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Cytotoxic naphthoquinones from *Diospyros fleuryana* leaves

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ABSTRACT

In the search for anti-cancer plants in Vietnam, the leaves of *Diospyros fleuryana* were selected for chemical investigation. Phytochemical analysis of the ethyl acetate (EtOAc) extract led to the isolation of two naphthoquinones isodiospyrin,[1] and 8'-hydroxyisodiospyrin,[2] and one isoflavone 7-*O*-methylbiochanin A.[3] The chemical structures of isolated compounds were determined by 1D-NMR (^{1}H, and ^{13}C-NMR), 2D-NMR spectra (HSQC, and HMBC), and MS spectroscopy. Compound 3 was isolated from genus *Diospyros* for the first time. Regarding the strong IC_{50} values of 2.27, and 8.0 μM against KB, and Hep cell lines respectively, cytotoxic examination suggested that compound 2 is a promising agent in anti-cancer treatment.

Keywords: *Diospyros fleuryana*; naphthoquinone; isoflavone, cytotoxicity

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INTRODUCTION

Genus *Diospyros* belongs to the family Ebanaceae, comprising of about 500 species and widely distributed in tropical and subtropical regions.[1] As a rich resource of active compositions, the plants of this genus are always seeking in both phytochemical and pharmacological aspects. A diversity of isolated metabolites such as polyphenols, terpenoids, hydrocarbons, lipids, benzopyrones, especially naphthoquinones has been reported.[2] In the meantime, *in-vitro*, *in-vivo*, and clinical studies dealt with the uses of *Diospyros* extracts, as well as their isolated compounds in treating anti-oxidant, anti-diabetic, anti-bacterial, anti-oxidant, anti-hypertensive, anti-inflammatory, cosmeceutical, enzyme-inhibitory, cardioprotective and neuroprotective activities.[2]

In Vietnam, *D. fleuryana* species, also known as Thi Dai La Rong, is now available from Thanhhoa to Nhatrang, and Tayninh.[3] To the best of our knowledge, there have not been studies on chemical composition and biological activity for *D. fleuryana* species. With the deepest aim to search for potentially bioactive compounds from Vietnamese plants,[4-12] we herein reported the chromatographic isolation, and structural elucidation of two naphthoquinones 1-2, and one isoflavone 3, together with their cytotoxic assay on four cancer lines KB, Hep, Lu, and MCF7.

RESULT AND DISCUSSION

From EtOAc extract of leaves, two naphthoquinones 1-2, and one isoflavone 3 were isolated from *D. fleuryana* species for the first time, and their chemical structures are discussed in detail.

Compound 1 was isolated as a yellow amorphous powder. The molecular formula of 1 was determined to be $C_{22}H_{14}O_6$, based on the quasi-molecular ion peak observed at *m/z* 375.0 $[M+H]^+$ in the positive ESI-MS spectrum. The ^{1}HNMR spectrum of compound 1 showed characteristics of a dimeric naphthoquinones (Table 1), in which each monomeric part included 3 proton signals [2 doublet protons H-2 (d_H 6.94, 10.5 Hz) and H-3 (d_H 6.92, 10.5 Hz), and 1 singlet proton H-8 (d_H 7.61); 2 doublet protons H-2' (d_H 6.72, 10.5 Hz) and H-3' (d_H 6.94, 10.5 Hz), and 1 singlet proton H-6' (d_H 7.30)]. The ^{1}HNMR spectrum in $CDCl_3$ was also composed of 2 methyl groups [7-CH_3 (d_H 2.01, s) and 7'-CH_3 (d_H 2.03, s)] in upfield, and 2 hydroxy groups [5-OH (d_H 12.05, s) and 5'-OH (d_H 12.43, s)] in downfield. The ^{13}CNMR spectrum of compound 1 featured 22 carbon signals assignable to 2 methyl carbons (d_C 20.4 and 20.6 ppm) 6 methine carbons (d_C 121.4-140.1), 10 aromatic quaternary carbons (d_C 113.2-162.0), and 4 carbonyl carbons (d_C 184.4-190.3). The chemical structure of compound 1 was further confirmed by 2D-NMR spectroscopies (HSQC and HMBC). As shown in

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Table 1 ^{1}H (500 MHz) and ^{13}C-NMR (125 MHz) data of compounds 1-2 in $CDCl_3$

Position	Compound 1		Compound 2	
	d_C	d_H (*J* in Hz)	d_C	d_H (*J* in Hz)
1	184.4		184.9	
1'	184.9		186.6	
2	139.6	6.94 (d, 10.5)	139.7	6.77 (d, 10.0)
2'	140.1	6.72 (d, 10.5)	147.3	
3	137.7	6.92 (d, 10.5)	138.0	6.94 (d, 10.0)
3'	138.8	6.94 (d, 10.5)	143.0	
4	190.1		190.1	
4'	190.3		186.1	
5	158.6		162.1	
5'	162.0		158.9	
6	135.1		125.7	7.30 (overlap)
6'	125.7	7.30 (s)	129.7	7.28 (overlap)
7	145.5		146.8	
7'	148.2		129.7	7.28 (overlap)
8	121.4	7.61 (s)	127.1	
8'	130.3		158.8	
9	128.6		129.2	
9'	128.8		112.2	
10	113.2		114.0	
10'	114.2		112.1	
7-CH_3	20.4	2.01 (s)	20.6	2.19 (s)
7'-CH_3	20.6	2.03 (s)	13.4	1.86 (s)
5-OH		12.05 (s)		12.34 (s)
5'-OH		12.43 (s)		12.31 (s)
8'-OH				12.66 (s)

Table 2 ^{1}H (500 MHz) and ^{13}C-NMR (125 MHz) data of compound 3 in CD_3OD

Position	Compound 3	
	δc	d_H (*J* in Hz)
1		
2	155.0	8.11 (s)
3	124.5	
4	182.2	
5	161.3	
6	100.2	6.24 (d, 2.0 Hz)
7	166.0	
7-OCH_3	56.1	3.91 (s)
8	94.8	6.37 (d, 2.0 Hz)
9	156.7	
10	105.6	
1'	124.6	
2', 6'	131.3	7.50 (d, 9.0 Hz)

Figure 1, naphtholquinone nuclei were established due to the key HMBC correlations H-3/C-1, C-2, C-4, and C-10, H-6/C-8, and C-10; H-2'/C-4', C-9', and C-10', and H-3'/C-1', and C-3', H-6'/C-8', and C-10'. Hydroxy groups located at carbons C-5 and C-5' because of HMBC correlations 5-OH/C-5, C-6, and C-10; 5'-OH/C-5', C-6', and C-10'. HMBC correlations 7-CH_3/C-7, and 7'-CH_3/C-7' allowed to determine the position of 2 methyl groups at carbons C-7, and C-7', respectively. Finally, the key HMBC cross peak between 7'-CH_3/C-6 indicated that two monomeric units connected through C-6–C-8' bridge. From the above findings and comparison with literature, compound 1 was to be a dimeric naphtholquinone, named isodiospyrin.[13]

Compound 2 was obtained as a red amorphous powder. Its molecular formula was found to be $C_{22}H_{14}O_7$ from the positive ESIMS quasi-molecular ion peak at *m/z* 391.0 $[M+H]^+$. The ^{1}HNMR spectrum of compound 2 (in $CDCl_3$) revealed the similar pattern of compound 1, with remarkable resonance signals of naphthoquinone nuclei at [H-2 (d_H 6.77, d, 10.0 Hz), H-3 (d_H 6.94, d, 10.0 Hz), and H-6 (d_H 7.30, overlap); H-6' and H-7' (d_H 7.28, overlap)], hydroxyl singlet signals at 5-OH (d_H 12.34), 5'-OH (d_H 12.31), and 8'-OH (d_H 12.66), methyl singlet signals at 7-CH_3 (d_H 2.19), and 2'-CH_3 (d_H 1.86). The ^{13}CNMR spectrum of compound 2 contained 22 carbon signals, including 2 methyl carbons, 5 methine carbons, 11 aromatic quaternary carbons, and 4 carbonyl carbons (Table 1). The chemical structure of compound 2 was also determined by 2D-NMR spectra (HSQC and HMBC). Naphthoquinone nuclei have associated with the key HMBC correlations H-2/C-3, and C-4, H-3/C-1, and C-2, H-6/C-8, and C-10, H-6'/C-8', and H-7'/C-5'; three hydroxy groups substituted at carbons C-5, C-5', and C-8' with the important HMBC cross peaks H-5/C-5, C-6, and C-10, H-5'/C-5', C-6', and C-10', and H-8'/C-7', C-8', and C-9'. Moreover, HMBC evidence H-7/C-6, C-7, and C-8, and H-2'/C-1', C-2', and C-3' implied that methyl groups located at carbons C-7, and C-2'. From these results and a comparison with a previous report,[14] the structure of compound 2 was assigned to be 8'-hydroxyisodiospyrin.

Compound 3 was precipitated out of EtOAc extract of *D. fleuryana* leaves as a yellow amorphous powder. The ^{1}H-NMR was the characteristics of isoflavone, in which methine proton H-2 was found to be associated with a singlet signal at δ_H 8.11, two double signals resonating at δ_H 6.24, and δ_H 6.37 were assigned to aromatic methine protons H-6, and H-8, respectively. Two methoxy group 7-OCH_3, and 4'-OCH_3 gave singlet signals at 3.91, and 3.85 ppm, respectively. Symmetric phenyl unit (ring B of flavone) was found to appear at δ_H 7.50 (2H, d,

Table 2 Continued

Position	Compound 3	
	δc	d_H (*J* in Hz)
3', 5'	114.9	7.01 (d, 9.0 Hz)
4'	159.7	
4'-OCH_3	55.8	3.85 (s)

Table 3 The results in cytotoxic assay

No	Concentration	Inhibitory percentage			
		KB	Hep	Lu	MCF7
EtoAc extract	128 (μg/mL)	90	86	85	86
	32	77	53	40	35
	8	37	21	26	11
	2	13	4	7	7
	IC_{50} (μM)	15.8	29.75	53.33	60.23
Compound 1	128 (μg/mL)	88	85	88	79
	32	62	50	30	34
	8	37	20	23	29
	2	20	16	15	19
	IC_{50} (μM)	20.48	32.0	65.1	66.13
Compound 2	128 (μg/mL)	88	90	92	84
	32	82	81	77	50
	8	79	50	33	34
	2	46	43	14	24
	IC_{50} (μM)	2.27	8.0	17.27	32.0
Compound 3	128 (μg/mL)	98	99	98	92
	32	19	24	20	32
	8	14	12	11	18
	2	9	4	0	11
	IC_{50} (μM)	69.67	64.84	68.92	57.79
Ellipticine	IC_{50}	0.31 ± 0.02	0.38±0.02	0.41±0.03	0.54±0.05

9.0 Hz, H-2', H-6'), and δ_H 7.01 (2H, d, 9.0 Hz, H-3', H-5'). Based on the ^{13}C-NMR/DEPT data [seven aromatic methines at δ_C 94.8-155.0 ppm, seven quaternary carbons at δ_C 105.6-166.0, and δ_C 182.2 (CO), together with two methoxy groups at δ_C 55.1 (7-OCH_3), and 55.8 (4'-OCH_3)], and compared with literature compound,[15] isolated compound 5 was unambiguously determined to be a isoflavone, which trivially named 7-*O*-methylbiochanin A. Despite the fact that this compound is now available in nature but this is the first time to found in genus *Diospyros*, to date.

Naphthoquinones derived *Diospyros* plants are recognized as useful agents in cytotoxic treatments. For instance, plumbagin and 3,3'-biplumbagin from *D. shimbaensis* exerted the IC_{50} values of 130.8, and 82.1 μM, respectively, against the growth of MDA-MB-231 breast cancer cell.[16] Our current assay emphasized on using constituents from Vietnamese plant *D. fleuryana* species for cytotoxic activities. As resulted in Table 3, EtOAc extract has generated the moderate IC_{50} values of 15.8 and 29.75 μM repellent the growth of KB and Hep cell lines, and the weak IC_{50} of values of 53.33 and 60.23 μM with regards to Lu and MCF7 cell lines, respectively. Similarly, isodiospyrin[1] was found to inhibit KB and Hep cell lines with the moderate IC_{50} values of 20.48 and 32.0 μM, respectively, but suppress Lu and MCF7 cell lines with the weak IC_{50} values of 65.1 and 66.13 μM. Of particular interest, respecting to the inhibition of KB and Hep cell lines, 8'-hydroxyisodiospyrin (2) has shown to consist of the strong IC_{50} values of 2.27 and 8.0 μM, respectively. Compound 2 also revealed the significant IC_{50} values of 17.27 and 32.0 μM towards Lu and MCF7 cell lines. Isoflavone 3 mostly controlled the growth of four tested cancer cell lines at a high concentration of 128.0 μg/mL, and induced a range of IC_{50} values of 57.79-69.67 μM. The currently cytotoxic record from *D. fleuryana* species is the first time, thereby expecting that the extensive evidence on mechanisms is willing.

EXPERIMENTAL

General experimental procedures

ESI-MS spectrum was obtained with Thermo Scientific LTQ Orbitrap XL spectrometer (USA). NMR spectra were recorded on a Bruker 500.13 MHz spectrometer, operating at 125.76 MHz for ^{13}C NMR and at 500.13 MHz for ^{1}H NMR. Silica gel (40-63 μm), Sephadex LH-20 (25-100 μm), and RP-18 (Cosmosil C18-Prep, Kyoto-Japan) were used for column chromatography (CC). TLC silica gel 60 F254 (Merck) was used for thin-layer chromatography (TLC). Compounds were visualized under UV radiation (254, and 365 nm), and by spraying plates with 10% H_2SO_4 followed by heating with a heat gun.

Plant materials

Leaves of *D. fleuryana* was collected in Ngoclac - Thanhhoa province in January 2018. A voucher specimen VN-310 was identified by taxonomist Dr. Nguyen Quoc Binh, Institute of Ecology and Biological Resources. The plant sample was deposited in Laboratory of Application Biochemistry, Institute of Chemistry.

Extraction and isolation

The dried ground leaves powder of *D. fleuryana* (1.16 kg) was ultrasonically extracted 3 times with EtOAc (10 L × 3) for 45 min at 45 °C, and the combined extract was then concentrated under vacuum (20~30 mbar) at 50°C to give a EtOAc

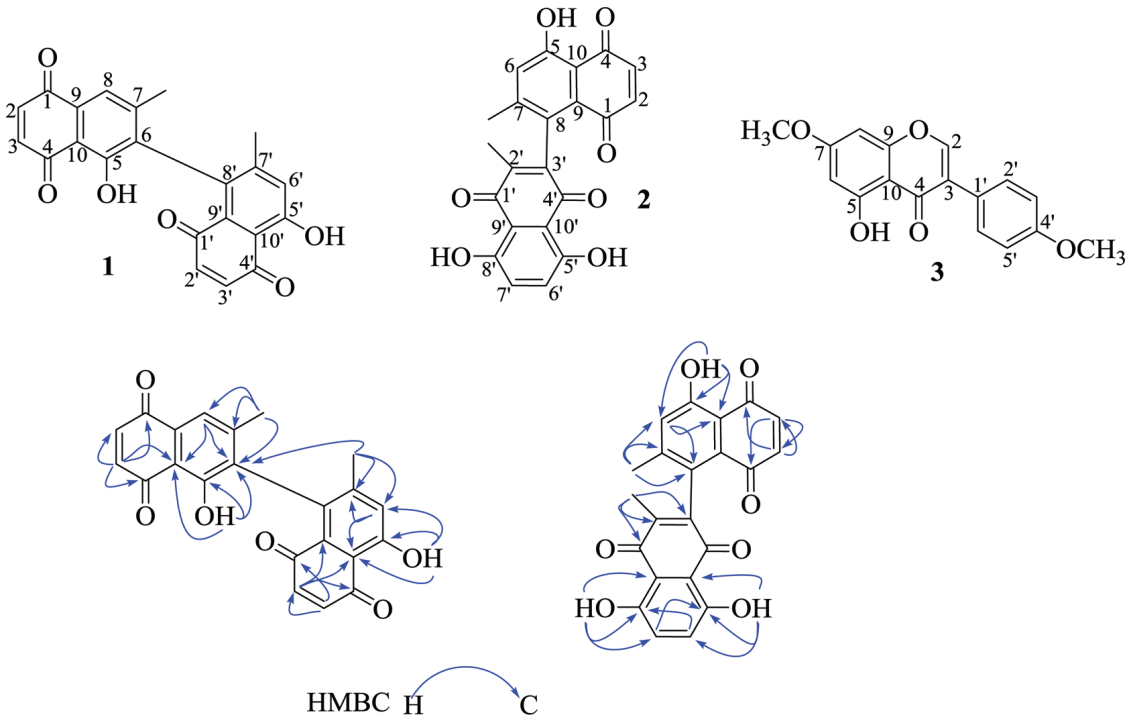

Figure 1 Chemical structures 1-3 and their key correlations in HMBC spectroscopy

residue (155.0 g). This residue was subjected to silica gel (10 × 50 cm, 300.0 g) column chromatography, eluting with a gradient of *n*-hexane-acetone (9:1→ 0:10, v/v) and acetone-methanol (9:1→0:10, v/v), to yield 15 fractions (Fr.1-Fr.15). The fraction Fr.3 (1.5 g) was then diluted by dichloromethane to afford the soluble part (Fr.31), and the insoluble residue (Fr.32). The fraction Fr.31 (0.15 g) was chromatographed on Sephadex LH-20, eluting with methanol-dichloromethane (9:1, v/v), to yield 3 fractions Fr.311-Fr.313. Compounds 1 (5.0 mg), and 2 (6.0 mg) were isolated from the fraction Fr.312 (20 mg) by preparative TCL (*n*-hexane-acetone, 8:2, v/v). Similarly, the fraction Fr.7 (3.2 g) was subjected to silica gel column chromatography, eluting with dichloromethane-methanol (6:1, v/v), to yield 6 fractions (Fr.71-Fr.76). Utilizing the reverse RP-18 column [methanol-water (1:1, v/v)] for the fraction Fr. 713 (50 mg), compound 3 (3.1 mg) was isolated as a pure compound.

Isodiospyrin (1): Yellow amorphous powder; ESI-MS (+): 375.0 $[M+H]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$): see Table 1.

8'-Hydroxyisodiospyrin (2): Red amorphous powder; ESI-MS (+): 391.0 $[M+H]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$): see Table 1.

7-O-Methylbiochanin A (3): Yellow amorphous powder; ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD): see Table 2.

Cytotoxic activity assay

MTT assay was used to determine the cytotoxic activity of EtoAc extract and isolated compounds 1-3 with human cancer cell lines (KB, LU-1, Hep-G2 and MCF-7) acquired from the American Type Culture Collection (ATCC).[17] Cells were cultured in medium DMEM supplemented with 10% Fetal bovine serum (FBS), 1% Penicillin and Streptomycin and 1% L-glutamine, under a humidified atmosphere of 5% CO_2 at 37°C. Compounds were dissolved in DMSO at 20 mg/mL and a series of dilutions for each compound was prepared to final concentrations of 128, 32, 8, 2 and 0.5 µg/mL. Cells were separated with trypsin and seeded in each well with 3 x10^4 cells per ml and were treated with sample different concentrations on 96-well plates. Untreated cells represented the controls. After 72h of treatment, an MTT solution (10 µl, 5 mg/mL) of phosphate buffer was added to each well for 4 hrs until intracellular purple formazan crystals are visible. Remove MTT and add DMSO solution (100 µL). The optical density of the solution was determined by a plate reader BIOTEK at 540 nm. The inhibition ratio was calculated based on the optical densities from the three replicate tests.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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