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Phytochemical Investigation of *Sonchus Oleraceus* (Family:Asteraceae) Cultivated in Iraq, Isolation and Identification of Quercetin And Apigenin

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Abstract

Objective: The aim of our study was to investigate chemical constituents of leaves of Sonchus oleraceus grown naturally in Iraq because in Iraq no phytochemical investigation had been previously made in Iraq.
Methods: Leaves of *S . oleraceus* were macerated in absolute methanol for 2 days, filtered and fractionated by petroleum ether, chloroform, ethyl acetate, and n-butanol. The ethyl acetate fraction was analyzed by high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) for its phenolic acid contents and phenolic acid were isolated from this fraction and identified by gas chromatography/mass spectrometry, infrared, ultraviolet, HPLC, and HPTLC.
Results: The different chromatographic and spectroscopic results revealed the presence of flavnoids.
Conclusion: The results of the current study showed the presence of quarcetin in the ethyl acetate fractionand and apigenin in n-butanol fraction of *S. oleraceus.*

Keywords: *Sonchus oleraceus,* flavnoids , High-performance liquid chromatography, High-performance thin-layer chromatography.

INTRODUCTION

Herbal products are complex mixtures of organic chemicals that can come from any raw or processed part of a plant, including leaves, stems, flowers, roots and seeds [1]. they usually contain a number of pharmacologically active compounds.

The plant Sonchus oleraceus belongs to the daisy family (Asteraceae)[2] is an upright, annual herb with simple branches. A special feature of these sow thistle is that the largest part of the plant is smooth and bare, without ant hair or bristles. The stems are hollowed out and have a milky sap and their lower part gets a purple color later in the spring. The leaves vary according to age, the old leaves are long-stalked and deeply lobed. The color of the leaves varies from light green to greenish blue and may have a jagged outline, but on spikes or hairs. The fruits are simple achenes, brownish in color and oval / oblong in shape. Plant has various uses such as cancer, digestion, laxative, emollient, blood purifier and also of strain of Rajsthan used as liver tonic[3].

S. oleraceus is native to Europe, North Africa and West Asia. It has spread to North and South America, India, China, southern Australia[4]. In New Zealand[5], S. oleraceus is estimated as a green vegetable that is usually cooked with meat. The genus Sonchus comprises about 60 species, and three of them have become common weeds around the world. These are S. arvensis, perennial Saw thistle and the two annual species S. oleraceus, common Saw thistle and S. asper, prickly Saw thistle. The main constituents of Sonchus L. were terpenes, steroids, flavones, coumarins , etc. It has hepatoprotective activity, antitumor effect, cardiovascular therapy, etc.[6].

Ten compounds were isolated and elucidated as luteolin(Ⅰ),luteolin-7-O-β-D-glucoside(Ⅱ),apigenin(Ⅲ),apigenin-7-O-β-D-glucuronide methyl ester(Ⅳ),apigenin-7-O-β-D-glucuronide ethyl ester(Ⅴ),apigenin-7-O-β-D-glucopyranuronide(Ⅵ),germanicyl acetate(Ⅶ),3β-hydroxy-6β,7α,11β-H-eudesm-4-en-6,12-olide(Ⅷ),oleanolic acid(Ⅸ) and 1-cerotol(Ⅹ) from Sonchus oleraceus.[7]. Caffeic acid is a type of polyphenol, -7-glucoside and apigenin-7-glucoside. In addition, flavonoids (luteolin, apigenin, kaempferol, and quercetin) and their glucoside derivatives were identified from whole plant extracts Recently, caftaric acid has also been identified from leaf extracts [8]. S.oleraceus L. (family Asteraceae) is known for its high content of antioxidants and antioxidant activity [9,10,11,12,13].The amount of polyphenol and the antioxidant activity in plants depend on environmental factors such as the growing season and location [14]. Some flavonoids isolated from *S. oleraceus* showed antioxidant activity [15].

Flavonoids are naturally occurring compounds as secondary metabolites in plants functioning as a plant's physiological survival by protecting against fungal infections and UV radiation [16,17].

Apigenin: A Flavones are the most common substances in the flavonoid group with antitumor activity.[18], anxiolytic properties[19], anti- inflammatory[20,21], antioxidant, and anticarcinogenic properties[22,23], antiviral, antibacterial, insecticidal, apoptotic, anti- aging, and antioxidant properties[24].**Apigenins** play an important role in human cancer: breast cancer[25], Cervical Cancer[26], Colon Cancer[27], Hematologic Cancer[28], Lung Cancer[29].

Quercetin is one of the important bioflavonoids which has anti-inflammatory[30], antihypertensive[31], anticarcinogenic properties[32], antibacterial[33] and antiviral activity[34] vasodilator effects, antiobesity, antihypercholesterolemic and antiatherosclerotic activities.[35,36].

METHODS

Collection of plant materials

The plant was collected from Almasyab -Babil - Iraq in May-2017. The plant was identified and authenticated by Dr .Ibrahim Saleh Abbas.

Equipment and chemical

The instruments used were rotary evaporator (BÜCHI Rotavapor R-205, Swiss), sonicator (Baranson Sonifier, USA), high-performance liquid chromatography (HPLC) (Sykam, Germany), and high-performance thin-layer chromatography (HPTLC) (Eike Reich/CAMAG-Laboratory, Switzerland). All chemicals and solvents used were of analytical grade and obtained from Riedel-de Haen, Germany, except trifluoroacetic acid, and methanol which are HPLC grade purchased from Sigma-Aldrich, Germany. The standard quercetin and apigenin were purchased from Chengdu Biopurify Phytochemicals, China (purity >97). Thin-layer chromatography (TLC) aluminum plates pre-coated with silica gel 60 F 254 (100 mm×100 mm, 0.2 mm thick) used were obtained from E. Merck Ltd., India.

Extraction

Leaves of S. oleraceus were thoroughly washed and dried in the shade. The dried plant was pulverized in a mechanical grinder. 500g S. oleraceus leaf powder were macerated in methanol for 3

days and filtered, and the filtrate was evaporated to dryness using a rotary evaporator under vacuum. 15 g of the residue was suspended in water and then fractionated by partitioning with petroleum ether, chloroform, ethyl acetate and n-butanol (100 ml x 3) for each fraction. The first three organic layers were dried over anhydrous sodium sulfate, filtered and evaporated to dryness.

Preparations of standards and samples for analysis

Standard solutions for the quarcetin and apigenin HPLC were prepared by dissolving 0.04 mg in 1 ml HPLC HPLC grade. Dried samples were prepared for HPLC analysis by dissolving in methanol and sonicating at 60 ° C for 25 minutes at 60 ° C, followed by centrifugation at 7500 rpm for 15 minutes. The clear supernatant of each sample was evaporated in vacuum. Residues were individually resuspended in 1 ml HPLC grade, homogenized using a vortex mixer and passed through a 2.5 µm disposable filter and stored at 4 ° C for further analysis. 20 µl of the sample was injected into the HPLC system for analysis.

Standards used for HPTLC analysis (quercetin and apigenin) were prepared by dissolving 1 mg of each standard in 1 ml of methanol while preparing samples by dissolving a few milligrams of each sample in 1 ml of methanol.

Preliminary phytochemical investigation

Test for flavonoids

Few milligrams of the ethyl acetate fraction were suspended in ethanol and few drops of 5% ethanolic KOH were added, and then, few drops of 5% HCl were added. The changes in colors were recorded.

Test for phenols

Few milligrams of methanol plant extract were treated with few drops of 1% FeCl3. Formation of dark greenish-blue color indicates the presence of phenols.

Isolation of *flavonoids* derivative by preparative TLC

A phenolic derivative was isolated by preparative TLC, utilizing ethyl acetate fraction , n-butanol fraction.

Preparation of stationary phase

Readymade silica gel GF254 plates with a layer thickness of 0.5 mm dimension 20×20 cm. The plates were reactivated by heating in the oven at 120°C for 15-20 minutes, left to cool, and used for application after allocation of the baseline and the solvent front.

Preparation of mobile phase (solvent system)

The constituents of the mobile phase for *flavonoids* (chloroform: acetone: formic acid - 75:16.5:8.5 V/V) were mixed in a conical flask and introduced in the jar. The jar was lined with a filter paper, closed tightly, and left for saturation.

Application of sample

About 1 g of the sample was dissolved in absolute methanol and applied on the baseline of TLC plates using a capillary tube.

Detection of separated spots

Detection was carried out by examination under UV light with two wavelengths, namely 254 and 366 nm. The purity of each band was checked by analytical TLC until a single spot on the TLC plate was obtained for identification with the reference standard.

HPLC analysis

HPLC technique (SYKAM,Germany) was applied for the detection of different constituents found in the ethyl acetate fraction and n-butanol fraction as, flavonoids, and phenolic acids using a mobile phase composed of methanol (Solvent A), and Solvent B was 0.05% trifluoroacetic at pH 2.5, gradient program from A=70%(0-5min),A=40%(5-8min),A=90%(8-15) with flow rate 1.0 ml/ min, wavelength 280 nm, and a column nucludar C-18-ODS(25cm*4.6mm), Autosampler model:S5200, Pump model:S2100 Quaternary Gradient pump ,Column Oven modl:S4115,Fraction Collector Model=Frac-950.

HPTLC analysis

The ethyl acetate fraction was also analyzed for its flavonoid and phenolic acid contents using HPTLC (Eike Reich / CAMAG Laboratory, Switzerland) using silica gel GF254 plates prepared in a mobile phase from chloroform: acetone: formic acid (75 :16.5: 8.5) at 280 and 366 nm wavelengths.

Identification of isolated flavonoids derivatives

The isolated flavonoids derivatives were identified by different spectroscopic and chromatographic techniques listed below:

• Gas chromatography/mass spectrometry (GC/MS): The GC/MS instrument used was GC/MS-QP 2010 ultra: SHIMADZU/GC GC 2010 Plus.
• Conditions used for GC/MS analysis are the followings:
• Column temperature: 50.0°C
• Injection temperature: 280.0°C
• Injection mode: Split less
• Sampling time: 1 minute
• Flow control mode: Pressure
• Pressure: 100.0 kPa
• Column flow: 1.2 ml/min
• Linear velocity: 45.4 cm/s
• Purge flow: 3.0 ml/min.
• Infrared (IR): IR spectra for quercetin and apigenin was recorded in KBr disk , the range of scanning 4000-400 cm−1
• UV: Few milligrams of quercetin and apigenin were dissolved in 1 ml absolute methanol, and its UV absorbance was scanned from 200 to 400 nm
• HPLC: As listed before
• HPTLC: As listed before.

RESULTS

Preliminary examination of the ethyl acetate fraction and n-butanol fraction results is shown in Table 1.

The HPLC results of analyzed ethyl acetate fraction and n-butanol show the presence of the listed compounds according to their retention times shown in the Table 2 and their chromatograms in Fig. 1,2compared with that of standard quercetin and apigenin shown in Figs. 3and 4, respectively.

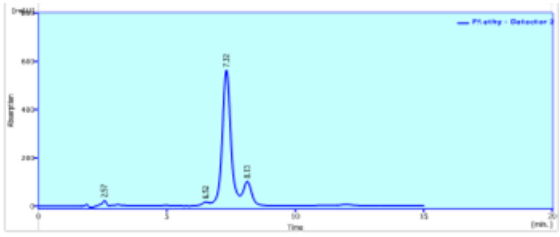


Fig. 1: High-performance liquid chromatography chromatogram of ethyl acetate fraction

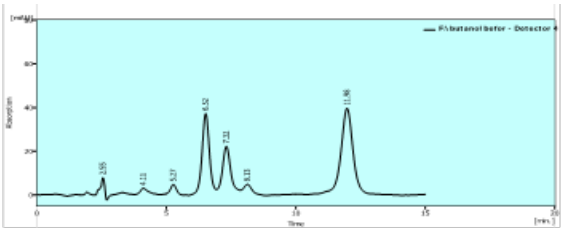


Fig.2: High-performance liquid chromatography chromatogram of n-butanol fraction

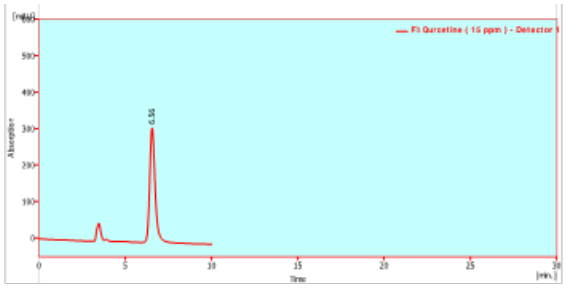


Fig. 3: High-performance liquid chromatography chromatogram standard quercetin

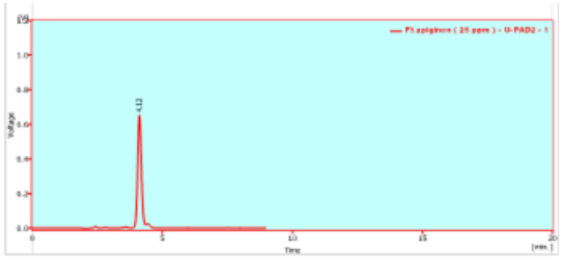
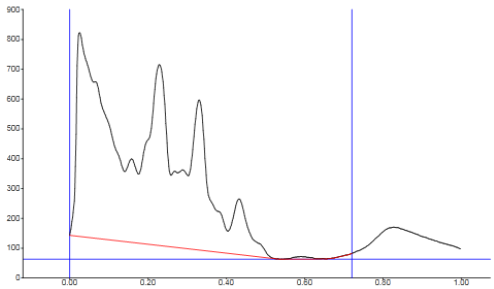
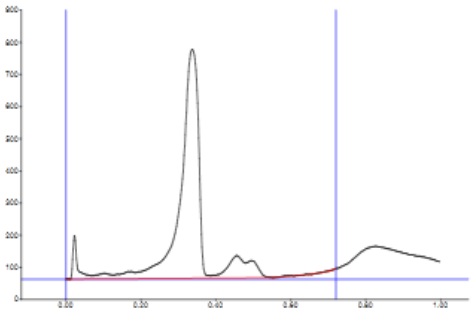


Fig.4: High-performance liquid chromatography chromatogram standard apigenin.



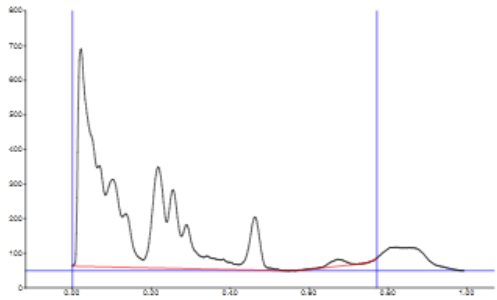
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height
1	0.00	10.5	0.03	683.1	27.08	0.14	235.0
2	0.14	235.3	0.16	279.7	11.09	0.18	231.5
3	0.18	232.6	0.23	606.2	24.03	0.26	240.0
4	0.28	250.6	0.29	263.0	10.43	0.30	245.2
5	0.30	246.3	0.33	503.5	19.96	0.40	74.4
6	0.41	75.1	0.43	186.8	7.41	0.52	1.2

(a)



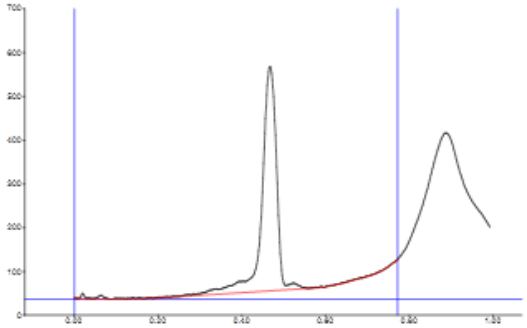
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height
1	0.01	0.7	0.02	135.0	13.92	0.07	10.6
2	0.20	21.1	0.34	711.5	73.39	0.38	8.6
3	0.41	10.8	0.46	69.6	7.18	0.48	46.2
4	0.48	46.4	0.50	53.3	5.50	0.55	1.1

(b)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height
1	0.00	1.8	0.02	628.6	32.69	0.06	286.1
2	0.08	207.5	0.10	253.2	13.17	0.13	145.9
3	0.13	145.9	0.14	155.1	8.07	0.18	22.3
4	0.18	22.7	0.22	292.1	15.19	0.24	132.9
5	0.24	133.6	0.26	227.0	11.80	0.28	93.1
6	0.28	95.1	0.29	127.3	6.62	0.33	34.0
7	0.33	34.1	0.34	37.8	1.97	0.38	27.5
8	0.38	27.5	0.38	29.4	1.53	0.41	15.6
9	0.42	14.6	0.46	152.9	7.95	0.52	3.6
10	0.63	1.1	0.67	19.8	1.03	0.72	1.2

(c)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height
1	0.01	0.2	0.02	11.8	2.09	0.04	0.5
2	0.35	11.1	0.40	26.1	4.62	0.41	24.7
3	0.41	25.1	0.47	512.2	90.80	0.51	10.0
4	0.51	10.0	0.52	14.0	2.48	0.56	0.2

(d)

Fig. 5: (a) Track 1: High-performance thin-layer chromatography (HPTLC) of ethyl acetate fraction. (b) High-performance thin-layer chromatography (HPTLC) of standard quercetin. (c)Track 2: HPTLC of n-butanol fraction. (d) Track 2: HPTLC of standard apigenin.

HPTLC results are shown in Fig. 5. Preparative TLC chromatograms used for isolation of quercetin are shown in Fig6. Preparative TLC chromatograms used for isolation of apigenin are shown in Fig7.

HPTLC plates used for isolation of quercetin are shown in Fig. 8. HPTLC chromatogram (a) at 366 nm and (b) at 254 nm.

HPTLC plates used for isolation of apigenin are shown in Fig. 9. HPTLC chromatogram (a) at 366 nm and (b) at 254 nm

GC/MS chromatogram of the isolated quercetin and apigenin

GC/MS chromatogram of the isolated quercetin are shown in Figs. 10.

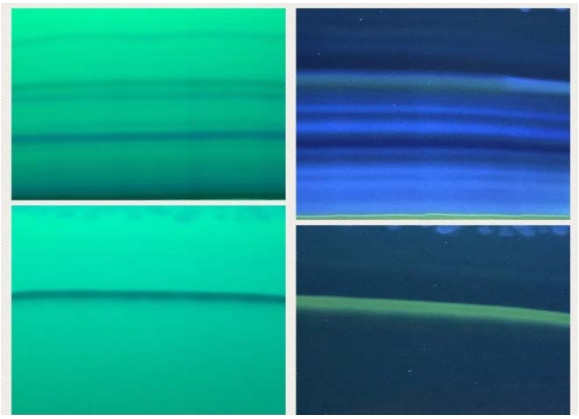
GC/MS chromatogram of isolated apigenin are shown in Figs. 11.

Table 1: Preliminary examination of the ethyl acetate fractionand n-butanol fraction for flavonoids.

Part used	Fraction	Flavonoids
Leaves	Ethyl acetate	+
Leaves	Butanol	+

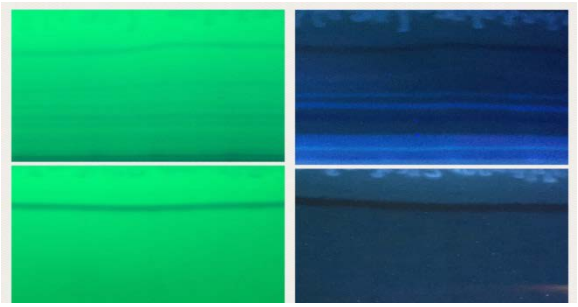
Table 2: Retention time in minutes of standard quercetin and apigenin

Standard material	Retention time
Quercetin	6.560
Apigenin	4.12



(a) (b)

Fig. 6: Preparative thin-layer chromatography for standard quercetin (1) and ethyl acetate fraction (2) and on silica gel GF254, detection by ultraviolet light (a) at 254 nm and (b) at 366 nm



(a) (b)

Fig. 7: Preparative thin-layer chromatography for standard apigenin(1) and n-butanol fraction (2) and on silica gel GF254, detection by ultraviolet light (a) at 254 nm and (b) at 366 nm

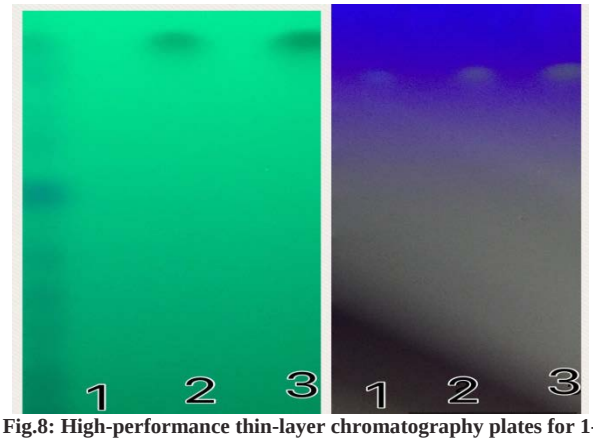


Fig.8: High-performance thin-layer chromatography plates for 1-ethyl acetate fraction and 2-isolated3-standard quercetin detection under ultraviolet light (a) at 254 nm and (b)at 366 nm

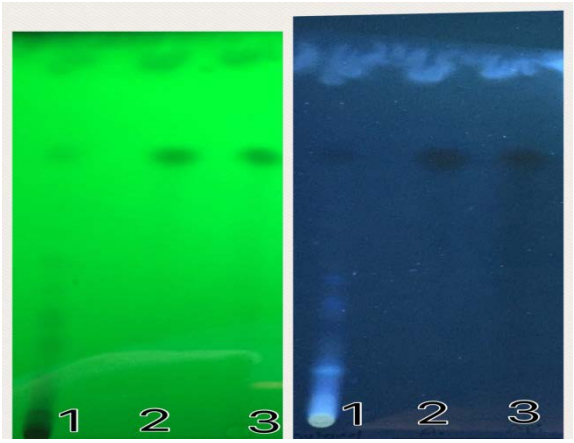


Fig.9: High-performance thin-layer chromatography plates for 1-n-butanol fraction and isolated3-standard apigenin, detection under ultraviolet light (a) at 254 nm and (b)at 366nm

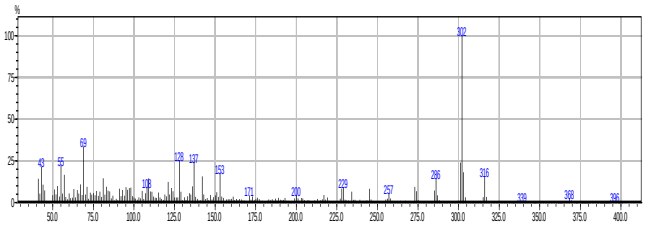


Fig. 10: Gas chromatography/mass spectrometry chromatogram of isolated quercetin

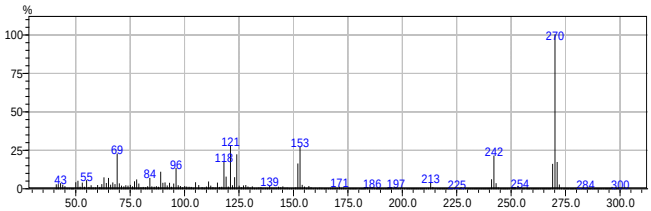


Fig. 11: Gas chromatography/mass spectrometry chromatogram of isolated apigenin

IR spectroscopy

The IR spectral analysis of the isolated **flavonoids compound (quercetin)** showed the peaks at 3333.10 , 3093.92,1697.41,1608.69, 1521.389,1261.49, 1014.59, 864.14 and680.89 cm−1.

IR spectral analysis of the isolated **flavonoids compound (apigenin)** showed the peaks at 2900-3400, 3012.91, 2856.67, 1649.19, 1554.68, 1442.80,1242.20,1178.55and 736.83 cm−1 .

The IR spectra of the isolated **flavonoids compound(quercetin , apigenin)**is shown in Figs. 12and 13, respectively.

UV spectrum of the isolated **flavonoids** compound is shown in Figs. 14 and 15, respectively.

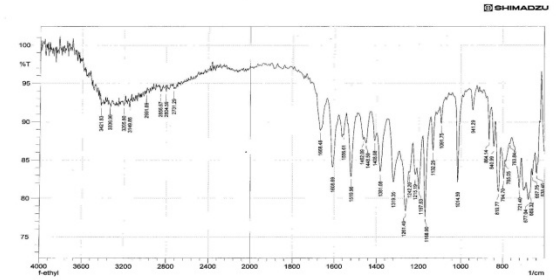


Fig. 12: Infrared spectrum of isolated quercetin

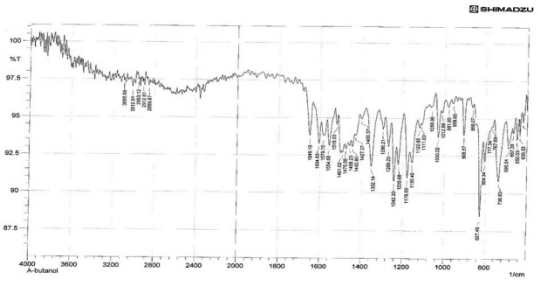


Fig. 13: Infrared spectrum of isolated apigenin

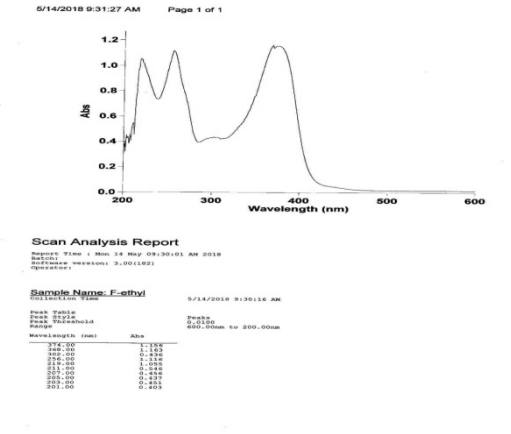


Fig. 14: Ultraviolet spectrum of isolated (F=quercetin)

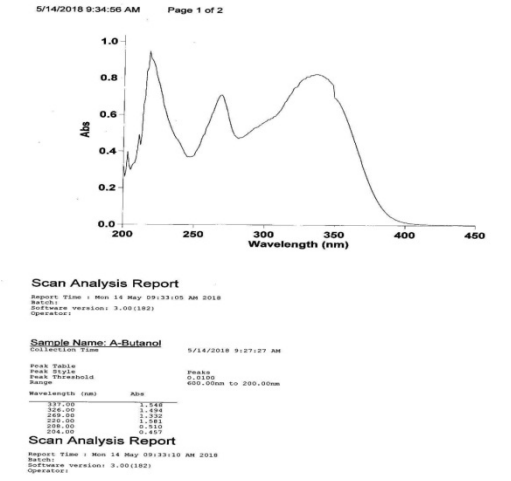


Fig. 15: Ultraviolet spectrum of isolated apigenin

DISCUSSION

Thin-layer chromatography (TLC) is a relatively old technique among the various chromatographic separation methods, but it shows some marked advantages over the other chromatographic techniques[37].

TLC or HPTLC is mainly used as a cost-effective method of separation, qualitative identification or semi-quantitative visual analysis of samples. Accordingly, TLC is often described as a pilot method for HPLC [38]. However, recent reviews show that the TLC and HPTLC techniques can be used to solve many qualitative and quantitative analytical problems in a variety of fields including medicine, pharmacy, chemistry, biochemistry, food analysis, toxicology, and environmental analysis [39].

Natural products were the only most prolific source of drug development. More than 100 new products are in clinical develoapment, especially as anti-cancer agents and anti-infective agents [40]. Natural products have played a key role in modern drug development, especially for antibacterial and antitumour drugs [41]. Most of these natural products are secondary plant metabolites. During the extraction solvent to diffuse into the solid plant material and solubilize compounds of similar polarity. The extraction and separation of chemical constituents of plants depends on selective solvents by standard techniques.

Quercetin is a class of natural compounds widely used in plants has an important role in health care and various traditional medical systems in the world. Quercetin has antioxidant and anti-inflammatory effects that can help reduce prostate inflammation [42,43].

Fractionation is the best way to separate each group of constituents alone if the plant contains several groups of constituents.

Preliminary phytochemical analysis confirmed the presence of flavonoids. The ethyl acetate extract of the cold process, which was further subjected to isolation, gave pale yellow crystals.

In the IR spectral analysis(**quercetin**), the peak at 3333.10 cm−1, a broad band is most probably the result of O-H stretching vibrations of phenol OH group. The peak at 2947.33 cm−1 showed C-H stretching due to –CH3. The peak at 1697.41 cm−1 indicates the presence of -C=O, carbonyl group . The peak at 1608.76 cm−1 showed the presence of -CH=CH group. The peak at 1521.89 cm−1 indicates the presence of benzene ring. The peak at 864.14cm−1showed the presence of disubstitution of benzene ring of isolated compound. The molecular weight of isolated quercetin detected in the GC/MS at302 m/z . The UV florescence, HPLC, and HPTLC results are similar to that of standard quercetin .All the above results confirm that the isolated compound is quercetin.

Quercetin, **flavonoids** derivative, has been found in many plant species and has various anti-inflammatory, antihypertensive, vasodilator effects, antiobesity, antihypercholesterolemic and antiatherosclerotic , antioxidant activities as well as pharmacological effects[44].

The IR spectra of the isolated compound (Fig. 13) showed a broad intermolecular OH stretch vibrations band at ~3333 cm^{-1}. There was an aromatic C–H stretch, 3040 cm^{-1}. There is a vibration band at 1646 cm^{-1} characteristic for flavone of conjugation between the C=O and double bonded of C2–C3, also, 1801 cm^{-1} for lactone ring. The IR spectra showed three vibration bands (1466, 1497, and 1578 cm^{-1}) for the ring C=C, while 1466 cm^{-1} denotes the characteristic of C–O–H stretch. The intensive band at 1024 cm^{-1} was most probably the result of C–O–C stretch from the central heterocyclic ring. The molecular weight of isolated apigenin detected in the GC/MS at270 m/z . The UV florescence, HPLC, and HPTLC results are similar to that of standard apigenin .All the above results confirm that the isolated compound is apigenin.

Apigenin showed a reduction in carrageenan-induced paw swelling throughout the duration of the experiment and induced strong anti-inflammatory activity. The effectiveness of apigenin at 1 and 3 hours indicates its antagonistic effect on prostaglandin, BK, histamine and serotonin. The production of histamine and serotonin occurs after one hour, while BK and prostaglandin are released after the injection of carrageenan after two and three hours, respectively.[45] High-dose apigenin was similar to the anti-inflammatory effect of diclofenac sodium. The results obtained are an indication that apigenin might be effective in acute inflammatory conditions. Since inflammation is also accompanied by pain, most anti-inflammatory drugs have an antinociceptive potential. The peripheral analgesic activity of the drugs could be via the inhibition of cyclooxygenases (COX) and / or lipoxygenases as well as other mediators, while the central

analgesic effect could be through the inhibition of central pain receptors.

Apigenin and their glycosides have been shown to have potent antibacterial effects[46], antileukemic activities, Apigenin-7-O-glucuronide is known to have anti-inflammatory activity .

The significance of this study is that it is the first study to confirm the presence of quercetin and apigenin in the Iraqi species of S. oleraceus.

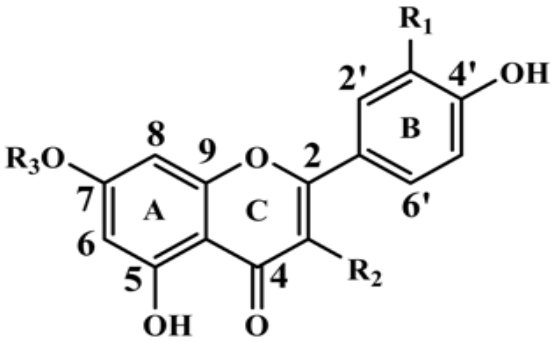


Fig.18 Structures of compound isolated from S. oleraceus.
R1=R2=OH, R3=H: quercetin ,, R1=H ,R2=H, R3= H: apigenin

CONCLUSION

The results of the current study showed the presence of **flavonoids** derivative, i.e., quercetin in the ethyl acetate fraction and apigenin in butanol fraction .

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