several illnesses, especially those involving the antioxidant system. Few studies have dealt with these plants in Iraq. Therefore, this study aims to determine, extract, and investigate the bioactivity of some plant extracts. The aerial parts of the plant were collected from Sulaimaani governorate, Iraq. The plant's methanolic extract was obtained using Soxhlet. The phenolic acid primary screening was conducted using phytochemical tests, followed by secondary screening using Reverse-Phase HPLC-UV-Vis. Two specific phenolic acid types, rutin and gallic acid, were isolated and identified using preparative liquid chromatography and HPLC, respectively, along with FTIR spectroscopy. Then, the antioxidant and IC50 value of the methanolic extract was measured. Several phenolic compounds were detected in variable volumes, including rutin (32.9 ppm), kaempferol (90.1 ppm), apigenin (51.4 ppm), gallic acid (71.4 ppm), quercetin (74.1 ppm), myricetin (44.3 ppm), and catechin (78.9 ppm). A high free radical scavenging activity for methanol extract (IC50 value of 381.189µg/mL), compared to the control (vitamin C: 416.667µg/mL), was obtained. These findings indicate that the methanolic extract exhibits superior antioxidant activity to vitamin C and shows the significance of the antioxidant effectivity of *Gundelia tournefortii,* which can be potentially used as an antioxidant supplement. It is essential to note that this study is the first to successfully isolate rutin and gallic acid from the aerial parts of the Iraqi *Gundelia tournefortii.*

Keywords: *Gundelia tournefortii,* Reverse Phase HPLC-UV-Vis*,* IC50, preparative liquid chromatography (PLC), Soxhlet extraction

INTRODUCTION

There has been a significant increase in interest in herbal supplements and functional food products, driven by their undeniable nutritional and health benefits [1, 2]. For decades, medicinal plants have been the primary ingredient in numerous medications used to treat various illnesses. Many kinds of plants utilized in herbalism are included in the category of medicinal plants [3]. *Gundelia tournefortii* L is considered a medicinal plant that is affiliated with the family of Asteraceae; this plant is typically known as akub, tumbleweed, and kanger in Arabic, English, and Kurdish, respectively, and is characterized in the semi-desert areas of many countries including Palestine, Lebanon, Syria, Iraq, Jordan, Iran, Armenia, Azerbaijan, and Turkey The leaves, roots, stem and undeveloped flower buds of *G. tournefortii* are eaten usually consumed either as fresh plant or after being cooked like artichoke and utilized medicinally for over 2,000 years [4-7] several studies have demonstrated that *G. tournefortii* has pharmacological properties such as antioxidant activity [8], antibacterial activity [9], anti-platelet activity [7], hepato-protective activity [10], hypolipemic activity, hypoglycemic activity [11], antiparasitic activity and anti-inflammatory activity. *G. tournefortii* also reduces some cardiovascular risk factors and decreases atherosclerosis [12]. The therapeutic effects of medicinal plants are closely related to their antioxidant capacities [8]. Reactive Oxygen Species (ROS), such as the superoxide ion (O2-), the hydroxyl radical (*OH), and hydrogen peroxide (H2O2), are well-known contributors to oxidative damage in the body. This damage is firmly associated with serious health issues, including cancer, neurodegenerative diseases, aging, and cardiovascular diseases. Among these, cancer has decisively become one of the most common and pressing health challenges in recent years, significantly impacting mortality rates globally [13,14]. Antioxidants are vital compounds that efficiently shield the body from harm caused by reactive oxygen species, reactive nitrogen species, and reactive chlorine species, thereby averting potential diseases [15]. Antioxidants play a crucial role in reducing the risk of cardiovascular diseases and strokes, as well as slowing the progression of DNA damage-induced cancer. Although the body contains diverse antioxidants that are found in the bloodstream, the immune system alone cannot neutralize all the free radicals generated within the body. Therefore, it is essential to obtain antioxidants from external sources, such as dietary intake. As a result, potent antioxidants with lower toxicity and higher effectiveness are a vital requirement [16]. Antioxidant compounds in plants are predominantly phenolic in nature. These powerful substances encompass tocopherols, carotenoids, phenolic acids (including benzoic acid derivatives and cinnamic acids), flavonoids, and diterpenes [17]. Secondary metabolites derived from plants, mainly phenolic compounds, can potentially clear free radicals in all plant parts, including the seeds, leaves, roots, fruits, and skin [18]. Phenolic acids exhibit distinct levels of antioxidant activity, primarily influenced by the number and arrangement of hydroxyl groups within their structures. Introducing additional ortho- or para-hydroxyl or methoxyl groups significantly enhances their antioxidant potency. Additionally, they exhibit significant anti-cancer properties, including the ability to inhibit cell proliferation effectively, disrupt angiogenic factors, and block oncogenic signaling cascades. Their mechanisms also prevent growth and differentiation, limit cellular migration and metastasis, and actively induce apoptosis [19]. Analyzing and quantifying phenolic compounds in diverse natural food products is effectively carried out through HPLC analysis. This complex process is attributed to the numerous phenolic compounds present in most natural food products

[20]. This work investigated *the components of G. tournefortii using HPLC-UV-Vis, isolated rutin and gallic acid by preparative liquid chromatography, and identified the isolated components using various techniques. It then studied the antioxidant efficacy* of *G. tournefortii.*

MATERIALS AND METHODS

Plant Materials

The aerial part of *G. tournefortii* was gathered from the Sulaymaniyah governorate in north Iraq. The fresh plant was confidently collected in May 2023, cleaned, and then shade-dried for several days at room temperature. It was subsequently crushed into medium-milled pieces and stored in airtight bottles.

Extraction of Phytoconstituents

Extraction Method for Different Phytoconstituents

Twenty grams of fine powder, plant defatted by petroleum ether. Using a Soxhlet apparatus, the alcoholic extract was extracted from the defatting plant using 250 ml of 80% methanol for 12 hours at 65°C. The resulting product was dried in a rotary evaporator. Finally, the products were saved in a dark glass container at 4°C [21]

Phytochemical Screening by Chemical Tests

According to a research database, the phytochemical tests screened the phenol and flavonoids in the extract [22, 23]

- Ferric Chloride Test

When a small quantity of the extract is combined with a few drops of a solution of neutral ferric chloride, the appearance of a blackish-green color unequivocally confirms the existence of phenolic compounds.

- Lead Acetate Test

After adding a few drops of a solution of aqueous basic lead acetate to the extract, the appearance of a yellow sediment confirms the presence of phenolic compounds.

- Shinoda Test

The addition of small pieces of magnesium ribbon and concentrated hydrochloric acid to the extract resulted in a red color after a few minutes, indicating the presence of phenolic compounds.

Detection of Different Phytoconstituents by Reverse Phase HPLC-UV-Vis

The HPLC-UV-Vis analysis effectively detected and estimated various compounds in the plant extracts. High-performance liquid chromatography (HPLC) was performed using a SYKAM HPLC system (Germany) equipped with a C18-ODS column (250 mm × 4.6 mm, 5 µm particle size). A sample volume of 100 µL was injected into the system. The mobile phase consisted of 95% acetonitrile with 0.01% trifluoroacetic acid (solvent A) and 5% acetonitrile with 0.01% trifluoroacetic acid (solvent B), both at a flow rate of 1 mL/min. The gradient program was as follows: 10% A, from 0 to 5 minutes, 25% A, from 5 to 7 minutes, and 40% A, from 7 to 13 minutes, followed by a return to initial conditions. Phenolic compounds were detected using a UV-Vis detector set to 278 nm[24]. The compounds were identified qualitatively by comparing the retention times obtained from the chromatographic settings of the tested samples with those of known standards.
rutin, kaempferol, apigenin, genestic acid, quercetin, myricetin, and catechin.

Isolation of Proposed Rutin and Gallic acid by PLC

Isolation and purification of the proposed rutin and gallic acid from the methanol extract of aerial parts of *Gundelia tournefortii* were carried out using Preparative liquid chromatography (PLC). This process consisted of the following steps:

- The plates used in preparative liquid chromatography are ready-made glass plates coated with silica gel GF254 and measured (20×20 cm). The plates' thickness, measured in isolation, was 0.5 mm, and they were used as the stationary phase.
- The best mobile phase to isolate rutin was ethyl acetate: methanol: water (8.1: 1.1:0.8) [25]. The mobile phase best for separating gallic acid was chloroform: ethyl acetate: formic acid (5:4:1)[26].
- A few milligrams of methanol extract were dissolved and arranged in a band on a plate using a capillary tube, and the band extended to the edge of the plate; the sample was removed once completely dry and examined using ultraviolet light (wavelength:254nm) while being marked with a pencil and scrapped with a spatula. After the plates had been used to separate the compounds, the entire powder produced was placed into a clean and dry conical flask.
- A sufficient quantity of methanol was added and left to stand for 24 hours. It was then double-filtered using filter paper and left to dry for several hours to isolate the pure constituents.

Identification and Characterization of Isolated Compounds

Reverse Phase High-Performance Liquid Chromatography- Ultra-Violet- Visible (HPLC-UV-Vis)

HPLC-UV-Vis analyzed isolated rutin and gallic acid to confirm the purity of isolated rutin and gallic acid using the same previous conditions (Detection of different phytoconstituents by Reverse Phase High-Performance Liquid Chromatography) by the same apparatus.

Fourier Transform Infrared (FT-IR) Spectroscopy

The isolated gallic acid and rutin samples, along with two commercially available standards (gallic acid and rutin), were analyzed using Fourier Transform Infrared (FTIR) spectroscopy (Bruker, USA). For pellet preparation, 2 mg for each sample was thoroughly mixed with 200 mg of anhydrous potassium bromide (KBr) and ground into a homogeneous powder. The mixture was compressed under a hydraulic

press at 7 tons for 3 minutes to form a translucent pellet. Before analysis, the pellet was dried at 50°C for 1 hour to minimize moisture interference.

Correlations between structural assignments and signature bands for many classes of chemicals have been established in the mid-infrared wavenumber range from 4000 to 400 cm^{-1}, with a spectral resolution of 4, and the spectrum takes about three minutes to be recorded [27].

Antioxidant Activity

The scavenging activity of the methanolic extract and vitamin C were detected by the 2,2-Diphenyl-1-Picrylhydrazyl Free Radical Scavenging Assay (DPPH) as follows:

1- DPPH solution: To create a solution containing 0.8 micromolar

2- Solution of vitamin C: Prepare a stock solution with a concentration of 2 mg/mL vitamin C to create five concentrations: 500, 400, 300, 200, and 100 µg/mL.

3- Sample solution: (2 mg/ml) of methanolic extract to make five concentrations: 500, 400, 300, 200, and 100 µg/mL.

The radical scavenging activity of plant extract was confidently assessed using the methodology outlined by [28]. One milliliter of methanolic extract at various concentrations (500, 400, 300, 200, and 100 µg/mL) was added to 1 mL of a DPPH solution, serving as the source of free radicals. The resulting mixture was brooded for 30 minutes at room temperature. The reduction in solution absorbance at a wavelength of 517 nm was measured to assess the sample's proton-donating activity. The reference standard employed in this study was vitamin C. The proportion of activity of DPPH radical scavenging was detected by using a subsequent form:

DPPH radical scavenging activity % $AA = [(A° - A^1) / A^1] \times 100$ equation (1)

where AA stands for antioxidant activity, A° is the "DPPH absorbance", and A^1 is the sample or standard absorbance

Statistical Analysis

The data are presented as the mean ± standard deviation (SD). We performed t-tests and two-way ANOVAs using the SPSS program to assess the DPPH scavenging activity assay results thoroughly. A p-value equal to or lower than 0.05 is definitive evidence of statistical significance.

RESULTS

Phytochemical Screening by Chemical Tests

Following the effective extraction of the aerial parts of the plant in the study, we confirmed through preliminary phytochemical analysis that the methanolic extract of *G. tournefortii* is rich in phenolic compounds. Furthermore, all conducted tests—lead acetate, ferric chloride, and the Shinoda test—returned positive results, demonstrating the extract's significant phytochemical properties.

Detection of Different Phytoconstituents by Reverse Phase HPLC-UV-Vis

The process of qualitative identification was successfully validated through a precise comparison of the retention times obtained under similar chromatographic conditions for the methanolic sample with authentic standards, including rutin, kaempferol, apigenin, genestic acid, quercetin, myricetin, and catechin. The results confirmed the presence of these compounds, as shown in Table 1 and Figures 1, 2, 3, 4, and 5, which agree with similar findings obtained by Dicle University Science and Technology Application and Research Center (DUBTAM) [29].

Table 1 Retention time (RT) values for the methanol sample of *Gundelia tournefortii* compared to standards.

Standards name	Rt value of the sample	Rt value of standards	Concentration(ppm)
rutin	3.80	3.88	32.9
kampeferol	5.18	5.22	90.1
apigenine	9.08	9.08	51.4
genestic acid	3.25	3.20	71.4
quercetin	8.02	8.12	74.1
myricetin	4.16	4.18	44.3
catechin	10.00	10.05	78.9

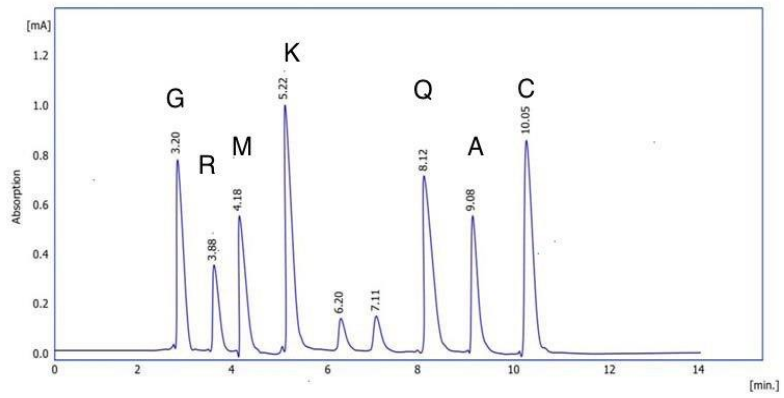

Fig. 1 HPLC-UV-Vis chromatogram for the methanol sample of *Gundelia tournefortii* (G) genestic acid, (R) rutin, (M) myricetin, (K) kaempferol, (Q) quercetin, (A) apigenine, (C) catechin.

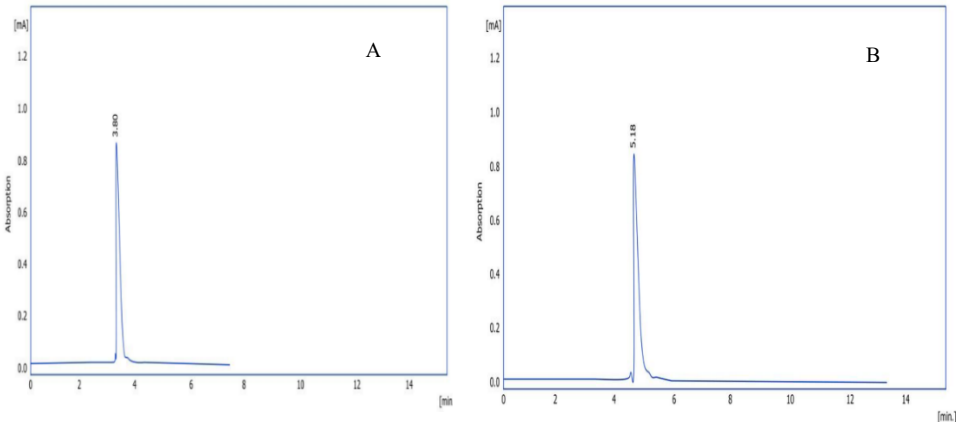

Fig. 2 HPLC-UV-Vis chromatogram for (A) rutin and (B) kaempferol standards.

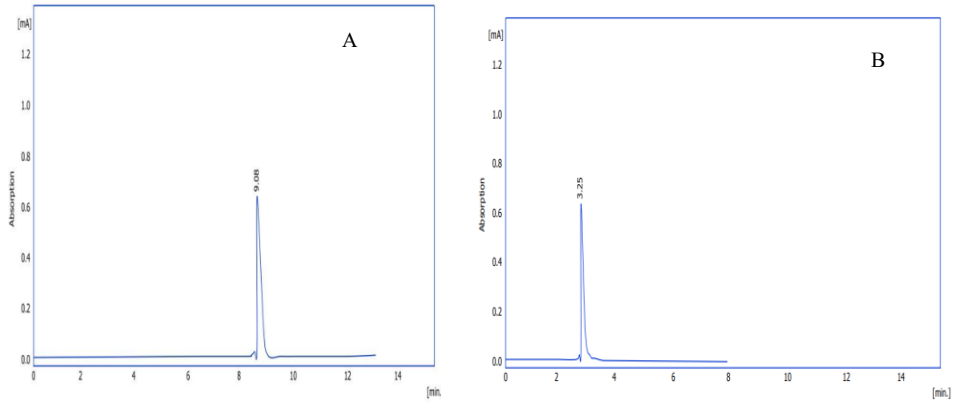

Fig. 3 HPLC-UV-Vis chromatogram for (A) apigenin and (B) genestic acid standards.

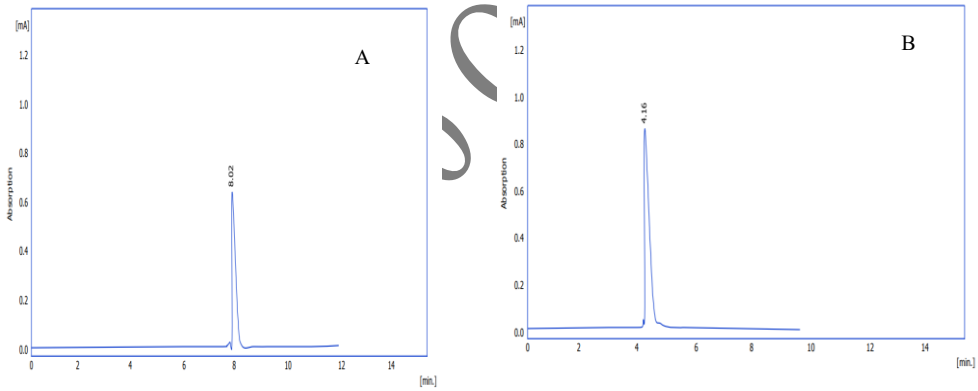

Fig. 4 HPLC-UV-Vis chromatogram for (A) quercetin, (B) myricetin standrads.

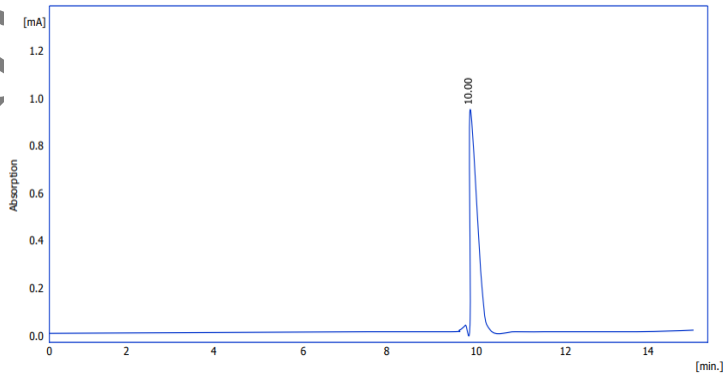

Fig. 5 HPLC-UV-Vis chromatogram for catechin standard.

Isolation of Rutin and Gallic Acid by Preparative Liquid Chromatography

The isolation and purification of rutin and gallic acid were performed using preparative liquid chromatography on the methanol extract obtained by Soxhlet extraction. A spot check was applied for further verification of the isolated components. Ethyl acetate: methanol:

water (8.1:1.1:0.8) was used as the mobile phase for rutin, while chloroform: ethyl acetate: formic acid (5:4:1) was used for gallic acid. UV 254nm was employed to visualize the isolated compounds, as shown in Table 2 and Figure 6

Table 2 Rf values of isolated and standard compounds.

compound	Solvent system	Rf Value of Standard	Rf Value of isolated compound
Rutin	Ethyl acetate: methanol: water 8.1: 1.1:0.8	0.3	0.315
Gallic acid	Chloroform: ethyl acetate: formic acid 5: 4: 1	0.60	0.599

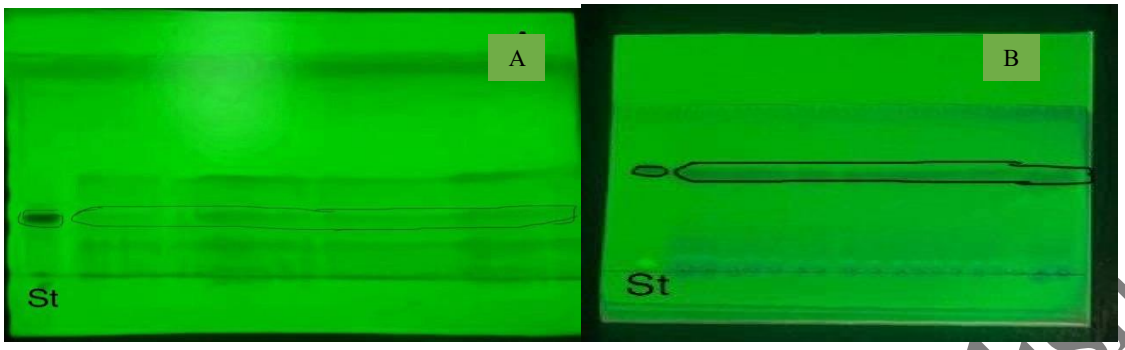

Fig. 6 Preparative layer chromatogram for methanol extract for isolation rutin(A) and isolation gallic acid (B) using 0.5mm silica gel GF254nm plate under UV 254 nm.

Quantity estimation of Rutin and Gallic acid

For quantitative analysis, the calibration curve was plotted using the area under the curve (AUC) versus four concentration levels for each rutin and gallic acid standard from which the concentration of the proposed phenolic phytoconstituents (rutin and gallic acid) in methanol was calculated by applying a straightline equation, as illustrated in figure 7

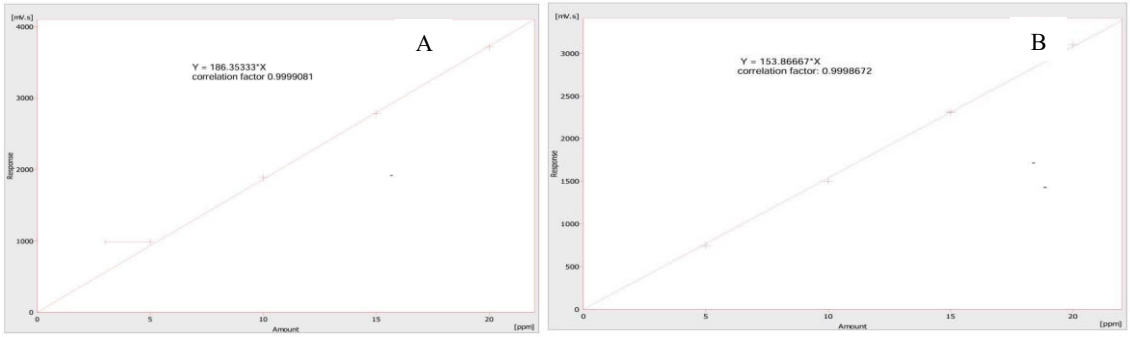

Fig. 7 Calibration curves for Rutin (A) and Gallic acid (B) by HPLC.

Identification and Characterization of Isolated Constituents by PLC

Identification and Characterization of Isolated Rutin

1. Reverse Phase HPLC-UV-Vis

The Isolation of rutin from the extract was further examined using Reverse Phase HPLC-UV-Vis to verify its identity. Retention times of isolated rutin obtained under identical chromatographic conditions were similar to the RT of rutin standards, as shown in Figure 8.

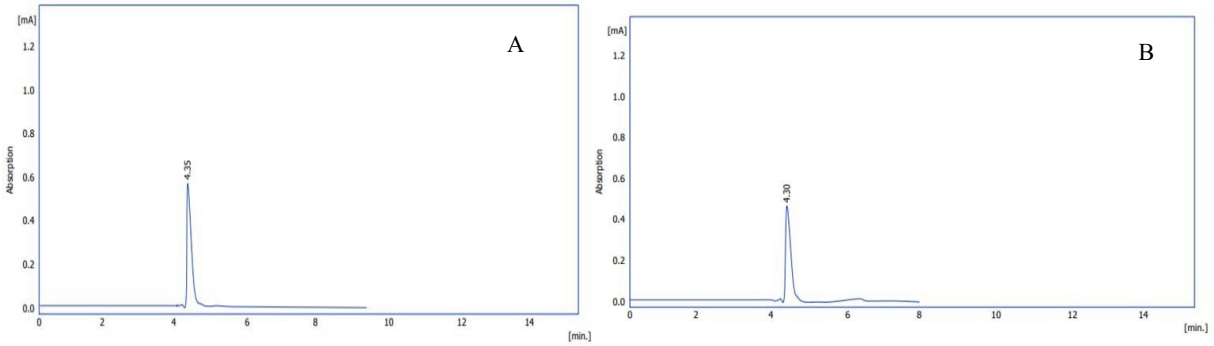

Fig. 8 HPLC-UV-Vis chromatogram of A= rutin standard and B= isolated rutin.

2. Fourier Transforms Infrared (FT-IR) Spectra

In phytochemical research, applying FT-IR spectroscopy as a fingerprinting tool to contrast a natural reference standard with a synthetic one is the most typical use of this technique. Therefore, compared with the standards, the IR spectra and the characteristic IR absorption bands of the isolated rutin produced the same results as shown in Table 3 and Figure 9.

Table 3 Characteristic FT-IR bands of absorption (cm-1) of the isolated rutin compared to the rutin standard.

Functional group	Frequency wave No. for isolated rutin	Frequency wave No. for standard rutin
O–H Stretching	3400	3423.65
C–H Stretching	2880	2929.87
C=O Stretching	1650	1650
C=C Aromating Stretching	1600	1602
C–O	1160	1163.25

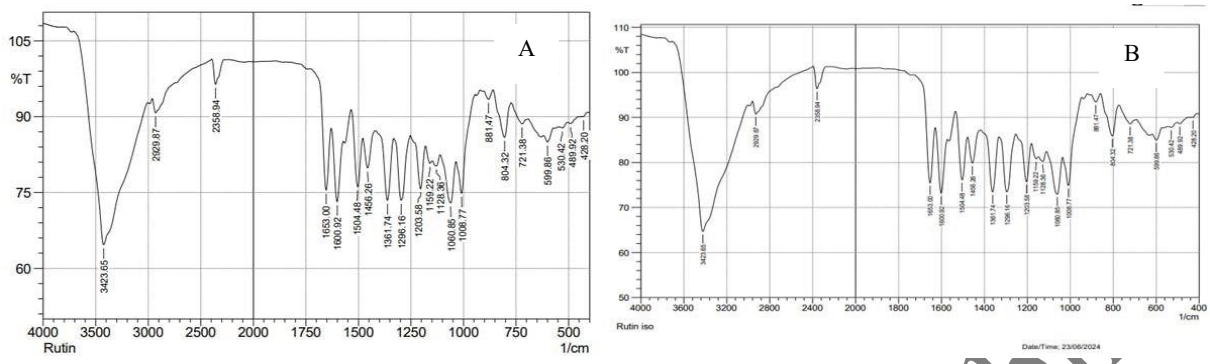

Fig. 9 FT-IR spectra of A= rutin standard and B= isolated rutin.

Identification and Characterization of Isolated Galic Acid

1. Reverse Phase HPLC-UV-Vis

The Isolation of gallic acid was further examined using Reverse Phase HPLC-UV-Vis to verify its identity. Retention times of isolated gallic acid obtained under identical chromatographic conditions were similar to the RT of gallic acid standards, as shown in Figure 10.

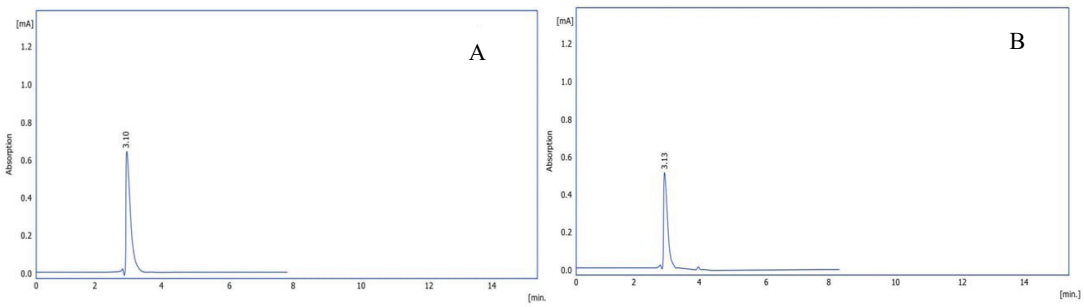

Fig. 10 HPLC-UV-Vis chromatogram of A= gallic acid standard and B= isolated gallic acid.

2. Fourier transforms infrared (FT-IR) spectroscopy

In phytochemical research, applying FT-IR spectroscopy as a fingerprinting tool to contrast a natural reference standard with a synthetic one is the most typical use of this technique. Therefore, compared with the standards, the IR spectra and the characteristic IR absorption bands of the isolated gallic acid produced the same results as shown in Table 4 and Figure 11.

Table 4 Characteristic FT-IR bands of absorption (cm-1) of the isolated gallic acid compared to the gallic acid standard.

Functional group	Frequency wave No. for isolated gallic acid	Frequency wave No. for standard gallic acid
O–H Stretching	3483.14	3356.78
C–O Stretching	1253.42	1253.17
C=O Stretching	1696.53	1696.42
C=C Aromatic Stretching	1616.87	1616.58

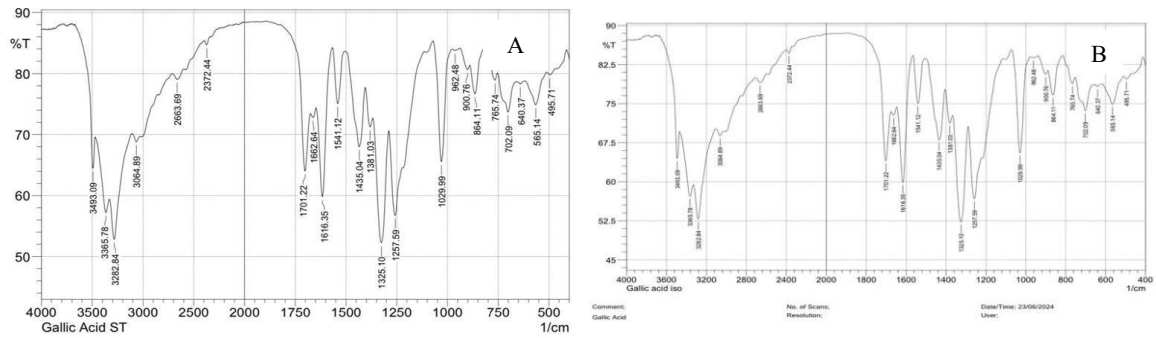

Fig. 11 FT-IR spectra of A= gallic acid standard and B= isolated gallic acid.

Antioxidant Activity (DPPH Assay)

The methanol extract of *G. tournefortii's capacity to scavenge radicals was quantified by measuring the reactivity of DPPH with the methanol extract at a wavelength of 517* nm, as described in previous studies. The capacity of methanolic extract to effectively eliminate free radicals was successfully established, as indicated by the IC50 value for methanol extract and standard reference vitamin C, which was found to be 381.189µg/ml and 416.667µg/ml, respectively, with p-value 0.706519849 (Fig. 12).

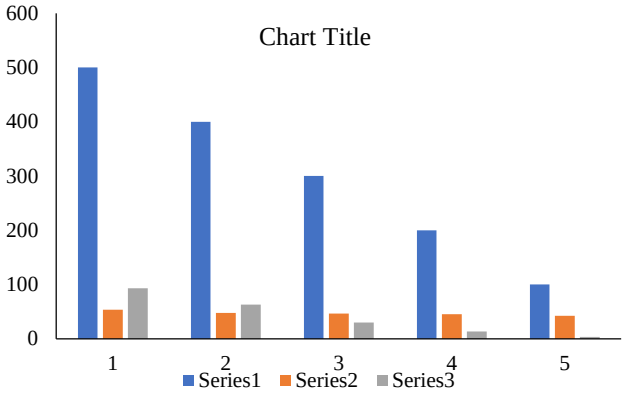

Fig. 12 2,2-Diphenyl-1-Picrylhydrazyl Free Radical Scavenging Assay DPPH.

DISCUSSION

Antioxidants are powerful substances that function as reductants in redox reactions, effectively neutralizing free radicals and other oxidants. They reduce one reactant while simultaneously oxidizing another, which is crucial in maintaining balance within biochemical processes[30].

The antioxidant activity of the methanol extract of *G. tournefortii* was evaluated using the DPPH method, a widely recognized and reliable method due to its accessibility and high sensitivity.[31]. In our study, the results of the DPPH method demonstrate that the methanol extract exhibits significant antioxidant potential, with an impressive IC50 value of 381.189 µg/ml. In comparison, the positive control, vitamin C, shows an IC50 value of 416.667 µg/ml, further highlighting the effectiveness of the methanol extract. The methanolic extract of *G. tournefortii's* remarkable ability to scavenge free radicals is directly attributed to phenolic compounds [32]. Many phenolic compounds exist in the methanolic extract of *G. tournefortii*, as shown in Table 1. Kaempferol is an exceptionally effective scavenger of superoxide. Its capacity to significantly lower superoxide levels at low concentrations is fundamental to its robust antioxidant activity. This is vital, as generating superoxide anions is essential for producing the most reactive oxygen and nitrogen species that contribute to oxidative stress [33]. Apigenin has demonstrated remarkable anti-mutagenic, antioxidant, and anti-tumor properties. The mechanism of apigenin is well-established and involves multiple pathways. It effectively inhibits oxidant enzymes, modulates critical redox signaling pathways such as NF-kB, Nrf2, MAPK, and PI3/Akt, and reinforces both enzymatic and non-enzymatic antioxidants. Additionally, apigenin plays a pivotal role in metal chelation and free radical scavenging, thereby further enhancing its protective effects [35, 34, 35]. A study [36] demonstrates that rutin and quercetin possess antioxidant properties, with quercetin exhibiting greater antioxidant effectiveness. Myricetin exhibits potent antioxidant properties and is an effective scavenger of free radicals. These remarkable activities contribute to a broad spectrum of useful outcomes, including anti-platelet aggregation, immunomodulatory action, antihypertensive effects, anti-inflammatory responses, anti-allergic effects, tranquilizer properties, and helpful in treating cancer[37]. Catechins exhibit potent antioxidant properties and effectively shield the skin from the sun's harmful UV rays [38]. (Fig. 12), which illustrates the comparative percentages of free radical scavenging exhibited by the methanol extract of *G.tournefortii* and the reference standard vitamin C at equivalent concentrations of methanolic extract, which indicates that the extract of *G. tournefortii* at concentrations of 500 and 400µg/mL has high inhibition percentage more than the standard (vitamin C), which mean that the methanolic extract demonstrates superior antioxidant activity compared to vitamin C.

CONCLUSIONS

Upon analyzing the data from the current investigation, it has been conclusively determined that the methanolic extract obtained from the aerial parts of Iraqi *G. tournefortii* contains a diverse range of phenolic compounds, including rutin, kaempferol, apigenin, genistein acid, quercetin, myricetin, and catechin, as detected by HPLC UV-Vis. This is the first study conducted in Iraq about the Isolation of rutin and gallic acid by PLC from the aerial parts of *G. tournefortii.* Different techniques, such as HPLC and FTIR, were employed to characterize the isolated components. The methanolic extract exhibits remarkably high antioxidant activity, as determined by the DPPH assay, due to the presence of these phenolic compounds, with an IC50 value of 381.189 µg/mL. This value surpasses that of vitamin C, which has an IC50 value of 416.667 µg/mL. Based on these results, *G. tourefortii* can potentially be used to prepare antioxidant supplements.

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