

FLAVONOID CONSTITUENTS OF *Reaumuria hirtella*.

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By
A.E.Abozeed

Medicinal and Aromatic Plants, Desert Research Center, El-Matarya, Cairo, Egypt

ABSTRACT

The present study aimed to investigate the total flavonoid compounds from the aerial parts and the roots of *Reaumuria hirtella* collected at different seasons of (2012-2013) from Ageba region. Also, it aimed to isolate and identify the flavonoid compounds from the aerial parts only using chromatographic and spectroscopic analysis: UV, MS, ¹H-NMR and ¹³C-NMR. In addition, these compounds were determined spectrophotometrically and calculated as kaempferol. The results showed that the total flavonoid contents of the aerial parts were higher generally than that of the roots. The spectroscopic analysis revealed the presence of seven flavonoid compounds: kaempferol, kaempferol-3-O- α -l-rhamnoside, kaempferol 7-O-diglucoside, quercetin, quercetin-3-O-rutinoside (rutin), quercetin-3-rhamnoside and quercetin-3-O-glucoside and one phenolic compound as ferulic acid.

Key words: flavonoids, phenolic acids, *Reaumuria hirtella*.

1. INTRODUCTION

The genus *Reaumuria* consists of 12 species belongs to the family *Tamaricaceae* and distributed in the deserts of Northern Africa, Asia and Southern Europe (Yang and Gaskin, 2006).

A sulphated flavonol has been isolated from the leaves of *R. mucronata* and identified as kaempferol 3,7-disulphate (Nawwar *et al.*, 1977).

Flavonol, kaempferol and quercetin, and their glycosides are major flavonoids, and also methylated flavonols, rhamnazin, rhamnetin, rhamnocitrin, kaempferide, tamarixetin, kaempferol 7,4'-dimethyl ether and dillenetin, and their glycosides are found in some species of the *Tamaricaceae* (El Sissi *et al.*, 1973; Nawwar *et al.*, 1975 and 1984; Wang *et al.*, 2009 and La *et al.*, 2011). Flavonols frequently occur as sulphates in the *Tamaricaceae* (Harborne, 1975; Saleh *et al.*, 1975; El Ansari *et al.*, 1976 and Tomás-Barberán *et al.*, 1990).

Ten flavonoids were isolated from the aerial parts of *Reaumuria soongarica* growing in the deserts of Mongolia (Tsukasa, *et al.*, 2012).

In this paper, we describe the isolation and identification of flavonoids and their chemical properties from the aerial parts of *Reaumuria hirtella* growing in Ageba region (northern coastal region).

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Source of plants

The fresh aerial parts and roots of *Reaumuria hirtella* were collected seasonally from Ageba region (Northern coastal region) during the period of investigation. The plant materials were cleaned, dried in an electric oven at 50°C and ground to fine powder and then kept for analysis.

2.1.2. Solvents

Ethyl alcohol, n-butanol, acetic acid, were obtained from petroleum ether (40-60°C), ether, chloroform and ethyl acetate.

2.1.3. Reagents

Sodium methoxide solution (2.5 ml was added cautiously to 100 ml dry methanol), sodium acetate, boric acid, hydrochloric acid (50 ml were mixed with 100 ml water) and aluminum chloride solution (96 gm anhydrous aluminum chloride in 1L ethanol).

2.1.4. Solvent systems

1. n. butanol-acetic acid-water (4:1:5 v/v/v)
2. Acetic acid-water (15:85 v/v)

2.1.5. Preparation of flavonoids extract

The dried aerial parts of *Reaumuria hirtella* were ground to fine powder and extracted with 70% aqueous MeOH several times, and evaporated under reduced pressure. The concentrated aqueous extract was fractionated

successively using Soxhelt apparatus, according to Lee *et al.*, (2004).

2.2. Methods

2.2.1. Quantitative estimation of the total flavonoids

Total flavonoids were estimated according to the method described by (Karawya and Aboutable, 1982). The flavonoid content was calculated as kaempferol.

Two grams of powder air - dried material , accurately weighed were defatted using petroleum ether (40-60 °C) and extracted with 95% ethanol till exhaustion. The ethanolic extract was adjusted to 50 ml. Five ml were treated under calibration curve, three determinations were carried out for each sample with reference to the standard curve, the corresponding amounts of kaempferol were found out.

2.2.2. Calibration curve

Different aliquots of ethanolic solution of kaempferol equivalent to 5-200 µg were separately introduced into test tubes, evaporated till dryness on a hot water bath (40-50 °C). Five ml of 0.1 M aluminium chloride reagent were added. The absorbance of the color developed was measured at $\lambda_{\text{Max}} = 266$ nm against a blank. Three determinations for each concentration of standard solution were carried out .

2.3. Chromatographic analysis

The concentrated aqueous extract was subjected to two dimensional paper chromatography technique using the solvent system n- butanol: acetic acid: water (4:1:5, v/v/v) and acetic acid: water 15:85, respectively. The developed chromatograms were air dried and examined under ultraviolet light. Elution started with benzene and the polarity was increased gradually by the addition of proportion of ether (10%, 20% and 30%) followed by chloroform, ethyl acetate and methanol solvents in the same trend. The received fractions were evaporated and similar fractions were collected together, evaporated and subjected to paper chromatography.

Preparative paper chromatography was applied on Whatmann No.3 paper chromatography using the solvent system AcOH-15% (Anderson and Sowers, 1998), preparative chromatography was applied on thin layer chromatography (TLC). The separated flavonoids and phenolic acids were purified on a Sephadex LH-20 column using methanol/water system.

2.4. Physical analysis

2.4.1. Ultraviolet spectrophotometry (UV)

The purified material (after passing through Sephadex LH-20) was dissolved in pure methanol then subjected to UV spectrophotometric measurements in quartz cavette (4ml capacity, 1cm thick) using Shimadzu UV 240 spectrophotometer. In the case of flavonoids, AlCl_3/HCl , $\text{NaOAc}/\text{H}_3\text{BO}_3$ and NaOMe reagents were separately added to the solution, and UV measurements were then carried out (Mabry *et al.*, 1970) .

2.4.2. ^1H - and ^{13}C - Nuclear magnetic resonance (NMR)

^1H - and ^{13}C -NMR are the most efficient method for identification and elucidation of the structure of various types of flavonoids. Chemical shift in ppm varies according to the proton and/or carbon position in the flavonoids nucleus, thus offering good criteria for structure elucidation. The type and position of the anomeric sugar proton, its "J" value in Hz are good signs for confirming the chemical structure (Mabry *et al.*, 1970).

The NMR measurement was carried out on A Jeol EX-270 NMR spectrometer apparatus (270 MHz for ^1H - NMR and 67.5 MHz for ^{13}C -NMR).

2.4.3. Mass spectrometry (MS)

The isolated purified compounds were subjected in most cases to fast atom bombardment (positive and negative) mass spectrometric analysis. (FAB - MS).

* Some other compounds were subjected to electron ionisation and/or chemical ionization mass spectrometric analysis (EL, CL-MS).

* The spectra were conducted using mass spectrometer varian Mat 711, Finnigan SSQ 7000 and MM7070E (Mabry *et al.*, 1970).

2.5. Chemical reactions

Analysis to determine the nature of aglycone and sugar was carried out according to standard procedures performed by (Harbone *et al.*, 1975).

2.5.1. Controlled (Mild) acid hydrolysis

A known weight of the purified compound was subjected to mild acid hydrolysis in aqueous HCl (0.1 N) at 100°C for 15 minutes. The reaction mixture was examined at definite intervals by comparative paper chromatography (CoPC) for tracing any intermediate that might be released during the course of hydrolysis.

2.5.2 Complete (Normal) acid hydrolysis

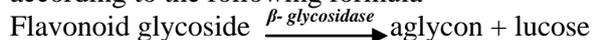
A known weight of the flavonoid material

under investigation was subjected to acid hydrolysis in either 2N HCl at 100°C for 2 hours or in 2N HCl in aqueous MeOH (1:1 v/v) in vacuum.

The obtained hydrolysate in both reactions was then extracted with ethyl acetate. The received AcOEt extract was washed with H₂O twice, concentrated under vacuum and subjected to (CoPC) using authentic reference markers. The remaining aqueous layer was then freed from acid through extraction with 20% N-methyl diethylamine in CHCl₃ and subjected to (CoPC) using authentic sugars as reference markers.

2.5.3. Enzymatic hydrolysis

The flavonoid glycosides were enzymatically hydrolysed through an enzyme which corresponds to the type of the sugar attaching the compound and the obtained aglycone was tested chromatographically with an authentic sample according to the following formula



One mg of the compound was dissolved in 2ml of 0.05 acetate buffer pH₅ with 1mg of the powdered β – glycosidase and the mixture was allowed to stand for over night at 37°C. The hydrolysate was subjected to CoPC against flavonoid authentic markers. Also, the sugar moieties were detected using authentic sugars markers.

3. RESULTS AND DISCUSSIONS

3.1. Determination of the total flavonoids

The total flavonoids of the aerial parts and the roots of *Reaumuria hirtella* at different seasons are given in Table (1). The results indicated that the total flavonoids of the aerial parts were higher than in the roots. Also, it showed that the total flavonoids reached its maximum value of 2.08% in the aerial parts

Table (1): Total flavonoids of the aerial parts and roots of *R. hirtella*.

Seasons	Total flavonoids % (g/100g. Dry wt.)	
	Aerial parts	Roots
Winter	2.08	0.32
Spring	2.02	0.33
Summer	1.58	0.29
Autumn	1.62	0.23

during winter and its maximum value of (0.33) in the roots during spring.

3.2. Investigation of phenolic compounds

Seven flavonoids and one phenolic compound were isolated, purified by Sephadex LH-20 column, PC and TLC chromatography and identified through UV shift reagents, ¹H and ¹³C- NMR. These compounds were named F₁-F₇ and P₁.

3.2.1. Kaempferol

The compound F₁ was obtained as yellow crystals, soluble in methanol, m.p.227oC, Rf-values and colour reactions are outlined in Table (2)

UV λ. _{Max} in MeOH (nm) : 253 (sh) , 268,324 (sh), 367 AlCl₃ : 266,305 (sh), 350,422 , AlCl₃/HCl : 266,305 (sh), 350,422 , NaOAc : 275,302 (sh) , 385, NaOAc/ H₃BO₃ : 267, 296 (sh), 320 (sh), 370 , NaOMe: 280 , 318 (sh), 420.

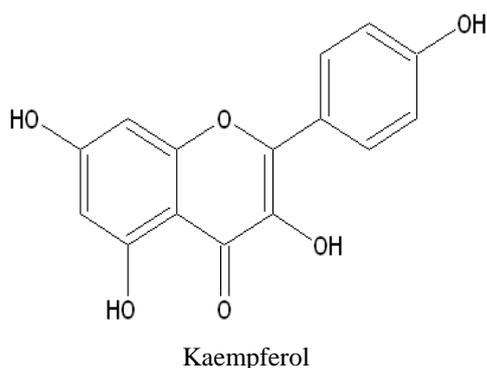
UV spectral data, λ max in NaOMe and shift reagents showed that :

1. Band I and II appeared at 367 and 268 nm, respectively, which indicates that it is a flavonol type with free OH at position 3.
2. Addition of NaOMe resulted a bathochromic shift in band I (+63 nm) which proved the presence of free OH group at position 4.
3. Bathochromic shift in band II (+7 nm) occurred on the addition of NaOAc, which indicates the presence of free OH at position 7. Addition of boric acid gave no shift indicating the absence of orthodihydroxy group at B-ring.
4. Bathochromic shift in band I (+53 nm) occurred on addition of AlCl₃ indicating the presence of free OH group at C-3 and C-5, this shift was not affected after the addition of HCl indicating the absence of orthodihydroxy group at B-ring.

¹H-NMR spectrum of compound F₁ in dimethyl sulfoxide acid DMSO-d₆, showed signals at:

δ (ppm) 8.0 (2H, d, J =8Hz, H2 and H6), δ 6.9 (2H, d, J = 8Hz, H3 and H5), δ 6.4 (1H,d,J = 1.5 Hz,H8) and δ 6.2 (1H,d,J = 1.5 Hz , H6).

From UV analysis, compound E1 is probably kaempferol. The structure of this compound was further confirmed by ¹H NMR spectrum in DMSO which showed the signals characteristic for kaempferol as described by Mabry *et al.* (1970).



3.2.2. Kaempferol-3-O- α -L-rhamnoside

Compound F₂ was obtained as yellow crystals, its m.p.228-230°C, R_F- values and color reactions are recorded in Table (2).

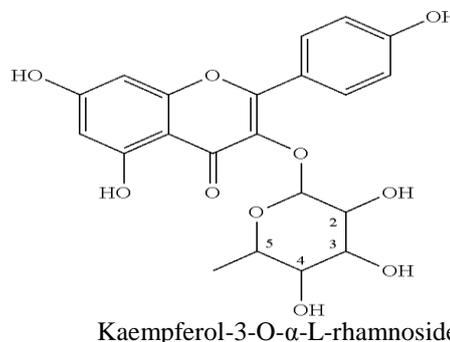
UV spectral data of compound F₂ in methanol and shift reagent, indicated that:

Compound F₂ may be kaempferol with substitution at position 3, as it gave band I in methanol at 350 nm and band II at 265 nm , the remaining UV spectral data were found to be similar to that of kaempferol type.

A known weight of this compound was subjected to partial and complete acid hydrolysis using 0.1 N and 2 N HCl, afforded kaempferol as the aglycone moiety and rhamnose as the sugar moiety.

El mass spectrum revealed the presence of molecular ion peak M⁺ at m/e 433 and other important ions m/e 303,287 (kaempferol).

From UV, acid hydrolysis, EI mass spectrum and by comparison with authentic sample, compound F₂ could be kaempferol-3-O- α -L-rhamnoside.



3.2.3.Kaempferol 7-O-diglucoside.

Compound F₃ was obtained as yellow crystals, its m.p. 241-243 °C, R_F- values and colour reactions are recorded in Table (2).

UV λ_{max} (nm): MeOH 256, 369; NaOMe decomposition; +AlCl₃ 267, 306sh, 360, 434; AlCl₃/HCl 265, 303sh, 360, 422; +NaOAc 257, 409; +NaOAc/ H₃BO₃ 255, 375. NaOMe 280-318 (sh), 420 .

El mass spectrum revealed the presence of molecular ion peak M⁺ at m/e 609 (molecular ion peak, kaempferol+2 mol glucose), and other important ions m/e 287 (kaempferol).

Table (2). R_F -Values and color reactions of the isolated compounds.

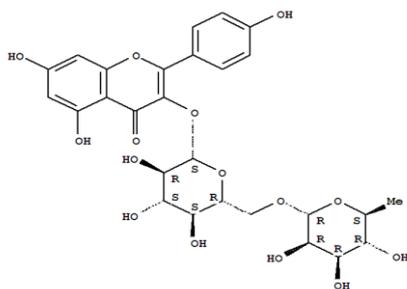
Compound	Solvent system	R _F -value	Color reactions		
			visible	UV	UV+HN3
F ₁	BAW	85	Yellow	Yellow	Yellow
	Ac-OH-15%	40	Yellow	Yellow	Yellow
F ₂	BAW	87	-	Deep purple	Yellow
	Ac-OH-15%	50	-	Deep purple	Yellow
F ₃	BAW	83	Yellow	Yellow	Bright yellow
	Ac-OH-15%	70	Yellow	Yellow	Bright yellow
F ₄	BAW	73	B.yellow	Deep purple	Yellow
	Ac-OH-15%	29	B.yellow	Deep purple	Yellow
F ₅	BAW	57	Yellow	Yellow	Yellow
	Ac-OH-15%	31	Yellow	Yellow	Yellow
F ₆	BAW	79	Yellow	Yellow	Yellow
	Ac-OH-15%	37	Yellow	Yellow	Yellow
F ₇	BAW	80	-	Brown	Brown
	Ac-OH-15%	60	-	Brown	Brown
P ₁	BAW	88	-	Bright blue	Fl. blue
	Ac-OH-15%	56	-	Bright blue	Fl. blue

F₁-F₇ flavonoids

P₁ phenolic compound

B. bright

Fl. fluorescence



Kaempferol 7-O-diglucoside

Flavonoid F₃ produced kaempferol and glucose by acid hydrolysis. UV spectral properties in addition to various shift reagents (MeOH, AlCl₃, AlCl₃/HCl, NaOAc, NaOAc/H₃BO₃ and NaOMe) according to Mabry *et al.* (1970) showed that F₃ is 7-substituted kaempferol. The attachment of 2 mol glucose to kaempferol was shown by MS survey, i.e., appearance of the molecular ion peak, *m/e* 609 and fragment ion peak, *m/e* 287. From the results described above, F₃ was characterized as kaempferol 7-O-diglucoside. No bathochromic shift in band II on addition of NaOAc.

3.2.4. Quercetin

This compound (F₄) was obtained as yellow crystals, soluble in methanol, its m.p. 322-324 °C, R_f values and colour reactions are recorded in Table (2).

UV. λ max (nm) MeOH: 258, 306 (sh), 370, AlCl₃: 272,328,454, AlCl₃/HCl: 268, 300 (sh), 362(sh) 428, NaOAc: 274,320 (sh), 428, NaOAc/H₃BO₃: 264, 292 (sh) 384, NaOMe: 262,332.

Compound F₄ was subjected to UV spectral analysis in methanol and shift reagents. The obtained result showed that:

- 1- Band I in methanol appear at 370 nm, indicating that the compounds is a flavonol type with free OH at position 4.
- 2- The bathochromic shift in band I (+70nm) with an increase of intensity by addition of NaOMe indicating the presence of free OH at position 4.
- 3- The bathochromic shift occurred in band I (+11 nm) on addition of NaOAc indicating the presence of free OH at position 7, which deleted by H₃BO₃ addition, indicates the presence of orthodihydroxy group (3,4 position).
- 4- The bathochromic shift in band I (+75 nm) appeared on addition of AlCl₃ indicated the presence of 3 and 5-OH free groups.

5- The hypthochromic shift of AlCl₃ spectrum in band I (-20 nm) after the addition of HCl indicates the presence of orthodihydroxy group in B-ring (3,4 position).

Thus from UV and R_f -values compound F₄ may be quercetin, this was confirmed by ¹H-NMR.

Spectrum of compound F₄ in DMSO- d₆, showed the following signals :

δ (ppm) 7.7 (1H,d, J=8.5 Hz, H2), δ 7.5 (1H, dd, J=8.5, J=2.5 Hz, H6), δ 6.8(1H, d, J= 8.5 Hz, H5), δ 6.5 (1H, d j = 1.5 Hz, H6) and δ 6.2 (1H, d, J=1.5 Hz, H-8).

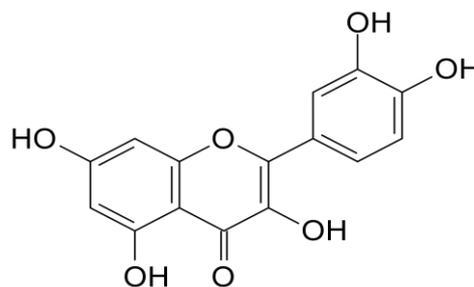
From the previously mentioned data and by comparing with published data, (Crowford and Mabry 1978) compound F₄ identified as quercetin.

3.2.5. Quercetin-3-O- rutinoside (Rutin)

Yellow crystals, m.p 190°C . R_f - values and colour reactions are recorded in Table (2).

UV λmax (nm), MeOH: 256,265(sh.), 290,355, AlCl₃:274,302(sh.), 330 (sh.), 432, AlCl₃ / HCl: 270, 298, 359, 399, NaOAc: 272, 324, 398, NaOAc /H₃BO₃:263,292 (sh.), 368, NaOMe: 272, 310,410. Band I in methanol at 355 nm indicated that this compound is a flavonol with 3-OH substitution.

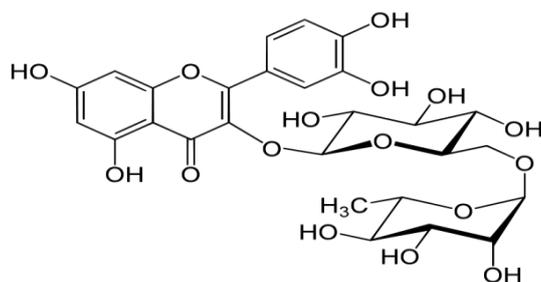
¹H-NMR (DMSO-d₆): δ 8.10 (1H, d, J=2.5 Hz, H2), δ 7.86 (1H, dd, J=8.5, 2.5Hz, H-6), δ 6.89 (1H,d,J=8.5Hz, H-5), δ 6.65 (1H, d, J=2.5



Quercetin

Hz, H-8), δ 6.5 (1H, d, J=2 Hz H6), δ 5.13 (1H, d, J=7.50 Hz H1 glucose), δ 4.55 (1H, d, J=2.5 Hz, H1 rhamnose), δ 3.47-3.87 (m, sugar protons), δ 1.23 (3H, d, J=6 CH3).

¹³C-NMR (methanol-D₆): δ ppm 174.3 (C-4), 164.5 (C-4), 164.5 (C-7), 161.2 (C-5), 156.6 and 156.4 (C-2 and C-9 respectively), 148.5 (C-4), 144.8 (C-3), 133.3 (C-3), 121.6 (C-6), 121.2 (C-1), 116.1 (C-2), 115.2 (C-5), 103.8 (C-10), 98.8 and 93.7 (C-6 and C-8, respectively), 100.7 (C-1"), 76.5 (C-3), 75.9 (C-5),74.1 (C-2),71.5 (C-4), 62.8 (C-6), 101.3 (C-1), 71.9 (C-4), 70.6 (C-2), 70.4 (C-3), 70 (C-5) and 17.6 (C-6).



Quercetin-3-O- rutinoside

3.2.6. Quercetin-3-rhamnoside

Yellow crystals, mp. 224-226°C, R_f values and colour reactions are recorded in Table (2).

UV λ max (nm) MeOH: 260.358 AlCl₃: 272,300 (sh.), 440, AlCl₃ / HCl: 272, 300 (sh.), 420 NaOAc: 275.300 (sh.), 395 NaOAc/ H₃BO₃: 260,325 (sh), 375, NaOMe: 272,320 (sh.), 430.

¹H-NMR (DMSO- d₆): δ 7.7 (1H, d, J=2.5 Hz, H2), δ 7.5 (1H, dd, J=8.5, 2.5 Hz, H6)

δ 6.8(1H, d, J=8.5, H5) , δ 6.5 (1H, d, J= 2.5 Hz, H8), δ 6.2 (1H, d, J=2.5 Hz, H6), δ 5.4 (1H, d, J= 2 Hz, H1 rhamnose), δ 3.5-4 (m, sugar protons) and δ 1.2 (3H, d, J=6Hz, CH₃ rhamnose).

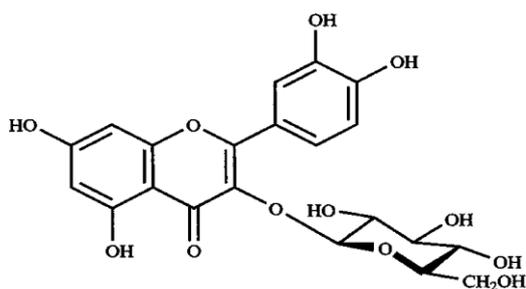
3.2.7. Quercetin-3-O-glucoside

This compound (F₇) was found to be yellow crystals, its mp. 228-230°C, R_f values and colour reactions are recorded in Table(2).

UV spectral data of compound F₇ in methanol and shift reagent, from which it can concluded that: Compound F₇ may be quercetin with substitution at position 3. UV λ max (nm) MeOH: 265,350, AlCl₃: 265, 300 (sh) 440, AlCl₃/HCl:265, 350, 420, NaOAc: 270,300,380, NaOAc/H₃BO₃: 270, 310, 373, NaOMe: 275, 330, 430 .

¹H-NMR_(DMSO-d₆): δ 7.2(2H,d,J= 8Hz, H2 and H6), δ 6.8 (2H,d,J= 8 Hz, H3 and H5), δ 5.8 (1H,d,J= 2.5 Hz, H8), δ 5.7 (1H,d,J= 2.5 Hz, H6), δ 5.4 (1H,d,J = Hz, H1 glucose).

From UV, ¹H-NMR spectrum and by comparison with authentic sample, compound E₆ could be identified as quercetin-3- glucoside.



Quercetin 3-O-glucoside (Isoquercitrin, 3).

3.2.8. Ferulic acid

This compound is soluble in benzene, chloroform and methanol. R_f values and color reactions of the compound P₂ were recorded in Table (2).

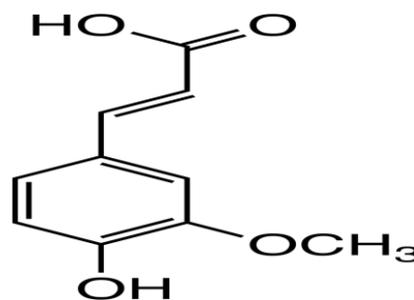
From UV spectral data of the compound P₂ exhibited absorption band I and II (285 and 312) which were characteristic of phenyl propanoids.

Bathochromic shift after addition of NaOMe in band I and II (345 and 290 nm, respectively) proved the presence of free OH.

The R_f values, color reactions and UV spectral data proved that, the compound P₂ seemed to be phenolic in nature and contain the free OH.

The ¹H-NMR spectral data (Table 2) showed two doublets at δ 7.54 and 6.25ppm, J=17Hz which are characteristic for trans olefinic double band (H-7 and H-8), respectively. The presence of two doublets at δ 7.15 and 6.95 and the doublet of doublet signal at δ 7.09 are corresponding to *ortho* and *meta* coupling which is a good evidence for the presence of trisubstituted benzene. The presence of singlet at δ 8.9 confirmed the UV analysis for the presence of free OH group. The presence of a signal at δ 3.85 for- OCH₃ group.

From the previous obtained data, the compound P₂ was identified as ferulic acid (4-hydroxy-3-methoxy).



Ferulic acid

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المكونات الفينولية لنبات المليح

عاطف السيد أبو زيد

قسم النباتات الطبية والعطرية – مركز بحوث الصحراء – المطرية – القاهرة – مصر

ملخص

يهدف هذا البحث الي دراسة الفلافونيدات الكلية لكل من الأجزاء الهوائية والجذور لنبات المليح والذي تم تجميعه في مواسم مختلفة خلال عامي (2012-2013) من منطقة عجيبة . فتم فصل وتعريف المركبات الفلافونيدية للأجزاء الهوائية فقط باستخدام التفاعلات اللونية والتحليل الكامل والجزئي بالحمض وقياس طيف الكتلة وتحليل اطياف الاشعة البنفسجية والرنين المغناطيسي لانبوية الهيدروجين والكربون . وقد اتضح من الدراسة ان نسبة الفلافونيدات الكلية في الأجزاء الهوائية كانت عموما اعلي منها في الجذور خلال المواسم الاربعة . وقد تم دراسة الفلافونيدات والفينولات دراسة تفصيلية في الأجزاء الهوائية للنبات أشارت الي وجود سبعة مركبات فلافونيدية وهي (كامفيرول ، كامفيرول -3- رامينوسيد ، كامفيرول -7- ثنائي جلوكوسيد ، كوارستين ، روتين ، كوارستين -3- رامينوسيد ، كوارستين -3- جلوكوسيد) ومركب واحد فينولي هو حمض الفريوليك .

المجلة العلمية لكلية الزراعة – جامعة القاهرة – المجلد (65) العدد الثاني (ابريل 2014):163-169.