

Separation, Characterization and Anticoagulant Activity of Coumarin and its Derivatives Extracted from *Melilotus officinalis*

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Coumarin is a phenolic natural product extracted from yellow clover *Melilotus officinalis* by maceration in ethanol solvent. Crude extract was analyzed by high performance liquid chromatography then divided into two portions, first one characterized by Fourier transform infrared spectroscopy (FTIR) and investigated for anticoagulation activity by prothrombin time (PT) while the second portion subjected for fractionation using column chromatography (CG) and fractions were characterized by FTIR, each fraction was detected as anticoagulant agent. As results, coumarin crude extract eluted with retention time (7.6 min.) as standard coumarin by HPLC, molecular spectrums of fractions obtained by column chromatography were measured using FTIR. Anticoagulation activity of crude extract results were dose dependent pattern .We concluded that *M. officinalis* is a good source for coumarin and coumarin derivatives as shown in (HPLC) analysis while using column chromatography (CG) and (FTIR) considered as valuable techniques for separation and characterization.

Keywords: *Melilotus officinalis*, coumarin, FTIR, HPLC, Anticoagulant, column chromatography.

Many years ago, plants were consumed for medicinal purposes which using as herbal or alternative homemade remedies, either as extracts, or as purified potential fractions with biological activities. The world health organization (WHO) establishes that 80% of human beings in growing counties (65% of the world's population) remain confidences in using bioactive derivatives utilized from plants (Karakas *et al.*, 2012). *M. officinalis* (yellow sweet clover) is being as one of important therapeutic plants which were utilized since long times ago by several herbalists and physicians as

a digestive, eye inflammation, hemorrhoids, stomach ulcer and disorders. Extract of dried petals of *M. officinalis* took part in pharmaceutical effects for the symptomatic treatment of venous-lymphatic in-sufficiency (Mahajan and Gajare, 2012). Coumarin considered as the major active compound in the *M. officinalis* crude extract and appear to function in various capacities in different plant defense mechanisms against insect herbivores and fungi (Murray *et al.*, 1982) , in addition, this component has biological properties due to obstacle blood clot formation in the vessel (Klein, 2011). In this study, two techniques were used in analysing and detecting the presence of coumarin compound in crude extract ; High performance liquid chromatography technique (HPLC) by pass a pressurized liquid "referred to mobile phase" and a

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sample mixture through out a column contain adsorbent particles, causing the separation of the sample compounds because they have various degrees of interaction with the absorbent; while the other technique was Fourier Transform Infrared Spectroscopy (FTIR) briefly by a moving mirror inside the apparatus changes the distribution of infrared light that passes through the interferometer. The signal directly recorded, named an "interferogram", represents light output as a function of mirror position. An apparatus records this raw data into the final result. Anti-coagulant activity measured by prothrombin time kit (PT).

MATERIAL AND METHODS

Preparation of plant extract

Leaves of *M. officinalis* were collected from gardens of Baghdad University, (10) gm of dried powdered plant sample defatted with (100) ml hexan for (3) hours. Then extracted in (100) ml of ethanol by maceration and crude extract evaporated at 40°C by using incubator and resultant was collected and stored at 4°C until use (Szewczyk and Koka, 2012).

Detection by High performance liquid chromatography analysis

Chromatographic separation of coumarins was performed by apparatus Shimadzu LC-10 system with LC-10AD pump, UV-variable wave length detector (at 203 nm) . Sample passes through a column C-18, (5m, 250 mm x 0.6 mm). Separations were carried out with isocratic mode, acetonitrile: water (40:60; v/v) utilized as a mobile phase at a flow rate of (1) mL min⁻¹; with an injection volume of 20 µL, The HPLC system was operated at room temperature (23 ± 10C). The sample was injected with concentration 10mg / 1 ml while standard coumarin as 1mg/ml (Celeghini, *et al.*, 2001).

Separation of coumarin derivatives by column chromatography

Separated active ingredients were added as three volumes of ether to one volume of crude extract in separating funnel then shaken very well , two layers obtained as upper ether layer and lower aqueous layer.

Column (3.1 x5) packed with suspension of silica gel and methanol, after the stability of the gel the column were washed with methanol then

added ether layer to the column. Washed with solvent system consist of diethyl ether with 10% acetic acid. Fractions were collected as 1 ml for each tube with flow rate 12ml/hr.

Fourier transform infrared spectroscopy (FTIR) analysis

Chemicals that used in this study were purchased from Merck, BDH, Sigma Aldrich and Fluka companies. Melting point have been estimated using digital stuart scientific SMP3 melting point apparatus. FTIR spectra were established by Shimadzu FTIR- 8400 Fourier transform infrared spectrophotometer using KBr discs in the (4000-600) cm⁻¹ spectral range (Sliverstien and Webster, 1998).

Prothrombin Time assay procedure

A test tube contains 0.1 ml of plasma was kept in incubator for 3 to 5 min at 37°C after that 0.2 ml of Biolabo reagent (biolabo SA, France) was added to the test tube after warming the reagent at 37°C. Vary concentrations of *M. officinalis* coumarin extract were added separately to the test tube, the coagulation time or prothrombin time (PT) was estimated in seconds by stopwatch in addition to recording coagulation time of +ve and -ve control. Each test concentration was repeated three times and data average used. Normal saline used instead of extract in -ve control while EDTA as a +ve control (Govindappa *et al.*, 2015)

RESULTS AND DISCUSSION

HPLC analysis

M. officinalis extract was analyzed by HPLC apparatus and coumarin compounds eluted retention time at 7.66 min. which were closed to retention time of Standard coumarin which appeared at 7.63 min. (Fig. 1 a & b).

As showed in (Fig.1 a), the concentration of coumarin components in the plant extract is low due to small peak of eluted compounds compared with high peak of standard coumarin in (Fig.1 b) although the concentration used of *M. officinalis* extract was 10 mg/ml while the concentration standard coumarin prepared with 1mg/ml.

Fourier transform infrared spectroscopy (FTIR) characterization of crude extract

M. officinalis crude extract confirmed the presence of phenolic and amino coumarin derivatives (7-hydroxy coumarin and 6-amino

coumarin) were afforded a pale deep green color and m.p (216-236 °C) also phenolic test and amino group test have confirmed the presence of phenol and amino groups (Ralph *et al.*, 2004). FTIR spectrum of these compounds indicate that t-H: (3375) cm^{-1} of hydroxyl group while a t- NH_2 : asym. (3375) cm^{-1} and sym. (3193) cm^{-1} , also the presence of tC-H aromatic at (3030) cm^{-1} , tC=O: (1737) cm^{-1} of carbonyl group, tC=C: (1622) cm^{-1} of olefinic group and tC-O-C at (1072) cm^{-1} (Silverstein *et al.*, 2005); FTIR spectral data crude extract coumarin shown by figure (2)

M. officinalis fractionated by column chromatography separation, collected and subjected for FTIR apparatus and the results are presents as following:-

a- FTIR spectrum of 3,3-methylene bis 4-hydroxy-coumarin (dicoumarol) compounds indicate that

tO - H : (3645) cm^{-1} while tC-H aromatic at (3097) cm^{-1} , tC - H aliphatic (2958 , 2927) cm^{-1} , t C=O : (1731) cm^{-1} and t C=C aromatic at (1568, 1550) cm^{-1} in addition to presence tC-O-C (asym. 1128, sym 1072) cm^{-1} (REF); FTIR spectral data by figure (3).

b- FTIR spectrum of 2-hydroxy-4-methylene-5-oxo-2, 3- dihydro furane [2-3a][1] benzo pyrane compounds indicate that tO - H : (3365) cm^{-1} while tC-H aromatic at (3076) cm^{-1} , t C - H aliphatic (2933) cm^{-1} and t C=C of olefin at (1625) cm^{-1} in addition to presence t C=C aromatic (1577, 1425) cm^{-1} and t C-O (1074) cm^{-1} (REF); FTIR spectral data by figure (4).

c- FTIR spectrum of 2-hydroxy-5-methylene-6-oxo-2 H,6H 3,4 dihydro pyronal [3,2-C][1] benzo pyranone compounds indicate that tO - H : (3429) cm^{-1} while tC-H aromatic at (3001) cm^{-1} , t C - H aliphatic (2945) cm^{-1} , t C=O :

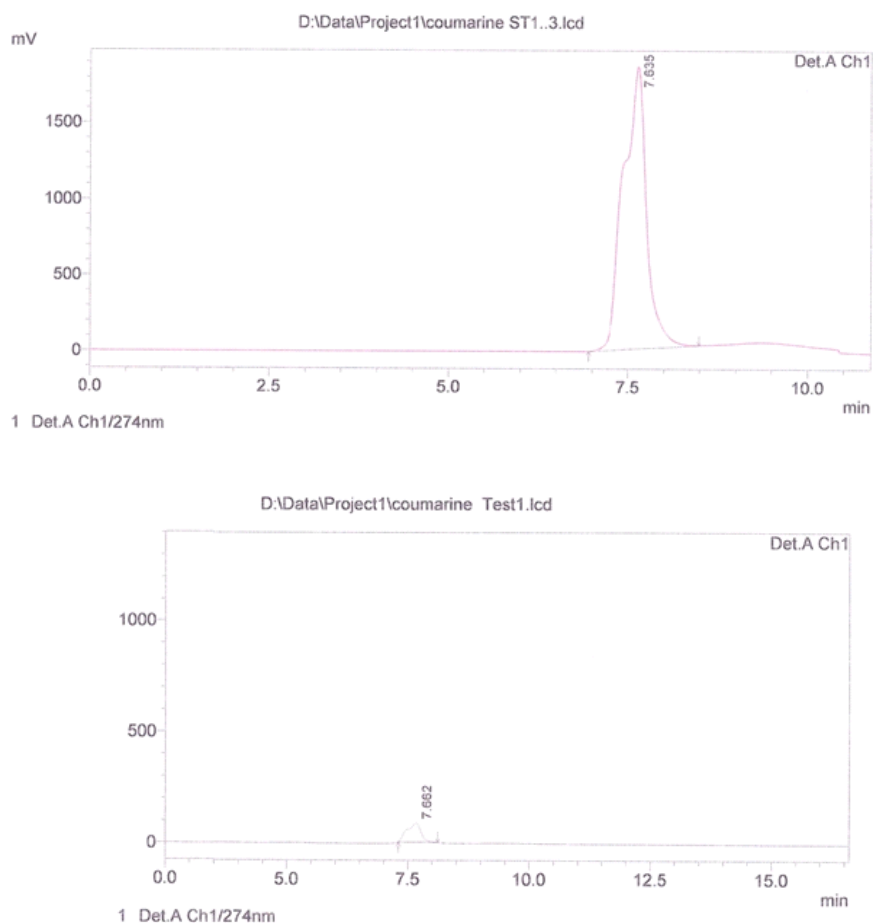


Fig. 1.

- (1699) cm^{-1} and $\text{tC}=\text{C}$ at (1641) cm^{-1} in addition to presence $\text{tC}=\text{C}$ aromatic (1562) cm^{-1} and $\text{tC}-\text{O}$ (1020) cm^{-1} (REF); FTIR spectral data by figure (5).
- d- FTIR spectrum of 2-hydroxy-5-oxo-3,4 dihydro furan [3-2 a][1] benzo pynone compounds indicate that $\text{tO}-\text{H}$: (3390) cm^{-1} while $\text{tC}-\text{H}$ aromatic at (3015) cm^{-1} , $\text{tC}-\text{H}$ alphatic (2927) cm^{-1} , $\text{tC}=\text{O}$: (1733) cm^{-1} and $\text{tC}=\text{C}$ aromatic at (1573, 1614) cm^{-1} in addition to presence $\text{tC}-\text{O}-\text{C}$ (asym. 1118) cm^{-1} (sym. 1074) cm^{-1} (REF); FTIR spectral data by figure (6).
- e- FTIR spectrum of 2-hydroxy-5-oxo-3,4 dihydro pyronal [3,2-C][1] benzopyrane compounds
- g- indicate that $\text{tO}-\text{H}$: (3479) cm^{-1} while $\text{tC}-\text{H}$ aromatic at (3045) cm^{-1} , $\text{tC}-\text{H}$ alphatic (2921) cm^{-1} , $\text{tC}=\text{O}$: (1735) cm^{-1} and $\text{tC}=\text{C}$ aromatic at (1581, 1461) cm^{-1} in addition to presence $\text{tC}-\text{O}$ (1099) cm^{-1} (REF); FTIR spectral data by figure (7).
- f- FTIR spectrum of 6-amino coumarin compounds indicate that tNH_2 (asym. 3415, sym. 3390) cm^{-1} while $\text{tC}-\text{H}$ aromatic at (3087) cm^{-1} , $\text{tC}=\text{O}$: (1731) cm^{-1} and $\text{tC}=\text{C}$ aromatic at (1622, 1544, 1461) cm^{-1} in addition to presence $\text{tC}-\text{O}$ (1122) cm^{-1} (REF); FTIR spectral data by figure (8).

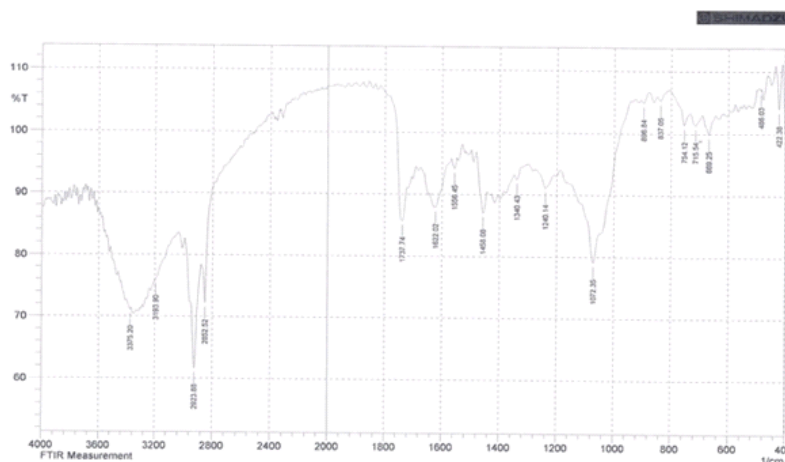


Fig. 2. FTIR spectrum of coumarin crude extract obtained from *Melilotus officinalis*

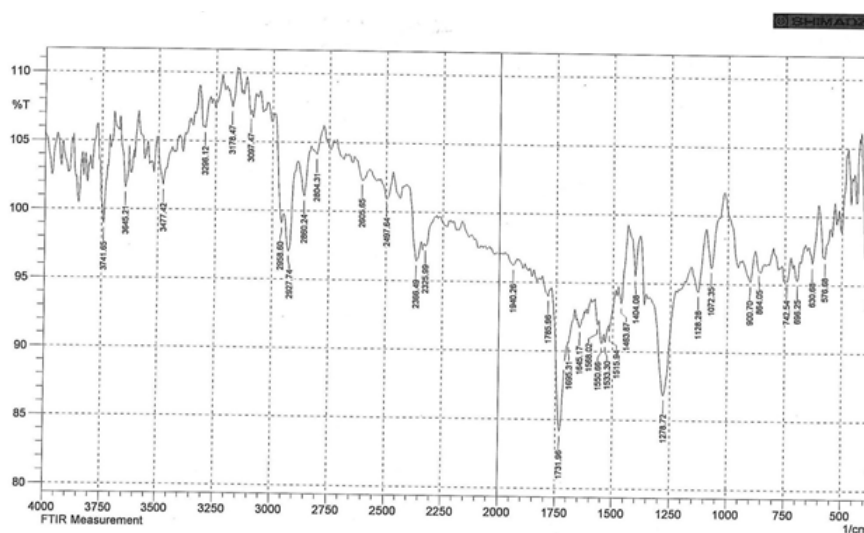


Fig. 3. FTIR spectrum of 3,3-methylene bis 4-hydroxy-coumarin (dicoumarol) compounds

- indicate that tC-H aromatic at (3051) cm^{-1} , t C = (2360) cm^{-1} , t C=O : (1737) cm^{-1} and t C=C aromatic at (1625, 1595, 1575) cm^{-1} in addition to presence tC-O (1097) cm^{-1} (REF); FTIR spectral data by figure (9)
- h- FTIR spectrum of 6-carboxylic coumarin compounds indicate that tO – H : (3452-2896) cm^{-1} while tC-H aromatic at (3080) cm^{-1} , t C=O : (1718) cm^{-1} and t C=C aromatic at (1577) cm^{-1} in addition to presence tC-O (1076) cm^{-1} (REF); FTIR spectral data by figure (10).
- i- FTIR spectrum of mixture contain 7-acetyl coumarin and 4-methyl-2,5-dioxo-3-phenyl-2H,5H-pyrano-[3,2-c][1]- Benzopyran compounds indicate that tC-H aromatic at (3004) cm^{-1} , t C – H aliphatic (2987) cm^{-1} , t

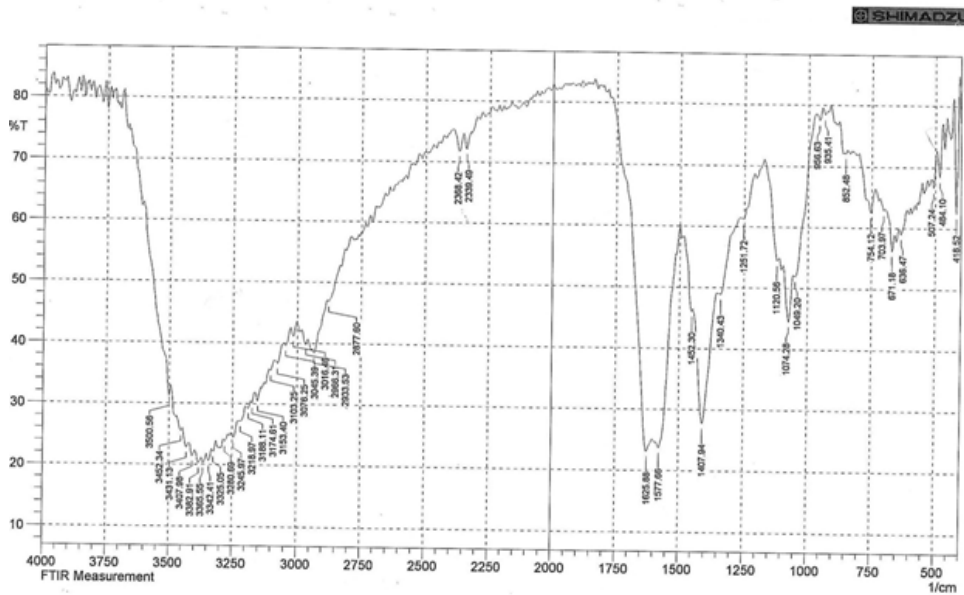


Fig. 4. FTIR spectrum of 2-hydroxy-4-methylene-5-oxo-2, 3- dihydro furane [2-3a][1] benzo pyrane compounds

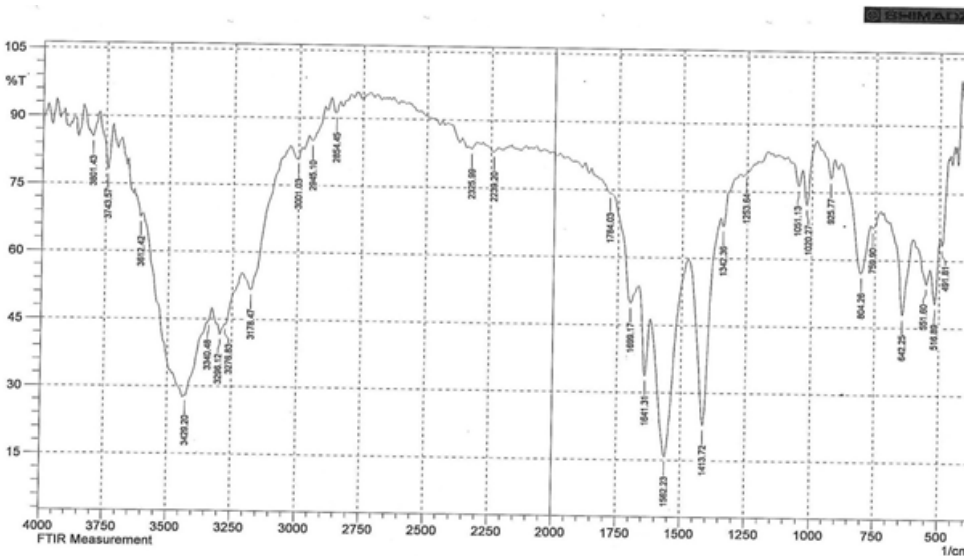


Fig. 5. FTIR spectrum of 2-hydroxy-5-methylene-6-oxo-2 H,6H 3,4 dihydro pyronal [3,2-C][1] benzo pyranone compounds

- j- FTIR spectrum of 4-hydroxy coumarin compounds indicate that tO – H : (3382) cm^{-1} while tC-H aromatic at (3091) cm^{-1} , t C=O : (1726) cm^{-1} and t C=C aromatic at (1625, 1552) cm^{-1} in addition to presence tC-O-C (1074) cm^{-1} (REF); FTIR spectral data by figure (12).

Anti-coagulant activity

In this study, anti-coagulant activity of the crude extract was investigated by prothrombin

time kit and results were showed by table (1) below:-

Prothrombin time of treated blood (46 Sec., 140 Sec. and No-clot) with different concentration of *M. officinalis* extract (25, 50 and 75 μl) respectively, showed anti-coagulation activity by dose-dependent manner compared with the negative control which was 14 seconds while treated blood with EDTA as +ve control prevent clot formation.

Normally, blood should be stay as a fluid inside the vascular system while forming clot

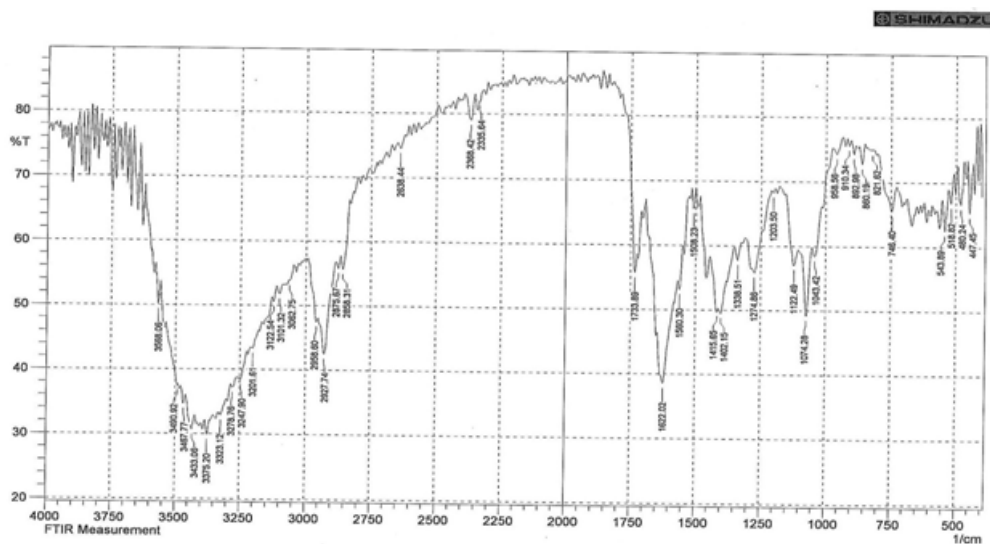


Fig. 6. FTIR spectrum of 2-hydroxy-5-oxo-3,4 dihydro furan [3-2 a][1] benzo pynone compounds

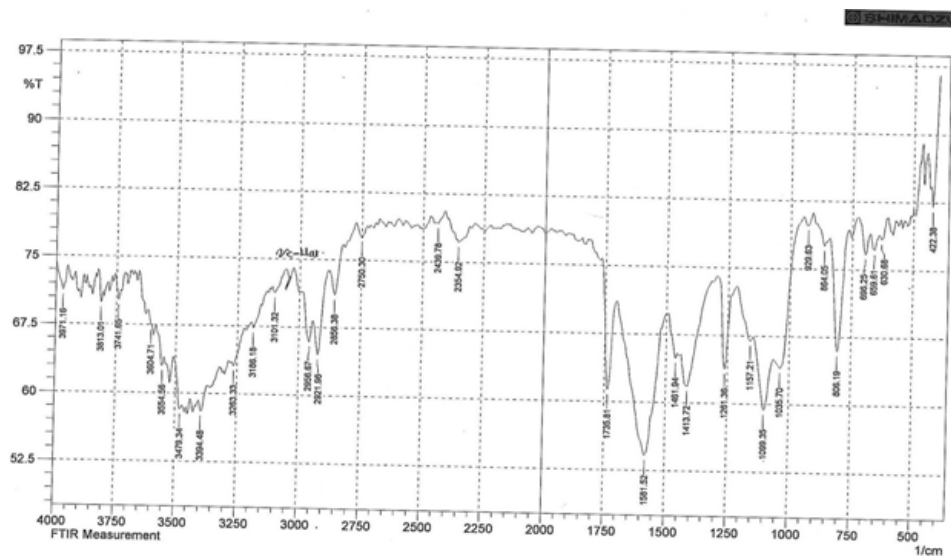


Fig. 7. FTIR spectrum of 2-hydroxy-5-oxo-3,4 dihydro pyronal [3,2-C][1] benzopyrane compounds

formation directly when the vascular exposing to injury or accident. The predominant agents for keeping blood fluidity such as parenteral anti-coagulant heparin and its derivatives promote a natural inhibitor of coagulant protease.

Fibrinolysis causing activate fluidity of blood due to lyse intravascular thrombi, increasing the stimulation of fibrinolysis may cause systemic destroying of fibrinogen and coagulation factors so minimal concentration of coumarin involved in

ethanolic extract of *M. officinalis* (sweet clover) block multiple steps of the coagulation cascade by being competitive inhibitors of Vitamin-K in the biosynthesis of prothrombin (Jain and Jos, 2012).

Anti-coagulant activity of coumarin derivatives fractions

M. officinalis crude extract fractionations by column chromatography were tested as anti-coagulant agents and the results shown by table (2):-

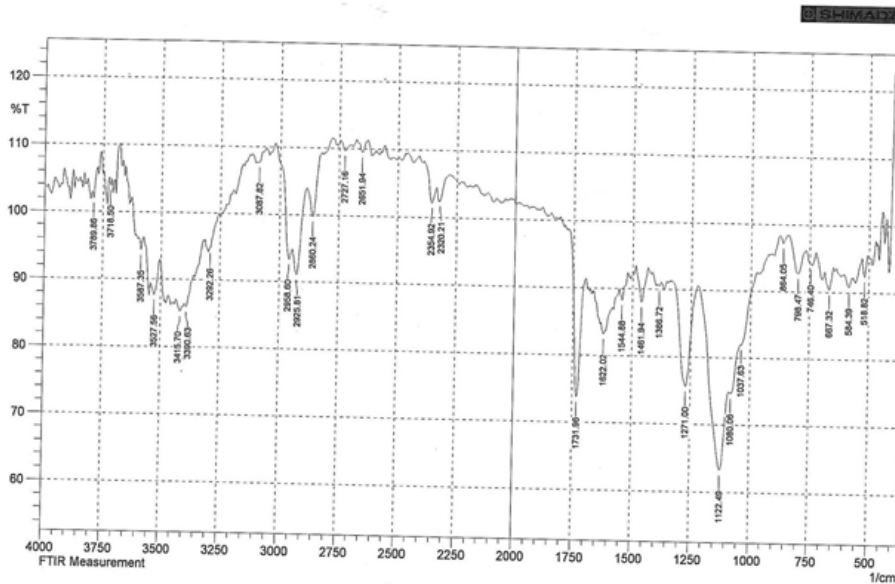


Fig. 8. FTIR spectrum of 6-amino coumarin compounds

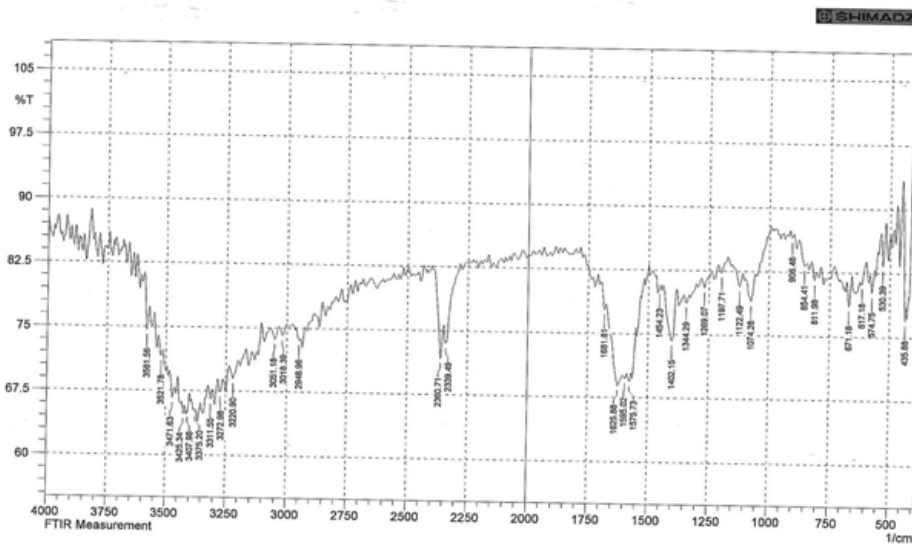


Fig. 9. FTIR spectrum of mixture consist of 6-amino coumarin and 6-cyano coumarin compounds

The results indicated that Dicoumarol or bis 4-hydroxy coumarin gave the higher anti-coagulant activity with (2800 sec.) due to presence of methoxyl group so the modification of 4-hydroxy coumarin by adding methoxyl group will increase anticoagulation effect (Venugopala, *et al.*, 2013& Mathur and Arora, 1963). dicumarol, inhibits the clotting mechanism by interaction the production process of an important factors which were

produced by liver and necessary for vitamin K activity (Asif, 2015).

2-hydroxy-4-methylene-5-oxo-2, 3-dihydro furane [2-3a][1] benzo pyrene compound and 2-hydroxy-5-methylene-6-oxo-2 H,6H 3,4 dihydro pyronal [3,2-C][1] benzo pyranone compound showed PT with (2000 and 1150 Sec.) respectively, Chmielewska & Cieglak (1958) analyzed the results of these compounds activity

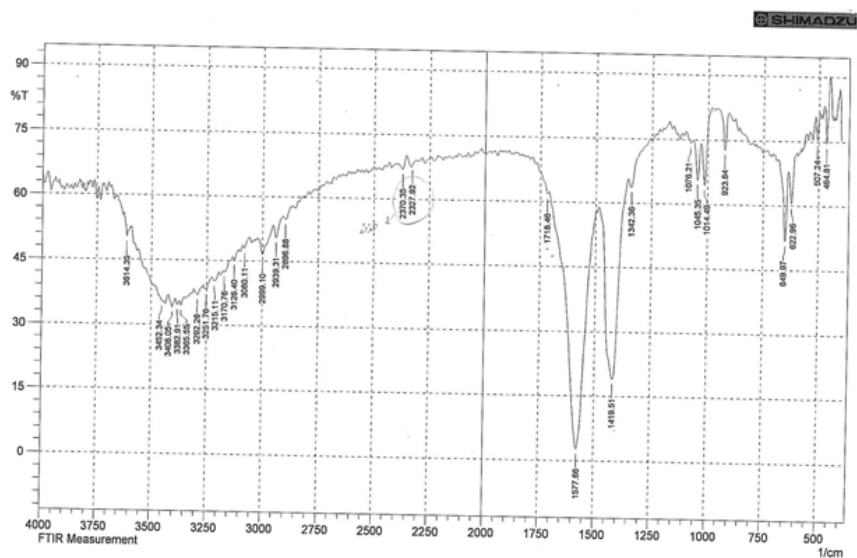


Fig. 10. FTIR spectrum of 6-carboxylic coumarin compounds

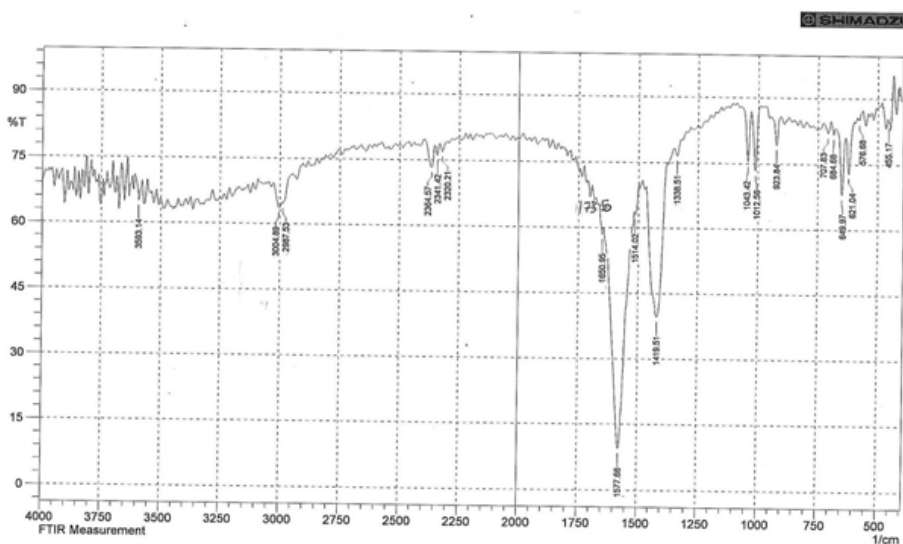


Fig. 11. FTIR spectrum of mixture contain 7-acetyl coumarin and 4-methyl-2,5-dioxo-3-phenyl-2H,5H-pyrano-[3,2-c][1]- Benzopyran compounds

according to structural similarity with vitamin K, while 2-hydroxy-5-oxo-3,4 dihydro furan [3,2 a][1] benzo pynone compound(1800 Sec.) or 2-hydroxy-5-oxo-3,4 dihydro pyronal [3,2-C][1] benzopyrane (950 Sec.) which were considered as anti-vitamin K due to structure contains cyclic hemiacetals.

Present data shows that the following compounds (6-amino coumarin, mixture of 6-amino coumarin and 6-cyano coumarin, and 6-carboxylic coumarin) compounds gave valuable activity with time as (935, 890 and 875 Sec. respectively) due to presence of nitrogen, cyano and carboxyl substituents in 6- position.

Mixture of 7-acetyl coumarin and 4-methyl-2,5-dioxo-3-phenyl-2H,5H-pyrano-[3,2-c][1]- Benzopyran result data have a considerable

example mention relation between activity and molecular shape refer to extra ring which were added to 4-hydroxy system Otherwise, 4-hydroxy coumarin have activity against clots as (800 sec.), support Link (1943)hypothesis about minimum structure required for anticoagulation.

Coumarin derivatives consuming orally is effective treatment for a wide range of clot dependent disorders. Investigations have shown that oral consuming treatment is medicinally reactive against intravascular clot formation in patients suffering from thromboembolism and atrial fibrillation disorders in blood stream; this is prevention stroke and mortality in cases of operations of myocardial and heart valves. Otherwise, anticoagulant medications using with cases suffering from heart disorders dependent rheumatism due to inhibiting variety proteins causing activation clot formation. Continuously treated with heparin causing osteoporosis beside uncomfortable situation for the patients so the coumarins are used instead of heparin (Goodman & Gilman's, 2006).

CONCLUSION

Coumarins naturally found in *M. officinalis* consider as important phenolic compounds due to their clinically and biologically

Table 1. Anticoagulant activity of *M. officinalis* crude extract

Sample	Concentrations	Prothrombin time/ sec.
<i>M. officinalis</i>	25 µl	80 Sec.
Crude extract	50 µl	140 Sec.
	75 µl	No-clot
-ve control	100 µl/ normal saline	30 Sec.
+ve control (EDTA)	100 µl	3100

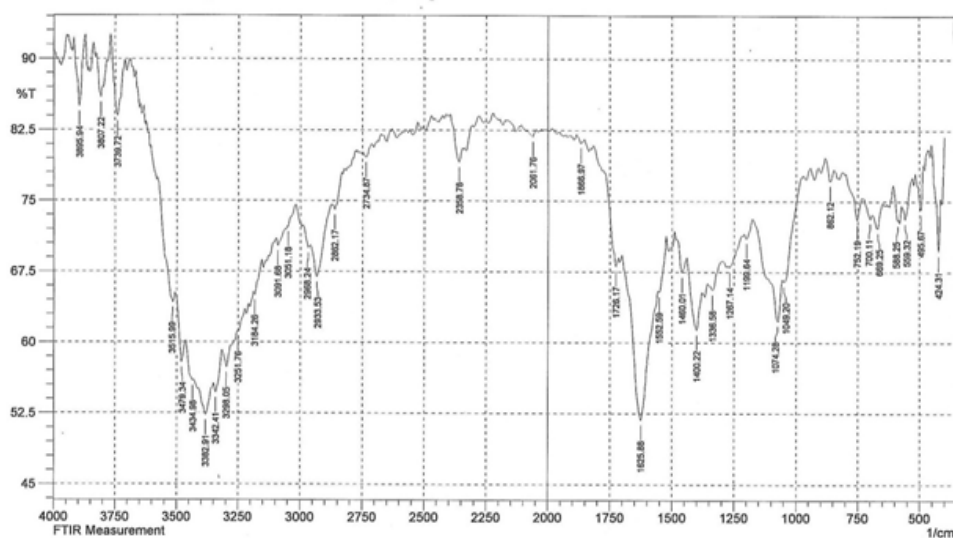


Fig. 12. FTIR spectrum of 4-hydroxy coumarin compounds

Table 2. Anticoagulant activity of Coumarin and coumarin derivatives separated from *M. officinalis* extract by column chromatography

Sample	Concen. /μl	Prothrombin time (PT)/Sec.
I. 3,3-methylene bis 4-hydroxy-coumarin (dicoumarol)	25	200
	50	350
	75	2800
II. 2-hydroxy-4-methylene-5-oxo-2, 3- dihydro furane [2-3a][1] benzo pyrane	25	110
	50	150
	75	2000
III. 2-hydroxy-5-methylene-6-oxo-2 H,6H 3,4 dihydro pyronal [3,2-C][1] benzo pyranone	25	190
	50	210
	75	1150
IV. 2-hydroxy-5-oxo-3,4 dihydro furan [3-2 a][1] benzo pynone	25	140
	50	220
	75	1800
V. 2-hydroxy-5-oxo-3,4 dihydro pyronal [3,2-C][1] benzopyrane	25	95
	50	150
	75	950
VI. 6-amino coumarin	25	80
	50	120
	75	935
VII. mixture of 6-amino coumarin and 6-cyano coumarin	25	75
	50	100
	75	890
VIII. 6-carboxylic coumarin	25	60
	50	90
	75	875
IX. 4-hydroxy coumarin	25	60
	50	80
	75	800

applications. HPLC proved as a useful tool for quality detection while FTIR is valuable for control molecular characterization of these compounds. Coumarins shows significant anticoagulant potential, especially purified coumarin derivatives compounds.

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