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ESTIMATION OF PHENOLIC CONSTITUENTS OF *ASCLEPIAS SINAICUS* (MUSCHL.)

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Asclepias sinaicus (Muschl.) was collected from natural habitat (El- Qusaima road at north Sinai) during the year of 2005. It is known in Arabic as ghalquit el- deeb or hargal or gheil.

Nine main flavonoids were isolated from the ethanolic extract of the plant aerial parts and three from the plant seeds. They were established by Nuclear magnetic resonance for hydrogen proton and carbon thirteen (^{1}H and ^{13}C-NMR) and different spectral data. Plant aerial parts contain quercetin, rutin, quercetin-3- galactopyranoside, quercetin-3-glucoside-7-rhamnoside-3$'$ methoxide, kaempferol, myricetin, myricetin 3-rutinoside, isorhamnetin and isorhamnetin 3-rutinoside. While plant seeds contain rutin, quercetin-3- galactopyranoside and kaempferol.

The aerial parts contain the coumarin compounds; aesculin and scopoletin while plant seeds contain scopoletin.

A quantitative estimation of the total flavonoids in the alcoholic extracts of both aerial parts and seeds was accomplished by spectrophotometric method during the four seasons of the year (2005). The percentage of total flavonoids of aerial parts reached maximum value of 3.96% collected in spring season.

Qualitative and quantitative estimation of the phenolic constituents were determined using HPLC techniques. There are fifteen and ten compounds identified in aerial parts and seeds of *A. sinaicus*, respectively. The highest concentration were *p*-coumaric acid (1.359 and 0.419 µgm/100gm) for plant aerial parts and seeds, respectively.

Keywords: *Asclepias sinaicus*, phenolic constituents, Nuclear magnetic resonance for hydrogen proton and carbon thirteen (^{1}H and ^{13}C-NMR).

Knowledge of the chemical constituents of plants is desirable, not only for the discovery of therapeutic agents, but also because such information may be valuable in disclosing new sources of such economic materials as tannins, oils, gums, precursors for the synthesis of complex substances, etc. In addition, the knowledge of the chemical constituents of plants would further be valuable in discovering the actual value of folkloric remedies (Mojab *et al*., 2003). Coumarins are group of the most common secondary plant metabolites.

Asclepiadaceae (milk weed family) is a large family with 347 genera and about 3850 species. *Asclepias sinaicus* (Muschl.), is one of the most common shrubs (Boulos, 2000).

Kretsu (1983) stated that the subterranean organs of the *Gomphocarpus fruticosus* contained quercetin, coumarins, cardiac glycosides, β-sitosterol and rutin, while the roots contained tannins, quercitin and the sugar moiety rhamnose.

El-Domiaty *et al*. (1993) isolated lupeol acetate, lupeol, β-sitosterol, β-sitosterol-O-glucoside, oleanolic acid and two flavonoidal compounds from *Gomphocarpus sinaicus*.

Meena and Renwick (1998) reported that the major stimulants were identified as quercetin-3-O-(2$^{\prime\prime}$-O-β-xylosyl)-β-D-galactoside and quercetin - 3-O-β-D-galactoside from *Asclepias syriaca* and *A. incarnata*, respectively.

Sikorska and Matlawska (2000) isolated quercetin and its glycosides, 3-O- galactoside, 7-O-glucoside, 3-O-β- D-xylopyranoside (1→2)-β-D-galactoside and 3-O-β-D-glucopyranoside (1→2)-β-D-galactoside from the flowers of *A. syriaca*.

Sikorska *et al*. (2000) isolated the phenolic acids; *p*-coumaric, protocatechuic and caffeic acids from the leaves and flowers of *A. syriaca* using TLC and HPLC techniques. Moreover, the flowers contained gallic acid and the leaves contained α-resorcylic, vanillic and chlorogenic acids.

Sikorska and Matlawska (2001) isolated kaempferol, kaempferol 7-O-β-glucoside, kaempferol 3-O-β-galactopyranoside, kaempferol 3-O-β-xylopyranosyl (1→2)-β-galactopyranoside, kaempferol 3-O-β-glucopyranosyl (1→2)-β-galactopyranoside, isorhamnetin, isorhamnetin 7-O-β-glucoside, isorhamnetin 3-O-β- xylopyranosyl (1→2)-β-galactopyranoside from the flowers of *A. syriaca*.

Sikorska *et al*. (2001) isolated kaempferol, kaempferol 3-O-β-galactopyranoside, 3-O-β-xylopyranosyl (1→2)-β-galactopyranoside, kaempferol-3-glucopyranosyl (1→2)-β-galactopyranoside, kaempferol rhamnopyranosyl (1→2)-β-galactopyranoside from the seed hair of *A. syriaca*.

MATERIALS AND METHODS

1. Plant Materials

Aerial parts and seeds of *Asclepias sinaicus* (Muschl.) were collected from El-Qusaima road at north Sinai during the vegetative growth seasons of the year 2005. Samples were air-dried in shade, ground to fine powder and stored for the following investigations.

2. Investigation of the Flavonoids

2.1. Isolation of the flavonoid compounds

The air-dried aerial parts (1kg) were collected from spring samples and seeds (200g) of the plant and were separately extracted exhaustively with 80% aqueous ethanol after defatting with petroleum ether using Soxhelet apparatus. The ethanolic extract was separately evaporated under reduced pressure and low temperature, then extracted with chloroform.

The obtained residue was treated with excess of ethanol and filtered to remove inorganic salts and non-phenolic compounds.

Chromatographic screening of each extract was done on paper chromatography using BAW system (butanol: acetic acid: water 4:1:5 v/v/v) and AcOH-15% system (acetic acid: water 15:85 v/v). The developed chromatograms were air dried, and examined under ultra violet (UV) light, then exposed to ammonia vapour and re-examined under UV light. For isolation of the obtained compounds, polyamide column was used. Elution was started with water and water/methanol in a gradual increasing of methanol amount. The received fractions were concentrated and subjected to preparative paper chromatography (PPC) on Whatman No.3 using BAW (Liu *et al.*, 1989). The collected separated bands were eluted in methanol, purified by repeated PPC (in AcOH-15%) and over Sephadex LH-20 column using methanol water system. For sugars identification, the glycosidic parts were examined with authentic sugar references on PC using BAW (4:1:5).

2.2. Physical tests

The purified isolated compounds were characterized by ultraviolet spectrophotometer, ^{1}H and ^{13}C-NMR (nuclear magnetic resonance), a JEOL Ex – 270 NMR spectrometer apparatus (270 MH_z for ^{1}H –NMR and 67.5 MH_z for ^{13}C – NMR) and mass spectrometric analysis (MS). Varian Mat 711, finningan SSQ 7000 and OMM 7070 were used for recording atom bombardment (Mabry *et al.*, 1970).

2.3. Chemical reaction

A known weight of the flavonoid material under investigation was subjected to controlled (mild) or complete (normal) acid hydrolysis and enzymatic hydrolysis (Harborne *et al.*, 1975).

2.4. Total flavonoids

Estimation of the total flavonoids in the ethanolic extract of the aerial parts and seeds of *A. sinaicus* was colourimetrically determined according to the method described by Karawya and Aboutabl (1982).

2.4.1. Calibration curve of the quercetin

Different aliquots of ethanolic solution of quercetin equivalent to 5-200μg were separately introduced into test tubes, evaporated to dryness on a water bath (40-50°C) then five ml of 0.1M $AlCl_3$ reagent were added. The absorbance of the developed color was measured at 445nm against a blank. Three determinations for each concentration of standard solution were carried out.

For preparation of the extracts; two grams of powdered plant aerial parts and seeds were separately defatted with petroleum ether and extracted with 96% ethanol till exhaustion. The ethanolic extract in each case was adjusted to 50ml. Five ml aliquots were separately introduced into test tubes, evaporated to dryness on a water bath. The five ml of 0.1M $AlCl_3$ were added. The absorbance of the developed color was measured at 445nm. Three determinations of each sample were carried out.

3. Investigation of Coumarins

The plant seeds (200gm) and aerial parts (750gm) were washed with clean water, dried at 40-50°C in oven and ground to fine powder. The powders of each case were exhaustively defatted by petroleum ether using Soxhlet apparatus. The defatted marc was extracted with acetone: water (5:1) by percolation until exhaustion. After stripping off the solvent under reduced pressure at ≅40°C using rotavapour apparatus, a brownish sticky residue was obtained in each case. The residue of each part (aerial parts and seeds) was separately suspended in 100ml water and extracted successively with chloroform and ethyl acetate. The solvent of each fraction was evaporated, separately, under reduced pressure to give chloroform fraction and ethyl acetate fraction that ready for further isolation and purification of the coumarin compounds on silica gel chromatoplates using chloroform: methanol (9:1) as a solvent system.

3.1. Isolation and purification of coumarin compounds

The isolation and purification of coumarin compounds were examined by thin layer chromatography (TLC) using suitable solvent systems (chloroform: methanol 9:1 v/v and chloroform : methanol 9.5: 0.5 v/v). The developed plates were air-dried and examined under both visible and UV light. The plates were then exposed to ammonia vapors and immediately re-examined to observe any changes in color or fluorescence. The separated constituents were eluted in the case of preparative TLC by scraping the different bands and eluted with an appropriate solvent.

3.2. Extraction and identification of phenolics by HPLC

Phenolic compounds of aerial parts and seeds were extracted according to the method outlined by Ben-Hammouda *et al.*(1995). Identification of individual phenolic compounds of the plant samples was performed on a Hewlett-Packard HPLC (Model 1100), using a hypersil C_{18} reversed - phase column (250x4.6mm) with 5μm particle size. Injection by

means of a Rheodyne injection valve (Model 7125) with 50µl fixed loop was used. A constant flow rate of 1ml min^{-1} was used with two mobile phases: (A) 0.5% acetic acid in distilled water at pH 2.65; and solvent (B) 0.5% acetic acid in 99.5% acetonitrile. The elution gradient was linear starting with A and ending with B over 35min, using an UV detector set at wavelength 254nm. Phenolic compounds of each sample were identified by comparing their relative retention times with those of the standards mixture on chromatogram. Sixteen standard phenolic compounds were obtained from Sigma (St. Louis, USA) and from Merck-Schuchardt (Munich, Germany) Chemical Companies. The concentration of an individual compound was calculated on the basis of peak area measurements, then converted to µg phenolic g^{-1} dry weight.

RESULTS AND DISCUSSION

1. Investigation of the Flavonoids

The ethanolic extracts of the aerial parts and seeds of *A. sinaicus* were found to be rich in phenolics and flavonoids. Several trials using polyamide column were carried out. Fractionation of each extract was carried out by polyamide column, PPC using BAW and Sephadex LH-20.

Nine and three flavonoid compounds were isolated from the plant aerial parts and seeds, respectively (Table 1).

Table (1). Distribution of the flavonoids in aerial parts and seeds of *Asclepias sinaicus*.

Flavonoid compounds	Name	Aerial parts	Seeds
F_1	Quercetin	+	–
F_2	Rutin	+	+
F_3	Quercetin-3-galactopyranoside	+	+
F_4	Quercetin-3-glucoside-7- rhamnoside-3′methoxide	+	–
F_5	Kaempferol	+	+
F_6	Myricetin	+	–
F_7	Myricetin 3-rutinoside	+	–
F_8	Isorhamnetin	+	–
F_9	Isorhamnetin 3-rutinoside	+	–

F_{1-9} = Flavonoid compounds + = Present – = Absent

1.1. Compound F_1

This compound was isolated as yellow needles with melting point 308-312 ^{0}C, R_f 0.7 in BAW and soluble in methanol and 70% ethyl alcohol and gave negative results with Molisch's test (Molisch, 1963)(Table 2). The UV spectral data (Table 3) showed that compound F_1 exhibited UV absorption maxima at 370nm (MeOH), which may be suggested as 3-substituted flavonol (Mabry *et al*., 1970 and Markham, 1982).

A rapid instability and decomposition on addition of sodium methoxide indicates the presence of 3,3',4'- trihydroxy substituted system. The UV absorption data before and after the addition of shift reagent are characteristic for the 3-substituted flavonol having free 5,7 and 4'-OH groups. The hypthochromic shift (-26nm) observed at band I in $AlCl_3$ /HCl spectrum relative to $AlCl_3$ spectrum indicated the presence of O-dihydroxy group in ring-B (C-3', 4'), which was further confirmed from the bathochromic shift (14nm) in band I with sodium acetate / boric acid. A free 7-hydroxyl group was detected from the bathochromic shift (16nm) in the presence of sodium acetate in band II.

The ^{1}H-NMR of compound F_1 was in agreement with quercetin skeletal pattern (Table 4), which was represented by the presence of two protons, a doublet at δ_H 6.17(J=1.5Hz) and a broad singlet at δ_H 6.37 assigned for H-6 and H-8, respectively. In addition, the doublet at δ_H 6.89-6.86 (J=8.4Hz) assigned for H-5' proton, the broad singlet at δ_H7.72 assigned for C-2' proton and the doublet of doublet at δ_H 7.64 (J=8.1, 1.6Hz) assigned for C-6'. These data are comparable to those of 3', 4' oxygenated flavonols as mentioned by Mabry *et al.* (1970). The compound was confirmed by direct comparison with the reference materials and by comparing to the published data (Mabry *et al.*, 1970 and Agrawal, 1989). The previous results confirmed the identification of compound F_1 as querectin.

Table (2). R_f-values and color reactions of the isolated compounds.

Compound	Solvent system	R_f-value	Color reactions		
			Visible	UV	UV+NH_3
F_1	BAW Ac-OH-15%	0.70 0.27	——	Yellow	Yellow fluresence
F_2	BAW Ac-OH-15%	0.55 0.60	——	Yellow	Yellow fluresence
F_3	BAW Ac-OH-15%	0.48 0.43	——	Deep purple	Yellow
F_4	BAW Ac-OH-15%	0.51 0.70	——	Purple	Yellow
F_5	BAW Ac-OH-15%	0.86 0.14	Yellow	Yellow	Yellow fluresence
F_6	BAW Ac-OH-15%	0.31 0.16	——	Brown	Yellow
F_7	BAW Ac-OH-15%	0.26 0.30	——	Brown	Yellow
F_8	BAW Ac-OH-15%	0.70 0.12	——	Dull yellow	Dull yellow
F_9	BAW Ac-OH-15%	0.50 0.63	——	Purple	Yellow
C_1 C_2	$CHCl_3$:MeOH (9:1) $CHCl_3$:MeOH(9.5:0.5)	0.72 0.35	——	Blue Blue	—— Blue

F_{1-9} = Flavonoid compounds C_{1-2} = Coumarin compounds

Table (3). Ultra violet spectral data of the isolated compounds.

Comp.	UV Shift reagents					
	MeOH	Me ONa	$AlCl_3$	$AlCl_3$/HCl	AcONa	AcONa/H_3BO_3
F_1	258, 306 (sh), 370	262, 332	272, 328, 454	268, 300 (sh), 362 (sh), 428	274, 320 (sh), 428	264, 292 (sh), 384
F_2	259, 266 (sh), 299 (sh), 359	272, 327, 410	275, 303 (sh), 433	271, 300, 340 (sh), 402	271, 325, 393	220, 298, 387
F_3	258, 302 (sh), 356	264, 324, 402	274, 298 (sh) 344 (sh), 430	268, 298 (sh), 364 (sh), 400	272, 298 (sh), 372	262, 292 (sh), 372
F_4	257, 355	270, 398	270, 310, 350, 410	270, 310, 350, 410	260, 360	262, 360
F_5	253 (sh), 268, 324 (sh), 367	280, 318 (sh), 420	266, 305 (sh) 350, 422	266, 305 (sh), 350, 422	275, 302 (sh), 385	267, 296 (sh), 320 (sh), 370
F_6	254, 272 (sh.), 374	262 (sh), 285 (sh), 322, 423	271, 316 (sh), 450	266, 275 (sh), 308 (sh), 366, 428	269, 335	258, 304 (sh), 382
F_7	255, 265 (sh), 265 (sh), 360	268, 325, 415	270, 315, 430	268, 305 (sh), 365 (sh), 400	270, 310, 395	258, 312, 390
F_8	253, 267 (sh), 306 (sh), 326 (sh), 370	240 (sh), 271, 328, 435	264, 301 (sh) 361 (sh), 431	242 (sh), 262, 271 (sh), 302 (sh), 357, 428	360 (sh), 274, 320, 393	255, 270 (sh), 306 (sh), 326 (sh), 377
F_9	254, 265 (sh), 305 (sh), 356	271, 328, 414	268, 278 (sh), 300 (sh), 369 (sh), 402	267, 275 (sh), 300 (sh), 359 (sh), 399	271, 320, 396	254, 267 (sh), 304 (sh), 360
C_1	228, 252 (sh), 260 (sh), 298 (sh), 344	-	-	-	228, 277, 420	-
C_2	260, 310, 350	-	-	-	260, 320, 410	-

F_{1-9} = Flavonoid compounds C_{1-2} = Coumarin compounds sh = shoulder

Table (4) . ^{1}H-NMR of some isolated compounds

Comp	δ ppm
F_1	δ ppm 7.72 (1H, br.s., J=2.1Hz, H-2′), 7.64 (1H, dd, J=8.1Hz, H-6′), 6.89 (1H, d, J=8.4Hz, H-5′), 6.37 (1H,br.s., J=2.5Hz, H-8) and 6.17 (1H, d, J=1.5 Hz, H-6).
F_2	δ ppm 7.72 (1H, br.s., J=2.1Hz, H-2′), 7.64 (1H, dd, J=8.1Hz, H-6′), 6.89 (1H, d ,J=8.4Hz, H-5′), 6.37 (1H, br.s., J=2.5Hz, H-8) and 6.17 (1H, d, J=1.5Hz, H-6). Sugar moieties: δ 5.6 (1H, d, J=2.5Hz, H-1′′ rhamnose), 5.3 (1H, d, J=8Hz, H-1‴ glucose), 3.2-3.9 (m-glucose protons) and 1.2 (3H, d, J=6Hz, CH_3 rhamnose).
F_3	δ ppm 7.54 (d, J=2.1 Hz, H-2′), 6.83 (d, J=8.4Hz, H-5′), 7.66 (dd, J=8.7Hz, H-6′), 6.39 (1H, d, J= 2.1Hz, H-8), 6.20 (1H, d, J=2.1Hz, H-6), 5.35 (1H, d, J=7.5Hz, H-1″), 3.84 (1H, d, J=2.8Hz, H-4″), 3.63 (1H, dd, J=5.96,11.23Hz, H-2″), 3.55 (1H, dd, J=9.62, 3.52Hz, H-3″) and 3.46 (1H, m, H-6″).
F_4	δ ppm at 7.95 (1H, d, J=8.5Hz, H-2′), 7.65 (1H, dd, J=8.5Hz, H-6′), 6.94 (1H, d, J=8.5Hz, H-5′), 6.75 (1H, d, J=2.5Hz, H-8) and 6.45 (1H, d, J=2.5Hz, H-6). Sugar moieties: δ 5.6 (1H, d, J=2.5 Hz, H-1″ rhamnose), 5.4 (1H, d, J=8.0 Hz, H-1‴ glucose), 3.2-3.9 (m), 3.92 (s, OCH_3) and 1.2 (3H, d, J=6.0 Hz, CH_3 rhamnose.
F_5	δ ppm at 8.0 H (2H, d, J=8.0Hz, H-2′ and H-6′), 6.9 (2H, d, J=8.0Hz, H-3′ and H-5′), 6.4 (1H, d, J=2.5Hz, H-8) and 6.2 (1H, d, J=2.5Hz, H-6) .
F_6	δ ppm at 6.25 (1H ,d ,J=2.5 Hz, H-6), 6. 33 (1H, d, J = 2.5Hz, H-8) and 7.32 (s, H-2′ and H-6′).
F_7	δ ppm of aglycone moiety at: 6.25 (1H, d, J=2.5Hz, H-6), 6.33 (1H, d, J=2.5Hz, H-8), 7.22 (s, H-2′ and H-6′). Sugar moiety: δ 5.88 (1H, d, J=7.5Hz, H-1″ glucose), 4.35 (d, J=2Hz, H-1‴ rhamnose), 1.15 (d, J=6Hz, CH_3 rhamnose), 3.1-3.9 (m, sugar proton).
F_8	δ ppm: 7.6 (1H, d, J=8.5Hz, H-2′), 7.5 (1H, dd, J=8.5Hz, H-6′), 6.89 (1H, d ,J=8.5Hz, H-5′), 6.4 (1H, d, J=2.5Hz, H-6), 6.2 (1H, dd, J=2.5Hz, H-8) and 3.9 (s,OCH_3) .
F_9	δ ppm 7.6 (1H, d, J=8.5Hz, H-2′), 7.4 (1H, d, J=8.5Hz, H-6′), 6.9 (1H, d, J=8.5Hz, H-5′), 6.7 (1H, d, J= 2.5 Hz, H-8), 6.4 (1H, d, J=2.5Hz, H-6). Sugar moiety: 5.6 (1H, d, J=8Hz, H-1″ rhamnose), 5.4 (1H, d, J=8Hz, H-1‴ glucose), 3.1-3.9 (m, sugar proton) and 3.9 (s, OCH_3).
C_1	δ ppm 7.63 (1H, d, J=9.5Hz, H-4), 6.91 (1H, s, H-5), 6.84 (1H, s, H-8), 6.26 (1H, d, J=9.5Hz, H-3) and (3H, s, OCH_3)
C_2	δ ppm 7.9 (1H, d, J=9.5Hz, H-4), 7.1 (1H, s, H-5), 6.7(1H, s, H-8) and 6.2 (1H, d, J=9.5Hz, H-3).

F_{1-9} = Flavonoid compounds C_{1-2} = Coumarin compounds sh= Shoulder
Hz = Hertz J = Coupling constant s = Singlet
d = Doublets dd = Double of doublets

1.2. Compound F_2

This compound gave positive Molisch's test indicating its glycosidic nature with m.p.185°C and has yellow to greenish crystalline powder or needle, partially soluble in water and soluble in ethanol and methanol, insoluble in chloroform and ether. TLC was applied with authentic marker using BAW and AcOH-15%)(Table 2).

UV spectral data of compound F_2 are similar to those reported for quercetin type compounds with 3-OH substitutions (Table 3).

Compound F_2 was further confirmed as rutin from a complete acid hydrolysis which gave the aglycone querecetin and the sugars were identified as glucose and rhamnose.

^{1}H-NMR of compound F_2 showed signals characteristic for quercetin with additional signals for sugar moieties (Table 4), two signals for two anomeric sugar protons at δ 5.6 (1H, d, J=2.5Hz, H-1$''$ rhamnose), δ 5.3 (1H, d, J=8Hz, H-1$'''$ glucose) and the remaining sugar protons m at δ (3.2-3.9) and signal at δ 1.2 (3H, d, J=6Hz,CH_3 rhamnose).

^{13}C-NMR spectrum of compound F_2 showed signals characteristic for quercetin with C-3 more upfield which indicates the presence of a substitution on this carbon. Two anomeric sugar carbons were detected at 102.08 and 103.1 for C-1" and C-1"', indicating the disaccharide nature of compound F_2. One CH_3 carbon of rhamnose was shown at δ ppm 18, while C-6 of glucose was observed at δ 62.70ppm, so the linkage is 1→6 (Harborne *et al.*, 1975). Thus compound F_2 was identified as rutin (quercetin – 3-O-α- L- rhamnoside 1→6 β-D-glucoside) (Table 5).

Table (5). ^{13}C-NMR of some isolated compounds.

Compound	δ ppm
F_2	148.2 (C-2). 134.34 (C-3), 176.2 (C-4), 159.92 (C-5), 98.3 (C-6), 163.7 (C-7), 94.81 (C-8), 157.16 (C-9), 103.63 (C-10), 123.91 (C-1$'$), 114.56 (C-2$'$), 145.28 (C-3$'$), 150.3 (C-4$'$), 116.22 (C-5$'$) and 122.0 (C-6$'$). Sugar moiety at δ ppm: 102.08 (C-1$''$), 72.43 (C-2$''$), 73.12 (C-3$''$), 73.70 (C-4$''$), 72.13 (C-5$''$), 67.4 (C-6$''$), 103.1 (C-1$'''$), 71.65 (C-2$'''$), 75.6 (C-3$'''$), 71.4 (C-4$'''$), 76.15 (C-5$'''$) and 62.7 (C-6$'''$).
F_5	146.8 (C-2), 135.4 (C-3), 175.9 (C-4), 161.0 (C-5), 98.6 (C-6), 164.2 (C-7), 93.8 (C-8), 156.4 (C-9), 103.7 (C-10), 121.9 (C-1$'$), 129.9 (C-2$'$), 115.8 (C-3$'$), 159.5 (C-4$'$), 115.8 (C-5$'$) and 129.9 (C-6$'$).
C_2	161.28 (C-2), 110.70 (C-3), 144.60 (C-4), 112.25 (C-5), 148.40 (C-6), 150.33 (C-7), 102.53 (C-8), 142.84 (C-9) and 110.7 (C-10).

F_2 and F_5 = Flavonoid compounds C_2 = Coumarin compound

The physiological functions of rutin include, inhibition of capillary permeability and enhancement of capillary resistance, anti-inflammatory action, antihydropic action, aldose reductase inhibiting action and improvement in glaucoma induced by diabetes. Rutin is classified as vitamin P which increases the strength of the walls of the blood capillaries and regulates their permeability. It is also known to have pharmacological action as an anti-inflammatory, antihistaminic and antiviral agents.

1.3. Compound F_3

This compound was obtained as yellowish orange powder, m.p.220-222°C, R_f 0.45 in BAW. It is soluble in methanol and water, insoluble in ether and chloroform and gave positive Molish's test indicating its glycosidic nature (Table 2).

Compound F_3 showed exhibited UV (MeOH) absorption maxima at 352nm, which suggested a 3- substituted flavonol skeleton (Table 3). The UV spectral analysis before and after the addition of different shift reagents are characteristic for the 3- substituted flavonol having free hydroxyl groups at C-5, C-7 and C-3$^{\prime}$, C-4$^{\prime}$ (Mabry *et al.*, 1970 and Markham, 1982). The hypsochromic shift (30nm) observed in band I in $AlCl_3$ spectra of compound (F_3) and after treatment with acid indicated the presence of O- dihydroxy groups in ring-B. The same conclusion may be reached from the bathochromic shift (16nm) in band I with sodium acetate/boric acid.

The ^{1}H-NMR data of compound F_3, showed the presence of two signals at δ 6.19 (d, J=1.8Hz, H-5) and 6.39 (d, J=1.8Hz, H-7) for ring A and three signals δ 7.54 (d, J=2.1Hz, H-2$^{\prime}$), δ 6.83 (d, J=8.4Hz, H-5) and δ7.66 (dd, J=8.7, 2.4Hz, H-6$^{\prime}$) for ring- B, which indicated the presence of 3$^{\prime}$, 4$^{\prime}$ disubstitutions (Table 4). From the above data and from the results of acid hydrolysis, the aglycone part was identified as querecetin. The presence of one anomeric proton at δ 5.35ppm (d, J=7.5Hz, H-1$^{\prime\prime}$) as well as anomeric carbon which indicating the presence of a single sugar unit, which is β-linked to the aglycone at C-3. Galactose was identified as the sugar part of quercetin. From the result of acid hydrolysis and by comparison with published data (Khaleel, 1995), the structure of compound F_3 was elucidated as querecitin -3-O-β- galactopyranoside (hyperoside).

1.4. Compound F_4

R_f values and color reactions of the compound F_4 were outlined in table (2). Acid hydrolysis of this compound using 2N HCl gave querecetin-3$^{\prime}$- methoxide and the sugars are identified as rhamnose and glucose.

UV spectral data of compound F_4 in methanol showed band (I) at 355nm indicating the presence of flavonol with 3-OH substitution (Table 3). Addition of NaOAc caused no shift, suggesting occupation of 7-position.

Addition of H_3BO_3 caused no shift, indicating the absence of any catecholic hydroxyl groups. Addition of NaOMe caused a bathochromic shift indicating the presence of free OH at 4$^{\prime}$-position.

^{1}H-NMR spectral data showed signals characteristic for quercetin (Table 4), beside two signals for 2 anomeric sugar protons. Thus, from R_f-values, color reactions, acid hydrolysis, UV and ^{1}H-NMR spectral data, compound F_4 was identified as quercetin -3-O-β-glucoside-7-O-α-rhamnoside-3$^{\prime}$-methoxide.

1.5. Compound F_5

Compound F_5 appears as light yellow crystals from methanol, with m.p.276-278°C and soluble in methanol and 70% ethyl alcohol. Its R_f-values are within the range of flavonoid aglycone (Harborne, 1984). The change of its color from yellow to fluorescence yellow (Table 2) when exposed to ammonia vapor under UV light indicated that compound F_5 is a flavonol accompanied with free 5-OH (Liu *et al.*, 1989).

Compound F_5 showed two major absorption bands in MeOH, band I at 367nm and band II at 268nm, which indicated a flavonol with free hydroxyl group at the 3 position (Harborne, 1984). Additiona of NaOMe resulted in a bathochromic shift in band I (+53nm), which proved the presence of a free OH group at $4'$-position (Mabry *et al.*, 1970). The presence of shoulder at 318nm in NaOMe along with the bathochromic shift for band II in NaOAc (+7nm) referred to the same band in MeOH suggested the presence of free hydroxyl group at C-7. The hypsochromic shift in band I (-15nm) after the addition of H_3BO_3 on NaOAc gave no shift which suggested the absence of any catecholic hydroxyls in compound F_5. A bathochromic shift in band I (+52nm) with $AlCl_3$ which was not affected with the addition of HCl indicates the presence of free hydroxyl group at C-3 and C-5 and this confirmed the absence of catecholic hydroxyls. The structure of compound F_5 was confirmed as kaempferol by ^{1}H-NMR (Table 4) and by ^{13}C-NMR (Table 5) which showed the signals characteristic for kaempferol (Mabry *et al.*, 1970).

1.6. Compound F_6

Compound F_6 appears as light yellow crystals, soluble in alcohol; slightly soluble in hot water; insoluble in chloroform or acetic acid. R_f values and color reactions of compound F_6 indicated the aglycone (Table 2). UV spectral data (Table 3) found to be similar to those for myricetin type compounds (Mabry *et al.*, 1970). The ^{1}H-NMR spectrum of this compound in methanol (Table 4) indicated that it is a flavonol with free OH at 3-position (Liu *et al.*, 1989). From R_f- values, color reaction, UV spectral data and ^{1}H-NMR, it was identified as myricetin ($3,5,7,3',4',5'$-hexa-hydroxy flavone) with molecular weight of 318.2. Myricetin is widely used in the field of pharmacy, healthy food and cosmetics. Also, it can treat diarrhoea, jaundice, fever, scrofula, chronic inflammation of the throat and modilises body defences in early stage of infection.

1.7. Compound F_7

R_f-values and color reactions of compound F_7 are indicative of diglycosides (Table 2). Complete acid hydrolysis of this compound gave myricetin as the aglycone and the sugar moiety was identified as glucose and rhamnose. Enzymatic hydrolysis proved the release of myricetin-3-O-glucoside, thus glucose attached to the myricetin nucleus and not rhamnose.

UV spectral data of compound F_7 was found to be similar to those reported for myricetin type compounds (Mabry *et al.*, 1970). Band I in methanol indicates that position 3 is occupied. UV spectral data revealed the presence of free OH at positions $3'$, $4'$ and 7 (Table 3). ^{1}H-NMR spectral data of compound F_7, showed signals characteristics for myricetin with additional signals for glucose at δ 5.0 and 3-5-3-7 (m), and at 5.35, 4.4 and 3.2-3.7 m (CH_3 rhamnose). From the above mentioned data this compound was identified as myricetin 3-O-rutinoside.

1.8. Compound F_8

R_f-values and color reactions of compound F_8 are indicative of aglycone. UV spectral data of this compound (Table 3) were found to be similar to those reported for quercetin type compounds (Mabry *et al*., 1970). Band I in methanol indicate that compound F_8 is a flavonol with free OH at position 3 (Liu *et al*., 1989). ^{1}H-NMR spectrum of this compound (Table 4) showed signals characteristics for isorhamnetin (Mabry *et al*. 1970). From the mentioned data, compound F_8 was identified as isorhamnetin.

1.9. Compound F_9

R_f-values and color reactions of compound F_9 are indicative of aglycone with diglycosidic compounds (Table 2). Complete acid hydrolysis of this compound gave isorhamnetin as the aglycone and the sugar moiety were identified as glucose and rhamnose. Enzymatic hydrolysis of compound gave isorhamnetin 7-glucoside proving that the glucose was attached to isorhamnetin nucleus. Band I in a methanol indicates that compound F_9 is a flavonol while position 3 is occupied. ^{1}H-NMR spectrum of this compound (Table 4) showed signals characteristics for isorhamnetin and signals for glucose and rhamnose at δ 5, 3.35-3.7 (m), 5.35, 3.2-3.7 (m) and 1.1 (CH_3 rhamnose). From R_f – values, color reactions, acid hydrolysis, enzymatic hydrolysis, UV spectral data and ^{1}H-NMR, compound F_9 was identified as isorhamnetin 3-O-rutinoside.

2. Investigation of Coumarins

2.1. Fractionation of the chloroform extract

The chloroform fraction of the aerial plant parts and seeds were found to be free from any fluorescent spots, while ethyl acetate extract showed two fluorescent spots and gave positive responses with ammonia and UV light. So, the ethyl acetate fraction was chosen for isolation and identification of coumarins.

2.2. Fractionation of the ethyl acetate extract

TLC analysis of the ethyl acetate fraction using the solvent system chloroform: methanol (9:1v/v) showed one spot of blue color (R_f = 0.72) under UV light (C_1) and another spot of blue color under UV light, R_f= 0.33 (C_2) in chloroform: methanol (9.5: 0.5 v/v). The aerial parts of *A. sinaicus* contain C_1 and C_2, while the plant seeds contain C_2 only.

2.2.1. Identification of compound C_1

Compound C_1 appears as white crystals from methanol, soluble in chloroform and methanol with m.p. 204-206°C and R_f =0.72 in solvent system (chloroform: methanol 9:1 v/v) in the range of 7-hydroxy -6-methoxycoumarin (Murray *et al*., 1982).

The UVλ_{max} nm in methanol were 228, 252 sh, 260 sh, 298 sh, 344 which characteristics for 7-hydroxy-6-methoxy coumarin. Addition of NaOAc causes bathochromic shift (+76nm) which indicates the presence of free OH at position 7. UV spectral data are similar to those reported for 7-

hydroxy-6-methoxy coumarin compounds with free OH at position 7. ^{1}H-NMR in DMSO showed signals characteristic for 7-hydroxy-6-methoxy coumarin (Murray *et al*., 1982). The presence of pair of doublets at 7.63 and 6.26ppm indicates that C_2 is unsubstituted coumarin (pyranone ring). Proton 6, being substituted by a methoxy group was confirmed by the appearance of H-5 and H-8 as singlet peaks; in addition to the presence of a sharp singlet at 3.95ppm integrated for 3 protons ascribed for methoxy proton.

Mass spectrum m/z was 193 (M^+, 100%), 192 ($[M-H]^+$, 35%), 178 ($[M-CH_3]^+$, 7%), 163 ($[M-OCH_3]^+$, 9%), 82($[M-4CO]^+$ 8%) and 69 ($[M-4CO-CH_3]^+$, 7%). Mass spectrum exhibited a molecular and a base peak at m/z = 193 ascribable to scopoletin, a peak at m/z 178, due to loss of methyl group, the remaining oxygen atoms are subsequently removed as CO to give the fragments m/z =163 ($[M-OCH_3]^+$, 9%), 82 ($[M-4CO]^+$, 8%) and 69 ($[M-4CO-CH_3]^+$, 7%).

Based on the obtained spectral data, m.p. and chromatography with reference compound (The Merk Index, 2001), C_1 could be identified as 7-hydroxy-6-methoxycoumarine (Scopoletin).

2.2.2. Identification of compound C_2

Compound C_2 exhibited a single spot (blue color in UV light before and after exposure to ammonia vapor), R_f 0.35 in solvent system chloroform: methanol (9.5: 0.5 v/v); being in the range of 6,7-dihydroxycoumarin (Murray *et al*. 1982).

The UV spectral data of C_2 are similar to those reported for 6,7-dihydroxycoumarin compounds with free OH at positions 6 and 7. Compound C_1 showed two major absorption bands in MeOH; band I at 350nm and band II at 260nm. A bathochromic shift was occurred on addition of NaOAc, which indicated the presence of free OH at position 7. The UV spectrum data of compound C_2 coincide with those published for aesculetin (Harborne, 1984).

^{1}H-NMR spectrum in DMSO d_6 showed signals at δ(ppm): 7.9 (1H, d, J=9.5Hz, H-4), 7.1(1H, s, H-5), 6.7(1H, s, H-8) and (1H, d, J= 9.0Hz, H-3). Data showed that the presence of two pair of doublets at δ 7.9 and δ 6.2ppm indicates a coumarin nucleus with unsubstituted pyrone ring (Murray *et al*., 1982).

^{13}C-NMR spectrum in DMSO d_6 showed signals at δ(ppm): 161.28 (C-2), 110.70 (C-3), 144.60 (C-4), 112.25 (C-5), 148.40 (C-6), 150.33 (C-7), 102.53 (C-8), 142.84 (C-9) and 110.70 (C-10) (El-Sayed *et al*.1999)

Based on the obtained spectral data, m.p. (267-270°C), compound C_2 could be identified as 6,7-dihydroxycoumarin (aesculetin) with molecular weight of 178.14 and molecular formula as $C_9H_6O_4$.

3. Estimation of Total Flavonoids

The total flavonoids present in *A. sinaica* aerial parts and seeds were determined spectrophotometrically and calculated as quercetin. Data in

(Table 6) indicated that the percentage of total flavonoids of aerial parts reached maximum value of 3.96% collected in spring season. So, the isolation and identification of the flavonoid compounds were done using spring plant samples (aerial parts). The percentage of total flavonoids of plant seeds collected from summer season was 2.53%.

Table (6). Percentages of total flavonoids in different seasons.

Seasons	Aerial parts	Seeds
Winter	3.87	0
Spring	3.96	0
Summer	2.01	2.53
Autumn	2.65	0

4. Qualitative and Quantitative Estimation of Phenolic Constituents Using HPLC

It was cleared from the obtained data (Table 7) that there are fifteen and ten compounds identified in aerial parts and seeds of *A. sinaicus*, respectively. The highest concentration were *p*- coumaric acid (1.359 µgm / 100gm) and (0.419 µgm/100gm) for plant aerial parts and seeds, respectively.

Phenolic binding occurs readily with proteins, lipids, metals, and carbohydrates, some of whose function as nutrients, enzymes, structural components, or pathogens can be altered by binding and impacting digestion.

The aerial parts of *A. sinaicus* contain a trihydroxyphenol (pyrogallic), dihydroxyphenol (resorcinol) and mono-hydroxyphenols (*p*- OH benzoic acid, vanillin and salicylic acid).

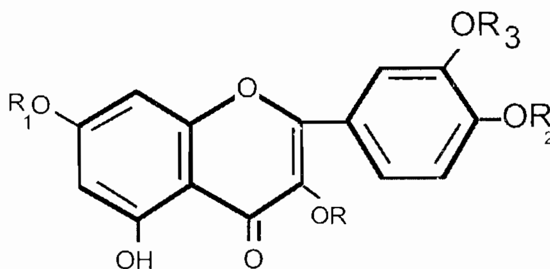
Table (7). Qualitative and quantitative estimation of phenolic constituents using HPLC.

Phenolic compound	µ gm /100gm	
	Aerial parts	Seeds
Pyrogallic	0.021	0.152
Resorcinol	0.024	0.064
p– OH-benzoic acid	0.632	-
Chlorogenic acid	0.017	0.036
Phenol	0.078	-
Vanillin	0.015	0.018
p – Coumaric acid	1.359	0.419
Ferulic acid	0.111	-
Salicylic acid	0.099	-
Rutin	0.148	0.028
O – coumaric	0.024	0.046
Myristin	0.004	-
Cinnamic acid	0.005	-
Quercetin	0.002	-
Kaempferol	0.001	0.001
Gallic acid	-	0.006
Catechin	-	0.270

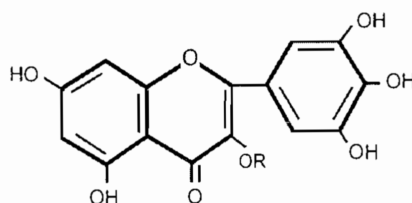
Pyrogallic acid $C_6H_3(OH)_3$ is a trihydroxyphenol, having a molecular weight of 126.11; melting point of 131-133 °C and boiling point of 309 °C. It is a powerful antiseptic by reason of its affinity for oxygen. Resorcinol ($C_6H_6O_2$) is a dihydroxylated phenol, molecular weight of 110.11, melting point of 110 °C and boiling point of 280°C. It is a skin, eye and mucous membrane irritant, used in the tanning and dyeing industries, in the manufacture of resin and resin adhesives, explosive. Resorcinol called 1,3-benzenediol and resorcin (Windholz, 1989).

Catechin is biflavonoids and has both antiviral and antioxidant qualities. It has been shown helpful in the treatment of viral hepatitis, it also appears to prevent oxidative damage to the heart, kidney, lungs and spleen. Salicylic acid (2-hydroxybenzoic acid), $C_6H_4(OH)CO_2H$, is a colourless, crystalline organic carboxylic acid that melt at 159°C and its molecular weight is 138.12. Salicylic acid and its derivates are toxic when consumed in large amount it is used in making aspirin, as a preservative (Windholz, 1989). *Para*-coumaric acid is a phenolic coumarin derivative that inhibits the development of stomach cancer. Gallic acid forms pyrogallol, or 1,2,3-trihydroxybenzene, $C_6H_3(OH)_3$, which are widely used in the manufacturing azo dyes and photographic developers and to treat certain skin disease.

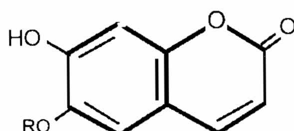
Structure of the isolated compounds:



Compound	R	R_1	R_2	R_3
F_1	H	H	H	H
F_2	Rhamnoglycosyl	H	H	H
F_3	Galactose	H	H	H
F_4	Glucose	Rhamnoglycosyl	H	CH_3
F_8	H	H	H	CH_3
F_9	Rhamnoglycosyl	H	H	CH_3



Compound	R
F_6	H
F_7	Rhamnoglycosyl



Compound	R
C_1	HCO_3
C_2	H

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تقدير المكونات الفينولية لنبات غلقة الديب (*Asclepias sinaicus*)

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تم تجميع نبات (*Asclepias sinaicus*) من بيئته الطبيعية بطريق القصيمة بشمال سيناء خلال عام ٢٠٠٥ وعرف النَبَات بالعربي باسم غلقة الديب أو الهرجل أو جهيل

تم فصل عدد ٩ من المركبات الفلافونيدية من المستخلص الكحولي للأجزاء الورقية لنبات غلقة الديب وثلاثة مركبات فلافونيدية فقط من البذور. كما تم تعريف كل مركب باستخدام طرق التحليل الطيفي المختلفة والرنين المغناطيسي لأنوية الهيدروجين ١ والكربون ١٣، فالأجزاء الورقية للنبات تحتوي على كيورستين ، رتين، كيورستين-٣-جالكتوبيرانوسيد، كيورستين -٣-جلوكوسيد-٧- رامنوسيد-٣-ميثوكسيد، كامبيفيرول، ميريستين ، ميريستين-٣-روتينوسيد، ايزورامنتين ، ايزورامنتين -٣- روتينوسيد . بينما بذور النبات تحتوي على الرتين، كيوريستين-٣- جالكتوبيرانوسيد والكامبيفيرول.

كما أوضحت النتائج أن الأجزاء الورقية للنبات تحتوى على مركبين من الكومارينات هما الاسكيولين والسكوبوليتين بينما احتوت البذور على سكوبوليتين فقط. وتم تقدير المحتوى الكلي للفلافونيدات بالمستخلص الكحولي باستخدام طريقة الامتصاص الطيفي خلال مواسم نمو النبات (٢٠٠٥) ونسبة الفلافونيدات الكلية في الاوراق اعلى قيمة لها هى ٣,٩٦% وكان ذلك في فصل الربيع.

كما تم تقدير المكونات الفينولية لأوراق وبذور النبات كميا وكيفيا باستخدام جهاز HPLC كما تم التعرف على ١٥ مركب في الاجزاء الورقية وعشر مركبات في بذور النبات.

كما اوضحت النتائج ان اعلى نسبة في حمض الكوماريك كانت (١,٣٥٩ و ٠,٤١٩ ميكروجرام لكل ١٠٠جم) للاجزاء الورقية والبذور على التوالي.