Indian Journal of Chemistry
Vol. 56B, December 2017, pp 1251-1255

1,6-Dihydroxy-3-methyl-9,10-anthraquinone: An anti-cancerous natural pigment from *Cassia sophera* Linn. (Caesalpiniaceae)

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Received 23 February 2017; accepted (revised) 4 September 2017

A biologically relevant natural pigment, 1,6-dihydroxy-3-methyl-9,10-anthraquinone **1**, widely produced by fungus from *Phoma* genus, has been isolated from *Cassia sophera* Linn. (Caesalpiniaceae) roots and subjected to detailed spectral studies, including 1D- and 2D-NMR. The compound **1** inhibits the proliferation of several cancer cell lines at different extents, showing minor effects on the growth of non-tumorogenic keratinocyte cells.

Keywords: 1,6-Dihydroxy-3-methyl-9,10-anthraquinone, *Cassia sophera*, spectral studies, anti-cancer activity

Cassia sophera Linn. (Caesalpiniaceae)[1-3], locally known as *Kulkasunda*, is used widely in traditional Indian medicine for the treatment of diseases such as asthma, allergy, inflammation, pain, arthritis, liver-infections, diabetes, and convulsions[4-8]. In continuation to our work for the search of new biologically active natural products[9-23], we unearthed the occurrence of an anthraquinone derivative in roots of *C. sophera*. Such plant-isolated natural product was determined to be 1,6-dihydroxy-3-methyl-9,10-anthraquinone **1** (Figure 1), from detailed spectral studies, which included 1D- and 2D-NMR analyses. Compound **1** was evaluated for the potential to inhibit cancer cells proliferation *in vitro*. Anthraquinone derivatives, both natural and synthetic, are considered as promising lead candidates in drug discovery program since they exhibit a wide range of biological and pharmacological properties that include antifungal, antimicrobial, antitumor, anti-plasmodium, and many more[24-31].

Results and Discussion

The 1,6-dihydroxy-3-methyl-9,10-anthraquinone **1** was obtained from *C. sophera* roots as an orange amorphous powder (m.p. 257-259°C) and molecular formula of $C_{15}H_{10}O_4$, deduced from elemental and HR-TOF-MS ($[M + Na]^+$, 277.0469) analyses. This anthraquinone derivative is known to be widely produced by fungi from the *Phoma* genus[32]. To the best of our knowledge, there is no evidence of the production of anthraquinone **1** in *C. sophera* tissues. Compound **1** exhibited UV-Vis peaks of maximum absorption at λ_{max} 232, 255, 279, 287 and 430 nm typical of substances that bear a hydroxy-9, 10-anthraquinone nucleus[33-35]. The IR spectrum of **1** showed important absorption peaks at 3437-3198 cm^{-1} (chelated OH), 3082-3045 cm^{-1} (Ar-H stretching), 2962-2918 cm^{-1} (aliphatic C-H stretching), 1674 cm^{-1} (α,β-unsaturated carbonyl) and 1628, 1620, 1614, 1603, 1483, 1456 cm^{-1} (aromatic unsaturation), thereby indicating the presence of methyl-substituted hydroxy-9,10-anthraquinone moiety in its structure[35-37]. Based on the IR bands at 1674 cm^{-1} (for carbonyl group) and 1628-1456 cm^{-1} regions (for aromatic core), one hydroxyl group is at α-position[38], while the other is at β-position[39]. Both hydroxyl groups are unlikely in the same aromatic ring, as no

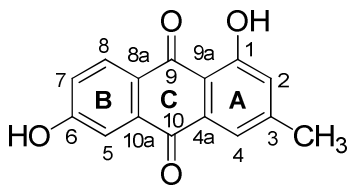

Figure 1 — Structure of 1,6-dihydroxy-3-methyl-9,10-anthraquinone **1**

doublets were observed in the ^{1}H NMR spectrum for the protons attached to each –OH and also no *ortho*-splitting was observed for the two protons attached to this aromatic ring; besides, the whole aromatic region would show no more than one singlet absorption in case of 1,2-dihydroxyl substitution pattern. This clearly indicates the substitution pattern of rings A and B in the structure of compound **1**. The ^{1}H NMR spectrum displayed signals at (i) δ 12.04 (s, 1H) and 11.93 (s, 1H) due to the two phenolic –OH at C-1 and C-6; (ii) δ 7.57 (s, 1H) and 7.02 (s, 1H) attributed to two protons at C-2 and C-4 of the aromatic ring-A ; (iii) δ 7.19 (s, 1H), 7.60 (d, J = 8.4 Hz, 1H) and 7.74 (d, J = 7.6 Hz, 1H) for the three ring-B aromatic protons attached to C-5, C-7 and C-8, and (iv) δ 2.39 (s, 3H) for the methyl group at C-3. As expected, the ^{13}C NMR spectrum of the anthraquinone derivative **1** recorded signals for 15 carbons, in which their nature was determined from DEPT-135 measurements. These ^{1}H- and ^{13}C NMR spectral data for **1** (shown with complete assignments in Table I) obtained from the high-resolution NMR spectrophotometer are fully in agreement with those reported elsewhere[35-37,40].

For further and thorough analysis of the proposed structure for 1,6-dihydroxy-3-methyl-9,10-anthraquinone **1**, we performed its detailed 2D-NMR (^{1}H-^{1}H-COSY-45, HMQC and HMBC) studies. All respective homo- and hetero-nuclear interactions are shown in Figure 2 and the results are summarized in Table I. As expected, ^{1}H-^{1}H-COSY-45 spectrum of **1** showed the interactions of H-7 (δ 7.60) with H-8 (δ 7.74) and H-8 (δ 7.74) with H-7 (δ 7.60). The HMQC spectrum also demonstrated the expected ^{1}H-^{13}C correlations between carbon atoms and the protons directly attached to them. Thus, H-2 (δ 7.57) correlates with C-2 (δ 121.30), H-4 (δ 7.02) with C-4 (δ 124.30), H-5 (δ 7.19) with C-5 (δ 124.49), H-7 (δ 7.60) with C-7 (δ 136.85), H-8 (δ 7.74) with C-8 (δ 119.87) and the methyl protons (δ 2.39) at C-3 with the methyl carbon at δ 22.21. The results from the hetero-nuclear multiple bond correlation (HMBC) spectral studies unambiguously confirmed the structure

Table I — 1D and 2D-NMR data for 1,6-dihydroxy-3-methyl-9,10-anthraquinone **1**

Carbon	^{1}H (δ, ppm)	^{13}C (δ, ppm)	DEPT-135	^{1}H-^{1}H COSY-45	^{1}H-^{13}C HMQC	^{1}H-^{13}C HMBC
C-1	—	162.67	C	—	—	—
C-2	7.57 (s, 1H, Ar-H)	121.30	CH	—	δ 7.57 (H-2) *vs* δ 121.30 (C-2)	δ 7.57 (H-2) *vs* δ 22.21 (C_3-CH_3), 113.69 (C-3), 124.30 (C-4), 181.92 (C-9)
C-3	—	113.69	C	—	—	—
C-4	7.02 (s, 1H, Ar-H)	124.30	CH	—	δ 7.02 (H-4) *vs* δ 124.30 (C-4)	δ 7.02 (H-4) *vs* δ 22.21 (C_3-CH_3), 121.30 (C-2)
C-5	7.19 (s, 1H, Ar-H)	124.49	CH	—	δ 7.19 (H-5) *vs* δ 124.49 (C-5)	δ 7.19 (H-5) *vs* δ 119.87 (C-8)
C-6	—	162.37	C	—	—	—
C-7	7.60 (d, 1H, J = 8.4 Hz, Ar-H)	136.85	CH	H-7 (δ 7.60) *vs* H-8 (δ 7.74)	δ 7.60 (H-7) *vs* δ 136.85 (C-7)	δ 7.60 (H-7) *vs* δ 133.60 (C-8a), 162.37 (C-6)
C-8	7.74 (d, 1H, J = 7.6 Hz, Ar-H)	119.87	CH	H-8 (δ 7.74) *vs* H-7 (δ 7.60)	δ 7.74 (H-8) *vs* δ 119.87 (C-8)	δ 7.74 (H-8) *vs* δ 115.82 (C-10a), 124.49 (C-5), 181.92 (C-9)
C-9	—	181.92	C	—	—	—
C-10	—	193.48	C	—	—	—
C-4a	—	149.29	C	—	—	—
C-8a	—	133.60	C	—	—	—
C-9a	—	133.23	C	—	—	—
C-10a	—	115.82	C	—	—	—
C_3-CH_3	2.39 (s, 3H, Ar-CH_3)	22.21	CH_3		δ 2.39 (CH_3) *vs* δ 22.21 (*C*H_3)	—
C_1-OH	11.93 (s, 1H, Ar-OH)	—	—	—	—	δ 11.93 (C_1-O*H*) *vs* δ 113.69 (C-3), 124.30 (C-4), 162.67 (C-1)
C_6-OH	12.04 (s, 1H, Ar-OH)	—	—	—	—	δ 12.04 (C_6-O*H*) *vs* δ 115.82 (C-10a), 124.49 (C-5), 162.37 (C-6)

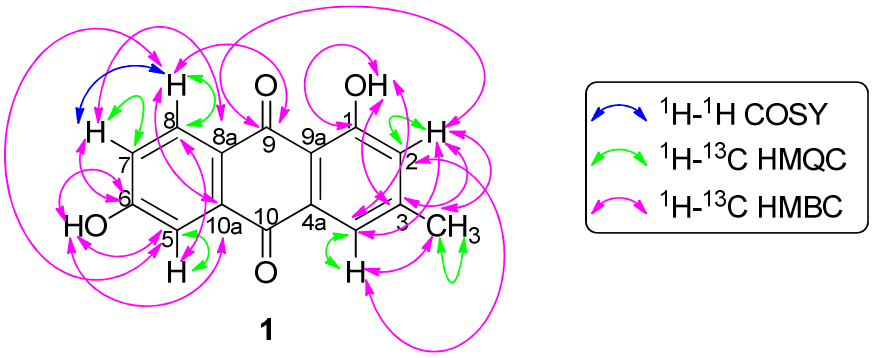

Figure 2 — ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMQC, and ^{1}H–^{13}C HMBC interactions for **1** (COSY, correlation spectroscopy; HMQC, hetero-nuclear multiple quantum coherence; HMBC, hetero-nuclear multiple bond correlation)

of molecule **1**. In the HMBC spectrum, H-2 at δ 7.57 showed interactions with C-3 methyl carbon at δ 22.21, C-3 at δ 113.69, C-4 at δ 124.30, and C-9 at δ 181.92, while H-4 (δ 7.02) exhibited such interactions with C-3 methyl and C-2 carbons at δ 22.21 and δ 121.30, respectively. Among the remaining three aromatic protons, H-5 (δ 7.19) suffered from long-range interaction with C-8 (δ 119.87), H-7 (δ 7.60) showed such interactions with C-8a (δ 133.60) and C-6 (δ 162.37), while H-8 (δ 7.74) recorded the HMBC correlations with C-10a (δ 115.82), C-5 (δ 124.49) and C-9 (δ 181.92). The C_1-hydroxyl proton at δ 11.93 showed expected HMBC interactions with C-1 (δ 162.67), C-3 (δ 113.69) and C-4 (δ 124.30); similarly, C_6- hydroxyl proton at δ 12.04 experienced HMBCs with C-5, C-6 and C-10a, at δ 124.49, 162.37 and 115.82, respectively. All these observed HMBC interactions, as depicted in Figure 2, are compatible with the structure proposed for **1**. Based on detailed 1D- and 2D-NMR spectral analyses, the proposed structure for compound **1** has herein been confirmed as 1,6-dihydroxy-3-methyl-9,10-anthraquinone.

A series of cancer cell lines, such as U251 (glioma), MCF-7 (breast), NCI-ADR/RES (multiple drugs-resistant ovarian), 786-0 (renal), NCI-H460 (lung, non-small cells) and HT-29 (colon), were then used to assess the potential anti-proliferative activity of anthraquinone **1** (Table II). The U251 cancer cell line was the most affected by **1**, regardless of the concentration used, while the growth of non-tumorogenic cells was marginally affected (less than 10%) by this natural product at concentrations equal or lower than 2.5 µg/mL (Table II). With exception of NCI-H460 cancer cells, compound **1** affected the growth of cancer cells at different extents exhibiting a cytostatic effect. Although anthraquinone **1** was not as effective as doxorubicin (used as the reference drug) on the cancer cell lines studied, the latter was not selective to cancer cells as it showed high toxicity to non-tumorogenic ones.

Table II — Percentage of growth inhibition of cancer and non-tumorogenic cells triggered by anthraquinone **1**

Entry	Cell line	Compd **1** (µg/mL)				Doxorubicin (µg/mL)			
		0.25	2.5	25	250	0.25	2.5	25	250
1	U251	17	32	34	34	59	–4	–35	–35
2	MCF-7	5	10	25	32	54	90	–3	–15
3	NCI/ADR-RES	11	13	21	28	16	51	97	–15
4	786-0	10	11	15	22	49	100	100	–52
5	NCI-H460	0	0	12	31	68	91	93	97
6	HT-29	11	14	14	17	27	70	91	92
7	HaCaT	7	9	18	28	46	75	100	–16

[a] Positive values stand for cytostatic activity while negative values indicate cytotoxic activity. The human cancer cell lines tested were glioma (U251), breast (MCF-7), multiple drugs-resistant ovarian (NCI-ADR/RES), renal (786-0), lung, non-small cells (NCI-H460) and colon (HT-29). Human keratinocyte cells (HaCaT line) were used as a reference of non-tumorogenic cell and doxorubicin was employed as a reference drug.

In conclusion, a natural anthraquinone (here referred to as **1**) was successfully isolated for the first time from *Cassia sophera* (Caesalpiniaceae) roots and its structure was unequivocally determined as 1,6-dihydroxy-3-methyl-9,10-anthraquinone from 1D- and 2D-NMR analyses. The potential to inhibit the proliferation of some cancer cell lines indicates that the anthraquinone **1** possesses an important structural motif that makes this plant natural product a lead compound in drug discovery programs[31,41].

Experimental Section

Chemicals and instrumentation

Chemicals used in this study were of analytical grade, and the solvents were dried before use following methods reported in the literature[42].

Melting points were recorded on a Chemiline CL-726 apparatus and are uncorrected. The infrared spectra were recorded on an FT-IR-8400S using KBr disc. The ^{1}H, ^{13}C and 2D-NMR spectra were obtained at 400 MHz and 100 MHz, respectively, on a Bruker DRX spectrometer using $CDCl_3$ as solvent. TMS was used as internal standard in the NMR measurements. Mass spectra (TOF-MS) were recorded on a QTOF Micro mass spectrometer. Elemental analyses were performed on an Elementar Vario EL III Carlo Erba 1108 microanalyzer instrument. Chromatography was carried on silica gel flash columns (Merck 60–120 mesh) and TLC was performed on silica gel 60 F254 (Merck) plates.

Plant materials

Cassia sophera Linn. (Caesalpiniaceae) plants were harvested during October-November, 2013 in the vicinity of Santiniketan, West Bengal, India. The plant material was identified by Dr. H. R. Chowdhury (Botany Department, Visva-Bharati University) and a voucher specimen (V/AM/7/2013) kept in the Laboratory of Natural Products and Organic Synthesis of this University.

Extraction and isolation of 1,6-dihydroxy-3-methyl-9,10-anthraquinone, 1

Air-dried, defatted and finely ground roots of *C. sophera* (1.5 kg) were extracted with ethyl acetate in a Soxhlet apparatus for about 70 h; the ethyl acetate extract (~4.5 L) was then concentrated under reduced pressure in a rotary evaporator to yield a greenish semi-solid mass (35 g). This reduced mass was subjected to column chromatography on silica gel (60-120 mesh, 400 g) using solvents of varying polarity; petroleum ether:ethyl acetate (97:3 *v*/*v*) eluent afforded 1,6-dihydroxy-3-methyl-9,10-anthraquinone (**1**; 90 mg; 0.006%) as an orange amorphous solid, R_f value: 0.92 (petroleum ether:ethyl acetate = 1:3), m.p. 257-259°C ($C_{15}H_{10}O_4$ requires C, 70.86; H, 3.96. Found: C, 70.81; H, 3.98). UV, FT-IR, ^{1}H NMR (400 MHz, $CDCl_3$), ^{13}C NMR (100 MHz, $CDCl_3$), DEPT-135, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMQC, and ^{1}H–^{13}C HMBC data are described in the text (also in Table I); HR-TOF-MS: *m/z* 277.0469 ($C_{15}H_{10}O_4Na$, $[M + Na]^+$ requires 277.0477). Anal. Calcd for $C_{15}H_{10}O_4$: C, 70.86; H, 3.96. Found: C, 70.81; H, 3.98%.

Antiproliferative assay

The human tumor cell lines U251 (glioma), MCF-7 (breast), NCI-ADR/RES (multiple drugs-resistant ovarian), 786-0 (renal), NCI-H460 (lung, non-small cells) and HT-29 (colon) were kindly provided by Frederick Cancer Research and Development Center - National Cancer Institute – Frederick, MD, USA. The human keratinocyte cell line HaCaT was kindly donated by Dr. Ricardo Della Coletta (FOP, UNICAMP, Piracicaba, SP, Brazil). Stock cultures were grown in RPMI 1640 (GIBCO BRL, Life Technologies) supplemented with 5% fetal bovine serum, 1 mg/mL penicillin and 200 U/mL streptomycin[43-45]. Cells in 96-well plates (100 µL cells/well) were exposed to the anthraquinone **1** (0.25 to 250 µg/mL) for 48 h at 37°C and 5% CO_2. Afterwards, cells were fixed with 50% trichloroacetic acid, assayed with sulforhodamine B and analyzed at 540 nm for determining cell proliferation[46]. Doxorubicin was used as a reference drug. Results presented are from two independent experiments, each done in triplicate.

Acknowledgement

GB is thankful to the Council of Scientific and Industrial Research, New Delhi for providing financial grant [No. 02(0260)/2016/EMRII].

Supplementary Information

Scanned copies of 1D- and 2D-NMR spectra of the anthraquinone derivative **1** are supplemented as Supplementary Information Electronic File.

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