

Phytochemical Study of some Flavonoids Present in the Fruits of Two *Ammi L. Species* Wildly Grown in Iraq

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Abstract

Ammi species belong to the family Umbellifereae that provide a host of bioactive compounds (mainly coumarins and flavonoids) of important biological activities, like prevention and treatment of heart and vascular disease and some types of cancer. Literature survey revealed that there was no study concerning *Ammi* flavonoids in Iraq. *Ammi majus* and *Ammi visnaga*, which are wildy grown in Iraq, were chosen for this study. This study concerned with extraction, identification, isolation, and purification of some biologically important flavonols quercetin and kaempferol from the fruits of *Ammi majus* and *Ammi visnaga*. Extraction of these flavonols was carried out using 85% methanol and 90% ethanol. Identification of these flavonols quercetin and kaempferol was done using thin Layer chromatography (TLC) where different solvent systems had been tried. Ultra violet (UV) Light and iodine vapor were used for detection. This identification was further augmented by using high performance Liquid chromatography (HPLC) and then these flavonols were isolated and purified. The most suitable extraction, isolation and purification procedures of flavonols were fully described in this study. The identification of isolated flavonols (quercetin & kaempferol) was carried out using melting point (M.P.), Thin Layer chromatography (TLC), and infrared spectroscopy (IR). This study confirms the presence of quercetin and kaempferol in *Ammi majus* & *an Ammi visnaga* fruit, the percentage of quercetin was higher in *Ammi visnaga* than *Ammi majus*, while the percentage of kaempferol was higher in *Ammi majus* than *Ammi visnaga*.

Key words : *Ammi majus*, *Ammi visnaga*, quercetin, kaempferol.

الخلاصة

جنس الخلة من العائلة المظلية (Umbellifereae) و هو يحتوي على الكثير من المركبات (منها الكومارين والفلافونويدات) ذات الفعالية الاحيائية التي تستخدم للوقاية وعلاج امراض القلب والشرابين وبعض انواع السرطانات. ونظراً لعدم وجود دراسات حول الفلافونويدات في نبات الخلة في العراق، لذلك اصبح من الاهمية دراسة بعض اصناف الخلة ومنها الخلة الشيطاني (*Ammi majus L.*) والخلة البلدي (*Ammi visnaga Lam.*) التي تنمو برياً في العراق. في هذه الدراسة تم استخلاص وكشف وفصل وتنقية بعض الفلافونويدات المهمة من الناحية الاحيائية وهما الكورستين والكامفيرول من ثمار النوع (*Ammi majus*) والنوع (*Ammi visnaga*) حيث تم الاستخلاص باستخدام المذيب العضوي ميثانول بنسبة 85% ومن ثم المذيب العضوي ايثانول بنسبة 90%. تم الكشف عن الفلافونويدات (الكورستين والكامفيرول) باستخدام تقنية كروماتوغرافيا الطبقة الرقيقة، باستخدام مذيبات مختلفة كوسيط ناقل والكشف عنها باستخدام الاشعة فوق البنفسجية وبخار الايودين، وكذلك تقنية كروماتوغرافيا الاداء العالي السائلة وبعدها تمت عملية الفصل والتنقية. كما تم في هذه الدراسة اختبار الطريقة المناسبة للاستخلاص والفصل والتنقية وشرحها بطريقة متكاملة. وكذلك استخدمت مجموعة من التقنيات للتحقيق من نوعية المركبات المفصولة (كورستين و الكامفيرول) ودرجة نقاوتها والتي شملت: درجة الانصهار للمركبات المفصولة وكروماتوغرافيا الطبقة الرقيقة وكذلك مطياف الاشعة تحت الحمراء. اثبتت هذه الدراسة وجود الفلافونويدات (الكورستين والكامفيرول) في ثمار الخلة الشيطاني و الخلة البلدي التي تنمو برياً في العراق. كما أظهرت هذه الدراسة أن كمية الكورستين كانت أكثر في الخلة البلدي من الخلة الشيطاني أما الكامفيرول فإن كميته أكثر في الخلة الشيطاني من الخلة البلدي.

Introduction

Ammi is a genus of 3-6 species of flowering plants in the Apiaceae Family; They are native in southern Europe, northern Africa and south west Asia^(1,2) *Ammi majus* and *Ammi visnaga* are one of the most important medicinal plant species in the world (figure 1, figure 2).⁽³⁾ In Iraq *Ammi majus*

usually found in fields and gardens and by the side of channels, often as weed of cultivation. It's collected from Kut, Baghdad, Hawija and many other areas. While *Ammi visnaga* are widely distributed in Erbil, Mousl, Baghdad, Sulaimania and Kirkuk in north of Iraq.⁽⁴⁾

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Figure (1) photography of *Ammi majus*



figure (2) photography of *Ammi visnaga*

Epidemiological data, clinical investigations, and animal studies provide strong evidence that the main active constituents of this genus are: Coumarin and their derivatives, flavonoids, volatile oil and fixed oil.⁽⁵⁾ All the flavonoids described in *Ammi* species can be classified into flavonols (quercetin, kaempferol, isorhamnetine) and flavones (apigenin, luteolin, chrysoeriol) these types of flavonoid have been shown to be powerful antioxidant and free radical scavengers.⁽⁶⁻⁸⁾ Among these flavonoids: quercetin which is a flavonol type of flavonoids that has been shown to help prevent the development of a variety of condition related to inflammation and free radical damage, including arthritis, allergies, macular degeneration, heart disease, gout, and various forms of cancer.^(9,10) The other flavonol found in these plants is the kaempferol, which is a strong antioxidant and help to prevent oxidative damage of our cells, lipids and Deoxyribonucleic acid (DNA). It seems to prevent arteriosclerosis by inhibiting the oxidation of low density lipoprotein and the formation of platelets in the blood. Studies have also confirmed that Kaempferol acts a chemopreventive agent which means that it inhibits the formation of cancer cell^(9,11)

Materials and methods

A. Plant materials

The plant materials (dried ripe fruits) of *Ammi majus* L. (Apiaceae) was collected during the months of March and April from local fields about 2 Km south of Kut. While the fruits of *Ammi visnaga* (Apiaceae) were collected from the botany garden in the College of Pharmacy, University of Baghdad. (Pharmacognosy department). Both of them were identified by the department of pharmacognosy, College of Pharmacy, University of Baghdad and authenticated by National Iraqi Herbarium, Botany Directorate at Abu – Ghraib. A 50 gm of powdered fruits of *Ammi majus* and *Ammi visnaga* were packed in a thimble of soxhlet extractors. 500 ml of petroleum ether (b.p 40 – 60 c⁰) was used in a soxhlet extractor for three hours to get rid of lipids and fat.⁽⁹⁾ The defatted powdered fruits after drying over night were extracted with 500 ml of 85% methanol for 12 hours by use of soxhlet apparatus for flavonoids extraction as free and glycosides.^(6,7) The methanolic extract was then filtered and then a portion had been taken and kept aside for further work. The remaining portion of filtrate was evaporated under reduced pressure at a temperature not exceeding 40 °C (F1). The methanolic extract (F1) residue was weighted and subjected for identification and purification procedures. To continue the extraction process, the previously extracted plant materials (fruits) of both *Ammi majus* and *Ammi visnaga* were dried at room temperature, and then were extracted again with 500 ml of 90 % ethanol for 12 hours by use of reflux apparatus for extracting the remaining flavonoids.^(6,7) The contents of the flask were filtered while hot and the ethanolic extract was allowed to cool at room temperature. A portion of ethanolic extract had been taken and kept aside for further work. The remaining portion of ethanolic extract was concentrated under reduced pressure to dryness (F2) and the dried extract (F2) was weighted and subjected for identification and purification procedures. Later on, equal volumes of methanolic extract and ethanolic extract of both *Ammi majus* and *Ammi visnaga* were mixed together to give methanolic ethanolic extract which also concentrated under reduced pressure to dryness (F3). The dry extract (F3) was weighted and subjected for identification and purification procedures. Figure (3 and 4) show Schematic procedure for the extraction method.

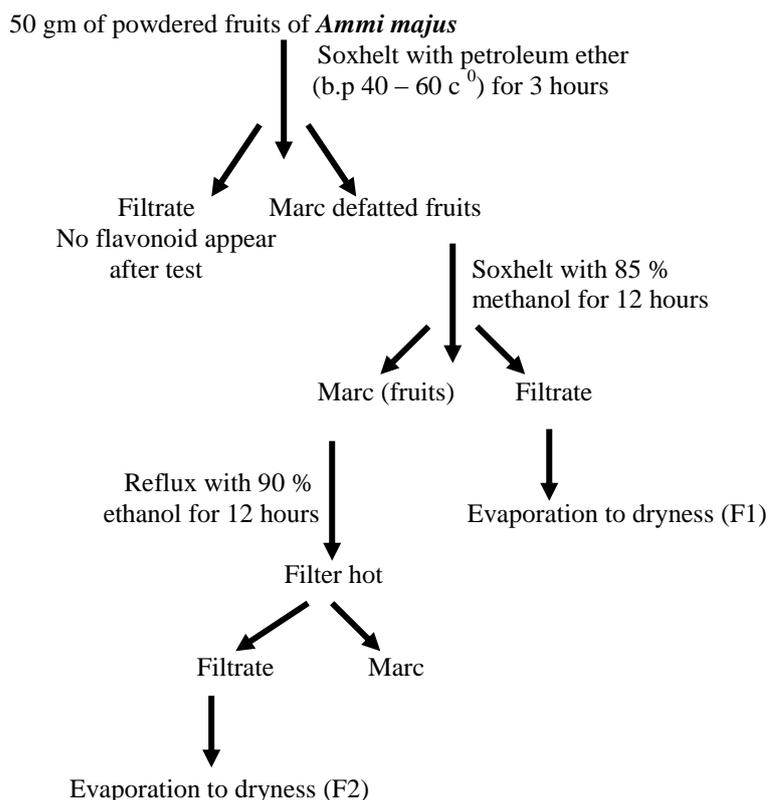


Figure 3 : Schematic procedure for the extraction method of flavonoids from *Ammi majus* fruits.

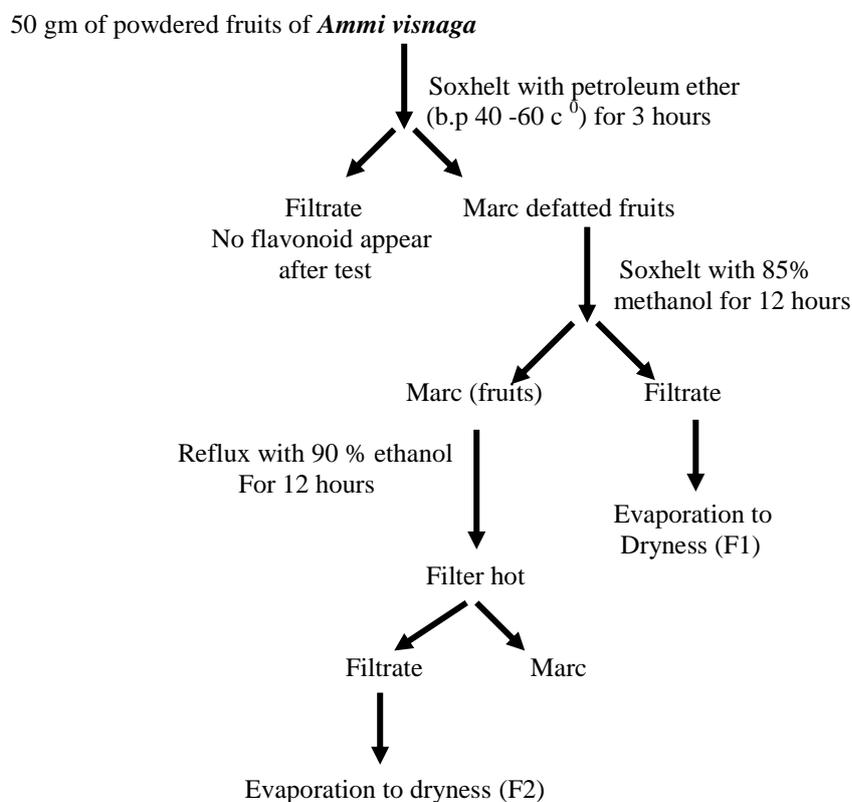


Figure 4 : Schematic procedure for the extraction method of flavonoids from *Ammi visnaga* fruits.

B. Identification of flavonoid

Identification of F1,F2,F3 were carried by thin Layer chromatography (TLC) using a ready made aluminum plates of silica gel GF254, two different detection methods, first by using UV light wave length 254 nm and 366 nm, second by using iodine vapor in the jar, in comparison with three different solvent systems S1,S2,S3.

Standard flavonoids:

Quercetin (FLUKA-Austria)

Kaempferol (Sigma-Aldrich,USA)

Different developing solvent systems that were:⁽¹²⁻¹⁵⁾

S₁= chloroform:Aceton:Formic acid(75: 16.5 : 8.5)

S₂ = chloroform: methanol (90:10)

S₃ = toluene: chloroform: Aceton (40: 25: 35)

C. Isolation and purification of quercetin and kaempferol

After locating of quercetin and kaempferol of the extract in comparison with standards, preparative thin layer chromatography was done to isolate and purify them. The portion of mixture methanolic-ethanolic extract (F3) was used to obtain the final product by applying it as a concentrated

solution in arrow of spots using capillary tube and the standard sample was applied in one side of the plate. the mobile phase used was S₁ = chloroform : Aceton : formic acid (75 : 16.5 : 8.5) the separated compound appear as a band identified using u.v light detection method. The band corresponding to the standard was scrapped out and collected in a beaker and eluted with gentle heating and filtered. Then the filtrate was evaporated to dryness under reduced pressure to give yellow precipitate. The precipitate then recrystallized using hot ethanol and maintained for TLC and measuring melting point and Infra red spectrum.

D. Qualitative and quantitative estimation of flavonoid using HPLC technique

Qualitative and quantitative estimations of quercetin and kaempferol were done by using Knauer/Germany High Performance Liquid Chromatography (HPLC) in which identifications were made by comparison of retention time obtained at identical chromatographic conditions of analyzed samples and authentic standards. The HPLC conditions are listed in the following table (1):

Table 1 : HPLC Conditions⁽¹⁶⁾

Sample	Mobile phase	Column	Flow rate	Detection
Quercetin	Acetonitrile: methanol : glacial acetic acid (70:30: 0.1)	C ₁₈ 5 mm x 150 mm	0.5 ml / min	UV. Detector at λ 306 nm
Kaempferol	methanol : water (7.5 : 92.5)	C ₁₈ ODS	1.5 ml / min	UV. detector at λ 308 nm

Results and Discussion

Three extraction portions were obtained from the experimental work in which methanolic extract (F1), ethanolic extract (F2) and the third extract portion, which is a mixture of methanolic – ethanolic extract (F3).

Results showed that the third extract portion was the best, because the amount of both extract and flavonoid were higher than the two other extract portions. As shown in (table 2).

Table 2 : Percentages of extract and flavonoids (quercetin, and kaempferol) in the fruits of *Ammi majus* and *Ammi visnaga*.

Plant	<i>Ammi majus</i>			<i>Ammi visnaga</i>		
	F1	F2	F3	F1	F2	F3
Extraction portions						
Percentage of extract	8.5	4.8	10.0	9.2	6.0	11.2
Percentage of quercetin	0.019	Traces	0.036	0.020	Traces	0.042
Percentage of kaempferol	0.025	Traces	0.045	0.018	Traces	0.035

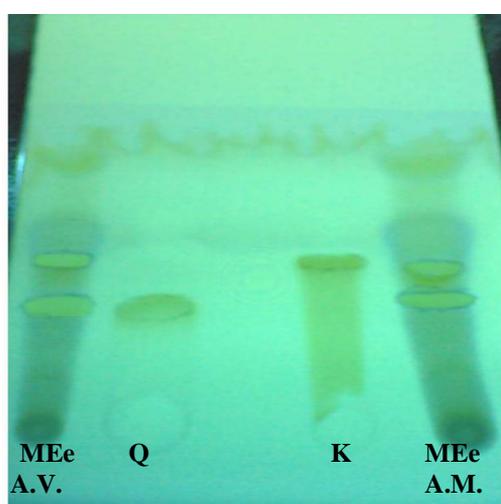
Identification of Flavonoids by TLC

TLC of the extracts (F1,F2,F3) obtained from dried ripe fruits of *Ammi majus* and *Ammi visnaga*, confirms the presence of

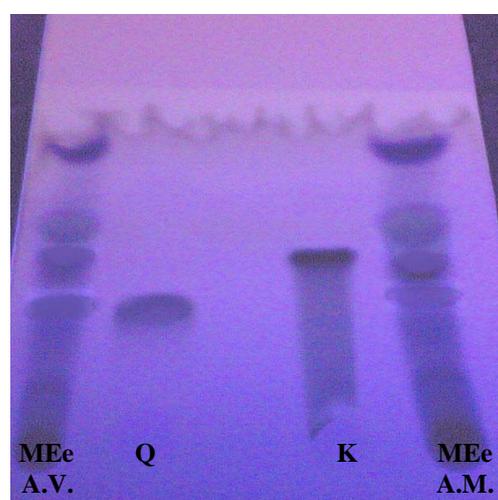
quercetin and kaempferol in all extraction portions in comparison with standards. As represented in table (3) and figure (5).

Table 3 : showed the R_f values of flavonoid (quercetin and kaempferol) and their standards in different developing solvent systems in TLC.

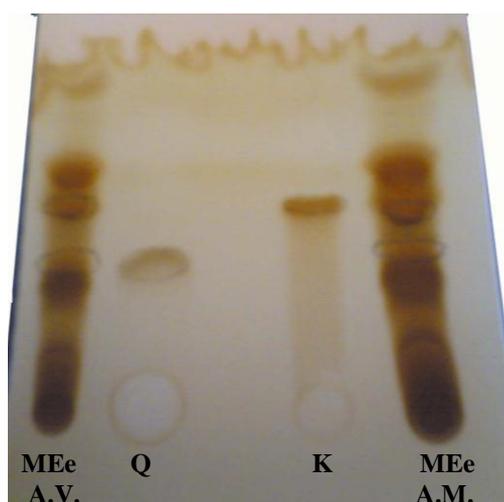
Solvent system	S ₁	S ₂	S ₃
R_f value of standard Quercetin	0.4	0.45	0.78
R_f value of quercetin in <i>Ammi majus</i>	0.38	0.43	0.76
R_f value of quercetin in <i>Ammi visnaga</i>	0.39	0.44	0.77
R_f value of standard kaempferol	0.52	0.6	0.85
R_f value of kaempferol in <i>Ammi majus</i>	0.51	0.61	0.84
R_f value of kaempferol in <i>Ammi visnaga</i>	0.49	0.59	0.86



(1)



(2)



(3)

Figure 5: TLC of fruits extracts of *Ammi majus* and *Ammi visnaga* obtained by extraction method using silica gel GF254 as adsorbent and (S1) as a mobile phase. Detection by UV-light at (1) 254 nm, (2) 366 nm, (3) iodine vapor. (A.M: *Ammi majus*, K : kaempferol standard, MEE: methanolic – ethanolic extract, A.V: *Ammi visnaga*, Q: quercetin standard)

Isolation and Quantitative determination of quercetin and Kaempferol by preparative TLC

From the investigation of the fruits extracts (fractions) of *Ammi majus* and *Ammi visnaga* on TLC plates, it was found that quercetin present in both fruits extracts but higher in *Ammi visnaga* while kaempferol also present in both fruits extracts but higher in *Ammi majus*. The percentage of both quercetin and kaempferol were obtained by weighing of the isolated compounds as shown in table (4)

Table 4 : Percentages of quercetin and kaempferol present in the fruits of *Ammi majus* and *Ammi visnaga*.

Plant fruits (total)	<i>Ammi majus</i>	<i>Ammi visnaga</i>
Quercetin	0.036 %	0.042 %
Kaempferol	0.045 %	0.035 %

Characterization of the isolated kaempferol and quercetin

TLC

Both isolated compounds appeared as a single spot having the same color and R_f value as that of reference standards.

Measuring melting points

The isolated compounds were identified to be quercetin and kaempferol from their sharp melting point. Since one of these compound showed a melting point of $314 - 316\text{ }^{\circ}\text{C}$ compared to quercetin melting point $316\text{ }^{\circ}\text{C}$. The other compound showed a melting point of $275 - 276\text{ }^{\circ}\text{C}$ compared to melting point $276 - 278\text{ }^{\circ}\text{C}$ for standard kaempferol.

IR.

The IR spectra of each isolated quercetin and kaempferol were recorded as KBr disc using IR spectra photometer BUCK scientific model 500, gave identical results were compared with authentic standard samples; which confirm that our isolated compounds are quercetin and kaempferol,⁽¹⁷⁾ as shown in figures (6-7).

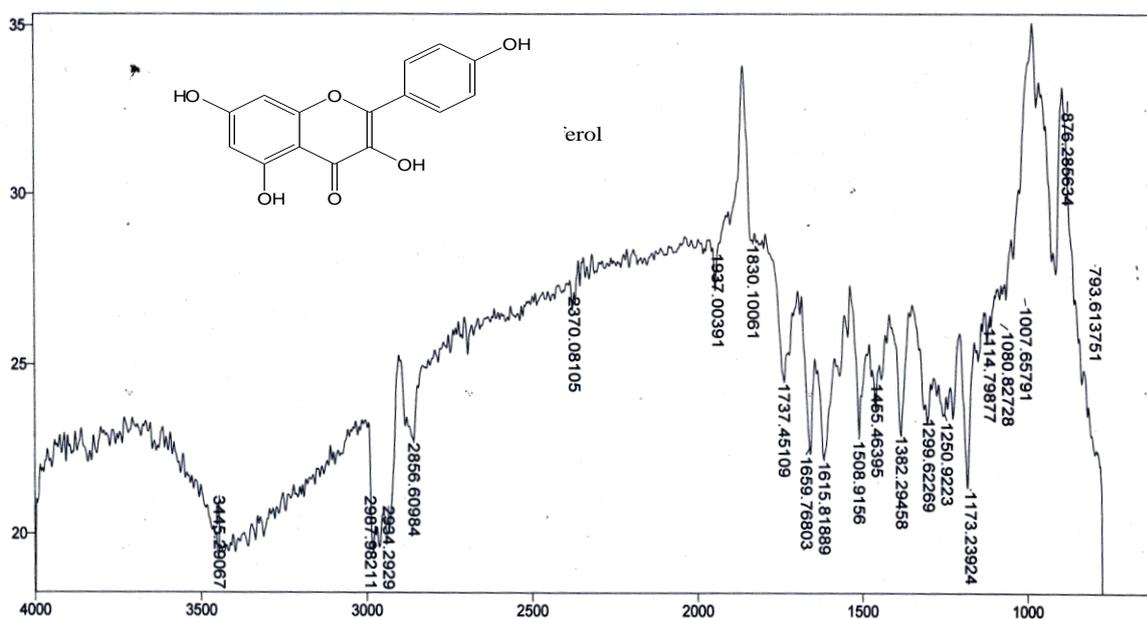


Figure 6 : IR Spectrum of isolated Kaempferol

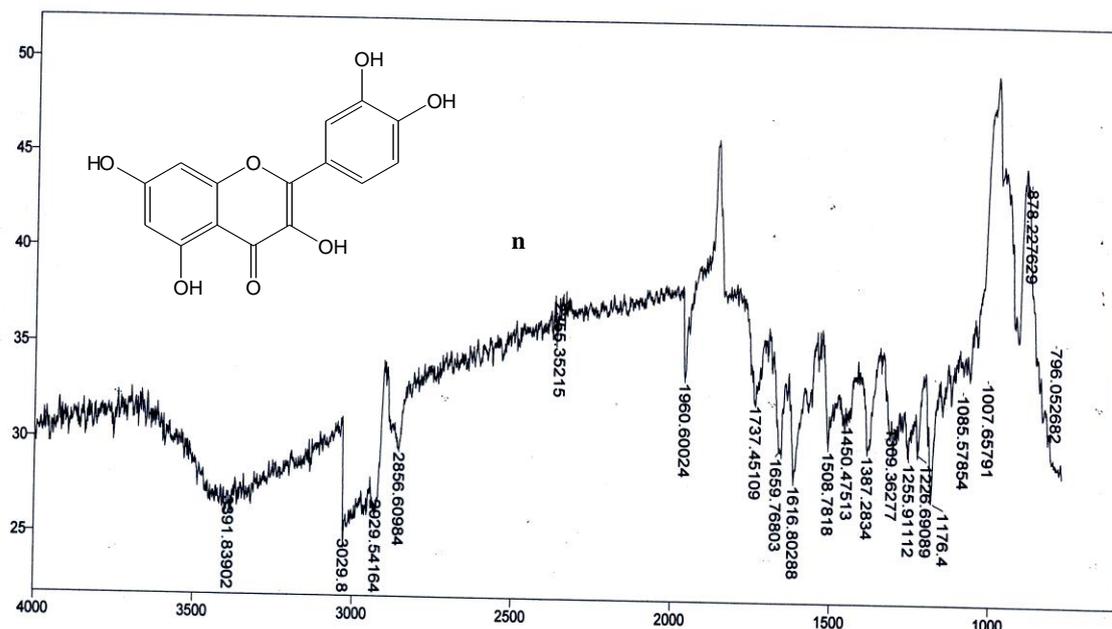


Figure 7 : IR Spectrum of isolated Quercetin

HPLC analysis

Generally, the percentage of quercetin and kaempferol is higher in methanolic – ethanolic extract than in methanolic extract and the methanolic extract contains higher amount of quercetin and kaempferol than ethanolic extract. In addition, *Ammi majus* had shown different percentages of quercetin and kaempferol than *Ammi visnaga*. Since the percentage of quercetin in *Ammi majus* was lower than the percentage of quercetin in

Ammi visnaga in all extracts. While the percentage of kaempferol in all *Ammi majus* extracts was higher than the percentage of kaempferol in *Ammi visnaga* extracts. The result indicates that the HPLC method was efficient for qualitative identification and quantitative determination of quercetin and kaempferol. as show in table (5) and figures (8-13).

Table 5 : Percentage of flavonols in *Ammi majus* and *Ammi visnaga*.

Extraction solvents	Percentage of the quercetin in the plant fruits.	Percentage of the kaempferol in the plant fruits.
methanolic extract of <i>Ammi majus</i> (F1)	0.026	0.037
methanolic extract of <i>Ammi visnaga</i> (F1)	0.033	0.025
ethanolic extract of <i>Ammi majus</i> (F2)	0.011	0.018
ethanolic extract of <i>Ammi visnaga</i> (F2)	0.015	0.012
methanolic – ethanolic extract of <i>Ammi majus</i> (F3)	0.045	0.052
methanolic – ethanolic extract of <i>Ammi visnaga</i> (F3)	0.050	0.043

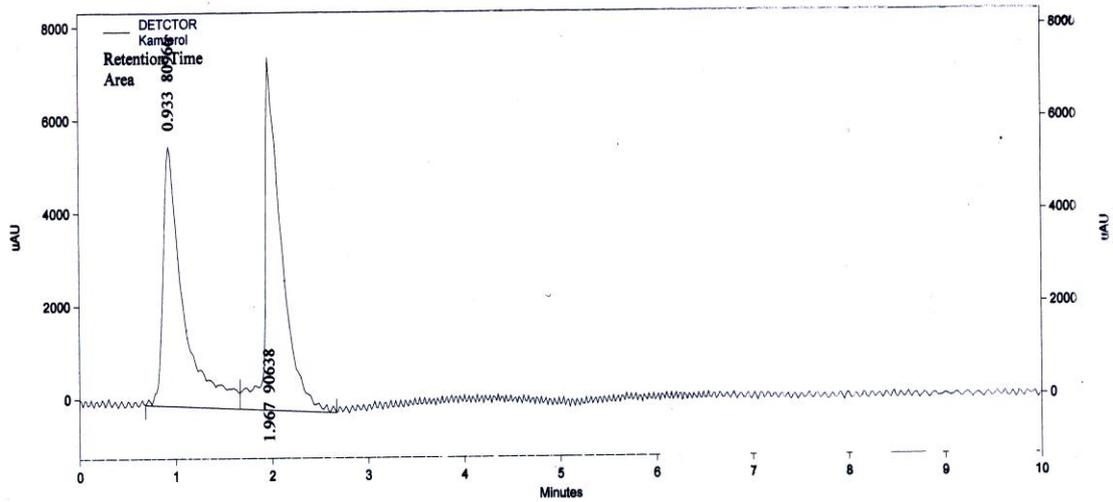


Figure 8 :HPLC analysis of kaempferol standard

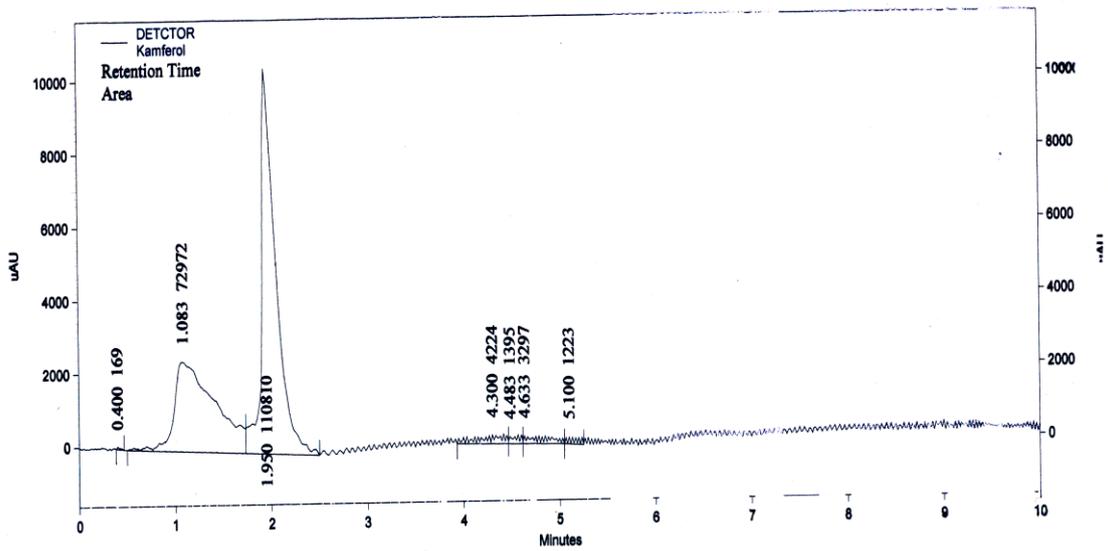


Figure 9 : HPLC analysis of methanolic-ethanolic extract of *Ammi majus*

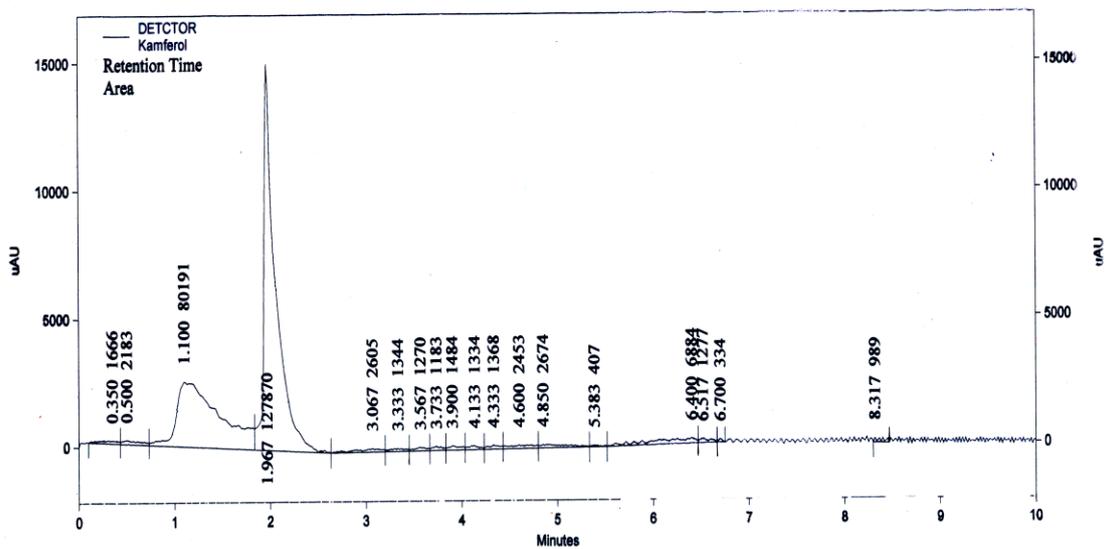


Figure 10 : HPLC analysis of methanolic-ethanolic extract of *Ammi visnaga*

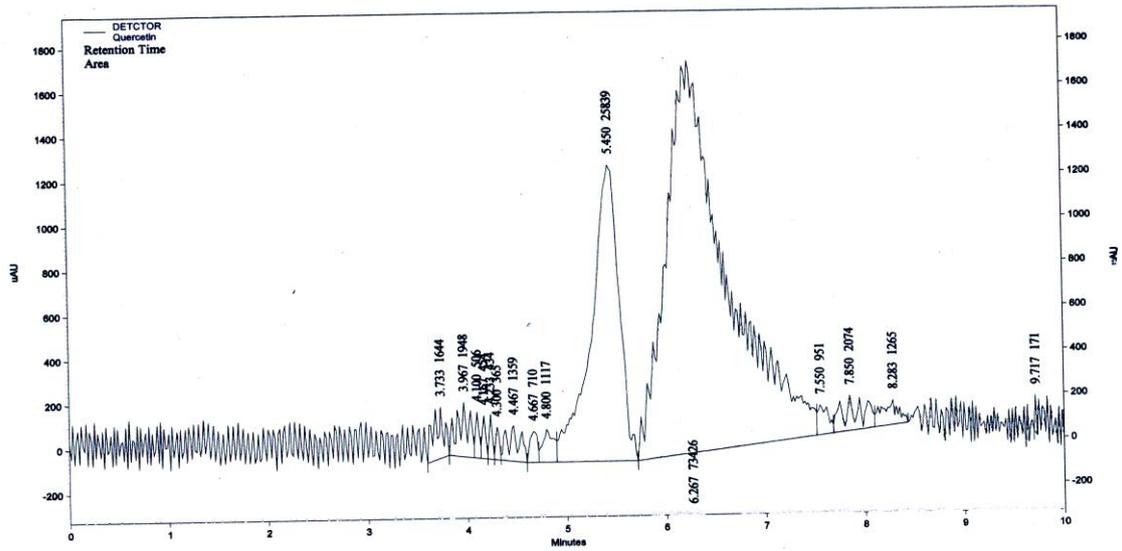


Figure 11: HPLC analysis of Quercetin standard

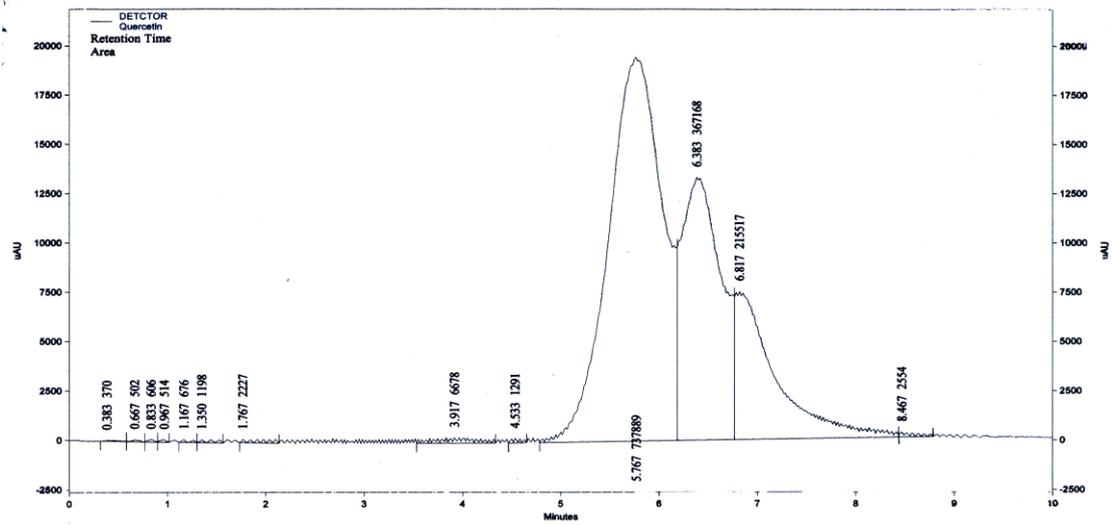


Figure 12 : HPLC analysis of methanolic-ethanolic extract of *Ammi majus*

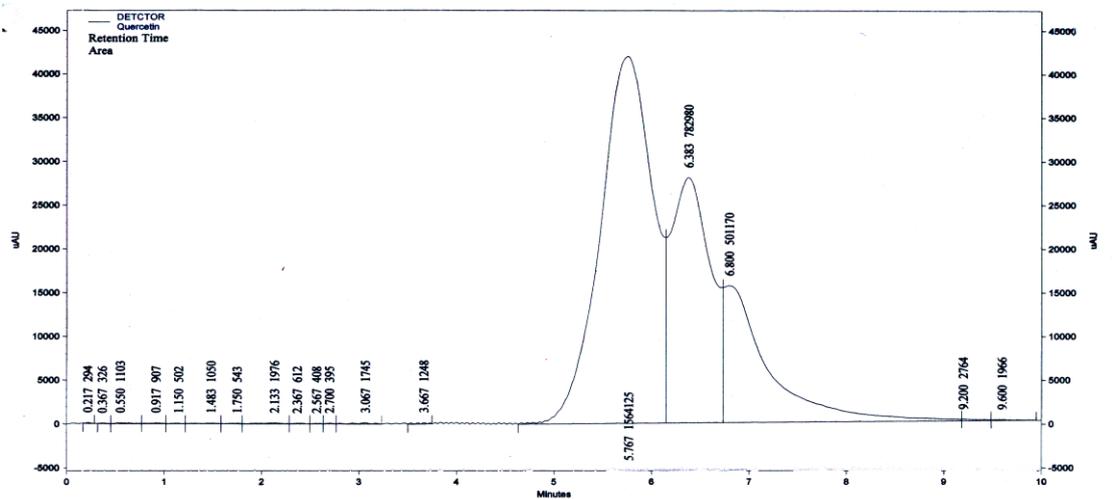


Figure 13 : HPLC analysis of methanolic-ethanolic extract of *Ammi visnaga*

Conclusions

Phytochemical investigation of *Ammi majus* and *Ammi visnaga* fruits, grown in Iraq revealed the presence of important group of medicinal natural products belong to flavonoid derivatives. Quercetin and kaempferol were isolated and identified in *Ammi majus* and *Ammi visnaga* fruits by using simple and reproducible TLC and HPLC method. The flavonoid, quercetin and kaempferol are found in the fruits of *Ammi majus* and *Ammi visnaga*, were quercetin present in large quantities in the fruits of *Ammi visnaga* than that of *Ammi majus*, while kaempferol present in the fruits of *Ammi majus* in large quantities than in *Ammi visnaga*.

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