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A New Naphthoquinone with Anti-inflammatory Activity from An Egyptian Collection of *Echiochilon fruticosum*

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Abstract – Phytochemical investigation of the roots of *Echiochilon fruticosum* resulted in the isolation of two naphthoquinone derivatives. Compound **1** was identified as anhydroalkanin while compound **2** was identified as a new derivative 5-hydroxy 8-methoxy 2-(4-methylpent-1,3-dienyl) naphthalene-1,4-dione named as echiochiloquinone. In addition, caffeic acid **3**, caffeic acid methyl ester **4** were isolated. The structures were determined by physical, chemical and spectral methods. The anti-inflammatory activity of the root extracts and compound **2** was evaluated utilizing both cotton pellet-induced and carragenin-induced rat paw edema. The ulcerogenic effect was also studied.
Keywords – *Echiochilon fruticosum*, Naphthoquinones, Echiochiloquinone, Caffeic acid, Anti-inflammatory, Ulcerogenic

Introduction

Several members of the family Boraginaceae are used in folk medicine as anti-inflammatory, antiseptic, and remedy for wounds (Li, 2002; Roeder, 1995). Antitumor, antimicrobial, anti-inflammatory, contraceptive and hypotriglyceridemic activities were reported for members of the family (Leyva *et al.,* 2000; Karyagina *et al.,* 2001; Kourounakis *et al.,* 2002; Singh *et al.,* 2003; Zhen *et al.,* 2002; Mats *et al.,* 1982). *Echiochilon fruticosum* is distributed in the sandy and stony ground of North Africa, Sinai, Syria and Arabia (Täckholm, 1974). The nutritive value of *E. fruticosum* has been investigated and the possibility of its use as forage for domestic animals were demonstrated (Boshra). A variety of chemical entities has been reported from *E. fruticosum*, including volatile components, Eugenol glycoside, vomifoliol and *trans* syringin. Several flavonoid derivatives such as 5-deoxy vitexin, 5,7-dimethoxy isoflavone, dihydro robinetin, garbanzol, 5,6,7-trihydroxy flavanone, naringenin 5-methyl ether and pincomberin-7-glycoside were also isolated (Hashem, 2003; Bergaoui *et al.,* 2004; Hammami *et al.,* 2004).

Experimental

General – Silica gel (230 - 400 mesh) and C_{18}-silica gel, E. Merck were used for column chromatography. Precoated TLC plates 0.25 mm, silica gel 60 (GF-254), and precoated TLC 0.20 mm, RP18 F_{254}, E. Merck, were used for TLC analysis. Melting points were determined on a Sturat SMP heating stage microscope. 1D and 2D NMR analyses were obtained using jeol 500 MHz spectrometer for ^{1}H and 125 MHz for ^{13}C NMR. Residual peaks of the deuterated solvents were used to reference the spectrum. EIMS was obtained on a Delsi-Nermag R30-10, while electrospray-ionization mass spectra were carried on a Finnigan LCQ. UV-VIS spectra were carried on a Helios α thermo spectronic, England, supported with software Vision 32.

Plant materials – *Echiochilon fruticosum* Desf. was collected from Omayed biosphere, 90 km west Alexandria, Egypt. The plant material was identified by Prof. Dr. Lotfy Boulos, National Research Center, Dokki, Cairo, Egypt. A voucher was kept in the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Egypt.

Extraction and Isolation – The powdered air-dried roots (400 g) were extracted by maceration with light pet. (1 × L 2). The combined extract was evaporated under reduced pressure to give 1.7 g of dark red semisolid residue. The marc left after extraction with light pet. was air dried then macerated in 70% EtOH (1 L × 2) followed by 50% EtOH (1 L × 2). Each extract was evaporated under reduced pressure to yield 3 and 6 g respectively.

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Part of the dried light pet. residue (1.5 g) was fractionated on a silica gel column (50 g, 30 × 2 cm) elution started with light pet.; polarity was gradually increased by CH_2Cl_2 followed by EtOAc. Fractions of 200 ml were collected, evaporated under reduced pressure and screened by TLC. The fraction eluted with 10% CH_2Cl_2 in light pet. (16 mg) was purified on C_{18} silica gel column (25 g, 3.5 × 20 cm) eluted with 95% MeOH to afford **1** (8 mg). Fraction eluted with 25% CH_2Cl_2 in light petroleum (100 mg) was crystallized from benzene to give **2** (80 mg).

The powdered air-dried aerial parts (2.2 Kg) were extracted at room temperature with 70% EtOH (7 L × 2). The combined EtOH extracts were evaporated under reduced pressure to give 62 g of dark green semisolid residue. The extract was dissolved in hot MeOH and allowed to settle to give 1.9 g precipitate. This precipitate was dissolved in CH_2Cl_2 to give 0.9 g CH_2Cl_2 soluble part. The CH_2Cl_2 soluble part was fractionated on a silica gel column. Gradient elution was performed using light pet. with increasing concentrations of EtOAc. Fraction eluted with 10% EtOAc in light pet. (400 mg) yielded 50 mg of compound **3** after crystallization from MeOH. Fraction eluted with 20% EtOAc in light pet. (30 mg) was purified on a silica gel column (15 g, 1 × 20 cm) elution was carried out using CH_2Cl_2 to yield 8 mg of **4**.

Anhydroalkannin (dehydroxy shikonin) 1 – $C_{16}H_{16}O_4$, orange red oil, UV-VIS (MeOH) λ_{max} 264, 283, 489, 510 and 558. ^{1}H NMR and ^{13}C NMR: Table 1. ESIMS *m/z* (rel. int.): 272 (M^+, 100), 203 (50).

5-Hydroxy 8-methoxy 2-(4-methylpent-1,3-dienyl) naphthalene-1,4-dione (Echiochiloquinone) 2 – $C_{17}H_{16}O_4$, orange red crystals, m.p. 158 - 160°C, UV -VIS (MeOH) λ_{max} 300, 510, 539, and 584 nm. ^{1}H NMR and ^{13}C NMR: Table 1. EIMS *m/z* (rel. int.): 284 (M^+, 100), 269 (284 - CH_3) (42), 256 (5), 253 (284 - OCH_3) (21), 225 (18), 203 (5).

Caffeic acid 3 – $C_9H_8O_4$, yellowish white amorphous solid; m.p. 194°C; ^{1}H NMR (δ_H, $CDCl_3$): 7.07 (1H, s, H-3), 6.87 (1H, d, *J* = 8 Hz, H-5), 7.01 (1H, br d, *J* = 8.4 Hz, H-6), 7.57 (1H, d, *J* = 16 Hz, H-7), 6.26 (1H, d, *J* = 15.3 Hz, H-8), 5.8 (1H, br s, OH), 5.7(1H, br s, OH); ^{13}C NMR (δ_c, CD_3OD): 146.3 (C-1), 143.8 (C-2), 115.6 (C-3), 127.7 (C-4), 122.5 (C-5), 115.9 (C-6), 144.8 (C-7), 114.5 (C-8), 167.9 (C-9). EIMS *m/z* (rel. int.): 180 (M^+, 100), 136 (18), 163 (180) (66).

Caffeic acid methyl ester 4 – $C_{10}H_{10}O_4$, white amorphous solid, m.p 159°C. ^{1}H NMR (δ_H, $CDCl_3$): 7.02 (1H, d, *J* = 1.5 Hz, H-3), 7.07, (1H, dd, *J* = 1.5, 7.6 Hz, H-5), 6.90 (1H, d, *J* = 8.4 Hz, H-6), 7.61 (1H, d, *J* = 15.3 Hz, H-7), 6.26 (1H, d, *J* = 16 Hz, H-8), 3.90 (3H,s). EIMS *m/z* (rel. int.): 194 (M^+, 91), 179 (23), 177 (100).

Table 1. ^{1}HNMR and ^{13}CNMR spectral data of **1** and **2** ($CDCl_3$).

	1		**2**	
H/C	δ_H	δ_C	δ_H	δ_C
1		183.2		189.4
2		134.6	6.11 (1H, d, *J* =10.7 Hz)	144.2
3	6.84 (1H, s)	151.6	6.82 (1H, d, *J*= 10.7 Hz)	120.2
4		183.16		183.9
5		162.8		152.1
6	7.20 (2H, s)	131.8	7.41 (1H,s)	125.8
7	7.20 (2H, s)	130.9		140.8
8		162.2		158.9
9		111.8		122.7
10		112.0		113.5
11	2.63 (2H, t, *J* = 7.6 Hz)	26.6	6.72 (1H, d, *J* =16 Hz)	121.6
12	2.31 (2H, q, *J* = 7.6 Hz)	29.8	7.21 (1H, dd, *J* =16, 11 Hz)	132
13	5.1 (1H, m)	129.2	6.87 (1H, d, *J*= 11 Hz)	137.3
14		133.8		141.9
15	1.69 (3H, s)	17.9	1.89 (3H,s)	26.6
16	1.60 (3H, s)	25.8	1.90 (3H,s)	19.0
OCH_3	–	–	3.79 (3H,s)	61.7
OH	12.47 (1H,s)	–	12.48 (1H,s)	–
OH	12.63 (1H,s)	–	–	–

Anti-inflammatory activity

Animals – Adult male Sprague-Dawley rats (120 - 150 g) were used. They were acclimatized one week prior to use and allowed unlimited access to rat chow and water. Prior to the start of experiment, the animals were randomly divided into groups (six rats each).

Cotton pellet- induced granuloma bioassay – Cotton pellet (35 mg ± 1 mg) were impregnated with 0.2 ml solution of the test samples in $CHCl_3$ or acetone (containing 10 μmol) and the solvent was allowed to evaporate. Each cotton pellet was subsequently injected with 0.2 ml of an aqueous solution of antibiotics (1 mg of penicillin G and 1.3 mg of dihydrostreptomycin/ml). Two pellets were implanted subcutaneously, one in each axilla of the rat, under mild general anesthesia. One group of animals has received the standard reference indomethacin (ind.) and the antibiotics at the same doses and serves as positive control. Pellets containing only the antibiotics were similarly implanted in the negative control group. After seven days the animals were sacrificed and the two cotton pellets, with the adhering granulomas, were removed, dried for 48 hours at 60°C and weighed. The increment in dry weight (difference between the initial and final weights) was taken as a measure of granuloma ± SE. This was calculated for each group and the percentage reduction in dry weight of granuloma from control value was also calculated. The ED_{50} values were determined through dose response curve, using doses of 4, 7, 10 and 15 μmol of the test samples (Suleyman *et al.*, 1999) (Table 2).

Carrageenin-induced paw edema in rats – The paw edema was induced by subplantar injection of 50 μl of 2% carrageenan solution in saline (0.9%). A similar volume of sterile saline was injected into the same region of the right hind paw. Ind. and the test materials were dissolved in DMSO and were injected subcutaneously in a dose of 10 μmol/kg body weight, one hour prior to carrageenan injection. DMSO was injected to the control group. Paw volume was determined before each treatment (basal volume) and 2, 4 and 6 h after carrageenin injection using a plethysmometer (Model7150, Ugo Basile, Varese, Italy). The drugs were administered *po* or *ip* at 2 h before the injection of carrageenin (N = 6 for each group) (Winter *et al.*, 1962).

The percentage protection against inflammation was calculated as follows:

$$V_c - V_d / V_c \times 100$$

Where V_c is the increase in paw volume in the absence of test compound (control) and V_d is the increase of paw volume after injection of test compound. Data were expressed as the mean ± S.E. Significant difference between the control and the treated groups was performed using ANOVA test and P values. The difference in results was considered significant when $p < 0.05$. The anti-inflammatory activity of the test compounds relative to that of ind. was also calculated. The results are recorded in Table 3.

Table 2. The anti-inflammatory activity (ED_{50}, μmol) using Cotton pellet- induced granuloma and ulcerogenic effects of the root extracts and **2**.

Test samples	ED_{50}, μmol	% ulceration
Control	–	0.0
Ind.	9.17	100
Light pet.	9.82	0
70% EtOH	9.85	0
50% EtOH	10.8	0
2	9.91	0

Table 3. The anti-inflammatory activity using Carrageenin-induced paw edema in rats of the root extracts and **2**.

Test material	Increase in paw edema (ml) ±SE [1,2]	% Protection	Activity relative to ind.
Control	0.87 ± 0.041	0.0	0.0
Ind.	0.21 ± 0.021	75.9	100
Light pet.	0.24 ± 0.031	72.4	95.5
70% EtOH	0.28 ± 0.030	67.8	89.4
50% EtOH	0.34 ± 0.012	60.9	80.3
2	0.31 ± 0.040	64.4	84.8

[1] SE: standard error.
[2] All data are significantly different from control ($P < 0.001$)

Ulcerogenic effects – Ulcerogenic effect was determined by the reported method of (Komatsu *et al.*, 1973). The tested materials were administered to fasted rats having free access to drinking water. Four hours after administration of the materials, the rats were sacrificed, the stomach was removed and, after incision along the lesser curvature, rinsed with a tap soaked in warm (37°C) saline and spread on a corkboard and pinned down. The mucosa of the glandular part of the stomach was inspected using a binocular microscope (10-fold magnification). The mucosal lesions were evaluated.

Results and Discussion

The molecular formula of compound **1** ($C_{16}H_{16}O_4$, *m/z* 272 EIMS) together with the UV-VIS spectra in methanol and the bathochromic shift after addition of NaOAc indicated a naphthazarin core with a close resemblance to

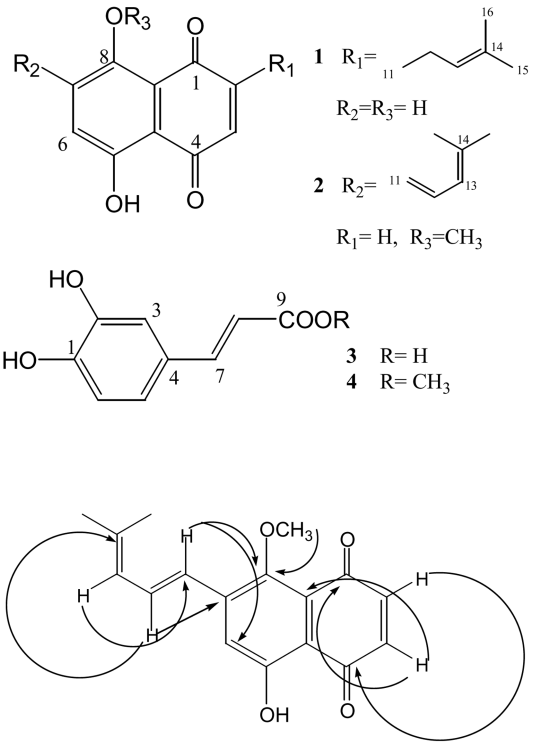

Fig. 1. HMBC correlations of **2.**

alkanin moiety (Khatoon and Mehrota, 2000; Thomson, 1971). In the ^{1}HNMR spectrum of **1**, the two singlets at δ_H 12.63 and δ_H 12.47 were assigned to two free hydroxyl groups at C-5 and C-8. The two singlet at δ_H 7.2 (2H) and δ_H 6.84 (1 H) were assigned to monosubstituted naphthazarin moiety. ^{1}H NMR spectrum also showed signals at δ_H 5.1 (1H, m), 2.63 (2H, t, J= 7.6 Hz), 2.31 (2H, q, J= 7.6 Hz), 1.59 (s, 3H) and 1.69 (s, 3H) assigned for a methylated prenyl side chain. The alkyl substituent was further supported by the presence of a peak at *m/z* 203 (272 - 69) in the MS/MS spectra. The data of **1** were identical with those published for anhydroalkannin (dehydroxy shikonin) previously isolated from *Alkanna hirsutissima*, *Alkanna tinctoria, Macrotemia cephalotes* and *Onosma heterophylla* (Afzal and Tofeeq, 1975; Inoue *et al.,* 1985; Papageorgiou, 1979; Han and Kaishim, 2008).

Compound **2** was isolated as orange rosette. Its UV-VIS spectrum in methanol showed maxima at λ_{max} 300, 510, 539, and 584 nm consistent with a naphthazarin nucleus (Khatoon and Mehrota, 2000; Thomson, 1971). Compared with **1** one of the hydroxyl group signals was replaced by a methoxyl group [δ_H 3.79 (3H, s), δ_C 61.7] in the ^{1}H NMR spectrum of **2**. In the EIMS of **2** the fragments at *m/z* 269 (284 - CH_3) and 253 (284 - OCH_3) were in full support of the methoxyl group. The aromatic signals at δ_H 6.11, 6.82 (each 1H, d, J= 10.7 Hz), and 7.41 (1H, s) were assigned for H-6, H-7, and H-3, respectively. These assignments were supported by HMBC correlation (Fig. 1). The extra carbon and proton signals were assigned to alkyl substituent (Table 1). The 3-bonds HMBC correlations of H-12 at δ_H 7.21 with C-7 at δ_C 140.8; H-11 at δ_H 6.72 with both C-6 at δ_C 125.8 and C-8 at δ_C 158.9 (Fig. 1) unambiguously indicated the attachment of the alkyl moiety to C-7. The $J_{11\text{-}12}$ = 16 Hz indicated their *trans*-orientation. On the other hand the $J_{12\text{-}13}$ = 11 Hz was diagnostic for *cis*-orientation. The M^+ at *m/z* 284 in the EIMS spectrum was in full agreement with the proposed structure. Furthermore, the fragments at 203 (284-81) and 81 were in support for the presence of the alkyl substituent. The above discussion enable the identification of **2** as a new naphthoquinone derivative 5-hydroxy 8-methoxy 2-(4-methylpent-1,3-dienyl)naphthalene-1,4-dione named as echiochiloquinone.

Evaluation of the anti-inflammatory activity of the different extracts and **2** was preformed applying two models of induced inflammations in experimental rats using ind. as a reference standard. In both the Cotton pellet- and carrageenin-induced paw edema all tested materials exhibited moderate anti-inflammatory activity as revealed from their ED_{50} values and percentage protection against inflammation. The light petroleum extract was the most active in the two assays followed by the 70% ethanol extract. The fact that **2** was less active than these two extracts clearly suggest that other active compounds still to be identified in the plant.

The tested extracts and compound **2** were evaluated for their ulcerogenic potential in rats. All the tested materials revealed a superior GI safety profiles (0 - 10% ulceration) in the experimental animals at oral doses of 30 mmol/kg/day, when compared with ind., the reference standard drug, which was found to cause 100% ulceration under the same experimental conditions (Table 2). Gross observation of the isolated rat stomachs showed normal stomach architecture for all compounds.

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