Isolation and Characterization of Isorhamnetin and Kaempferol from *Elaeagnus Angustifolia* (F:Elaeagnaceae) Cultivated in Iraq

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Abstract:

Elaeagnus angustifolia, is from Elaeagnus genus and a member of the Elaeagnaceae family which is reported to have many species that are distributed throughout the world. The plant acquired a considerable importance because it is a rich source of vitamins such as tocopherol, carotene, vitamin C, thiamine B1 and minerals like calcium, magnesium, potassium, iron and manganese. from the phytochemical point of view, the plant was reported to contains: flavonoids, alkaloids, terpens, sterols and saponin. TLC fingerprinting was used to identify the ethyl acetate extract containing flavonoids. The isolation and purification afforded crystalline powder which were subjected to spectral identification by ^{1}H-NMR and FT-IR. The compounds were identified as Isorhamnetin and Kaempferol.

Key words: *Elaeagnus angustifolia* ,1H-NMR, ethyl acetate extract.

فصل وتشخيص مادتي الايزورامنتين والكامفيرول من نبات تمر العجم (عائلة اليكيناسيا)المستزرع في العراق

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الخلاصة:

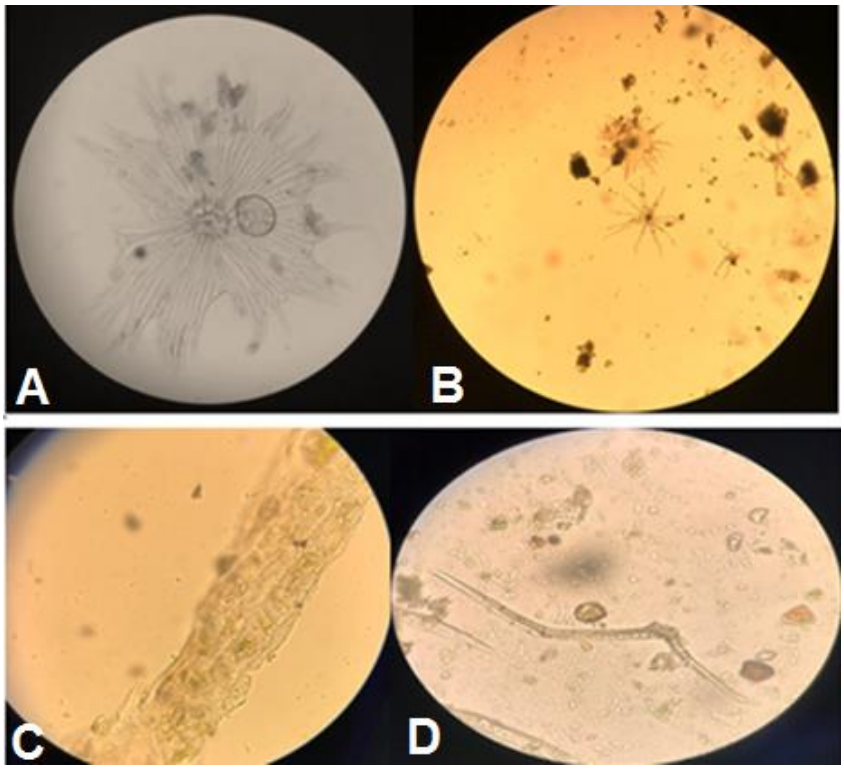
تمر العجم هو من جنس Elaeagnus وينتمي الى عائلة Elaeagnaceae التي اثبت ان لديها العديد من الانواع المنتشرة حول العالم .النبات اكتسب اهمية بالغة لأنه مصدر غني بالفيتامينات مثل التوكوفيرول، الكاروتين فيتامين سي، ثايمين والعديد من المعادن مثل الكالسيوم، المغنيسيوم، البوتاسيوم ،الحديد والمنغنيز.من الناحية الكيمونباتية، اثبت ان النبات يحتوي على الفلافينويد، القلويد، التربين ،الستيرول والسابونين. وجد في تقنية استشراب الطبقة الدقيقة ان مستخلص الاثيل اسيتيت يحتوي على الفلافينويدات والتي تم فصلها وتنقيتها والحصول عل مسحوق بلوري الذي تم تعيين الشكل الكيميائي بواسطة طيف الرنين النووي المغناطيسي والاشعة تحت الحمراء .اثبت الكشف ان المركبين هما الكامفيرول والايزورامنتين.

الكلمات المفتاحية: تمر عجم ، الرنين المغناطيسي ،مستخلص الاثيل اسيتيت.

Introduction:

Elaeagnus angustifolia **is** a member of Elaeagnaceae family that is generally known as Oleaster or Russian olive. The Ealaeagnus family is completely unrelated to the actual olive family" oleaeuropaea" [1]. *Elaeagnus* plants are deciduous or evergreen shrubs or small trees. [2] The genus Elaeagnus consist of about 70-80 species with an ability to grow under a

wide range of environmental conditions [3]. The microscopical examination of the of the plant leaf shows anomocytic stomata with multicellular trichomes, calcium oxalates in addition to starch were presented [4] as shown in figure1. The decoction and infusion of the fruit, flower, leaf and bark of E.angustifolia L. have been used traditionally to treat a variety of illnesses and their symptoms. In Iranian folk medicine, fruits have been used for the relief of pain and inflammation in patient with rheumatoid arthritis and for accelerating the wound healing process in an injured area [5,6]. Recent pharmacological studies have shown that E. angustifolia L. has anti-inflammatory, antimicrobial, anti-oxidant, anti-tumor and cardiovascular effect [7] Alarge number of phytochemical components have been derived from Russian olive and made this plant source of flavonoids, alkaloids, saponin, terpens, complicated and sterols. [8]



Figure(1): microscopical examination of E.angustifolia leaves
A-Star trichomes shape 40x B- Star Trichome Shape 10x C-Anomocytic Stomata 10x D-Calcium Oxalate and Starch.

The aim of this study was qualitative-quantitative analysis of chemical constituents of Elaeagnus angustifolia leaves since no phytochemical investigation had been done previously in Iraq.

Materials and Methods

Plant Material

Elaeagnus angustifolia leaves were collected from Al-Musayyib area, Babil province, Iraq. leaves were collected during March and dried in shade at room tempreture, grinded as powder and weighed.

Extraction and Detection of Flavonoid:

The powdered Arial part of E.angustifolia (100g)were defatted with hexane(700ml) then the defatted plant material was further extracted with 80% ethanol(700 ml) using soxhlet extractor .The ethanolic extract was concentrated by evaporation under reduced pressure using rotary evaporator. Then distilled water (35 ml) was added to the ethanolic extract, and the extract partitioned with ethyl acetate (50 ml) and

allowed to settle down. The lower aqueous layer was collected and labeled as fraction A, while the upper ethyl acetat layer was collected and labeled as fraction B

Indirect Detection of Flavonoid Glycosides by Acid Hydrolysis

In order to investigate the flavonoids after hydrolysis, Hydrochloric acid (35% ,7 ml) was added to (100 ml) of fraction (A) and was refluxed for 1.5 hour. The hydrolyzed extract was partitioned with ethyl acetate (3×100 ml).the lower aqueous layer was collected and labeled as fraction C and the upper ethyl acetate layer was labeled as fraction D which is dried with anhydrous magnesium sulphate, then Benedict test was performed to both aqueous layers before and after acid hydrolysis (fraction A and C) to detect the presence of sugar. Fraction A and C (2mL) were placed in two separated test tubes and then Benedicts' reagent (4mL) was added to each test tube. The solutions were heated in a boiling water bath for 10 minutes.

Thin layer chromatography

Ethyl acetate layer after and before hydrolysis fractions using silica gel GF254Pre-coated aluminum plates developed with following mobile phases:

•S1: Toluene: chloroform: acetone (40: 25: 35).

•S2: chloroform; methanol (90: 10).

The flavonoids contents of these fractions were compared with standards of Kaempferol and Isorhamnetin. (9)

Isolation of phytochemicals by preparative layer chromatography (PLC)

-For isolation and purification of aglycon I, 2 gm of ethyl acetate fraction before hydrolysis was conducted on preparative TLC and it was developed in S2 solvent system. The band was Detected under UV light at 254nm and 366nm

-aglycon II was isolated from ethyl acetate fraction after hydrolysis of the leaves utilizing the same procedure applied to ethyl acetate fraction before hydrolysis but applied 1.30gm of fraction on plate and using S1 mobile phase. the band was Detected under UV light at 254nm and 366nm.

Identification of Phytochemicals by High Performance Liquid Chromatography (HPLC):

The ethanol extract of E.angustifolia was analyzed by HPLC. Qualitative analysis was applied on liquid chromatography SYKNM Germany equipped with binary delivery pump model S 5200, the eluted peaks were monitored by UV-Vis 3240, column: nuclear C18-0DS, 2µm particle size (25x 4.6mm x5 µm). Mobile phase: linear gradient of solvent A 20% methanol and 2 %acetic acids in deionized water, solvent B was 70 % mehanol and 2 %acetic acids in deionized water. Gradient program from 0% B to 100% B for 11 minutes, flow rate 0.8 mL/ min, detection was done by UV at 280 nm.

Structure Elucidation of Organic Compounds by Spectroscopy:

1H- NMR:

The 1H-NMR spectra were performed at The University of Jordan, Faculty of Science, and Department of Chemistry. Instrument Model:Bruker 500 MHz-Avanc III. chemical shift is in part per million (ppm) with reference to the chemical shift of the deuterated solvent or the internal standard tetramethylsilane (TMS).

FTIR:

Fourier transform infrared spectroscopy is a technique for material analysis it offers quantitative and qualitative analysis of the sample. FTIR identified chemical bands in molecules, the range of scanning 4000-400 cm-1. IR radiation is passed through a sample. Some of the infrared radiation is absorbed by the sample, and some is transmitted. The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. IR spectra was done using Nicolet NEXUS 670 FT-IR.

Results and Discussion

Two flavonoid aglycones were isolated from the ethyl acetate fraction before and after hydrolysis ,Analytical TLC of ethyl acetate phase (fraction B)confirmed the presence of kaempferol(aglycon I) ,while ethyl acetate phase (fraction D) confirmed the presence of isorhamnetin(aglycon II) .these compounds appears as a single spot in three different mobile phase against kaempferol and isorhamnetin reference standard ,Table(1)shows Rf values of kaempferol and isorhamnetin standard and *E.angustifolia* extract

Table (1): Rf Values of Standard Isorhamnetin and Kaempferol Compared with Isolated Aglycons I&II in Different Mobile Phases.

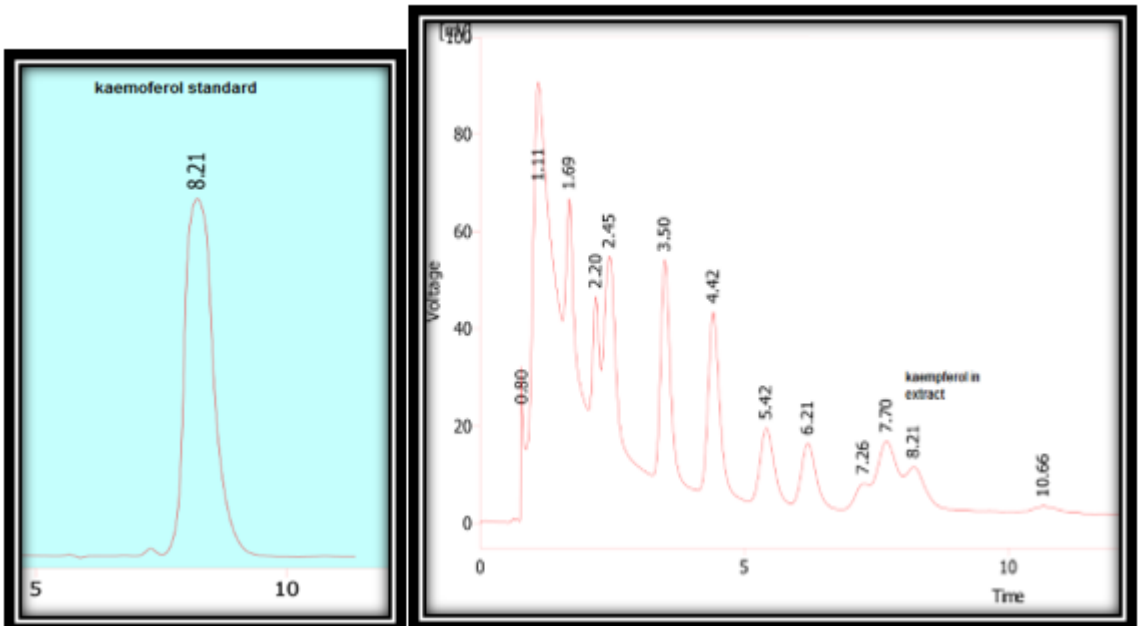
Mobile Phase No.	R_f value of Standard Kaempferol	R_f value of Isolated Aglycon I	R_f value of Standard Isorhamnetin	R_f value of Isolate aglycon II
S1	0.62	0.54	-	-
S2	-	-	0.73	0.73

High performance liquid chromatography (HPLC)

The HPLC results of the standards and the analyzed fractions with their retention times are shown in table 2 and the HPLC chromatogram of standard isorhamnetin and kaempferol with isolated compounds are shown in figure (2) and (3).

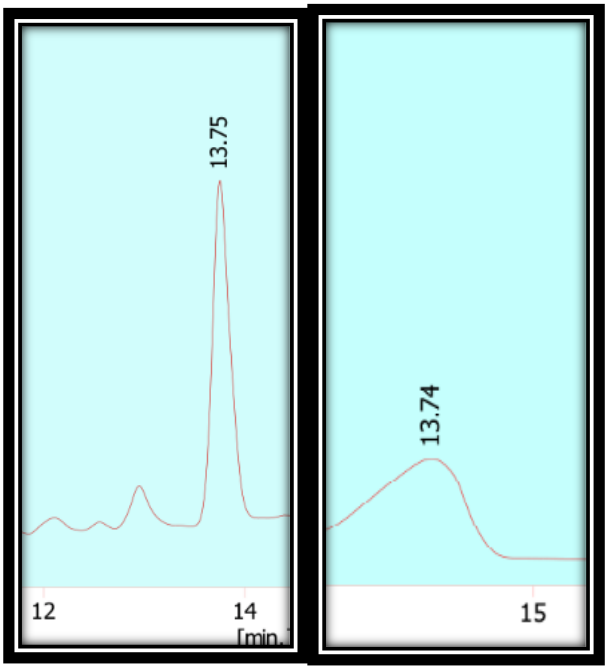
Table (2): Retention Times in Minutes for Standard Isorhamnetin and Kaempferol with Isolated Compounds.

Standard	Retention Time of Standard	Retention Time for Flavonoids in EtOAc Fraction
Kaempferol	8.210	8.21
Isorhamnetin	13.753	13.740



(A) (B)

Figure (2) HpLC chromatogram (A) flavonoid standards(kaempferol); (B) for *E.angustifolia* extract.



(A) (B)

Figure (3) HPLC Chromatogram (A) Isorhamnetin Standards; (B) for *E.Angustifolia* Extract.

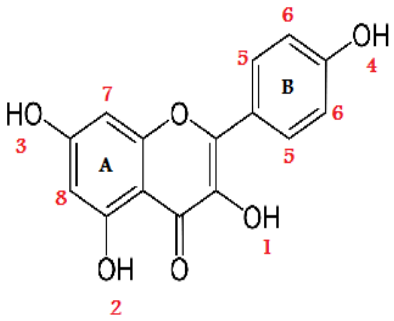
TLC with 0.5 mm thickness was conducted on 2 g of extract corresponding to 100 g of the plant to give 75 mg of kaempferol (0.08 %) while in ethyl acetate fraction after acid hydrolysis show the presence of Isorhamnetin which was confirmed by comparing with standard. Isorhamnetin was isolated by preparative TLC.

Structure Elucidation of Isolated Compound

1H-NMR

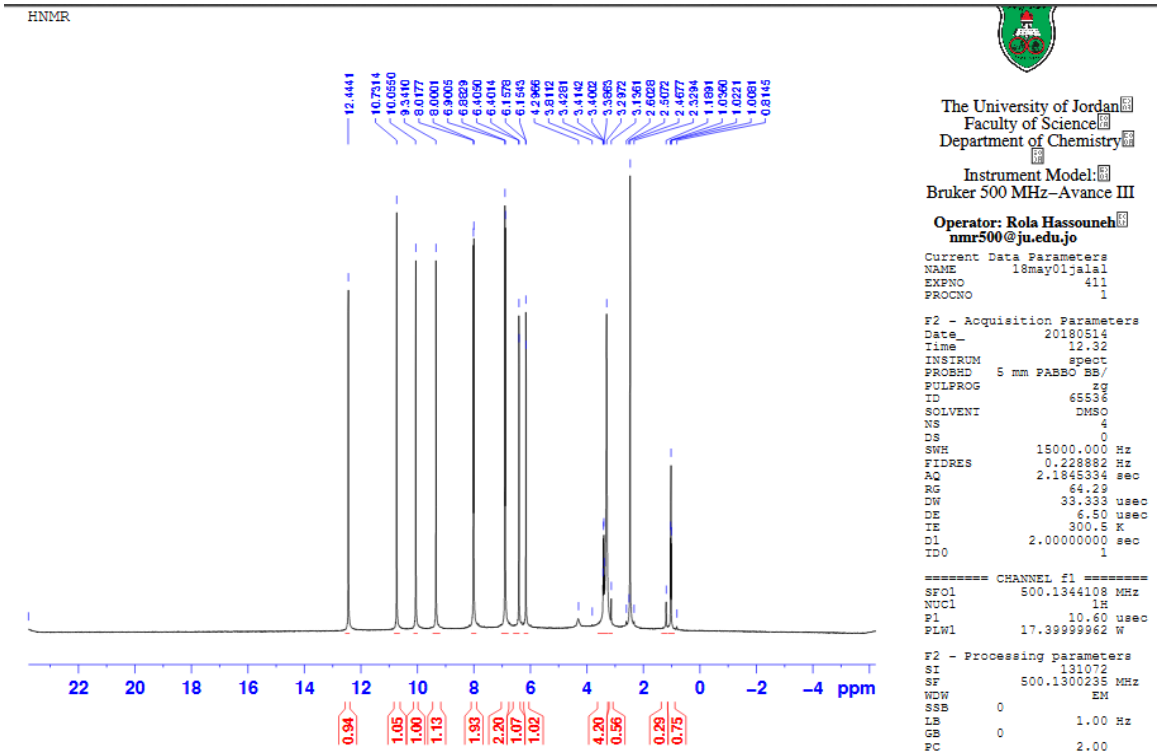
(Kaempferol)The spectra were recorded in DMSO solvent as in figure (4). The value of chemical shifts has been discussed according to the reference book [10] as shown in table (3)

Table (3):^{1}H-NMR Data and Their Interpretation of Kaempferol



3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one

No.	Chemical shift (ppm)	No. of H	Interpretation
1	12.4441	S,1H	Proton of Hydroxyl group 1
2	10.7314	S,1H	Proton of Hydroxyl group at 2
3	10.0550	S,1H	Proton of Hydroxyl group at 3
4	9.3410	S,1H	Proton of Hydroxyl group at 4
5	8.01	D,2H	Proton of aromatic ring B
6	6.90	D,2H	Proton of aromatic ring B
7	6.40	S,1H	Proton of aromatic ring A
8	6.15	S,1H	Proton of aromatic ring A

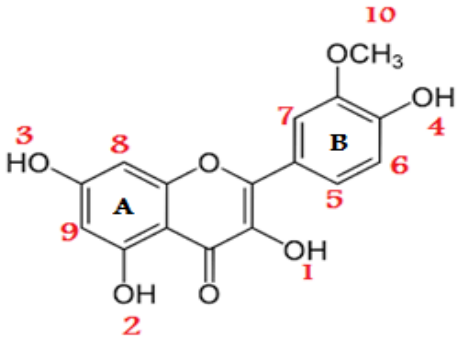


Figure(4): ^{1}H-NMR chromatogram of kaempferol

Isorhamnetin

The spectra were recorded in DMSO solvent as shown in figure(5).The values are chemical shifts have been discussed according to the reference books.(10) summarized in table(4).

Table (4):1H-NMR Data and their Interpretation of Isorhamnetin



3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H-chromen-4-one

No.	Chemical shift(ppm)	No.of H	Interpretation
1	12.42	S,1H	Proton of Hydroxyl group
2	10.73	S,1H	Proton of Hydroxyl group
3	9.69	S,1H	Proton of Hydroxyl group
4	9.37	S,1H	proton of Hydroxyl group
5	7.72	D,1H	proton of aromatic ring B
6	7.64	D,1H	Long coupling with another proton
7	6.91	S,1H	Proton of aromatic ring A
8	6.44	S,1H	Proton of aromatic ring A
9	6.16	S,1H	Proton of aromatic ring A
10	3.81	S,3H	Proton of CH_3

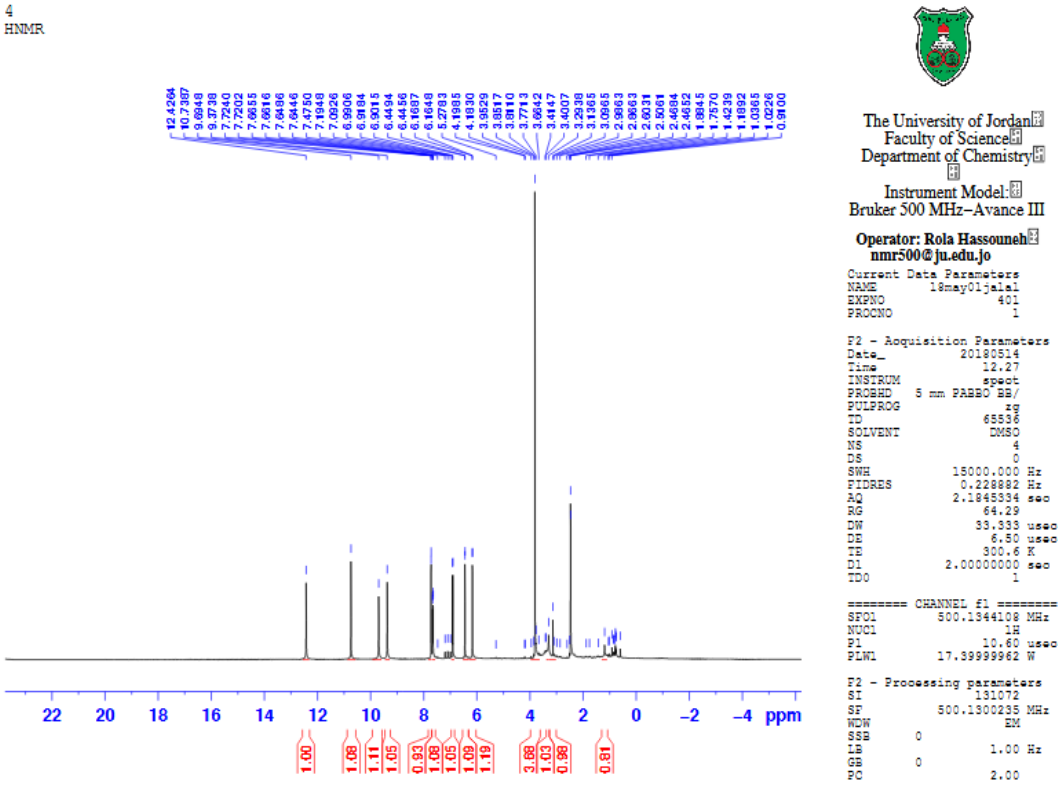


Figure (5): ^{1}H-NMR chromatogram of Isorhamnetin

FT-IR Spectrum:

Kaempferol

The FT-IR spectra of the kaempferol showed the characteristic absorption bands by which its functional groups were identified as in figure (6). The values of the characteristic bands of these spectra have been discussed according to the references book [10] and summarized in table (5).

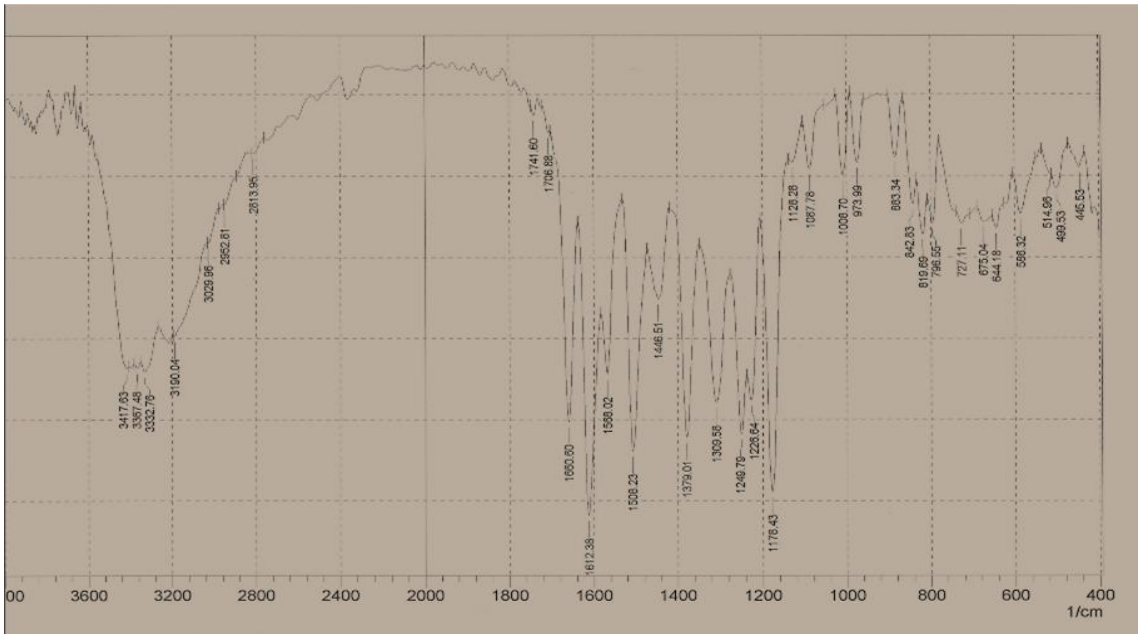
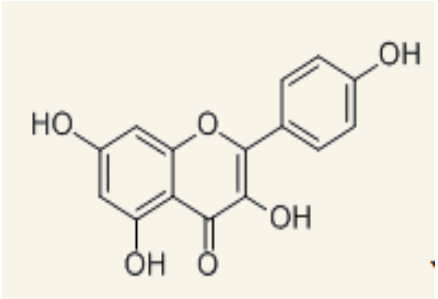


Figure (6): FT-IR Spectra of Kaempferol

Table (5): FT-IR Data and Their Interpretation of Kaempferol

Compound	Bands (cm^{-1})	Interpretation
	3417.63 – 3190.04	Broad band for stretching vibration of OH groups
	3029.96	Stretching vibration of CH aromatic
	1660.60	Stretching vibration for conjugated carbonyl group (α β unsaturated).
	1612.38,1568.02 ,1508.23	Stretching vibration of C=C of aromatic ring
	1249.79	Stretching vibration of C-OH of phenolic

Isorhamnetin

characteristic bands of these spectra shown in figure (7) have been discussed according to the references book [94] and summarized in table (6).

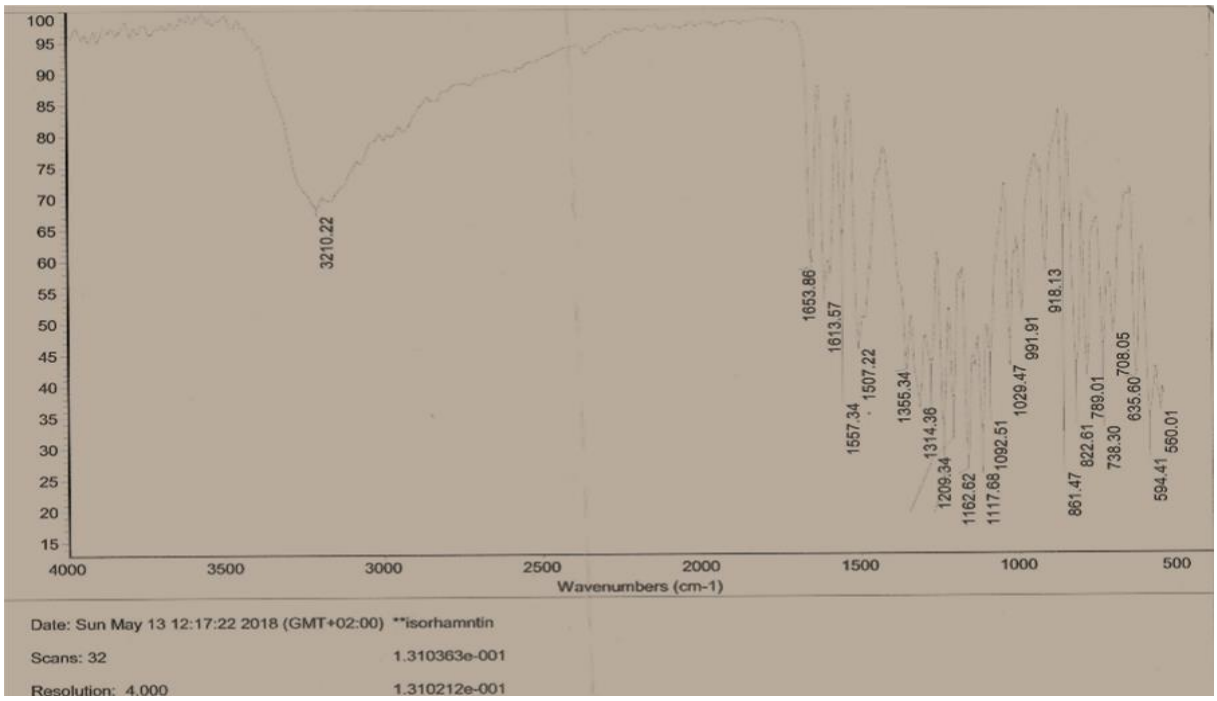


Figure (7): FT-IR spectrum of Isorhamnetin

Table (6): FT-IR Data and their Interpretation of Isorhamnetin.

Bands (cm^{-1})	Interpretation
3210.22	Broad band concentrated at 3210 for stretching vibration of OH groups
3080	Stretching vibration of CH aromatic
2880&2980	Stretching vibration of CH alkane
1653.86	Stretching vibration of conjugated carbonyl group (α β unsaturated)
1613.57,1557.34,1507.22	Stretching vibration of aromatic C=C
1209.34	Stretching vibration of C-OH phenol

Conclusion

From this study we confirmed that aglycon I am Kaempferol and aglycon II is Isorhamnetin also we conclude the leaves of Russian olive possess a good content of Kaempferol (0.08 %) and isorhamnetin. The structure of the isolated compounds was identified on the basis of spectroscopic method 1H-NMR and FT-IR spectrum.

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