Isolation of umbelliferone from leaves of *Conocarpus erectus* L. cultivated in Iraq

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Abstract:

Conocarpus erectus L. is a perennial, evergreen shrub belonging to Combretaceae family. In this study we investigate the phytoconstituents in the leaves of C.erectus. Preliminary tests of alcoholic extract proved the presence of flavonoids, coumarins, saponin, terpens, tannin and absence of alkaloids. Umbelliferone was detected in the ethyl acetate fraction of the extracted plant by TLC and isolated as a light yellowish powder by preparative TLC. Identification of it was done by HPLC, HPTLC and chemical structure elucidated by IR and UV.

Key words: coumarin , umbelliferone, TLC, HPLC, IR

عزل الامبيليفيرون من اوراق نبات الدمس المستزرع في العراق

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الخلاصة:

نبات الدمس هي شجيرة معمرة دائمة الخضرة تنتمي إلى العائلة القمبرطية. في هذه الدراسة قمنا بدراسة المكونات النباتية في أوراق نبات الدمس ، وقد أثبتت الاختبارات الأولية للمستخلص الكحولي وجود مركبات الفلافونويد والكومارين والصابونين والتريربينات والتانين وعدم وجود القلويات. تم اكتشاف أومبيليفرون في جزء أسيتات الإيثيل من النبات المستخلص بواسطة TLCالتحضيري وتم عزله كمسحوق مصفر خفيف بواسطة TLC التحضيري وتم التعريف عليه بواسطة تقنية كروماتوغرافيا السائل عالي الاداء HPLC و وجهاز الاستشراب بالطبقة الرقيقة عالي الاداء HPTLC التركيب الكيميائي بواسطة مطياف الاشعة التحت الحمراء IR ومطياف الاشعة الفوق البنفسجية.

الكلمات المفتاحية: كومارين ,امبيليفيرون , TLC التحضيري, كروماتوغرافيا السائل عالي الاداء HPLC, الاشعة التحت الحمراء IR

Introduction

Historically, natural products have been utilized for ancient times for the treatment of many illnesses and diseases. Natural product chemistry methodologies allow a broad collection of bioactive secondary metabolites from terrestrial and marine sources to be discovered.[1]Thus natural products are considered an important source of drug progress upon different diseases.[2] According to the World Health Organization (WHO), medicinal plants would be the best origin to receive a variety of drugs. Approximately 80% of people from developed countries use traditional herbs which have medicinally active constituents obtained from plants. [3]

Conocarpus erectus is a perennial, evergreen shrub or tree height of up to 40

feet involving in Combretaceae family Consist of 20 genera and 300 species. The conocarpus L. is genus, comprises of two different species *c.lancifolius* and *c.erectus* exist through the world such as southern Florida, Brazil and tropical West Africa. Commonly, *C.erectus* known as button mangrove or button wood.(4) Leaves are simple spiral, opposite or whorled , lanceolate, petiolate, entire in long 2 to 10 cm, Sometimes with a pair of petiolar glands ,paniculate spikes and Inflorescence axillary as seen in figure 1.[5]

Figure (1): Leaves of *C.erectus*

Furthermore, the genus of this plant are reported to have numerous traditional uses as in anemia, tumors, bleeding, catarrh, diabetes, headache diarrhea, conjunctivitis, gonorrhea, syphilis, antipyretic and anti-inflammatory in the treatment of fever (Decoction of leaves) and swellings.[6] Its bark, as well as fruits, are utilized in the management of hemorrhoids, wounds.(7) *C.erectus* L. is composed of a collection of constituents such as: primarily phenolic acids (gallic acid and ellagic acid ,while minor phenolic acid one is 3,4,3-Trimethoxyellagic acid and Brevifolin carboxylic acid)[8]. Catechin, Rutin, quercetin, Apigenin, Myricetin, Syringetin, Quercetin-3-glycoside, are flavonoids that have been reported to isolated and characterized from the extract of *C.erectus* plant. ، triterpenes, tannin and lignan. [9]

Pharmaologicaly, the plant reported to have been use as anticancer, antioxidant, antimicrobial and hepatoprotective activity. [10]

Umbelliferone is a 7-hydroxycoumarin and it is a benzopyran in nature that is a medically active compound. [11] It is created using the phenylpropanoid pathway. Furthermore, is a precursor for other coumarins and heterocycles with enhanced biological activities. It is extensively spread within the Rutaceae and Apiaceae (Umbelliferae) families and is efficiently extracted by using methanol. Also, it is a fluorescing constituent used as a sunscreen agent. Pharmacologically, it is reported to have antibacterial, antifungal activities and, good antioxidant activity. [12] Other reported activities are anti-inflammatory and anti-cancer, anti-hyperglycemic activities. [13]

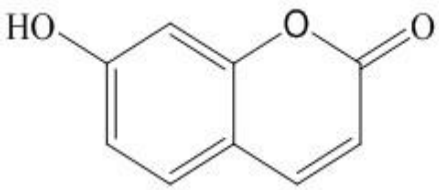


Figure 2: chemical structure of umbelliferone

Aim of study was to investigate the phytochemical constituents of Iraqi leaves of *C.erectus* and isolate of the umbelliferone (7-hydroxycoumarin) after detection in the ethyl acetate layer.

Methods

Collection of plant materials

The Leaves of *C.erectus* plant were collected from the botanical home of AL-Mustansiriyah college of Pharmacy .Authentication of plant carried out by national herbarium in Botany Directorate at Abu-Ghraib. The plants leaves were collected during the month of April (2019) and dried under shade conditions at room temperature during 12 days before grinding and weighing as a powder.

Extraction

The powdered leaves of *C.erectus* L. (100 g) were defatted with hexane (1000mL). The defatted plant material was further extracted with methanol 85% (1200 mL)

using soxhlet extractor. The methanolic extract was concentrated by evaporation under reduced pressure using rotary evaporator. Then distilled water (35mL) was added to the methanolic extract, and the extract partitioned (3time) with ethyl acetate (50mL) and allowed to settle overnight. The lower aqueous layer was collected and labeled as fraction A, while the upper ethyl acetate layer was collected and labeled as fraction B.

Preliminary phytochemical investigation

The extracts of *C.erectus* leaves were screened according to standard procedures of qualitative investigation to identify the major classes of natural secondary metabolites.[14][15]

Test for saponins (Foam Test):

Methanolic extract of the leaves (1mL) was mixed with distilled water (5mL) in a test tube and then shaken vigorously until persistent foam observed at least 1cm in height.

Test for terpenoids:

Alcoholic extract of the plant (5mL) was mixed with chloroform (2mL) and carefully added concentrated sulphuric acid (3mL). A reddish –brown color of interface is formed to indicate the presence of terpenoids.

Test for alkaloids:

Alcoholic extract (3.0 mL) was acidified with few drops of hydrochloric acid. Then a few drops of Wagner's reagent (that is prepared by dissolving of Iodine in Potassium Iodide) were added. The appearance of reddish -brown precipitate indicates the presence of alkaloid.

Tests for flavonoids:

Alcoholic KOH (2-3mL) were added to 1 mL of methanolic extract of the plant. A yellow color is detected if flavonoids compounds are present.

Test for tannin:

0.25g of plant extract was dissolve in (10ml) of distilled water and filtered.1% aqueous ferric chloride (FeCl3) solution was added to the filtrate, the appearance of dark green, dark blue or black color indicate the presence of tannins.

Coumarin test:

Few drops of methanol extract of leaves of C.erectus L. were placed on filter paper then addition of one to three drops of NaOH (1N) solution, and then the filter paper is subjected to UV light at 366nm for the presence of yellow to blue fluorescence.

TLC Detection of umbelliferone:

Detection of umbellefirone by TLC in comparison with their standards was performed for ethyl acetate fraction. A small amount was applied on analytical TLC plate (20x20cm of 0.25 mm thickness of aluminum silica gel) and dried the spots before development in covered glass jar that is saturated for 30 minutes with different solvent systems ;[Toluene: ether (saturated with 10% acetic acid)][(16)], [chloroform: methanol(80: 20)] and [Toluene: chloroform: acetone (40: 25: 35)]. Finally, the developed spots were examined under ultra-violate light at 254 and 366 nm.

The Rf value of ethylacetate fraction contents were compared with Rf value of umbelliferone standard**.**

Isolation of coumarin by preparative TLC

3.32 gm of ethyl acetate fraction was conducted on preparative TLC and it was developed in chloroform: methanol (80: 20) solvent system for umbelliferone. The band was detected under UV light at 254nm and 366nm then the Purification of compounds was checked by applying the separated compound on another preparative TLC and using toluene: ether (saturated with 10% acetic acid) solvent

system for umbelliferone and ensures the purity of isolated compounds.

Identification of isolated coumarin:

HPLC analysis:

Analysis was performed for estimation of isolated coumarin by (HPLC) method with UV detection. The HPLC analysis was carried out by prominence HPLC system (shimadzu) with a degasser (DGU-20A) and the separation was performed in reverse phase (RP) C18 column (250 x4.6 mm i.d), the mixture was passed through 0.45 µm disposable filters and then 100µL of sample was injected into the HPLC system. The separation was done by elution with isocratic mixture of methanol and water containing 0.1% v/v formic acid in the ration of 30:70. Flow rate was set at 1ml/min for 20 minutes, detected by UV at 366 nm. The umbelliferone was detected according to retention time of their standards. [17]

HPTLC analysis

HPTLC finger print analysis was conducted to confirm the isolated umbelliferone. HPTLC analysis was done by using Pre-coated silica gel 60 F 254 plates (10x20 cm) with layer thickness of 150 micron were used as a stationary phase. The standards and extract were applied automatically on the plate by CAMAG Linomat 5. The plate was automatically submerged into automatic developing chamber (ADC2 CAMAG) using solvent systems for ethyl acetate fraction (toluene: ethylacetate: formic acid 36:12:5) with migration distance about 7.5cm. The plates were air dried after development and scanned under UV (366 and 245nm) using CAMAG TLC scanner 4. The data were processed using win CATS software.

Fourier Transform Infrared Spectrometry (FTIR):

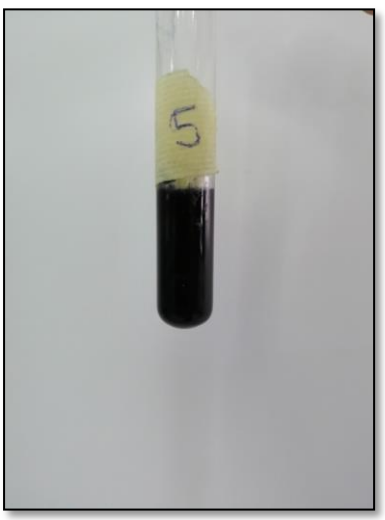
The FTIR method was carried out on a spectrophotometer system and recorded as KBr disc, which was used to identify the functional groups of isolated hydroxylated coumarin**.**

Results and discussion

Preliminary phytochemical examination of crude leaves extracts revealed the presence of flavonoids, coumarins, saponin, terpens, tannin and absence of alkaloids). The results of these tests are summarized in table 1 and figure 3.

Table (1): Qualitative estemation of the phytoconstituants found in the leaves of *C.erectus cultivated in Iraq:*

Tests	Flavonoids	Saponin	Coumarin	Terpenoids	Tannins	Alkaloids
Result	+ve	+ve	+ve	+ve	+ve	-ve



Flavonoid **Tannin**

Terpenoid **Saponin**

Figure (3): preliminary phytochemical investigation of *c.erectus*.

TLC Detection of umbelliferone:
Preliminary tests and analytical TLC of fraction B (ethyl acetate phase) confirmed the presence of hydroxylated coumarin in the plant extract in comparison with standards of Umbelliferone using 3 mobile phases as represented in Table 2 and figure 4.

Table (2): TLC Detection of umbelliferone using 3 solvent systems

Solvent system	Rf value of umbelliferon standard	Rf value of umbelliferone
chloroform: methanol (90 :10)	0.67	0.67
Toluene: ethylacetate: formic acid (36: 12: 5)	0.88	0.87
toluene: ether (saturated with 10% acetic acid)	0.45	0.45

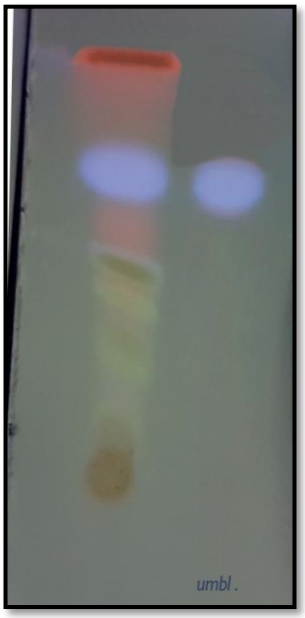


Figure (4): TLC plate for detection of umbelliferone.

Isolation of coumarin by preparative TLC

3.32 gm of ethyl acetate fraction was conducted on preparative TLC and it was developed in chloroform: methanol (80: 20) solvent system yield 63mg (1.08%) of umbelliferone. The band was detected under UV light at 366nm as in figure 5.

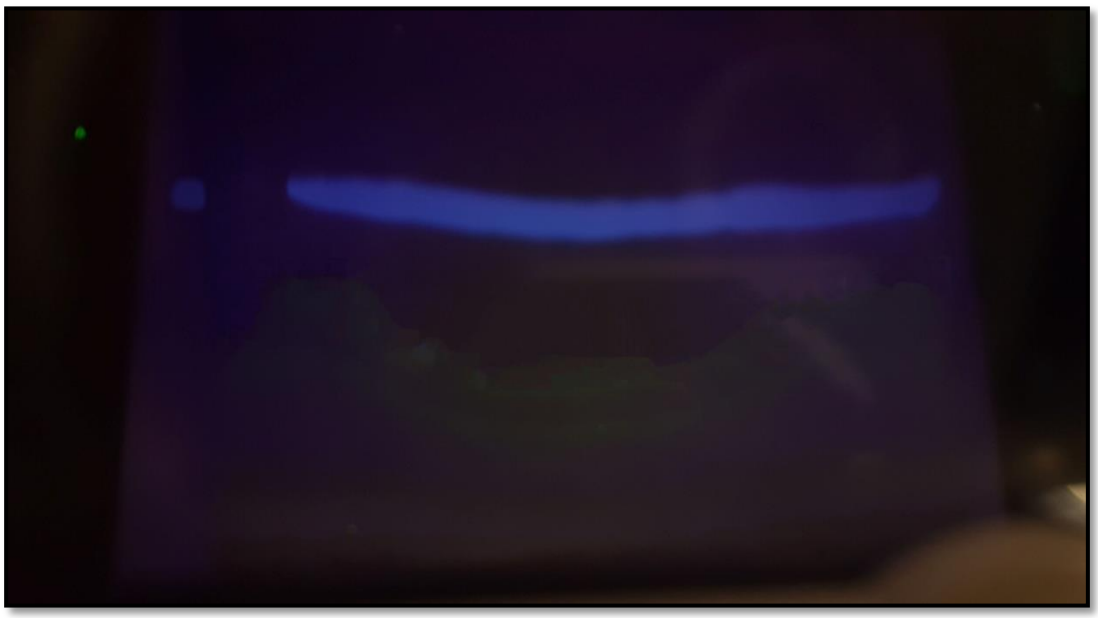
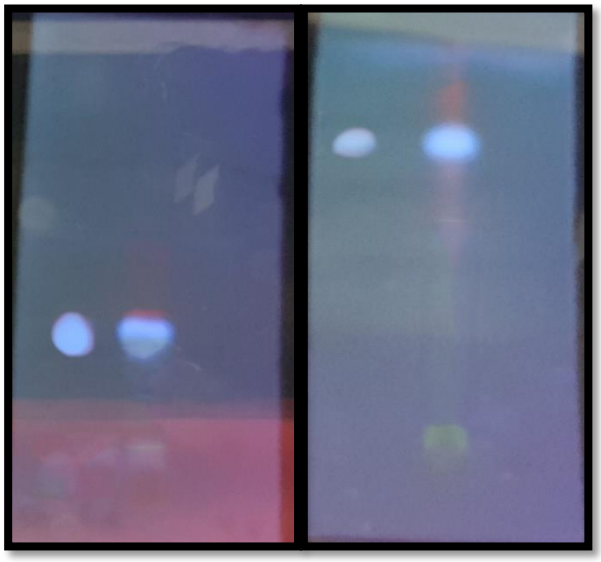


Figure (5):preperative TLC for isolated umbelliferoneof under UV light at 366nm

Toluene: ether (saturated with 10% acetic acid) solvent system used for purification of umbelliferone. The figure (6) represents analytical TLC for pure isolated compound with two solvent systems



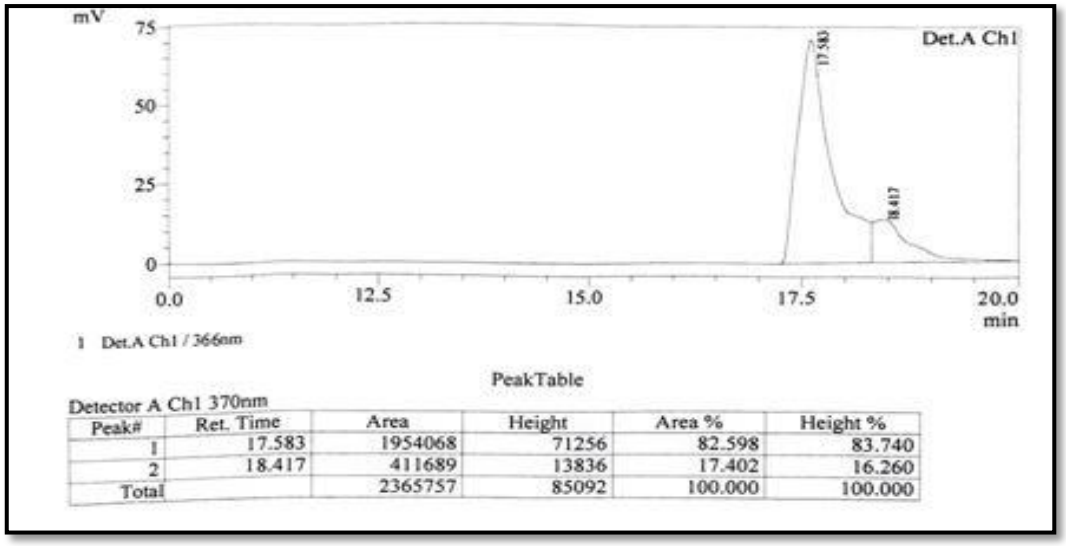
(X) **(Y)**

Figure (6): TLC plates for isolated hydroxylated coumarins under UV light at 366nm using 2 Solvent system.

Identification of isolated coumarin

HPLC analysis

HPLC analysis for coumarin in ethyl acetate fraction with isocratic elution method revealed the presence of umbelliferone in the leaves of plant. umbelliferone was determined by comparison of the retention time (Rt) of isolated compound to retention time of their standard. Figure 7 represent HPLC chromatogram of isolated compound, showed the presence of sharp peak has retention time (17.58 min.) compared with Rt of umbelliferone standard (17.46min) as seen in figure 8

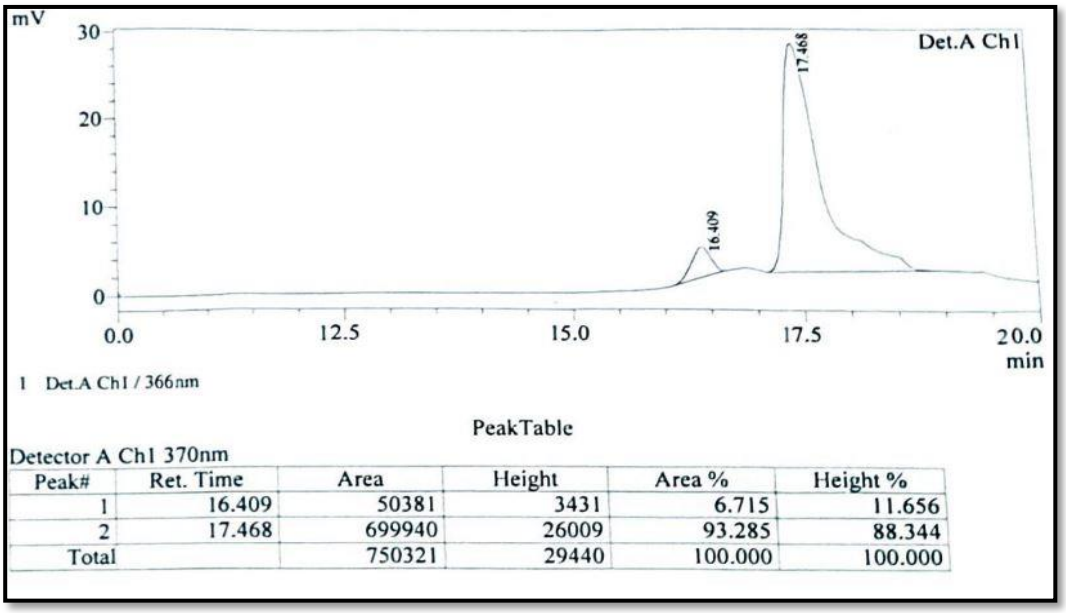


PeakTable

Detector A Ch1 370nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	17.583	1954068	71256	82.598	83.740
2	18.417	411689	13836	17.402	16.260
Total		2365757	85092	100.000	100.000

Figure (7): HPLC chromatogram of isolated umbelliferone.



PeakTable

Detector A Ch1 370nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	16.409	50381	3431	6.715	11.656
2	17.468	699940	26009	93.285	88.344
Total		750321	29440	100.000	100.000

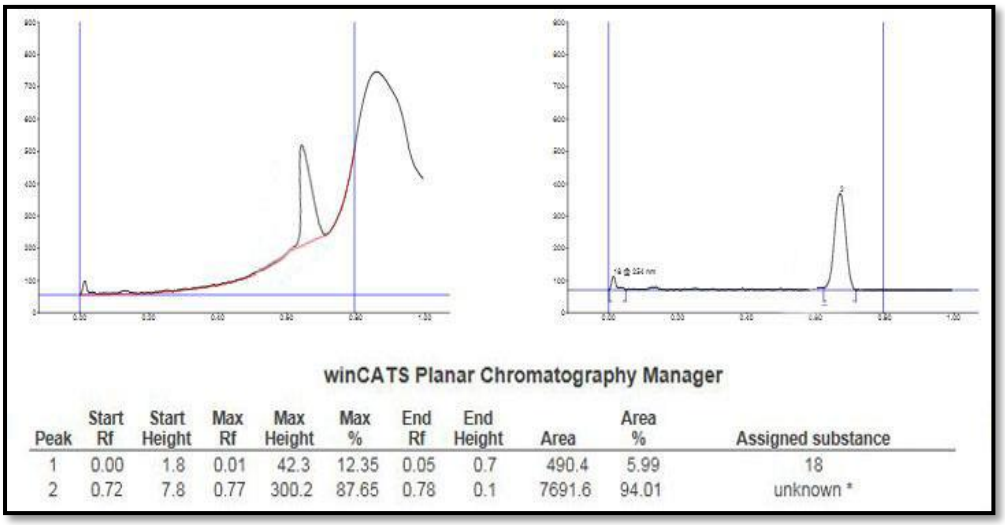
Figure (8): HPLC chromatogram of umbelliferone standard

HPTLC analysis for compounds isolated from ethyl acetate fraction

The ethyl acetate fraction of Iraqi cultivated conocarpus leaves was subjected to HPTLC along with umbelliferone standard and isolated compound, developed under UV light at 366 can be seen in Figure ٩

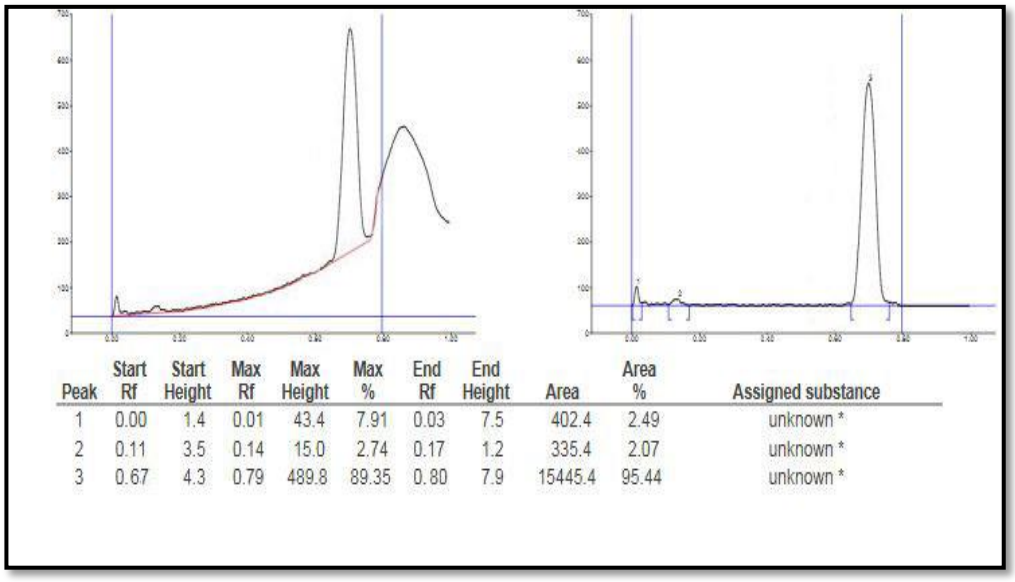
Figure (9): HPTLC image at 366 nm in S6 solvent system were A. E.A fraction,B. umbelliferon standard and C.isolated umbelliferon.

HPTLC analysis showed that standard of umbelliferone has maximum Rf value = 0.77 as in figure 10 in compared with well-defined peak of isolated compound at maximum Rf = 0.79 as shown in Figure 11.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.00	1.8	0.01	42.3	12.35	0.05	0.7	490.4	5.99	18
2	0.72	7.8	0.77	300.2	87.65	0.78	0.1	7691.6	94.01	unknown *

Figure (10) HPTLC chromatogram of umbelliferone standard.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.00	1.4	0.01	43.4	7.91	0.03	7.5	402.4	2.49	unknown *
2	0.11	3.5	0.14	15.0	2.74	0.17	1.2	335.4	2.07	unknown *
3	0.67	4.3	0.79	489.8	89.35	0.80	7.9	15445.4	95.44	unknown *

Figure (11): HPTLC chromatogram of isolated compound.

IR spectrophotometry:

Structure elucidation by FT-IR for isolated umbelliferone showed the presence of hydroxyl groups of aromatic rings as a broad band centered at 3335 cm-1, and presence bands at 1716 cm-1 of carbonyl of ester group in addition to C-H of aromatic ring; out of plane; strong band at 873-671 cm^{-1} , can be seen in figure 12

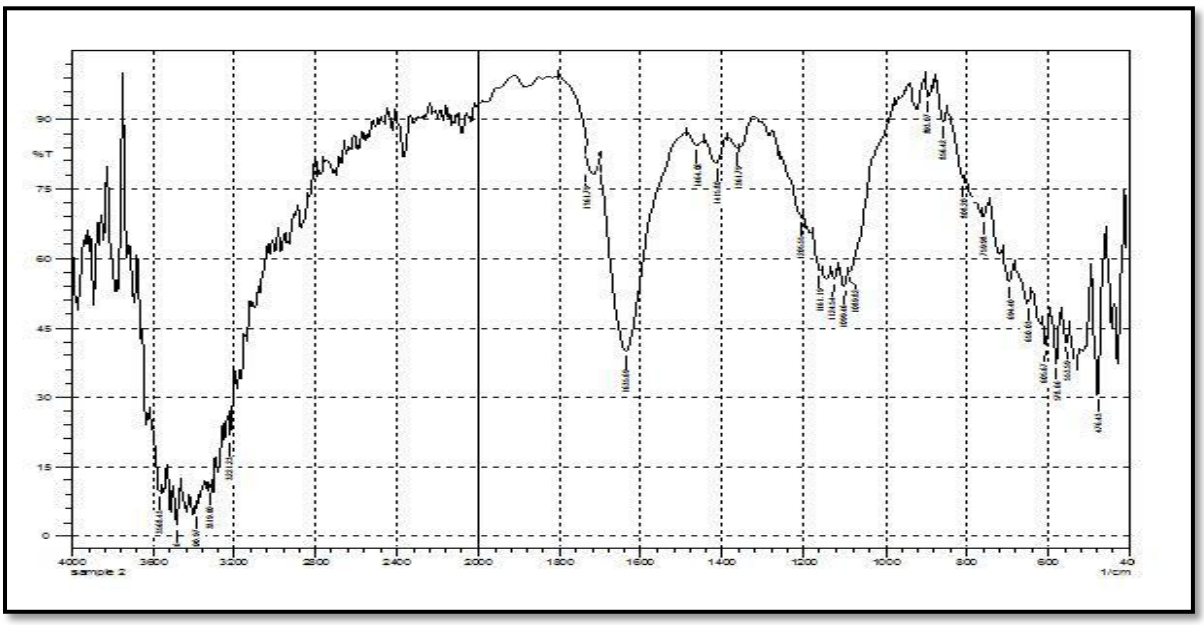


Figure (12): IR spectrum of isolated umbelliferone

Ultraviolet spectroscopy (UV) for isolated umbelliferone compound

UV spectrum was recorded between 240 to 400 nm, the maximum absorbance of isolated compound detected at a wavelength of 324 n m as seen in figure 13.

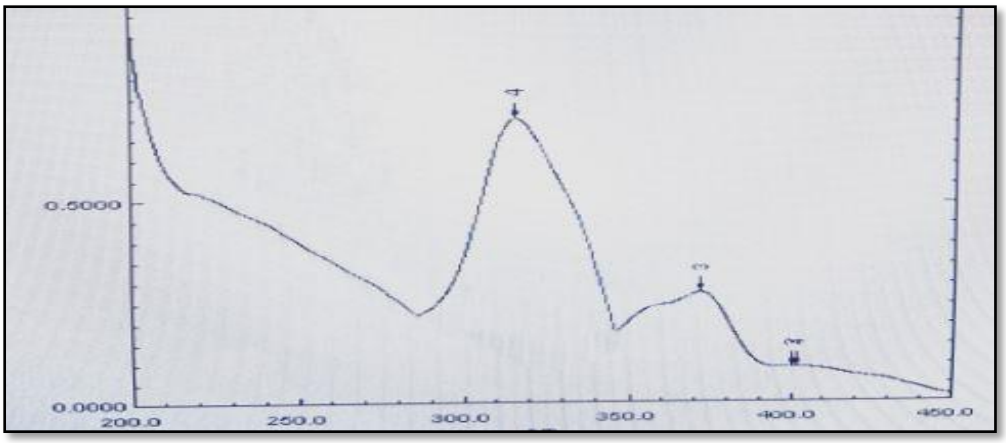


Figure (13): UV spectrum of isolated compound

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