SUPPLEMENTARY MATERIAL

Carbazole-pyranocoumarin conjugate and two carbazole alkaloids from the stems of *Clausena excavata*

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Abstracts

A carbazole-pyranocoumarin conjugate, carbazomarin B (**1**) and two carbazole alkaloids, 6-methoxymukonidine (**2**) and 2-hydroxy-3-methoxycarbazole (**3**), together with 27 known compounds (**4–30**), were isolated from the stems of *Clausena excavata*. Their structures have been elucidated by spectroscopic analyses. Compound **2** showed moderate cytotoxicity to HuCCA-1, MOLT-3 and HepG2 cancer cell lines with IC₅₀ values of 15.09–28.50 µg/mL, but none to A549 cell line. Heptaphylline (**6**) and nordentatin (**23**) were found to show moderate cytotoxic activity against HepG2 cell line with IC₅₀ values of 12.33 and 11.33, respectively, while clausine K (**27**) exhibited strong cytotoxicity with IC₅₀ value of 1.05 µg/mL, better than a standard drug (etoposide, IC₅₀ 13.40 µg/mL).

Keywords: *Clausena excavata*; carbazole alkaloids; Rutaceae; cytotoxic activity

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Experimental

Extraction and Isolation for all pure compounds

Chopped-dried stems of *C. excavata* (4.73 kg) were immersed in CH₂Cl₂ (16 l) at room temperature for 4 days. After evaporation, a dark brown gum of CH₂Cl₂ extract (23.4 g) was subjected to QCC over silica gel and eluted with a gradient of CH₂Cl₂–hexane (1:1, v/v), CH₂Cl₂ and MeOH to furnish 12 fractions (A–L). Fraction B was further purified by CC over silica gel and eluted with CH₂Cl₂–hexane (3:7, v/v) to give 9 fractions (B1–B9). Subfraction B2 was purified by CC over silica gel and eluted with EtOAc–hexane (3:97, v/v) to afford 13 fractions (B2A–B2M). Subfraction B2K was further purified on prep. TLC eluting with EtOAc–CH₂Cl₂–hexane (1:2:17, v/v) to give **4** (murrayafoline A) (3.4 mg). Subfraction B3 was purified by CC over silica gel and eluted with CH₂Cl₂–hexane (3:7, v/v) to afford 12 fractions (B3A–B3L). Subfraction B3D was further purified on prep. TLC eluting with CH₂Cl₂–hexane (3:7, v/v, 2 runs) to give **5** (girinimbine) (2.1 mg). Subfraction B3H was further purified on prep. TLC eluting with EtOAc–hexane (1:24, v/v, 2 runs) to give **6** (heptaphylline) (11.6 mg). Fraction E was further purified by CC over silica gel and eluted with EtOAc–hexane (3:17, v/v) to give 7 fractions (E1–E7). Subfraction E3 was purified by CC over silica gel and eluted with Me₂CO–hexane (3:17, v/v) to afford 5 fractions (E3A–E3E). Subfraction E3A was purified by CC over silica gel and eluted with Me₂CO–hexane (3:17, v/v) to afford 6 fractions (E3A1–E3A6). Subfraction E3A5 gave **7** (dentatin) (16.3 mg). Subfraction E3B was purified by CC over silica gel and eluted with Me₂CO–hexane (3:17, v/v) to afford 7 fractions (E3B1–E3B7). Subfraction E3B7 was purified by CC over silica gel and eluted with Me₂CO–hexane (3:17, v/v) to afford 7 fractions (E3B7A–E3B7G). Subfraction E3B7D gave **8** (mukonidine) (4.8 mg). Subfraction E4 was purified by CC over silica gel and eluted with Me₂CO–hexane (3:17, v/v) to afford 8 fractions (E4A–E4H). Subfraction E4H was purified by CC over silica gel and eluted with Me₂CO–hexane (3:17, v/v) to afford 6 fractions (E4H1–E4H6). Subfraction E4H4 was further purified on prep. TLC and eluted with Me₂CO–hexane (3:17, v/v, 4 runs) to give **9** (xanthoxylatin) (1.2 mg). Subfraction E4H5 was further purified on prep. TLC and eluting with CH₂Cl₂–hexane (4:1, v/v, 4 runs) to give **10** (mukonine) (6.0 mg) and **11** (mukonal) (4.0 mg). Subfraction E6 gave **12** (murrayanine) (62.0 mg). Fraction F was purified by CC over silica gel and eluted with EtOAc–hexane (3:17, v/v) to give 16 fractions (F1–F16). Subfraction F5 gave **2** (79.1 mg). Subfraction F8 was further purified on prep. TLC, eluting with CH₂Cl₂–hexane (4:1, v/v, 4 runs) to give **13** (lansine) (6.4 mg). Subfraction F10 was purified by CC over silica gel and eluted with Me₂CO–hexane (1:4, v/v) to give 5 fractions

(F10A–F10E). Subfraction F10C was purified by CC over silica gel and eluted with Me₂CO–hexane (1:3, v/v) to give 5 fractions (F10C1–F10C5). Subfraction F10C2 was further purified on prep. TLC, eluting with CH₂Cl₂–hexane (9:1, v/v, 3 runs) to give **14** (3-formylcarbazole) (3.1 mg) and **15** (dictamine) (1.1 mg). Subfraction F11 was purified by CC over silica gel and eluted with CH₂Cl₂–hexane (7:3, v/v) to give 6 fractions (F11A–F11F). Subfraction F11B was further purified on prep. TLC, eluting with EtOAc–hexane (1:4, v/v, 5 runs) to give **16** (7-methoxy methyl carbazole-3-carboxylate) (1.3 mg). Subfraction F13 gave **17** (*O*-methylumkonal) (83.8 mg). Fraction G was further purified by CC over silica gel and eluted with EtOAc–hexane (1:4, v/v) to give 23 fractions (G1–G23). Subfraction G12 and G14 were further purified on prep. TLC, eluting with CH₂Cl₂–hexane (17:3, v/v, 4 runs) to give **18** (7-methoxymukonal) (4.5 mg) and **19** (hortiamide) (4.8 mg). Subfraction G20 was purified by CC over silica gel and eluted with CH₂Cl₂–hexane (3:2, v/v) to afford 8 fractions (G20A–G20H). Subfraction G20C gave **3** (1.0 mg). Subfraction G21 was purified by CC over silica gel and eluted with CH₂Cl₂–hexane (4:1, v/v) to afford 14 fractions (G21A–G21N). Subfraction G21L and G21M were further purified on prep. TLC, eluting with CH₂Cl₂–hexane (7:3, v/v, 5 runs) to give **20** (3-formyl-2,7-dimethoxycarbazole) (2.2 mg) and **21** (clausine L) (8.5 mg). Fraction H was further purified by CC over silica gel and eluted with Me₂CO–hexane (1:3, v/v) to give 22 fractions (H1–H22). Subfraction H4 was purified by CC over silica gel and eluted with Me₂CO–hexane (3:17, v/v) to afford 4 fractions (H4A–H4D). Subfraction H4B was separated by CC with Sephadex LH–20, eluted with CH₂Cl₂ to afford 6 fractions (H4B1–H4B6). Subfraction H4B4 was further purified on prep. TLC, eluting with Me₂CO–hexane (1:9, v/v, 8 runs) to give **22** (kinocoumarin) (1.3 mg) and **23** (nordentatin) (1.3 mg). Subfraction H5 gave **24** (7-hydroxy-8-(1,1-dimethylallyl)citrusarin) (76.8 mg). Subfraction H8 was purified by CC over silica gel and eluted with EtOAc–hexane (3:7, v/v) to afford 8 fractions (H8A–H8H). Subfraction H8E was further purified on prep. TLC, eluting with EtOAc–hexane (3:7, v/v, 3 runs) to give **1** (2.0 mg). Subfraction H12 gave **25** (clausine H) (20.0 mg). Subfraction H22 was further purified on prep. TLC, eluting with Me₂CO–hexane (3:7, v/v, 4 runs) to give **26** (isomukonidine) (0.6 mg) and **27** (clausine K) (1.1 mg). Fraction I was purified by CC over silica gel and eluted with Me₂CO–hexane (1:3, v/v) to afford 15 fractions (I1–I15). Subfraction I11 was purified by CC over silica gel and eluted with EtOAc–hexane (3:7, v/v) to afford 13 fractions (I11A–I11M). Subfraction I11F was further purified on prep. TLC, eluting with EtOAc–hexane (3:7, v/v, 5 runs) to give **28** (valencic acid) (4.5 mg). Fraction J was purified by CC over silica gel and eluted with

Me₂CO–hexane (3:7, v/v) to afford **29** (clausenarin) (799.3 mg). Fraction K was purified by CC over silica gel and eluted with Me₂CO–hexane (3:7, v/v) to afford **30** (*O*-methylclausenolide) (92.4 mg).

Figure S1. Selected HMBC correlation (H → C) of compounds **1–3**.

Table S1. NMR Spectroscopic Data in CDCl₃ (¹H NMR (300 MHz) and ¹³C NMR (125 MHz)) for Compound **1**.

Carbazole Unit			Pyranocoumarin Unit		
position	δ _C , type	δ _H , (<i>J</i> in Hz)	position	δ _C , type	δ _H , (<i>J</i> in Hz)
1a	137.3, C		2'	161.2, C	
1	107.2, C		3'	110.7, CH	6.06, d (9.7)
2	148.5, C		4'	139.2, CH	7.84, d (9.7)
3	114.3, C		4'a	103.9, C	
4	120.9, CH	7.70, s	5'	150.3, C	
4a	118.6, C		6'	109.8, C	
5a	123.4, C		7'	156.3, C	
5	102.7, CH	7.38, d (2.6)	8'	116.5, C	
6	154.0, C		8'a	152.9, C	
7	113.9, CH	6.95, dd (8.8, 2.6)	2''	77.7, C	
8	111.2, CH	7.14, d (8.8)	3''	38.5, CH ₂	2.34, dd (14.3, 9.4)
8a	134.3, C				2.28, dd (14.3, 9.6)
3-CH ₃	16.4, CH ₃	2.49, s	4''	24.9, CH	5.07, dd (9.6, 9.4)
6-OCH ₃	56.1, CH ₃	3.88, s	5''	23.4, CH ₃	1.36, s
NH		7.26, (br s)	6''	29.4, CH ₃	1.58, s
			1'''	41.3, C	
			2'''	150.6, CH	6.40, dd (17.6, 10.3)
			3'''	108.3, CH ₂	5.00, d (10.3, 1.2)
					5.01, d (17.6, 1.2)
			4'''	29.9, CH ₃	1.76, s
			5'''	30.0, CH ₃	1.79, s

Table S2. NMR Spectroscopic Data in CD₃COCD₃ (¹H NMR (300 MHz) and ¹³C NMR (75 MHz)) for Compounds **2** and **3**.

Compound 2			Compound 3		
position	δ _C , type	δ _H , (<i>J</i> in Hz)	position	δ _C , type	δ _H , (<i>J</i> in Hz)
1a	146.2, C		1a	141.1, C	
1	96.6, CH	6.89, s	1	94.2, CH	7.09, s
2	160.5, C		2	134.4, C	
3	104.6, C		3	147.8, C	
4	122.7, CH	8.59, s	4	104.8, CH	7.51, s
4a	117.1, C		4a	115.8, C	
5a	124.0, C		5a	123.5, C	
5	103.0, CH	7.69, d (2.4)	5	119.1, CH	7.93, d (8.0)
6	154.6, C		6	118.2, CH	7.07, td (8.0, 1.2)
7	114.3, CH	6.99, dd (8.7, 2.4)	7	123.7, CH	7.24, td (8.0, 1.2)
8	111.4, CH	7.36, d (8.7)	8	110.4, CH	7.40, d (8.0)
8a	130.5, C		8a	140.2, C	
NH		10.37, br s	NH		10.01, br s
2-OH		11.05, br s	2-OH		7.14, s
3-CO ₂ CH ₃	171.3, C		3-OCH ₃	55.6, CH ₃	3.93, s
3-CO ₂ CH ₃	51.6, CH ₃	3.99, s			
6-OCH ₃	55.2, CH ₃	3.89, s			

Table S3. Cytotoxic activities (IC₅₀, µg/mL, mean ± SD, n = 3) of isolated compounds.

Compounds	Cytotoxic activity ^a			
	HuCCA-1	A549	MOLT-3	HepG2
2	22.60±1.98	>50	15.09±0.88	28.50±2.12
6	14.80±3.33	19.40±2.26	1.99±0.20	12.33±2.52
7	>50	>50	7.82±0.79	26.50±1.29
8	>50	>50	>25	>50
9	>50	>50	31.36±3.34	>50
11	44.18±3.29	46.00±5.66	7.27±0.25	34.00±4.36
12	37.60±0.28	48.40±2.26	9.20±0.33	28.75±3.50
17	26.80±7.35	15.90±4.36	3.96±0.28	20.25±6.50
18	17.20±4.53	>50	3.54±0.14	26.75±6.99
20	>50	>50	2.81±0.17	>50
21	32.50±0.85	30.90±2.69	13.52±1.99	19.00±1.00
22	17.90±8.49	11.70±0.25	7.49±0.42	16.33±4.04
23	10.85±0.85	17.50±9.32	3.34±0.06	11.33±0.58
24	>50	27.12±3.71	8.74±0.31	15.00±2.65
25	>50	>50	31.05±2.11	29.25±6.99
27	12.45±0.21	13.20±0.99	6.00±0.79	1.05±0.83
29	>50	>50	>50	>50
30	>50	>50	43.99±3.97	>50
Doxorubicin ^b	0.23±0.08	0.13±0.08	ND	0.26±0.08
Etoposide ^b	ND	ND	0.022±0.005	13.40±1.52

^a Cytotoxicity was tested against the following cell lines: MOLT-3 = T-lymphoblast (acute lymphoblastic leukemia) cell line; A549 = human lung carcinoma cell line; HuCCA-1 = human cholangiocarcinoma cancer cells, and HepG2 = Human hepatocellular liver carcinoma cell line.

^b Etoposide and doxorubicin were used as the standard drugs.

Figure S2. ^1H NMR spectrum of carbazomarin B (**1**) (300 MHz, CDCl_3).

Figure S3. ^{13}C NMR spectrum of carbazomarin B (**1**) (125 MHz, CDCl_3).

Figure S4. ¹H NMR spectrum of 6-methoxymukonidine (**2**) (300 MHz, CD₃COCD₃).

Figure S5. ¹³C NMR spectrum of 6-methoxymukonidine (**2**) (75 MHz, CD₃COCD₃).

Figure S6. ¹H NMR spectrum of 2-hydroxy-3-methoxycarbazole (**3**) (300 MHz, CD₃COCD₃).

Figure S7. ¹³C NMR spectrum of 2-hydroxy-3-methoxycarbazole (**3**) (75 MHz, CD₃COCD₃).