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FLAVONOIDS AND PHENOLIC CONSTITUENTS OF LEAVES AND STEMS OF *Ballota undulata* (FRESEN.) BENTH

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ABSTRACT

The chromatographic investigation of the phenolic and flavonoid content of *Ballota undulata* stems and leaves, collected from North Sinai, Al-Arish, at km 8, El-Qusaima road, revealed that, the leaves contain four flavonoids namely, kaempferol, quercetin, rutin and myricetin beside five phenolic acids namely, 2, 3-dihydroxy benzoic acid (O-protocatechuic acid), ferulic, chlorogenic, p-hydroxy benzoic and p-coumaric acids. The stems of *Ballota undulata* contain three flavonoids namely, kaempferol, quercetin and rutin beside four phenolic acids namely, 2, 3-dihydroxy benzoic acid (O-protocatechuic acid), ferulic, p- coumaric and chlorogenic acids.

These phenolic and flavonoid compounds were isolated through polyamide column chromatography, purified by using a sephadex LH-20 column and identified through R_f-values and colour reactions, UV spectral data, ^{1}H-NMR, ^{13}C-NMR spectra and mass spectrum. The leaves contain the highest percentages of total flavonoids.

Key words: *Ballota undulata, lamiaceae, mass spectrum, ^{1}H and ^{13}C NMR.*

1.INTRODUCTION

Lamiaceae (Labiatae), the mint family is a large family with

221 genera and 5600 species, cosmopolitan in distribution, but especially in the Mediterranean region and central Asia. Plants in this family are shrubs, subshrubs or herbs, but rarely trees, often glandular containing ethereal oils. Several species have medicinal value and others are used as pot herbs and flavouring agents and yield oils for perfumes. In addition to the volatile oils other constituents of the family include diterpenoids and triterpenoids saponins, a few pyridine and pyrrolidine alkaloids, polyphenols and tannins, iridoids, quinines, furanoids, cyclitols, coumarin and the sugars raffinose and stachyose (Ghazanfar, 1994).

Ballota is a genus of about 35 species of clump-forming perennials and subshrubs. These species are found in the Mediterranean, Europe, and western Asia where their natural habitat is rocky areas and waste grounds. Characteristically they exhibit opposite, toothed, scalloped leaves and whorls of two-lipped flowers with funnel-shaped calyxes.

Ballota undulata (Fresen.) Benth. Syn. *Marrubium undulata* Fresen. is a perennial herb, 30-60cm high; stems green, richly branched, frutescent, spreadingly hairy, leaves villous on both sides, orbicular 1.5-3 cm broad, crisp-wrinkled, crenate. Flowers in numerous globular whorls, many-flowered, distinct; bracts linear-spathulate, usually not exceeded by the leaves. Calyx limb broad, 0.8-1.3cm broad, leaf-like wrinkled crenate; corolla light pink (Boulos, 1995).

Ballota undulata is a rare herb found in the isthmic desert (*i.e.* El- Tih and the region North of Wadi Tumilat), the part of the Arabian desert from Qena-Qossier road and in Sinai proper (South of El-Tih desert). Also in the Mediterranean coastal strip from the border with Libya near El-Salloum to Port-Said and entire Sinai peninsula including the coastal Mediterranean strip and El-Tih desert east Suez canal (Boulos, 1995 and Täckholm, 1974).

Savona *et al.* (1982) isolated one diterpenoid identified as hispanolone from aerial parts of *Ballota andreuzziana*, while B. *pseudodictamnus* contained ballonigrin, 18-hydroxyballonigrin and (as traces) marrubenol, as well as the flavone 7, 4'-di-O-methylapigenin.

Boulos, (1983) mentioned that the flowering branches of *Ballota nigra* are antispasmodic, emmenagouge, tonic, vermifuge and

are used for treatment of tinea and as tranquilizer.

Darbour *et al.*, (1986) isolated an O-heteroside and a C-heteroside of apigenin from the leaves of *Ballota foetida (nigra)* that were considered to be responsible for the spasmolytic activity.

Tomas-Barberan *et al.*, (1992) reported that the occurrence of flavonoid p-coumaroylglucosides and 8-hydroxy flavone 7-allosylglucosides (in aerial parts), in the Labiatae, were restricted, to some genera of subfamily Lamioideae. Flavonoid p-coumaroylglucosides were present in all the species studied of the genera *Ballota, Phlomis* and *Marrubium*, they were valuable markers for chemotaxonomic studies.

Various flavonoids, such as diosmin, isoquercetin, rutin and quercetin have been identified in some species of *Teucrium* (Family: Labiatae). Four flavonoids; 6-methoxygenkwanin, salvigenin, cirsiliol and luteolin have been identified from *Teucrium polium* (Ghazanfar, 1994). The same author mentioned that flavonoids in the leaves of *Thymus vulgaris* (Labiatae) may account for its anti-spasmodic activity and that *Melissa officinalis* contains caffeic, chlorogenic, protocatechuic acids.

Ezer, *et al.*, (1999) detected the presence of flavonoids, phenylpropanoid heterosides, tannins, saponins, volatile oil, terpenic compounds and sugars in the aerial parts of *Ballota nigra* subsp. *anatolica* which is endemic in Turkey and used in folk medicine.

2.MATERIALS AND METHODS

2.1. Plant materials

Ballota undulata (Fresen.) Benth., family Labiatae (Lamiaceae) grows widely in El-Arish, (North Sinai) at El-Qusaima road, Km 8, was collected during the four seasons, winter, spring, autumn and spring (2003). The isolation of flavonoid compounds and phenolic acids was carried out using spring samples (April).The leaves and stems of the plant were separately cleaned, dried in an oven at 50 °C, ground to fine powder and kept for investigation.

2.2. Solvents

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

2.3. Reagents

2.3.1. Reagents for UV spectroscopic analysis

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

2.3.2. Reagents for carboxylic acids

Aniline/glucose (Smith, 1960). Two gm glucose were dissolved in 2ml water and 2ml aniline was dissolved in 20ml ethanol. The two solutions were mixed and completed to 100ml by n-butanol.

2.3.3. Reagents for hydroxy flavonoids

a) Aluminium chloride.About 1% methanolic solution (Markham, 1982).

b) Tetra phenyl diboroxide ethanol amino complex. 1% ethanolic solution (Markham, 1982).

2.3.4. Reagents for phenolics

a) Ferric chloride, 1% ethanolic solution (Smith, 1960).

b) Gibb's reagent, a freshly prepared N-2 trichloro-p-benzoquinone-4-monoimin (0.5%) methanolic solution and saturated aqueous sodium bicarbonate solution (Neish, 1960).

2.3.5. Reagents for sugars

Aniline/hydrogen phthalate (Stahl, 1969). Aniline (0.9gm) and O-phthalic acid anhydride (1.6gm) were dissolved in 100ml n-butanol saturated with water, the chromatogram was sprayed and heated in an oven at 100-105 °C for 10 minutes. The developed colour ranged from brown, yellowish brown to red for sucrose, the chromatograms were heated, after spraying with reagent for about 5 minutes at 110 °C.

2.4.Solvent systems

2.4.1. n-Butanol : Acetic acid : Water (4:1:5 v/v/v) (B.A.W.).

2.4.2. Acetic acid : Water (15:85 V/V) (AcOH-15%).

2.5.Methods

Leaves and stems powder of *Ballota undulata* collected from

spring season (April) was separately extracted with 80% aqueous ethanol. The ethanolic extract was separately evaporated under reduced pressure and low temperature. then extracted with chloroform. The obtained residue treated with excess of ethanol and filtered to remove inorganic salts and non-phenolic compounds.

2.5.1.Chromatography

Each alcoholic extract of the leaves and stems of *B. undulata* was separately chromatographed on Whatman No. 1 paper chromatography alongside with the available authentic samples using the solvent system BAW for the first way and solvent system Ac-OH 15% for the second way. The developed chromatograms were air dried, and examined under ultra violet (UV) light, then exposed to ammonia and re-examined under UV light.

The concentrated ethanolic extracts of the leaves and stems of *B. undulata* were applied separatily on the top of a polyamide column. Elution was started with distelled water followed by a mixture of water/ethanol and finally pure ethanol was used. The received fractions were evaporated and similar fractions were collected together, evaporated and subjected to paper chromatography (Liu *et al.*, 1989). Preparative paper chromatography was applied on Whatman No.3 paper chromatography using the solvent system BAW. The separated flavonoid compounds and phenolic acids were purified on a sephadex LH-20 column using methanol / water system.

The pure isolated compounds were spotted on Whatman No.1 paper chromatography, the chromatograms were irrigated using solvent systems (1) and (2). The developed chromatograms were air-dried, examined under UV light. Carboxylic acids, hydroxy flavonoids, phenolics and sugars were detected by spraying the dried chromatograms with the corresponding reagent.

2.5.2. Physical tests

2.5.2.1. Ultraviolet spectrophotometric analysis

Chromatographically pure materials were dissolved in pure methanol and subjected to ultraviolet spectrophotometer UV- 240. In the case of flavonoids, $AlCl_3$/HCl, NaOAc/H_3BO_3 and NaOMe were used.

2.5.2.2. ^{1}H and ^{13}C nuclear magnetic resonance analysis (NMR)

The NMR measurements were carried out on Buruker AMX-500ml, Varian Inova-500 and/or JEOL EX-270 NMR spectrometer apparatus as described by Mabry *et al.*, (1970).

2.5.2.3. Mass spectrometric analysis (MS)

The isolated chromatographically pure 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 impact and/or chemical ionization mass spectrometric analysis (EI, CI-MS). The spectra were conducted using Mass Spectrometer Varian Mat 711, Finnigan SSQ 7000 and MM 7070 E (Mabry *et al.*, 1970).

2.5.3.Chemical reactions

2.5.3.1. Controlled (Mild) acid hydrolysis

The pure material was hydrolysed using 0.1 N HCl under reflux for 1 hour. The resultants were traced chromatographically every 5 minutes using comparative paper chromatography (Harborne *et al.*, 1975).

2.5.3.2. Complete (normal) acid hydrolysis

The pure material was hydrolysed using 2N HCl under reflux for 1 hour. The released aglycone and sugar were subjected to comparative paper chromatography (CoPC) using authentic reference markers (Harborne *et al.*, 1975).

2.5.3.3. Enzymatic Hydrolysis

The flavonoid glycoside was enzymatically hydrolyzed through an enzyme corresponding to the type of the sugar in the compound in 0.05 acetate buffer (pH=5.1).

The mixture was left at 37-40°C for 24 hours. The hydrolysate was examined through (CoPC) against authentic reference markers as described by Harborne *et al.*, (1975).

2.6.Total flavonoids

Estimation of the total flavonoids in the ethanolic extract of extract of leaves and stems of *Ballota undulata* during the four

seasons was colourimetrically determined according to the method described by Karawya and Aboutabl,(1982).

2.6.1.Calibration curve

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), five ml of 0.1M aluminum chloride reagents were added. The absorbance of the color developed was measured at 445nm (wave length of maximum absorbance) against a blank. Three determinations for each concentration of standard solution were carried out (Karawya and Aboutabl, 1982).

2.6.2.Estimation of flavonoid content

The powdered air-dried material (2gm) of the leaves and stems were defatted with petroleum ether and extracted with 96% ethanol till exhaustion. The ethanolic extract was adjusted to 50ml. Five ml aliquots were, separately, introduced into test tubes, evaporated to dryness on a water bath. Five ml of 0.1M aluminium chloride were added. The absorbance of the colour developed was measured at 445nm. Three determinations for each sample were carried out.

3.RESULTS AND DISCUSSION

The developed chromatograms of the phenolic and flavonoid constituents of the leaves and stems of *Ballota undulata*, after drying, exposing to ammonia and subjected to ultraviolet light revealed that the phenolic and flavonoid constituents of the leaves and stems are not identical.

3.1. Isolation of the main phenolic and flavonoid constituents

When the concenterated extracts of the leaves and stems of *Ballota undulata* were applied on the top of polyamide column using methanol / water system, column of leaves gave 9 bands, while column of stems gave 7 bands.

Each band was subjected to preparative paper chromatography using Whatman No.3 paper chromatography using the solvent system BAW. The separated flavonoid compounds and phenolic acids were purified on a sephadex LH-20 column using methanol/water system.

Table (1): R_f-values and colour reactions of the isolated compounds.

Compound	Solvent	R_f- values	Reagent	Colour	
				Visible light	UV
F 1	BAW	0.86	Untreated	Yellow	Yellow
	AcOH-15%	0.14	NH_3	Yellow	Yellow fluorescence
F 2	BAW	0.71	Untreated	-	Yellow
	AcOH-15%	0.27	NH_3	-	Yellow fluorescence
F 3	BAW	0.55	Untreated	-	Yellow
	AcOH-15%	0.62	NH_3	-	Yellow fluorescence
F 4	BAW	0.31	Untreated	-	Brown
	AcOH-15%	0.16	NH_3	-	Yellow
P 1	Water	0.56	Untreated	-	Fluorescence blue
	BAW	0.87	Aniline/ glucose	-	Purple
	AcOH-15%	0.69			
P 2	BAW	0.88	Untreated	-	High blue
			NH_3	-	Fluorescence blue
	AcOH-15%	0.33	$FeCl_3$	-	Deep blue green
P 3	BAW	0.93	Untreated	-	Fluorescence blue
	AcOH-15%	0.44	NH_3	-	Yellowish green
			$FeCl_3$	-	Blue
P 4	BAW	0.97	Untreated	-	Brown
	AcOH-15%	0.72	NH_3	-	Brown
			$FeCl_3$	-	Bluish
			Aniline/ glucose		Dark brown
P 5	BAW	0.92	Untreated	-	Pale blue
	AcOH-15%	0.65	NH_3	-	Mauve
			$FeCl_3$	-	Bluish

Then the purified compounds were subjected to two dimensional paper chromatography using BAW for the first run and AcOH-15% for the second run. The obtained chromatograms revealed that *Ballota undulata* leaves contain compounds F1, F2, F3, F4, P1, P2, P3, P4 and P5, while stems of the plant contain F1, F2, F3, P1, P2, P3 and P5 (Where F for flavonoid compound and P for phenolic acids).

3.2.Isolation and identification of compounds (F 1)

The band No. (1) on paper chromatography was eluted with methanol / water, dried under reduced pressure and purified on sephadex LH-20 column using methanol / water (1:1) (Johnston, *et al.*, 1968). TDPC, of fraction (1), using the solvent system BAW followed by solvent system AcOH-15% revealed the presence of one major spot of flavonoid nature (compound F1). Its R_f-values were outlined in Table (1), are within the range of flavonoid aglycone (Harborne, 1984). The change of its colour from yellow to fluorescence yellow when exposed to ammonia vapour under UV light (366 nm) indicated that compound F1 is a flavonol accompanied with free 5-OH (Liu *et al.*, 1989). The obtained results of UV spectral data are outlined in Table (2).

3.2.1. ^{1}H -NMR spectral data

The spectra of compound (F1) in DMSO are represented in Table (3).The purified compound (1) appeared on paper chromatography as yellow colour changed to yellow fluorescence with ammonia under UV light. R_f-values and color reactions (Table 1) showed close similarity with kaempferol. Compound F1 was obtained as yellow crystals, soluble in methanol and 70% ethyl alcohol.

3.2.2. ^{13}C-NMR spectral data

The ^{13}C-NMR spectrum of compound F1 in DMSO showed signals at δ 175.9 (C-4), 164.2 (C-7), 161 (C-5), 159.5 (C-4′), 156.4 (C-9), 146.8 (C-2), 135.4 (C-3), 129.9 (C-2′ and C-6′), 121.9 (C-1′), 115.8 (C3′ and C-5′), 103.7 (C-10), 98.6 (C-6), 93.8 (C-8).

3.2.3. EI-Mass spectra of compound FI

The EI-Mass spectra of compound FI revealed the presence of molecular ion peak (M^+) as the base peak at m/z 286 and other

Table (2): UV spectral data, λ_{max} nm of the isolated compounds.

Compound	MeOH	MeONa	NaOAc	NaOAc + H_3BO_3	$AlCl_3$	$AlCl_3$ + HCl
F 1	253 (sh), 268, 324 (sh), 367	280, 318 (sh), 420	275, 302 (sh), 385	267, 296 (sh),320(sh), 370	266, 305(sh), 350, 422	266, 305 (sh), 350, 422
F 2	255,270(sh), 300(sh), 370	265, 328 (sh), 440	258, 332, (sh), 385	260, 300 (sh), 384	217, 318, 446	265, 305 (sh), 355 (sh), 425
F 3	255,270(sh), 300(sh), 370	265, 328 (sh), 440	258, 332, (sh), 385	260, 300 (sh), 384	217, 318, 446	265, 305 (sh), 355 (sh), 425
F 4	254, 272, (sh), 374	262 (sh), 285 (sh), 322, 423	269, 335	258, 304 (sh), 382	271, 316,(sh), 450	266, 275 (sh), 308 (sh), 366, 428
P 1	285, 308	-	-	-	-	-
P 2	285, 312	250, 290, 319	-	-	-	-
P 3	245, 300 (sh), 336	239, 265, 310 (sh), 382	-	-	-	-
P 4	250, 325 (sh)	275, 335 (sh)	-	-	-	-

important fragment ions; m/z 287, 258 (M-CO), 229, 153, 121 and 93.

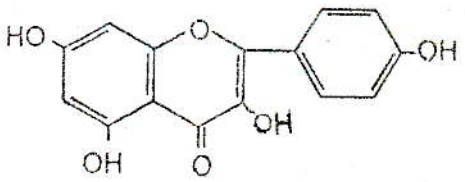
UV spectral analysis of compound F1 in methanol and after addition of different reagents (Table 2), showed the flavonoid nature of compound F1. Compound F1 showed two major absorption bands in MeOH, band I at 367nm and band II at 266nm, which indicated a flavonol with free hydroxyl group at the 3 position (Harborne, 1984).

The addition of sodium methoxide resulted in a bathochromic shift in band I (+53nm), with increasing in the intensity followed by decomposition of the band, 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 sodium acetate (+7nm) referred to the same band in MeOH suggested the presence of free hydroxyl group at C-7.

The hypothochromic shift in band I (-15) after the addition of H_3BO_3 on the NaOAc suggested the absence of any catecholic hydroxyls in compound F1.

A bathochromic shift in band I (+55nm) with aluminum chloride, which was not affected with the addition of hydrochloric acid, indicated the presence of free hydroxyl group at C-3 and C-5 and this confirmed the absence of catecholic hydroxyls. From the UV analysis, compound F1 is probably kaempferol. The structure of compound F1 was further confirmed as kaempferol by ^{1}H-NMR spectrum in DMSO which showed the signals characteristic for kaempferol (Mabry *et al.*, 1970).

Meanwhile, the mass spectrum fragments, molecular ion and other fragments indicated that compound F1 is kaempferol.



Kaempferol

3.3.Isolation and identification of compound F 2

The eluated band No.(2) when evaporated and purified on a

sephadex LH-20 column, using methanol / water (1:1) as described by Johnston *et al.*, (1968) and subjected to TDPC using the solvent system BAW, followed with solvent system AcOH-15%, revealed the presence of one major spot of flavonoid nature.

The compound F2 was obtained as yellow crystals, soluble in methanol and 70% ethyl alcohol. Its R_f -values and color reactions are outlined in Table (1) while the obtained results of UV spectral data are outlined in Table (2).

3.3.1. ^{1}H -NMR spectral data

^{1}H-NMR spectra of compound F2 in DMSO are represented in (Table 3).

The R_f values of compound F2 are within the range of flavonoid aglcone. The change of its colour from yellow to fluorescence yellow when exposed to ammonia vapour under UV light (366 nm) indicates that F2 may be a flavonol accompanied with free 5-OH (Harborne, 1984 and Liu *et al.*, 1989). The R_f - values and color reactions of compound F2 showed close similarity to that of quercetin. UV spectral analysis in methanol showed two major UV bands; band I at 370 nm and band II at 255 nm, which are typical to the flavonol nucleus with free hydroxyl group at position 3 (Harborne, 1984 and Liu *et al.*, 1989). Formation of a new band at (328nm.) resulted in a bathochromic shift (70 nm) in band I followed by decomposition, with sodium methoxide which proved that position 4 has a free OH group.The presence of a shoulder at 328 nm in NaOMe suggested the presence of a free 7-OH group.

A bathochromic shift of band I (+76nm.) in $AlCl_3$, indicated the presence of 3 and 5-OH group. The hypothochromic shift with $AlCl_3$-HCl in band I (-21nm.) after addition of HCl, indicated the presence of O-dihydroxyl group in B-ring.

Bathochromic shift in band I (+15nm.) with sodium acetate / boric acid spectra, was an additional proof of the presence of O-dihydroxyl group in B-ring. From UV analysis, compound F2 is probably quercetin.

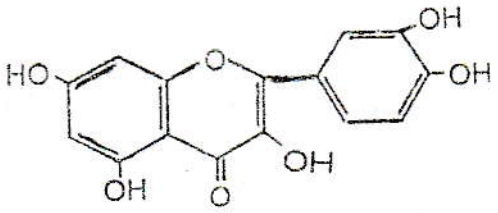
The structure of compound F2 was further confirmed as quercetin by ^{1}H-NMR, which showed signal charcteristic for quercetin (Mabry *et al.*, 1970).

The presence of signals at δ 7.6 (1H, d, j = 8.5, H-5), δ 7.5 (1H,

d, j = 8.5, H-6) and δ 6.89 (1H, d, j = 8.5, H= 5), indicated the presence of aromatic ring with two substitutions, m, p-substitution δ 6.4 (1H, d, j = 2.5 H-6) and δ 6.2 (1H, d, j = 2.5, H-8). Thus compound F2 was identified as quercetin.

3.3.2. Electron Impact (EI) Mass spectral data of compound F2

The EI- mass spectral data of compound F2 revealed the presence of a molecular ion peak (M^+) as the base peak at m/z 302 in addition to other important fragment ions at m/z 273, 245, 229, 137 and 69.



Quercetin

3.4. Isolation and identification of compound F3

The band No. (3) on paper chromatography after elution with methanol / water, drying under reduced pressure and purified on sephadex LH-20 column, using methanol / water (1:1) system, was subjected to TDPC using the solvent system BAW in the first way and AcOH-15% in the second way, revealed the presence of one major spot of flavonoid nature. Its R_f -values and color reactions were outlined in Table (1). TLC on silica gel was applied with authentic marker using BAW and AcOH-15%. From Table (1), compound F3 was identified as rutin.

F3 was further confirmed as rutin from a complete acid hydrolysis, UV spectral data, ^{1}H-NMR and ^{13}C-NMR spectrum.Complete acid hydrolysis gave the aglycone quercetin and the sugars are identified as glucose and rhamnose.

The obtained results of UV spectral data were outlined in Table (2). UV spectral data are similar to those reported for quercetin type compounds with 3-OH substitution.

3.4.1. ^{1}H-NMR spectral data

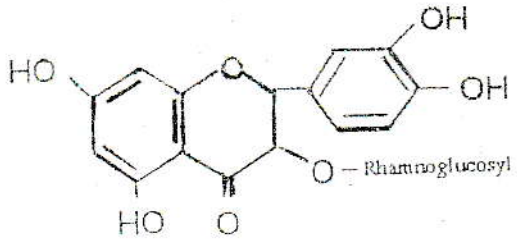
^{1}H-NMR spectrum of compound F3 in DMSO is presented in Table 3. ^{1}H-NMR spectral data showed signals characteristic for quercetin with additional signals for sugar moieties, two signals for 2 anomeric sugar proton at δ 5.6 (1H, d, j = 2.5 Hz, H-1″ rhamnose), δ 5.3 (1H, d, j =8 Hz, H-1‴glucose) and the remaning sugar protons m at δ (3.2-3.9) and signal at δ 1.2 (3H, d, j=6 Hz, CH3 rhamnose).

3.4.2. ^{13}C-NMR spectral data

^{13}C-NMR spectrum of compound F3 showed signals at δ ppm 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 (C-6′). Sugar moiety: 102.08 (C-1″),72043 (C-2″), 73.12 (C-3″), 73.7 (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‴).

^{13}C-NMR spectrum of the compound (F3)showed signals characteristic for quercetin with C-3 more upfield which indicates the presence of a substitution on this carbon. Two anomeric sugar carbons at 102.08 and 103.1 for C-1″ and C-1‴,indicating the disaccharide nature of compound F3,One CH3 carbon of rhamnose was shown at 18.

In compound (F3), C-6 of glucose at 62.70 ppm, so the linkage is (1→6) (Harborne et *al.*, 1975). Thus F3 is rutin (quercetin-3-O-α L-rhamnoside (1→6) β-D-glucoside).



Rutin

3.5. Isolation and identification of compound F4

R_f -values and colour reaction of compound (F4) are indicated of aglycone (Table 1). UV spectral data of compound F4 (band 4) was

Table (3): ^1H nuclear magnetic resonance (NMR) of some isolated compounds.

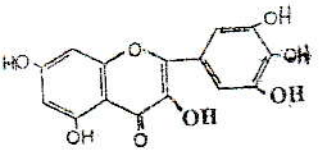
Compound	Signals at δ (ppm)
Kaempferol (F1)	8.0 (2H, d, J= 8 Hz, H-2` and H-6`), 6.9 (2H, d, j= 8Hz, H-3` and H-5`), 6.4 (1H, d, j= 2.5 Hz, H-8), 6.2 (1H, d, j= 2.5 Hz, H-6).
Quercetin (F2)	7.6 (1H, d, J=2.5 Hz, H-2), δ 7.5 (1H, dd, j = 8.5 Hz, H-6), δ 6.89 (1H, d, j = 8.5 Hz, H-5), δ 6.4 (1H, d, j = 2.5 Hz, H-6) and δ 6.2 (1H, d, j = 2.5 Hz, H-8).
Rutin (F3)	7.6 (1H, d, J=8.5 Hz, H-2'), δ 7.5 (1H, dd, j = 8.5 Hz, H-6'), δ 6.89 (1H, d, j = 8.5 Hz, H-5'), δ 6.4 (1H, d, j = 2.5 Hz, H-6) and δ 6.2 (1H, d, j = 2.5 Hz, H-8).
Myricetin (F4)	6.25 (1H, d, j = 2.5 Hz, H-6), δ 6.33 (1H, d, j = 2.5 Hz, H-8), δ 7.32 (S, H-2' and H-6').
2, 3-dihydroxy benzoic acid (P1)	6.8 (t, j = 7.5 Hz, 5-H), 7.1 (dd, j = 7.5 Hz and j = 2.5 Hz, 6-H), 7.4 (dd, j = 7.5 Hz and j = 2.5 Hz, 4-H).
Ferulic acid (P2)	8.9 (s, -OH), 7.5 (1H, d, j = 17 Hz, H-7), 7.15 (1H, d, j = 2.5 Hz, H-2), 7.05 (1H, dd, j = 7.5 Hz and 2.5 Hz, H-6), 6.95 (1H, d, j = 7.5 Hz, H-5), 6.25 (1H, d, j = 17 Hz, H-8), 3.85 (3H, s, OCH_3).
Chlorogenic acid (P3)	9.5 (s-OH), 9.0 (s-OH), 7.45 (1H, d, j = 17 Hz,- H-7), 7.1 (1H, d, j = 2.5 Hz, H-2), 6.95 (1H, dd, j =7 Hz and 2.5 Hz, H-6), 6.75(1H, d, j = 7.0 Hz, H-5), 6.15 (1H, d, j = 17.0 Hz, H-8), 5.55 (1H, broad, H-3),3.9 (1H, broad, H-5), 3.5 (1H, board, H-4), 1.9-2.1 (4H, multiblet, CH_2-2 and CH_2-6).
P-hydroxybenzoic acid (P4)	7.6 (2H, d, j =8 Hz, H-2 and H-6), 6.5 (2H, d, j =8 Hz, H-3 and H-5), 8.7(s, -OH)

found to be similar to those for myricetin type compounds (Mabry *et al.*, 1970). The obtained results of UV spectral data were outlined in Table (2).

3.5.1. ^{1}H-NMR spectral data

The spectrum of compound (F4) were outlined in Table (3). ^{1}H-NMR spectral data of compound (F4), showed signals characteristics for myricetin.

Band 1 from UV spectral data in methanol indicated that compound (F4) is a flavonol with free OH at 3- position (Liu *et al.*, 1989). From R_f -values, colour reaction, UV spectral data and ^{1}H - NMR, compound (F4) was identified as myricetin.



Myricetin

3.6. Isolation and identification of compound P1

Two–dimensional paper chromatography of band (5), revealed the presence of a major fluorescent blue spot under UV light which gave purple colour with aniline / glucose spray reagent (Smith, 1960), specific for carboxylic acids.

An amorphous pure sample of (P1) was obtained by applying the the preparative paper chromatography technique for fractionating the material of fraction (5) on Whatman paper No. 3MM, using BAW as solvent for separation, led to the isolation of a chromatographically pure sample of (P1).

Crystallization of the obtained amorphous material of (P1) from water gave colourless prisms of pure (P1), (m.p.202°C) which exhibited no optical activity when dissolved in $(Me)_2CO$.

R_f -values and UV spectral data of component (P1) were illustrated in Tables (1 and 2).

3.6.1. ^{1}H-NMR spectral data

δ (ppm): 6.8 (t, j = 7.5 Hz, 5-H), 7.1 (dd, j = 7.5 Hz and j = 2.5 Hz, 6-H), 7.4 (dd, j = 7.5 Hz and j = 2.5 Hz, 4-H)(Table 3).

3.6.2. ^{13}C-NMR spectral data

δ (ppm): 172.83 (C = O), 121.4 (C-1), 151.06 (C-2), 146.63 (C-3), 119.7 (C-4), 113.3 (C-5), 121.4 (C-6).

3.6.3. Alkali fusion

fifteen mg of component (5) were fused in an ignition tube together with 3 pellets of KOH for 2 minutes. The fusion product was left at room temperature, dissolved in 10 ml water and the aqueous solution obtained was rendered acidic by aqueous1.5 N HCL. Extraction in a separating funnel of the acidified aqueous solution by ether followed by CoPC of the ether extract proved the presence of 3, 4-dihydroxy benzene, catechol (UV λmax, nm, in MeOH: 285,308 nm).

The crystalline optically inactive material (P1) has showen chromatographic (Table1) and colour properties of a phenolic carboxylic acid (dark purple colour with aniline / xylose) which possess at least two ortho phenolic groups (blue colour with $FeCl_3$). It exhibited UV spectral data (Table 1) identical with those reported for O- protocatechuic acid, a phenolic acid of rare natural occurrence. This view was supported by Elctron Impact– Mass Spectrum (El-MS) analysis of compound P1.

3.6.4. Elctron Impact – Mass Spectrum (EI-MS) analysis of (P1)

m / z (%): 154 (M^+, 9), 136 (37), 110 (100), 92 (14), 81 (15), 64 (42), 52 (17), 44 (61).

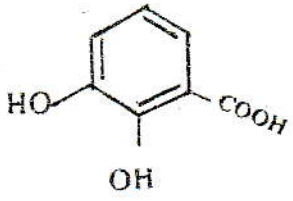
The received spectrum showed a molecular ion peak at m/z 154 and a fragmentation pattern characteristics for phenolic carboxylic acids. The recovery of component (P1) unchanged after alkaline hydrolysis in addition to its conversion to 2, 3- dihydroxybenzene (catechol) on fusion at 245°C for 2 minutes, CoPC cofirmed the assumed structure of compound (P1).

Further confirmation of the identification was then received through ^{1}H- NMR analysis of (P1). The spectrum showed only a pattern of aromatic proton signals typical for 1,2, 3-

triunsymmetrically substituted benzene (t, j = 7.5 Hz, at δ (ppm) 6.8, H-5; dd, j = 7.5 Hz and 2.5 Hz at 7.1, H-6 and dd, j = 7.5 Hz and 2.5 Hz at 7.4, H-4), thus confirming the structure of component (P1) as 2, 3-dihydroxy benzoic acid, O-protocatechuic acid.

^{13}C-NMR spectrum of (P1) also, was recorded and assigned, the spectrum showed a carbonyl carbon signal at δ (ppm) 172.82.

This recognizable downfield shift (on comparison with about 168 δ ppm) for phenolic acids lacking ortho free hydroxyl group, e.g. 2-methoxybenzoic acid (Breitmaier and Voelter, 1974), gallic acid and 3, 4-dihydroxy benzoic acid (Nawwar *et al.*, 1982) might be due to the hydrogen bonding with the adjacent free OH group. This hydrogen bonding also might affect the resonance of C-2 which was found resonating at δ (ppm) 151.06. The second oxygenated carbon (C-3) revealed its signal at δ (ppm) 146.63. The chemical shift values of the remaining four carbon signals are in consistence with the achived structure of (P1) as 2,3- dihydroxy benzoic acid.



2,3-dihydroxy benzoic acid (O-protocatechuic acid)

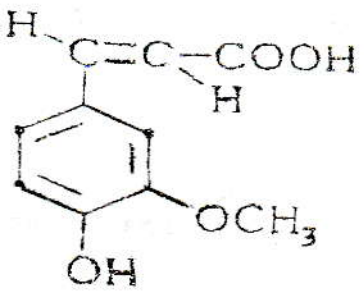
3.7. Isolation and identification of compound P2

The band No. (6) on paper chromatography after eluted with methanol/water, drying under reduced pressure and purified on sephadex LH-20 column, using methanol/water (1:1) system and subjected to TDPC using the solvent system BAW in the first way followed by the system AcOH-15%, revealed the presence of one major spot of phenolic nature. Its R_f -values and color reactions were outlined in Table (1).

UV analysis of compound P2 in methanol (Table 2) showed bands at 285nm and 312nm which are characteristic for phenylpropanoids, also with adding sodium methoxide, gives a

bathochromic shift (250 nm, 290, 319nm) proved the presence of free hydroxyl group.

^{1}H-NMR spectra of compound P2 (Table 3) showed two doublets at (7.5 and 6.25ppm), j=17Hz, characteristic of trans olefenic double bond H-7 and H-8, respectively. The two doublet at 7.15 and 6.95 (j = 7.5 Hz) and the doublet of doublet signal at dd, j = 7.09 Hz, which is corresponding to ortho and meta coupling and it is a good evidence for the presence of trisubstituted benzene. The presence of singlet at 9.15ppm confirmed the UV analysis for the presence of free OH group. The ^{1}H-NMR showed signals at 12.2 (broad and singlet) for – COOH proton and 3.85 for 3 protons of OCH_3 group. These data suggest that compound P2 is 3-methoxy-4-hydroxy cinnamic acid (Ferulic acid). The latter suggestion was confirmed by Electron Impact (EI) Mass which showed a molecular ion peak M^{+1} 195, which proved that compound (P2) 3-methoxy-4-hydroxy cinnamic acid (Ferulic acid).



Ferulic acid (3-methoxy-4-hydroxy cinnamic acid)

3.8. Isolation and identification of compound P3

Elution of band No. (7) from paper chromatography was performed with methanol/water, dried under reduced pressure, purified on sephadex LH-20 column, as described by Johnston *et al.*, (1968), using methanol and subjected to TDPC using the solvent systems (BAW and AcOH-15%), where one major spot of phenyl propanoid nature was detected (compound P3). Its Rf -values and color reactions were outlined in Table (1). The change of its colour from blue to yellowish green when exposed to ammonia vapour under UV light suggested that P3 is chlorogenic acid. The UV spectral data

of compound P3 outlined in Table (2) confirmed the presence of the absorbtion bands characteristic for phenylpropanoids. The bathochromic shift upon addition of NaOMe proved the presence of free hydroxyl groups.

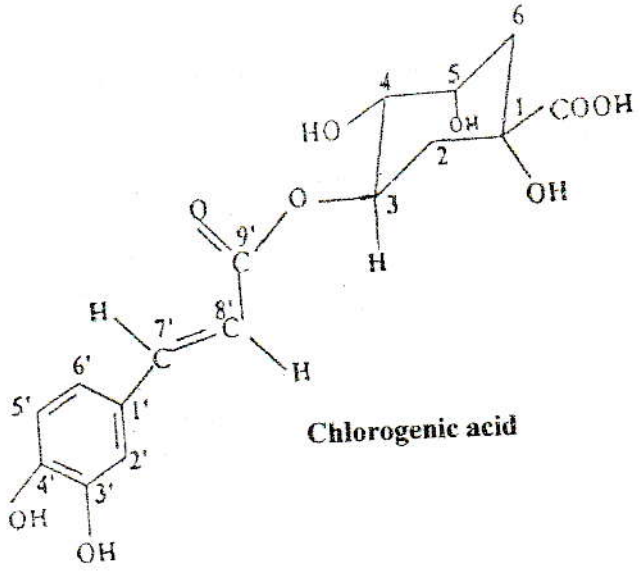
3.8.1. ^{1}H-NMR spectral data

δ (ppm): 9.5 (s-OH), 9.0 (s-OH), 7.45 (1H, d, j = 17 Hz, α – H), 7.1 (1H, d, j = 2.5 Hz, H-2), 6.8 (1H, dd, j =7 Hz and 2.5 Hz, H-6), 6.7 (1H, d, j = 7.0 Hz, H-5), 6.3 (1H, d, j = 17.0 Hz, B-H), 5.5 (s-OH), 1.8, 2.2, 3.7, 4.5, 7.0 (different protons of quinic acid; CI-MS m/z rel. int. %) 36: 355 (M^{+1}, 1.52 %), 175 (quinic acid moiety, $C_7O_5H_{11}$, 66%), 217 (M^+ , $C_8H_7O_2$, 100 %), 157 (175-H_2O 70 %). Its ^{1}H-NMR spectrum showed all protons characteristic for 3, 4-dihydroxy-cinnamic acid beside the proton for quinic acid moiety.

The purified compound (P3) gave blue florescence under UV light changing to yellowish green, when exposed to ammonia, and re-examined under UV light compound (P3) gave blue colour with $FeCl_3$ reagent, proving the phenolic nature. R_f -values and color reactions indicate the phenolic nature compound (P3).

UV spectral data of compound (P3) showed two major bands at 245, 330nm, in addition of sodium methoxide gave a bathochromic shift indicating the presence of free hydroxyl groups.

The structure of the compound (P3) is further confirmed as chlorogenic acid by proton NMR and mass spectrum.



Chlorogenic acid

3.9. Isolation and identification of compound P4

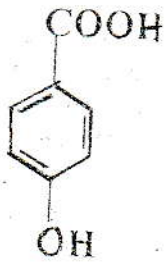
The band No. (8) on paper chromatography when eluted with methanol/water, dried under reduced pressure, purified on sephadex LH-20 column, and subjected to TDPC using the solvent systems BAW followed by the system AcOH-15%, revealed the presence of a single spot of phenolic nature (compound P4). Its R_f-values and color reactions were outlined in Table (1).

It is white amorphous powder, m.p.190-192°C, soluble in methanol and acetone, brown colour under UV, bluish colour with $FeCl_3$, dark brown colour with aniline/glucose reagent indicating its phenolic carboxylic acid nature.

UV analysis of compound (P4) in methanol showed a band at 250nm and a shoulder at 325 nm which is similar to benzoic acid with – OH group (Silverstein *et al.*, 1981). The bathochromic shift after the addition of sodium methoxide, a shift at 275nm (Table 2), proved the presence of free hydroxyl group.

IR ν max (kBr) Cm^{-1}; 3388 (OH), 1677 (O=C); while ^{1}H-NMR (DMSO-d_6) showed two doublets at 7.6 ppm (2H, d, j = 8Hz), 6.6 ppm (2H, d, j = 8 Hz), 8.3 ppm (s-COOH), 4.5 ppm (bs-OH).

EI-MS (Electron Impact – Mass Spectrum) m/z (rel. int. %) 139 (M^{+1}, 75.1 %), 138 (M^+, 49.1 %), 120 ($M^+ - H_2O$, 100%), 93 (M^+ - COOH, 86.06%) and 65 (C_6H_5, 90.2%), which proved that compound (P4) could be P-hydroxybenzoic acid.



P-hydroxybenzoic acid.

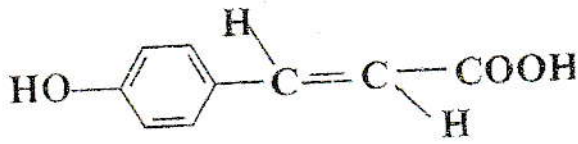
3.10.Isolation and identification of compound P5

The band No. (9) on paper chromatography when eluted with methanol / water, dried under reduced pressure, purified on sephadex LH-20 column, and subjected to TDPC using the solvent systems

BAW followed by the system AcOH-15%, revealed the presence of a single spot of phenolic nature (compound P5). Its R_f-values and color reactions were outlined in Table (1).

Compound (P5) was obtained as white powder, soluble in methanol and acetone.

The R_f -values and color reactions were outlined in Table (1), indicated that it has a phenolic nature. The compound was directly compared along with an authentic sample of p–coumaric acid in BAW and AcOH-15% systems using paper chromatography, the R_f -values and color reactions were identical. Thus the compound (P5) can be identified as p–coumaric acid (4-hydroxy cinnamic acid).



p-coumaric acid (4-hydroxy cinnamic acid).

3.11.Estimation of total flavonoids

The total flavonoids present in the leaves and stems of *Ballota undulata*, were determined spectrophotomitrically and calculated as quercetin.

Data presented in Table (4) indicate that the leaves contain the highest percentages of flavonoids than stems. It is obvious also that spring samples contain the highest value of total flavonoid followed by winter samples in both the stems and leaves of the plant, so the isolation of the previous kinds of flavonoids and phenolic constituents were attained using spring samples.

Table (4): Percentages of total flavonoids in the stems and leaves of *Ballota undulata*.

Seasons	Stems	Leaves
Winter	1.54	1.80
Spring	1.70	1.93
Summer	0.24	0.55
Autumn	1.23	1.42

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الفلافونيدات و المكونات الفينولية لاوراق و سيقان نبات الغصة

فاطمة على أحمد على

قسم النباتات الطبية و العطرية - مركز بحوث الصحراء- المطرية - القاهرة

ملخص

تم باستخدام طرق التفريد الكروماتوجرافي لسيقان و أوراق نبات الغصة و الذى تم جمعه من صحراء شمال سيناء طريق القصيمة – العريش عند الكيلو ٨ للتعرف على محتواها من الفلافونيدات و الاحماض الفينولية.

تم التعرف على اربعة مركبات فلافونيدية فى اوراق النبات وهى كامفيرول؛ وكوارسيتين؛ وروتين وميريسيتين بجانب خمسة احماض فينولية ، هى حمض ٢-٣ ثنائى هيدروكسى بنزويك؛ حمض الفيريوليك؛ حمض الكلوروجينك؛ حمض بارا هيدروكسى بنزويك و حمض كيوماريك.

كما تم التعرف على ثلاثة مركبات فلافونيدية فى سيقان النبات هى كامفيرول؛ وكوارسيتين وريوتين بجانب خمسة احماض فينولية هى حمض ٢-٣ ثنائى هيدروكسى بنزويك؛ حمض الفيريوليك؛ حمض الكلوروجينك و حمض كيوماريك.

تم تعريف كل مجموعة باستخدام التفاعلات اللونية و التحليل الكامل و الجزئى بالحامض و قياس طيف الكتلة و تحليل اطياف الاشعة البنفسجية و الرنين المغناطيسى لانوية الهيدروجين ١ و الكربون ١٣.

اوضحت النتائج أن المحتوى الكلى من الفلافونيدات يختلف فى السيقان عنه فى أوراق النبات و احتوت الاوراق على اعلى نسبة مئوية من الفلافونيدات الكلية خلال فصل الربيع.

المجلة العلمية لكلية الزراعة – جامعة القاهرة – المجلد (٥٥) العدد الرابع (أكتوبر ٢٠٠٤) :٦١٩-٦٤٤.