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Anthraquinones with Antibacterial Activities from *Crucianella maritima* L. Growing in Egypt

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Abstract – From the extracts of *Crucianella maritima* L. (Rubiaceae), five new anthraquinones namely; 1-hydroxy-2-methyl-6-methoxy anthraquinone, 6-methoxy-2-methyl quinizarin, 6-methyl-anthragallol-2,3-dimethyl ether, 6-methyl-anthragallol-2-methyl ether, and 1-hydroxy-2-carbomethoxyanthraquinone were isolated and identified. In addition, deacetyl asperulosidic acid 6'-glucoside sodium salt, a new iridoid diglucoside, along with twelve known anthraquinones, three flavonols, three sterols, and one triterpene were also isolated and identified for the first time from this plant. Their chemical structures were established by physical, chemical and spectroscopic data, including UV, MS, ID- and 2D-NMR analyses. The antimicrobial, cytotoxic activities and a preliminary clinical trial of the crude extracts and some isolates are also presented. Chemotaxonomical aspects are briefly discussed.
Keywords – *Crucianella maritima* L., Rubiaceae, anthraquinones, flavonols, iridoid diglucoside, biological activities, preliminary clinical trial, chemotaxonomy.

Introduction

Crucianella (Rubiaceae) is a small genus widely distributed in the Mediterranean region; Middle Europe and Western Asia (Plowden, 1968). It is represented in Egypt by four species of which the most common is *Crucianella maritima* L. (Tackholm, 1968). Our investigation on this plant indicated the accumulation of substantial amounts of anthraquinones in the roots and flavonoids in the aerial parts. Moreover, the plant extracts in different solvents exhibited highly pronounced antimicrobial activities. We previously reported the isolation of quercetin, rhamnetin, isorhamnetin, isorhamnetin-3-*O*-glucoside and isorhamnetin-3-*O*-neohesperidoside from the aerial parts (Sabri *et al.*, 1988). From the roots, five anthraquinones were isolated (El-Lakany *et al.*, 2001) of which three were identified as rubiadin, lucidin, and 1,6,7-trimethoxy-2-methyl-anthraquinone. Chemical structures of the remaining two anthraquinones could not be completely elucidated. These findings prompted us to continue the exploration of the plant.

Experimental

Plant material – Flowering and fruiting plants of *Crucianella maritima* L., growing wildly in sandy districts of the Mediterranean coastal strip, 70 to 80 kilometers west of Alexandria, were collected in March of the years 2000 and 2001. The plant was identified by comparison with a voucher sample deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University. The tested microorganisms were: 1-Gram +ve: *Bacillus substilis* ATCC 7972, *Micrococcus luteus* ATCC 10240. *Sarcina lutea* ATCC 9341, and *Staphylococcus aureus* ATCC 6538. 2-Gram-ve: *Bordetella bronchiseptica* ATCC 4617, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 12264, *Proteus mirabilis* ATCC 3126, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhi* ATCC 3112, *Serratia marcescens* ATCC 990, and *Shigella sonnie* ATCC 9290. Standard microorganisms and clinical bacterial strains used in this study were obtained respectively from the Department of Botany and Microbiology, Faculty of Science and the Department of Surgery, Faculty of Medicine, Alexandria University, Egypt. The antibiotics used were: 1-Penicillins: penicillin G, ampicillin, and piperacillin. 2-Cephalosporins: cephradine, cephamanadol, and cephotaxime. 3-Aminoglycosides: gentamycin and kanamycin. 4-Macrolides: erythromycin. 5-Fluoroquinolones: ciprofloxacin. 6-Glycopeptides: vancomycin.

General. M.p: uncorrected. UV: Perkin Elmer 550, MS: VG 7070 E-HF, NMR: ^{1}H- and ^{13}C-NMR (NOESY, HMBC, and HMQC): Jeol 500 and 125 MHz. Bruker

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Avance 300 and 75 MHz for NMR of iridoid. CC: Silica gel (70-230, ASTM) Merck, and Rp-18 silica Merck). TLC & pTLC: silica gel G Merck and pre-coated plates, silica gel 60 F 254 (0.25 mm) Merck. Spots detection: UV lamp, Exposure to NH_3 vapors, spraying with 10% alcoholic KOH, anisaldehyde-sulphuric acid, and/or 12% HCl.

Extraction and isolation: The concentrated alcohol extracts of each of the air-dried and pulverized roots (5 kg) and aerial parts (5 kg) of *C. maritima* L. were successively fractionated with petroleum ether, $CHCl_3$, and EtOAC.

The roots – Petroleum ether extract (12 g) was fractionated over a silica gel column, eluted with a mixture of petroleum ether-EtOAc with increasing polarity. Fractions eluted with 2.5% then 7% EtOAc in petroleum ether were purified by pTLC (Solvent: petroleum ether-EtOAc, 9.5:0.5 and 9:1) to give compounds **1**, **2**, and **3**, respectively. PTLC of fractions eluted with 10% EtOAc in petroleum ether (Solvent: toluene-EtOAc, 9.5:0.5) and 15% EtOAc in petroleum ether (petroleum ether-EtOAc, 1:1) afforded compounds **4**, **5**, **6**, and **7**, respectively. Fractions eluted with 17% EtOAc in petroleum ether gave rubiadin. Isolation of compound **8** detected in fractions eluted with 19% EtOAc in petroleum ether, could only be achieved by pTLC using Rp-18 silica (Solvent: MeOH-H_2O, 8:2). Fractions eluted with 20% EtOAc in petroleum ether were subjected to pTLC (Solvents: petroleum ether-ether, 6:4) and afforded compounds **9,** and **10** respectively. Eight grams from the $CHCl_3$ extract were chromatographed over silica gel column using gradient CH_2Cl_2-MeOH. Fractions eluted with 100% CH_2Cl_2, afforded compound **11**. Fractions obtained by 4% MeOH in CH_2Cl_2 afforded lucidin. Purification of the mother liquor by pTLC (Solvent: C_6H_6-MeOH, 9.5: 0.5) furnished compound **12**. The 10% MeOH in CH_2Cl_2 elutes, when subjected to pTLC (Solvent: $CHCl_3$-MeOH, 9.5: 0.5) gave quercetin (25 mg). The EtOAc extract (7g), was column chromatographed using silica gel and $CHCl_3$-MeOH gradients. PTLC of fractions eluted with 4% and 15% MeOH in $CHCl_3$ yielded compounds **13** and **14** respectively. The *n*-BuOH extract (2.5 g), was fractionated over Rp-18 silica gel column, using distilled water gradually mixed with MeOH. Fractions eluted with 20% MeOH in $CHCl_3$ were subjected to further separation on another Rp-18 silica column, followed by pTLC (EtOAc-MeOH-H_2O, 30:5:4) to afford compound **18**.

The aerial parts – Petroleum ether extract (10 g) was subjected to column chromatography using silica gel and petroleum ether-EtOAc gradient. Fractions eluted with 2.5, 7, 15 and 20% EtOAc in petroleum ether mixtures, furnished compounds **1, 2, 5, 6** and rubiadin, respectively. PTLC (Solvent: $CHCl_3$-MeOH; 7:3) of the 17% EtOAc in petroleum ether fractions yielded compounds **15-17**. EtOAc extract (8 g) was chromatographed over silica gel column and eluted with $CHCl_3$-MeOH gradient. Fractions eluted with 12% MeOH in $CHCl_3$ gave 45 mg of quercetin. Re-chromatography of the 25 and 40% MeOH in $CHCl_3$ elutes afforded respectively 10 mg of quercetin-3-*O*-glucoside and 12 mg of quercetin-3-*O*-rutinoside. β-sitosterol, β-sitosterol acetate, stigmasterol, and lupeol were also isolated from both aerial parts and roots.

1-Hydroxy-2-methyl-6-methoxyanthraquinone (**2**) (42 mg): Yellow needles, mp 157-160°C ($CHCl_3$). UV (λ_{max}, MeOH): 270, 405 nm, (MeOH + KOH) 265, 320, 495 nm. EIMS, *m/z* (rel. int.): 268 [M^+, $C_{16}H_{12}O_4$], (100), 253 (27.6), 240 (60.0), 225 (47.0), 211 (40.0), 197 (65.0), 183 (12.1), 140 (29.1) ^{1}H NMR and ^{13}C NMR data listed in Table 1.

6-Methoxy-2-methylquinizarin (**3**) (6 mg): Red needles, mp 165-168°C ($CHCl_3$), UV (λ_{max}, MeOH): 275, 480, 493 (sh.) nm, (MeOH + KOH) 284 (sh.), 524, 565 nm. EIMS, *m/z* (rel. int.): 284 [$C_{16}H_{12}O_5$] M^+ (64.3), 269 (17.4), 266 (6.1), 256 (7.1), 241 (8.2), 228 (6.1), 210 (5.1), 181 (8.2), 157 (8.2), 129 (33.7) ^{1}H NMR and ^{13}C NMR data listed in Table 1.

6-Methyl-anthragallol-2,3-dimethyl ether (**4**) (3 mg): Yellow needles, mp 175-178°C ($CHCl_3$), UV (λ_{max}, MeOH): 250, 409 nm, (MeOH + KOH) 252, 307, 505 nm. EIMS, *m/z* (rel. int.): 298 [$C_{17}H_{14}O_5$] M^+ (12.5), 283 (4.5), 255 (6.8), 227 (2.2), 210 (2.3), 166 (15.9), 136 (14.2), 121 (20.5), 95 (14.8) 57 (100) ^{1}H NMR and data are listed in Table 2.

6-Methyl-anthragallol-2-methyl ether (**8**), (9 mg): Yellowish-orange crystals, mp 190-193°C (MeOH). UV (λ_{max}, MeOH): 285, 408 nm, (MeOH + KOH) 312, 480 nm. EIMS, *m/z* (rel. int.): 284 [$C_{16}H_{12}O_5$] M^+ (100), 269 (9.9), 255 (10.5), 241 (30.8), 210 (16.7), 181 (7.9), 139 (17.6), 89 (7.0) ^{1}H NMR and ^{13}C NMR data are listed in Table 2.

1-Hydroxy-2-carbomethoxyanthraquinone (**16**) (4 mg): Yellow prisms, mp 210-213°C ($CHCl_3$: MeOH). UV (λ_{max}, MeOH): 325, 413 nm, (MeOH + KOH) 327, 502 nm. EIMS, *m/z* (rel. int.): 282 [$C_{16}H_{10}O_5$] M^+ (72.8), 251 (56.1), 250 (66.3), 224 (10.2), 223 (9.3), 222 (25.5), 194 (10.2), 167 (39.1), 139 (35.7) ^{1}H NMR and ^{13}C NMR data are listed in Table 2.

Deacetyl asperulosidic acid 6′-glucoside sodium salt (**18**) $C_{22}H_{31}O_{16}Na$ (10 mg): Creamy matrix $[\alpha]^D_{25}$ -27.9° (*c* = 0.9, MeOH). FABMS *m/z* (rel. int.): 597 [(M + Na)$^+$, $C_{22}H_{31}O_{16}Na$ + Na, 10], 569 (12), 566 (10), 544 (25), 529 (70), 525 (75). ^{1}H-NMR (300 MHz) δ: 7.40 (1H, d, *J* = 1.4 Hz, H-3), 3.31 (1H, m, H-5), 5.96 (1H, d, *J* = 1.6 Hz, H-7), 2.52 (1H, br. t, *J* = 8 Hz, H-9), 4.20 (1H, d, *J* = 15 Hz, Ha-10), 4.45 (1H, d, *J* = 15 Hz, Hb-10), 3.40-3.80 (m, 2Glc.). ^{13}C-NMR (75 MHz) δ: 98.4 (C-1), 158.2 (C-3), 111.6 (C-4), 36.3 (C-5),

75.4 (C-6), 130.0 (C-7), 148.4 (C-8), 44.2 (C-9), 60.5 (C-10), 173.4 (C-11), 97.1 (C-1'), 71.7 (C-2'), 75.5 (C-3'), 68.3 (C-4'), 75.1(C-5'), 68.0 (C-6'), 96.6 (C-1"), 71.5 (C-2"), 74.8 (C-3"), 68.1 (C-4"), 75.0 (C-5"), 61.3 (C-6").

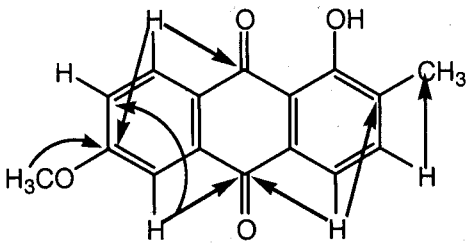
Biological Studies

The day chronic toxicity test – This test was performed on different plant extracts and all the isolated anthraquinones using *Daphnia magna* (Biesinger *et al.*, 1987 and Miller, G 1966).

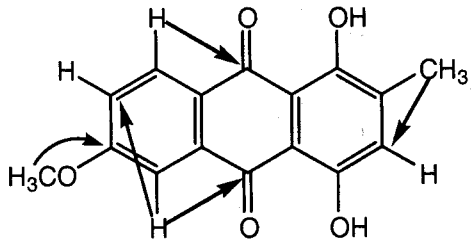
Antimicrobial activity test – It was conducted on certain anthraquinones against standard microorganisms using the disc diffusion method (Lorian, 1980) and fusidic acid discs (30 ug/disc) for comparison.

Preliminary clinical study – MICs (Lorian 1980) of plant extracts and some selected anthraquinones were determined using the clinical strains with and without combination of antibiotics (1:1).

Cytotoxic activity – It was determined against P388 cells (IC_{50} = μg/mL), for only five anthraquinones. It was kindly performed by Prof. Dr. H. Itokawa, Tokyo College of Pharmacy, Japan.



Compound **2**



Compound **3**

Fig. 1. The most important ^{1}H- and ^{13}C- correlations as observed from HMBC of compounds **2** and **3**.

Results and Discussion

In continuation of our investigation of the biologically active extracts of *Crucianella maritima* L. grown in Egypt, five new and twelve known anthraquinones were isolated. The known anthraquinones were characterized as; 1-hydroxy-2-methyl anthroquinone **1** (Thomson, 1971), anthragallol-2, 3-dimethyl ether **5** (Burnett and Thomson, 1968), lucidin-ω-ethyl ether **6** (El-Gamal *et al.*, 1995), 2-carboethoxy xanthopurpurin **7** (Lee and Chen, 1994), soranjidiol **9** (Adeside and Adesogan, 1972), 5-hydroxy-6-methyl-anthragallol-2-methyl ether **10** (Koyama *et al.*, 1993), alizarin **11** (Kuiper and Labadie, 1981), lucidin-ω-methyl ether **12** (Koyama *et al.*, 1992), 1,3,6-trihydroxy-2-methyl anthraquinone **13** (Itokawa *et al.*, 1983), lucidin-3-*O*-glucoside **14** (Yang *et al.*, 1998), anthragallol-3-*O*-methyl ether **15** (Burnett and Thomson, 1968), and anthragallol-2-methyl ether (**17**) (Thomson, 1971). Characterization and identification of the new antraquinones **2**, **3**, **4**, **8**, and **16** are described herein. Chemical structures of anthraquinones **2** and **3** were previously tentatively assigned as 1-hydroxy-6-methoxy-2-methyl anthraquinone or 1-hydroxy-7-methoxy-2-methyl anthraquinone and 1,4-dihydroxy-6-methoxy-2-methyl anthraquinone or 1,4-dihydroxy-7-methoxy-2-methyl anthraquinone respectively (El-Lakany *et al.*, 2001). In both pigments, the site of methyl group on C-2 was based on biogenetic grounds, while the methoxyl group was designated at either C-6 or C-7. In this work, their final structures were confirmed from new data furnished by HMBC and NOE experiments. In HMBC experiment (long-range correlation) of compound **2**, H-8 was correlated with C-9 and C-6, while H-5 was correlated with C-7 and C-10 (Table 1, Fig. 1). On the other hand, the CH_3 group was correlated with C-1, and C-4, supporting the structure of compound **2** as 1-hydroxy-2-methyl-6-methoxy derivative. In addition, irradiation of the methoxyl singlet at δ 3.39, in NOE experiment, resulted in a measurable enhancement of the doublet at δ 7.72 (H-5), confirming the site of this group to be at C-6. Similarly, compound **3** was found to be 6-methoxy-2-methyl quinizarin (Table 1, Fig. 1).

Compounds **4** and **8** were found to be anthragallol derivatives. Their ^{1}H-NMR spectra indicated a similar substitution pattern; ring A being monosubstituted, while ring C was 1, 2, 3-trisubstituted. This was revealed from the one proton singlet at δ 7.46 and 7.25 attributed to H-4 of **4** and **8**, respectively. In addition, the spectra of both compounds exhibited a one-methyl singlet (Table 2). The difference between both quinones was observed from their MS spectra. The molecular ion peak of compound **4** was observed at *m/z* 298 and that of compound **8** was found at *m/z* 284, i.e. less by 14 units, equivalent to a methylene group. This was evident from the ^{1}H-NMR spectra that showed, in compound **4**, closed signals at δ 4.02 and 4.03

Table 1. NMR spectral data (ppm) of compounds **2** and **3**

#	δ C **2** [$CDCl_3$]	HMBC correlations	δ C **2** [$CDCl_3$]	δ C **3** [$CDCl_3$]	HMBC correlations	δ C **3** [$CDCl_3$]
1			160.97			157.79
C-1-OH	13.1 (1H, s)	160.97 114.98 135.05		12.93 (1H, s)	157.79, 128.29, 111.62	
2	7.52		114.98			128.29
3	(1H, d J = 7.2 Hz)	160.97	136.58	7.16 (1H, s)	16.70, 111.62,	111.62
	7.72	119.00			157.24	
	(1H, d, J = 2.8 Hz)	131.37				
4	7.28	114.98	119.00			157.24
	(1H, dd, J = 2.8, 8 Hz)	182.51				
	8.25	135.05				
5	(1H, d, J=8 Hz)	120.97	110.06	7.78	121.41, 127.14	121.41
		182.51		(1H, d, J = 2.5 Hz)		
6			164.73			164.72
7	7.28	110.06	120.97	7.35 (1H, dd,	127.01, 130.89	127.01
	(1H, dd, J = 2.8, 8 Hz)	126.65		J = 2.5, 7,5 Hz)		
8	8.25	164.73	129.37	8.32 (1H, d,	164.72, 186.61,	130.89
	(1H, d, J=8 Hz)	188.13		J = 7.5 Hz)	135.99	
9			188.13			186.51
10			182.51			186.51
11			135.95			127.14
12			126.65			135.99
13			131.37			135.26
14			135.05			141.38
C-2-$\underline{C}H_3$	2.35 (3H, s)	160.97 117.98 119.00	16.19	2.40 (3H, s)	157.79, 128.29, 135.26	16.70
C-4-OH				13.50 (1H, s)	111.62, 157.24, 141.3	
C-6-CH_3		164.73	55.99			
C_6-O$\underline{C}H_3$	3.93 (3Hs)	135.95		4.02 (3H, s)	164.72	56.11

Solvent peaks were used as internal standards.

for two methoxyl groups, but only one was observed at δ 3.85, in compound **8**. Moreover, the UV spectra revealed absorption of a 1-hydroxy pattern in compound **4** and 1,3-dihydroxy pattern in compound **8**. The location of different substituents, in compound **4,** was provided from the NOESY spectrum, which correlated the two methoxyl signals to each other and to the singlet corresponding to H-4, consequently, the methyl should be at ring A. In the ^{13}C-NMR of compound **8**, the low-field chemical shift of the methyl signal at δ 21.24, indicated clearly that it must be located at C-6. This finding was also in accordance with those published for almost all anthragallol derivatives. Accordingly, compounds **4** and **8** were identified as 6-methyl-anthragallol-2,3-dimethyl ether and 6-methyl-anthragallol-2-methyl ether, respectively.

The molecular formula of compound **16** was found be $C_{16}H_{10}O_5$, based on ^{1}H-, ^{13}C-NMR and MS (M^+, *m/z* 282) data. The ^{1}H-NMR spectrum (Table 2) revealed six aromatic protons, two being *o*-coupled, and four for an AA' BB' system suggesting an anthraquinone with a disubstituted ring C. The 1-hydroxy pattern was deduced from the two signals in the ^{13}C-NMR spectrum for both chelated and non-chelated carbonyls, in addition to the shift in the UV spectrum upon adding alkali. The other substituent was found to be a carbomethoxyl group, as observed from both ^{1}H- and ^{13}C-NMR data (Table 2), as well as prominent fragment ions characteristic for methyl esters of aromatic acids appeared at *m/z* 251 [56.1%, M^+- OCH_3], 250 [66.3%, M^+- $HOCH_3$] and 222 [25.5%, M^+- CH_3COOH]. HMQC correlation as well as comparison with published data (Okyama *et al.*, 1990), for related compounds, resulted in identification of **16** as 1-hydroxy-2-carbomethoxy derivative. Compounds **2, 3, 4, 8** and **16** appear to be new anthraquinones, as revealed from literature survey.

The molecular formula of compound **18** ($C_{22}H_{31}O_{16}Na$) was established as the sodium salt of deacetyl asperulosidic acid 6'-*O*-glucoside; an iridoid isolated for the first time as a natural product. Its identity was established based on chemical and spectral evidence. Compound **18** gave a blue color with 12% HCl and a positive Molisch's test, suggesting

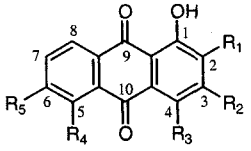
Table 2. NMR spectral data (ppm) of compounds **4**, **8** and **16**

#	δH **4** [$CDCl_3$]	δH **8** [DMSO-d_6]	δC **8** [DMSO-d_6]	δH **16** [DMSO-d_6]	δC **16** [DMSO-d_6]
1			*157.48		163.73
2			145.51		123.90
3			*158.48	8.18 (1H, d, *J*=8 Hz)	136.90
4	7.46 (1H, s)	7.25 (1H, s)	108.98	7.79 (1H, d , *J*=8 Hz)	116.71
5	8.07 (1H, br. s)	7.93(1H, br. s)	126.58	8.22-8.31 (m)	125.67**
6			135.07	7.77-7.81 (m)	133.82***
7	7.57 (1H, br. d)	7.66(1H, dd, *J*=8, 0.5 Hz)	132.84	7.77-7.81 (m)	134.25***
8	8.16 (1H, d, *J* = 7.8 Hz)	8.05(1H, d, *J*=8 Hz)	128.98	8.22-8.31 (m)	125.84**
9			186.57		187.33
10			181.48		181.10
11			**130.75		133.42*
12			**133.30		134.70*
13			127.05		117.90
14			108.16		131.82*
C-2-$\underline{C}H_3$					
C-2-O$\underline{C}H_3$	4.02 (3H, s)	3.85(3H, s)	56.08		
C-2-$\underline{C}$O-O					169.10
C-2-COO$\underline{C}H_3$				3.92 (3H, s)	51.44
C-3-O$\underline{C}H_3$	4.03 (3H, s)				
C-6-$\underline{C}H_3$	2.52 (3H, s)	2.37 (3H, s)†	21.24		
C-1-OH	12.81 (1H, s)	12.85(1H,s)		13.54 (1H, s)	

†Detected in $CDCl_3$ + CD_3OD.
*, **, *** Exchangeable values within the same column.
Solvent peaks were used as internal standard.
In compound **16** the assignment of H-3 and H-4 was confirmed based on HMQC.

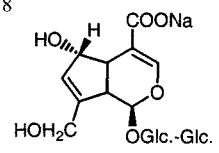
an iridoid glycoside. Acid hydrolysis and ^{13}C-NMR spectra demonstrated the presence of two glucose moieties. In the ^{1}H-NMR spectrum, the two doublets resonating at δ 7.4 (*J* = 1.4 Hz) and δ 5.9 (*J* = 1.6 Hz), indicated the presence of double bonds at C-3 and C-7. The two proton doublets observed at δ 4.20 and 4.45 (*J* = 15 Hz) were assigned to C-10 hydroxymethylene group, and the broad triplet resonated at δ 2.52 (*J* = 8 Hz) was due to H-9. ^{13}C-NMR and DEPT spectra indicated the presence of three oxymethylene groups, confirming the presence of two sugar moieties, one is due to the oxymethylene of the iridod nucleus and the other two could be attributed to two glucose moieties. The downfield shift of -CH_2O signal at δ 68.0, compared to the other two, was an evidence of the glycosidic linkage at 6'-OH. In addition, the spectrum revealed the presence of three oxygenated carbons, as well as one carbonyl (δ 173.4). The α-configuration of the OH at C-6 was based on the chemical shift of this carbon at δ 75.4 (Boros and Stermitz, 1990). The spectral data of compound **18** were found to be almost similar to those of asperulosidic acid with the exception of the extra glucose moiety (Leticia *et al.*, 1980). The FABMS displayed a peak at *m/z* 597 for $[M + Na]^+$ indicating the existence of compound **18** as sodium salt.

In addition to the previously isolated flavonols (Sabri *et al.*, 1988), quercetin was also obtained from the roots, whereas, the aerial parts furnished quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside (Agrawal, 1989). β-Sitosterol



	R_1	R_2	R_3	R_4	R_5
1	CH_3	H	H	H	H
2	CH_3	H	H	H	OCH_3
3	CH_3	H	OH	H	OCH_3
4	OCH_3	OCH_3	H	H	CH_3
5	OCH_3	OCH_3	H	H	H
6	CH_2-O-C_2H_5	OH	H	H	H
7	COO-C_2H_5	H	H	H	H
8	OCH_3	OH	H	H	CH_3
9	CH_3	H	H	H	OH
10	OCH_3	OH	H	OH	CH_3
11	OH	H	H	H	H
12	CH_2-O-CH_3	OH	H	H	H
13	CH_3	OH	H	H	OH
14	CH_2-OH	O-Glc.	H	H	H
15	OH	OCH_3	H	H	H
16	COO-CH_3	H	H	H	H
17	OCH_3	OH	H	H	H

Compound 18



The isolated compounds

acetate, β-sitosterol, stigmasterol and lupeol were also isolated from the plant.

Results of the Day Chronic toxicity test indicated that, all the tested extracts and anthraquinones were non-toxic to *Daphnia* up to 100 μg/mL/24 hrs. Antimicrobial activities study showed that, the different plant extracts responded positively, but with variable degrees of activities, towards the 12 test organisms used, especially *Staphylococcus aureus*. Moreover, compound **3** exhibited strong antibacterial activity against *Micrococcus Luteus* and *Proteus mirabilis*. Combination of each of the total alcohol extract, compounds **6, 7, 14,** and **16** with antibiotics enhanced their activities against most of the tested microorganisms.

In the preliminary clinical study carried out on 12 strains isolated from the chronic foot ulcers of diabetic patients, each of the alcoholic extract of roots, compounds **14** and **16** when combined with antibiotics, produced pronounced synergistic effect against the different strains. The most sensitive were *Streptococcus pyogenes, Staphylococcus aureus, Pseudomonas aeruginosa, E. coli* and *Proteus vulgaris*. These results appeared to be interesting, since such combination of antibiotics with safe, non-toxic natural products might be useful to overcome problems of resistant strains.

Cytotoxicity tests, carried out on P388 cells (IC_{50} = μg/mL) using compounds **3, 6, 7, 13**, and **14**, were found to be 26, 18, 5.5, 14, and 17 μg/mL respectively. Compound **7** was found to be the most active.

Chemotaxonomical significance – The chemical structures of the anthraquinones isolated from *Crucianella maritima* were found to be closely related to those previously reported for the genera, *Galium, Rubia* as well as *Morinda*, demonstrating the strong chemotaxonomical relationship between these four genera. Moreover, the presence of compound **18**, an iridoid related to asperuloside, previously found in the same three genera (Maya *et al.*, 1996; Briggs and Nicholls, 1954) sustained the above suggestion, which is in accordance with the latest taxonomic classification of the Rubiaceae (Willis, 1988).

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