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Chemical constituents of *Murraya siamensis*: three coumarins and their anti-tumor promoting effect ☆

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Abstract

Isolation and structure elucidation of three coumarins, murrayacoumarins A, B, and C, together with eight known coumarins, from the leaves of *Murraya siamensis* Craib collected in Thailand are described. Results of a primary screening of inhibitory effects of seven of these compounds on 12-*O*-tetradecanoylphorbol-13-acetate-induced Epstein–Barr virus early antigen activation in Raji cells are also presented.

Keywords: *Murraya siamensis*; Rutaceae; Coumarins; Anti-tumor promoting effect; Epstein–Barr virus activation test

1. Introduction

As a part of our systematic investigation of the chemical constituents of Rutaceous plants, we have identified various compounds in *Murraya* plants (Furukawa et al., 1985; Ito and Furukawa, 1987a,b; Ito et al., 1993) used in folk medicine in China and other Asian countries as analgesics, astringents, anti-dysenterics or febrifuges (Kan, 1972). In previous papers, we reported the isolation and identification of many natural coumarins from *Murraya paniculata* (L.) Jack (Ito and Furukawa, 1987b; Ito et al., 1990) and *Murraya exotica.* L (Ito and Furukawa, 1987c). Further, in a primary screen for novel anti-tumor promoters, we found that several 8-substituted 7-methoxycoumarins and furanone-coumarins showed potent inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells (Ito et al., 1999, 2000).

This paper describes the isolation and identification of three new coumarins, murrayacoumarins A (**1**), B (**2**), and C (**3**) from the leaves of *Murraya siamensis* Craib collected in Thailand. In the course of our continuing study, we also carried out primary screening of seven coumarins isolated from this plant by examining their inhibitory effects on EBV-EA activation.

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2. Results and discussion

2.1. Isolation and structure elucidation

The acetone extract of the leaves of *M. siamensis* was fractionated by silica gel column chromatography and preparative TLC to obtain three new and eight known coumarins (see Fig. 1).

Murrayacoumarin A (1) was isolated as a colorless oil, $[\alpha]_D^{24}$ +7.9° (*c* 0.114, MeOH). The molecular formula was determined as $C_{19}H_{24}O_6$ by HREIMS. The UV spectrum was similar to that of 5,7-dihydroxycoumarin (7) (Ito et al., 1991), and IR bands appeared at ν_{max} 3567, 3284*br* and 1717 cm^{-1} (hydroxy groups and an α,β-unsaturated lactone). The ^1H NMR spectrum (acetone-d_6, see Section 3) showed AB-type doublets at δ 6.00 (H-3) and 7.95 (H-4), and *meta*-coupled doublets at δ 6.38 (H-6) and 6.29 (H-8). These results, coupled with the observation of the H-4 proton signal at δ 7.95, at lower field compared with that of coumarin lacking a C-5 oxygen function (Murray et al., 1982), indicated the presence of a 5,7-dioxygenated coumarin nucleus in the molecule. The ^1H NMR signals at δ 5.52, 4.66, 3.24, 2.33, 2.10, 1.75, 1.71, 1.37, and 1.08 along with two hydroxy groups at δ 3.54 and 3.31, suggested that this new coumarin (1) contains an oxygenated C_{10} terpenoid side-chain [–OCH_2CH=$C(CH_3)$–$CH_2CH_2CHOHC(CH_3)_2OH$]. The observation of significant mass fragment ions (EIMS) at *m/z* 231 and 178 resulting from cleavage at C-3′/C-5′ and 5-O/C-1′ with a hydrogen transfer, respectively, also support the structure of the side-chain. The E configuration of the C-2′ → C-3′ double bond was suggested by the ^{13}C NMR chemical shift value of the C-4′ (δ_C 16.8) and an NOE between H-1′ and H-4′. The location of the side-chain at C-5 (not at C-7) was based on the observation of long-range C–H correlation between the methylene protons (H-1′) and the oxygenated carbon (C-5) having a correlation with the characteristic H-4 (δ_H 7.95) in the HMBC analysis, coupled with an NOE between H-1′ and H-6. Other HMBC correlations are shown in Fig. 2. Unfortunately, the synthesis of (*R*)- and (*S*)-MTPA ester for the application of Mosher's method did not afford a sufficient quantity of (*R*)- and (*S*)-MTPA ester to establish the C-7′ stereocenter in **1**. On the basis of these

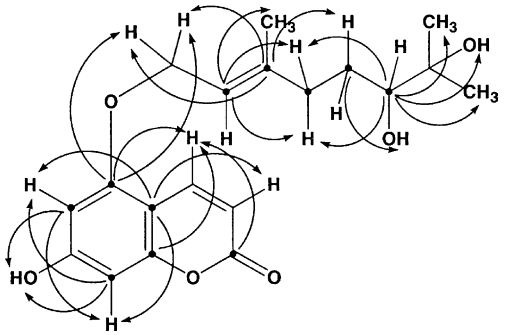

Fig. 2. C–H long-range correlations in the HMBC spectrum of murrayacoumarin A (**1**).

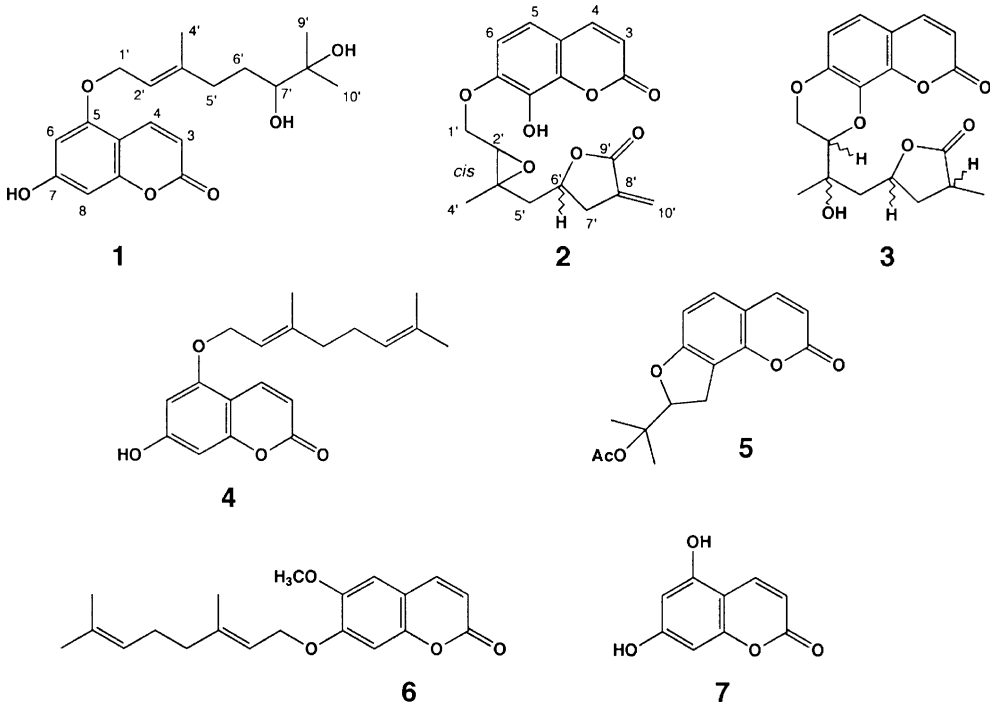

Fig. 1. Structures of coumarins from *Murraya siamensis*.

data, the structure of murrayacoumarin A is proposed as shown by the formula **1**, except for the configuration at C-7′.

Murrayacoumarin B (**2**) was obtained as a colorless oil, $[\alpha]_D^{24}$ +64.1° (*c* 0.103, MeOH) and its molecular formula, $C_{19}H_{28}O_7$, was determined by HREIMS. Strong UV bands at λ_{max} 260 and 316 nm, an IR band at ν_{max} 1727 cm^{-1}, and two pairs of doublets at δ_H 6.21, 7.86 and δ_H 7.12, 7.10 in the ^1H NMR spectrum (Table 1) indicated the presence of a 7,8-dioxygenated coumarin nucleus. IR bands at ν_{max} 1763, and ^1H and ^{13}C NMR signal patterns [δ_H 4.81, 3.20, 2.72, 5.67, 6.06; δ_C 75.3, 34.4, 136.2, 170.1, 121.5] were in good agreement with those of a 5-substituted α-methylene-γ-lactone ring, similar to the side-chain of clauslactone F (Ito et al., 2000), except for some chemical shift differences. Further, correlations of these protons with signals at δ_H 1.91 and 2.00 in the COSY spectrum indicated the linkage of C-5′ to the γ-lactone ring. The remaining proton signals for the side-chain appeared as a 3H-singlet at δ_H 1.45 and an ABC pattern at δ_H 4.20, 4.52, and 3.22. The ^1H NMR chemical shifts values of the 3H-singlet (δ_H 1.45) and the methine double–doublet (δ_H 3.22) coupled with ^{13}C NMR chemical shifts values of the methine carbon at δ_C 61.7 (C-2′) and a fully substituted carbon at δ_C 58.6 (C-3′) suggested the presence of a trisubstituted oxirane ring in the molecule. The connectivity of these signals was elucidated by HMBC analysis. A methine carbon (C-2′) showed a three-bond correlation with a methylene proton at δ_H 1.91 (H-5′) and 2.00 (H-5′) along with a quaternary methyl proton at δ_H 1.45 (H-4′). Further, a correlation between this methylene proton signal (H-5′) and the methyl carbon at δ_C 17.2 (C-4′) was also observed. The connection of the side-chain to C-7 on the coumarin nucleus was revealed by observation of a three-bond correlation between the methylene proton signals (H-1′) and carbon C-7 at δ_C 150.3 on the 7-oxycoumarin nucleus. In the NOE experiment, irradiation of the methyl proton at δ_H 1.45 (H-4′) resulted in 5% and 3% area increases of H-1′ (δ 4.20) and H-1′ (δ 4.52), supporting the relative stereochemistry (*cis*) of the trisubstituted oxirane ring. An attempt to determine the absolute configuration at C-6′ of the α-methylene-γ-lactone by means of the CD spectrum was unsuccessful, because the Cotton effect attributable to the n → π* transition of γ-lactone chromophore overlapped with that of the coumarin chromophore. On the basis of these results, the structure of murrayacoumarin B is proposed as shown in **2**, except for the absolute stereochemistry.

Murrayacoumarin C (**3**) was obtained as a colorless oil, $[\alpha]_D^{24}$ +79.0° (*c* 0.371, MeOH), and its molecular formula was determined as $C_{19}H_{20}O_7$ by HREIMS. IR bands at ν_{max} 3567 and 3386*br* cm^{-1} indicated the presence of a hydroxyl group. The presence of a 7,8-oxygenated coumarin nucleus in this new coumarin was

Table 1
^1H and ^{13}C NMR spectroscopic data of murrayacoumarin B (**2**) and murrayacoumarin C (**3**)

	Murrayacoumarin B (**2**) (acetone-d_6)			Murrayacoumarin C (**3**) ($CDCl_3$)		
	δ_C	δ_H	HMBC	δ_C	δ_H	HMBC
C-2	160.6 (*s*)		H-3, H-4	160.7 (*s*)		H-3, H-4
C-3	114.2 (*d*)	6.21 (*d*, 9.5)		113.4 (*d*)	6.28 (*d*, 9.5)	
C-4	144.9 (*d*)	7.86 (*d*, 9.5)		143.9 (*d*)	7.63 (*d*, 9.5)	
C-4a	115.0 (*s*)		H-3, H-6	113.3 (*s*)		H-3, H-6
C-5	119.2 (*d*)	7.12 (*d*, 8.8)	H-4	119.8 (*d*)	6.98 (*d*, 8.8)	H-4
C-6	110.5 (*d*)	7.10 (*d*, 8.8)		113.6 (*d*)	6.85 (*d*, 8.8)	
C-7	150.3 (*s*)		H-5, H-1′	146.5 (*s*)		H-5, H-1′
C-8	135.0 (*s*)		H-6	131.3 (*s*)		H-6
C-8a	145.0 (*s*)		H-4, H-5	143.8 (*s*)		H-4, H-5
C-1′	69.6 (*t*)	4.52 (*dd*, 11.4, 2.9) 4.20 (*dd*, 11.4, 6.6)		65.1 (*t*)	4.10 (*dd*, 11.0, 9.2) 4.63 (*dd*, 11.0, 2.2)	H-2′
C-2′	61.7 (*d*)	3.22 (*m*)	H-1′, H-4′, H-5′	77.4 (*d*)	4.15 (*dd*, 9.2, 2.2)	H-1′, H-4′
C-3′	58.6 (*s*)		H-4′, H-5′	71.9 (*s*)		H-2′, H-4′, H-5′
C-4′	17.2 (*q*)	1.45 (3H)	H-5′	23.1 (*q*)	1.41 (3H)	H-2′, H-5′
C-5′	45.4 (*t*)	2.00 (*dd*, 14.4, 5.5) 1.91 (*dd*, 14.4, 7.7)	H-4′	43.5 (*t*)	2.31 (*dd*, 15.0, 3.3) 1.87 (*dd*, 15.0, 9.9)	H-4′, H-7′
C-6′	75.3 (*d*)	4.81 (*m*)	H-5′	74.8 (*d*)	4.75 (*m*)	H-5′, H-7′
C-7′	34.4 (*t*)	2.72 (*ddt*, 17.2, 5.9, 2.9) 3.20 (*ddt*, 17.2, 7.7, 2.9)	H-5′, H-10′	38.3 (*t*)	2.63 (*m*) 1.62 (*brd*, 10.6)	H-5′, H-8′, H-10′
C-8′	136.2 (*s*)		H-7′	35.1 (*d*)	2.66 (*m*)	H-7′, H-10′
C-9′	170.1 (*s*)		H-7′, H-10′	178.9 (*s*)		H-8′, H-10′
C-10′	121.5 (*t*)	6.06 (*t*, 2.9) 5.67 (*t*, 2.9)	H-7′	14.8 (*q*)	1.29 (3H, *d*, 6.2)	H-7′, H-8′
Others		8.48 (*br*, 8-OH)			2.93 (*br*, OH)	

Values in (δ_H and δ_C) ppm. All signals correspond to 1H, unless otherwise stated. Figures in parentheses are coupling constants (*J*) in Hz.

deduced by the presence of ^{1}H NMR spectroscopic signals (Table 1) for two pairs of 1H doublets at δ_H 6.28 and 7.63 and δ_H 6.98 and 6.85, which were readily assignable to H-3 and-4, and H-5 and-6 on the coumarin skeleton, respectively (Ito et al., 1991). The IR spectra showed an additional C=O stretching band at ν_{max} 1769 cm^{-1} along with the coumarin carbonyl band in the vicinity of ν_{max} 1727 cm^{-1}, indicating the presence of a γ-lactone ring. Analysis of the ^{1}H and ^{13}C NMR spectra, including COSY and HMQC, suggested the presence of a C_{10} terpenoid side-chain consisting of a methyl group (δ_C 23.1; δ_H 1.41) attached to a quaternary carbon (δ_C 71.9) bearing a hydroxyl group, a methylene adjacent to a methine linked to two oxygen functions [δ_C 65.1; δ_H 4.10, 4.63; δ_C 77.4; δ_H 4.15], a methine linked to two methylenes and an oxygen atom [δ_C 43.5; δ_H 2.31, 1.87; δ_C 74.8; δ_H 4.75; δ_C 38.3; δ_H 2.63, 1.62], a methine linked to secondary methyl group [δ_C 35.1; δ_H 2.66; δ_C 14.8; δ_H 1.29], and a lactone carbonyl group [δ_C 178.9]. The linkage of these partial structural units was elucidated by HMBC spectroscopy (Table 1). Long-range correlations of a methyl carbon signal at δ_C 23.1 (C-4′) with proton signals at δ_H 4.15 (H-2′), 1.87 (H-5′), and 2.31 (H-5′), together with correlations of C-2′ (δ_C 77.4) with H-4′ (methyl, δ_H 1.41) suggests the linkage of structural units as shown in structure **3**. A correlation between the lactone carbonyl carbon at δ_C 178.9 (C-9′) and secondary methyl protons at δ_H 1.29 (H-10′) revealed the structure of a γ-lactone having a substituent at C-6′. Further, the orientation of the linkage between the side-chain and the 7, 8-dioxygenated coumarin nucleus was proposed by the observation of a three-bond hetero correlation of the carbon signal at δ_C 146.5 (C-7) on the coumarin skeleton with the methylene proton signals at δ_H 4.10 (H-1′) and 4.63 (H-1) on the side-chain. These data, together with observed significant mass fragment ions (EIMS) at *m*/*z* 204 and 247 resulting from cleavage at C-2′/C-3′ with a hydrogen transfer and C-3′/C-5′, respectively, led to the assignment of structure **3** to murrayacoumarin C. The relative and absolute configurations at C-2′, C-3′, C-6′, and C-8′ of this coumarin remains to be determined.

Coumarins **2** and **3** are the first examples of 7,8-dioxygenated coumarins having this unique side-chain and ether linkage in *Murraya* sp.

Eight known coumarins, 5-geranyloxy-7-hydroxycoumarin (**4**) (Ito et al., 1996), columbianetin acetate (**5**) (Hata et al., 1971), 6-methoxy-7-geranyloxycoumarin (**6**) (Ito et al., 1990), 5,7-dihydroxycoumarin (**7**) (Ito et al., 1991), umbelliferone (Ito et al., 1987c), clauslactone B (Ito et al., 2000), clauslactone A (Ito et al., 2000), and clauslactone E (Ito et al., 2000) were isolated and identified by published spectroscopic data.

2.2. Inhibitory effects on EBV-EA induction

The seven coumarins (**1–7**) were tested for their anti-tumor promoting activity by using a short-term in vitro assay of TPA induced EBV-EA activation in Raji cells. Their inhibitory effects on the activation of the virus-genome and the viabilities of Raji cells, and the 50% inhibitory concentration (IC_{50}) values are shown in Table 2. All coumarins showed potent dose-dependent inhibitory effects on EBV-EA induction by TPA. Among these coumarins, three coumarins (**1, 4** and **6**) bearing a C10 geranyloxy moiety showed 8.7–11.0% inhibitory activity even at a lower concentration (1×10 mol ratio/TPA), and fully blocked TPA-induced EBV-EA activation with only weak cytotoxicity against Raji cells at high concentration (1×10^3 mol ratio). These values corresponded to an IC_{50} of 230–331 mol ratio/TPA, and the IC_{50} values were lower than that of

Table 2
Inhibitory effects of coumarins from *Murraya siamensis* on TPA-induced EBV-EA activation[a]

Compound	EBV-EA-positive cells (% viability)				IC_{50}[b]
	compound concentration (mol ratio/32 pmol TPA)				(Mol ratio/ 32 pmol TPA)
	1000	500	100	10	
Murrayacoumarin A (**1**)	0.0 ± 0.6 (60)	30.2 ± 1.4 (>80)	64.5 ± 2.1 (>80)	91.3 ± 1.1 (>80)	230
Murrayacoumarin B (**2**)	15.2 ± 0.7 (60)	49.1 ± 1.8 (>80)	77.2 ± 2.0 (>80)	100.0 ± 0.6 (>80)	465
Murrayacoumarin C (**3**)	13.5 ± 0.7 (60)	42.6 ± 1.5 (>80)	73.2 ± 1.9 (>80)	100.0 ± 0.5 (>80)	442
5-Geranyloxy-7-hydroxycoumarin (**4**)	0.0 ± 0.5 (60)	28.8 ± 1.6 (>80)	74.2 ± 2.0 (>80)	89.0 ± 1.5 (>80)	331
Columbianetin acetate (**5**)	17.4 ± 0.8 (60)	52.6 ± 1.1 (>80)	78.5 ± 2.2 (>80)	100.0 ± 0.4 (>80)	504
7-Geranyloxy-6-methoxycoumarin (**6**)	0.0 ± 0.7 (60)	29.1 ± 1.0 (>80)	75.0 ± 1.6 (>80)	90.2 ± 0.3 (>80)	312
5,7-Dihydroxycoumarin (**7**)	17.7 ± 0.7 (30)	48.3 ± 2.3 (>80)	81.4 ± 2.1 (>80)	100.0 ± 0.3 (>80)	477
β-Carotene[c]	9.1 ± 0.5 (60)	34.3 ± 1.1 (>80)	82.7 ± 1.8 (>80)	100.0 ± 0.2 (>80)	400

[a] Mole ratio/TPA (32 pmol = 20 ng/mL), 1000 mol ratio = 32 nmol, 500 mol ratio = 16 nmol, 100 mol ratio = 3.2 nmol, and 10 mol ratio = 0.32 nmol. Values are EBV-EA activation (%) ± SD in the presence of the test compound relative to the positive control (100%). Values in parentheses represent the surviving Raji cells measured with Trypan Blue staining. A minimum 60% survival rate of Raji cells two days after treatment with the compounds is required for an accurate result.

[b] IC50 represents mol ratio to TPA that inhibits 50% of positive control (100%) activated with 32 pmol of TPA.

[c] Reference compound.

β-carotene (IC_{50} 400), a vitamin A precursor commonly used in cancer preventive field as a standard reference (Murakami et al., 1996). Murrayacoumarin A (1) bearing an oxygenated geranyloxy side-chain exhibited the most potent activity (IC_{50} 230). On the other hand, the two novel furanone-coumarins (**2** and **3**) and two known coumarins (**5** and **7**)were much less active (IC_{50} 442–504) than β-carotene.

These results suggest that some coumarins isolated from *M. siamensis* might be valuable as anti-tumor promoters in chemical carcinogenesis. Investigation into the inhibitory mechanisms of these coumarins on the tumor-promotion is now in progress.

3. Experimental

3.1. General

^1H and ^{13}C NMR, COSY, HMQC, HMBC ($J = 8$ Hz), and NOE were measured on JNM A-400, A-600 and/or ECP-500 (JEOL) spectrometers. Chemical shifts are shown in δ (ppm) with tetramethylsilane (TMS) as an internal reference. All mass spectra were run under EI conditions, unless otherwise stated, using HX-110 (JEOL), and/or JMS-700 (JEOL) spectrometers with a direct inlet system. UV spectra were recorded on a UVI-DEC-610C double-beam spectrophotometer (JASCO) in MeOH, IR spectra on an IR-230 (JASCO) in $CHC1_3$. Preparative TLC was done on Kieselgel 60 F_{254} (Merck).

3.2. Plant material

The plant material used in this study, *M. siamensis* Camb. was collected at the Sakaeraj Environmental Research Station, Nakorn-rachasima Province, Thailand during March 1998. Authentication was achieved by comparison with the herbarium specimen at the Royal Forest Department, Ministry of Agriculture and Cooperative, Bangkok, Thailand. A specimen has been deposited at the Barbosa Rodrigues Herbarium under voucher number NSR-092515.

3.3. Extraction and separation

The dried leaves (395 g) of *M. siamensis* were extracted with acetone (2 L × 3) at room temperature and the solvent evaporated under reduced pressure to give the acetone extract (19.0 g). The acetone extract was subjected to silica gel CC, eluting sequentially with hexane–acetone (4:1, 3:1, 2:1, 1:1), acetone, and MeOH gradients (each 0.5 L), successively, to obtain fractions 1 to 15.

Fraction 5 (hexane–acetone (4:1), 1.7 g) was applied to a silica gel column, eluting with $CHCl_3$–acetone (96:4), to obtain fractions from 5–1 to 5–12. Fraction 5–3 (622 mg) was further purified on silica gel, eluting with hexane–acetone (2:1), to yield columbianetin acetate (**5**, 13.4 mg) and 7-geranyloxy-6-methoxycoumarin (**6**, 2.1 mg), which were purified by preparative silica gel TLC (PTLC), developed with benzene–acetone (96:4). Fraction 5–8 (127 mg) was subjected to PTLC, developed with hexane–acetone (2:1), to afford 5-geranyloxy-7-hydroxycoumarin (**4**, 24.9 mg).

Fraction 7 (hexane–acetone (3:1), 333 mg) was purified on silica gel, eluting with hexane-iPr$_2$O (1:5), to yield umbelliferone (1.0 mg), which was purified further by PTLC, developed with $CHCl_3$–acetone (96:4).

Fraction 11 (hexane–acetone (2:1), 4.6 g) was applied to a silica gel column, eluting with $CHCl_3$–acetone (5:1), to yield murrayacoumarin C (3, 11.7 mg) which was purified by PTLC, developed with $CHCl_3$–acetone (96:4).

Fraction 12 (hexane–acetone (1:1), 590 mg) was subjected to silica gel CC, eluting with hexane–acetone (2:1), to obtain fractions from 12–1 to 12–7. Fraction 12–2 (357 mg) was purified on a silica gel column, eluting with $CHCl_3$–acetone (96:4), to yield clauslactone A (13.0 mg), which was purified further by PTLC, developed with $CHCl_3$–MeOH (30:1). Fraction 12–3 (43 mg) was also purified by PTLC, developed with $CHCl_3$–MeOH (20:1), to afford clauslactone E (7.1 mg). Fraction 12–4 (129 mg) was subjected to PTLC, developed with $CHCl_3$–MeOH (20:1), to afford clauslactone B (33.5 mg), which was purified further by PTLC, developed with $CHCl_3$–acetone (3:1).

Fraction 13 (acetone, 433 mg) was applied to a silica gel column, eluting with $CHCl_3$–acetone (5:1), to obtain fractions from 13–1 to 13–8. Fraction 13–2 (49 mg) was subjected to PTLC, developed with $CHCl_3$–MeOH (96:4), to afford murrayacoumarin B (**2**, 14.4 mg). Fraction 13–6 (205 mg) was also purified by PTLC, developed with $CHC1_3$–MeOH (96:4), to afford murrayacoumarin A (**1**, 9.0 mg) and 5,7-dihydroxycoumarin (**7**, 1.0 mg).

Identification of the test compounds was done by direct comparison of the spectroscopic data and TLC with authentic samples, or by comparison with reported NMR spectroscopic data.

3.4. Murrayacoumarin A (*1*)

Colorless oil; $[\alpha]_D^{24}$ +7.9° (c 0.114, MeOH); UV (MeOH) λ_{max} 206, 226*sh*, 250, 258, 332 nm; IR ($CHC1_3$) ν_{max} 3567, 3284*br*, 1717, 1612 cm^{-1}; ^1H NMR (acetone-d_6) δ 9.44 (1H, *brs*, 7-OH), 7.95 (1H, *d*, $J = 9.7$ Hz, H-4), 6.38 (1H, *d*, $J = 1.7$ Hz, H-6), 6.29 (1H, *d*, $J = 1.7$ Hz, H-8), 6.00 (1H, *d*, $J = 9.7$ Hz, H-3), 5.52 (1H, *m*, H-2′), 4.66 (2H, *d*, $J = 6.6$ Hz, H-1′), 3.54 (1H, *brd*, $J = 5.1$ Hz, 7′-OH), 3.31 (1H, s, 8′-OH), 3.24 (1H, *m*, H-7′), 2.33 (1H, *m*, H-5′), 2.10 (1H, *m*, H-5′), 1.75 (3H, *s*, H-4′), 1.71 (1H, *m*, H-6′), 1.37 (1H, *m*, H-

6′), 1.08 (6H, *s*, H-9′, 10′); ^{13}C NMR (acetone-d_6) δ 162.9 (C-7), 161.2 (C-2), 157.8 (C-5), 157.7 (C-8a), 142.8 (C-3′), 139.5 (C-4), 119.8 (C-2′), 110.7 (C-3), 103.8 (C-4a), 97.0 (C-6), 95.9 (C-8), 78.3 (C-7′), 72.8 (C-8′), 66.5 (C-l′), 37.4 (C-5′), 30.4 (C-6′), 25.8 (C-9′), 25.2 (C-10′), 16.8 (C-4′); Differential NOE: Irradiation of H-4′ (δ 1.75) – 4% enhancement of H-1′ (δ 4.66); Irradiation of H-1′ (δ 4.66) – 9% and 5% enhancement of H-6 (δ6.38) and H-4′ (δ 1.75), respectively; EIMS *m*/*z* 348 (M^+, 5), 231 (17), 178 (100), 153 (49), 135 (18); HREIMS *m*/*z* 348.1541 (calcd for $C_{19}H_{24}O_6$, 348.1573).

3.5. Murrayacoumarin B (**2**)

Colorless oil; $[\alpha]_D^{24}$ +64.1° (*c* 0.103, MeOH); UV (MeOH) λ_{max} 206, 260, 316 nm; IR ($CHCl_3$) ν_{max} 3546, 1763, 1727, 1616 cm^{-1}; for ^{1}H and ^{13}C NMR spectra, see Table 1; Differential NOE: Irradiation of H-1′ (δ4.20) – 8% enhancement of H-6 (δ 7.10); Irradiation of H-1′ (δ 4.52) – 9% enhancement of H-6 (δ 7.10); Irradiation of H-4′ (δ 1.45)-5% and 3% enhancement of H-1′ (δ4.20) and H-1′ (δ 4.52), respectively; EIMS *m*/*z* 358 (M^+, 29), 236 (17), 204 (100), 189 (25), 175 (67); HREIMS *m*/*z* 358.1057 (calcd for $C_{19}H_{28}O_7$, 358.1053).

3.6. Murrayacoumarin C (**3**)

Colorless oil; $[\alpha]_D^{24}$ +79.0° (*c* 0.371, MeOH); UV (MeOH) λ_{max} 206, 226*sh*, 262, 318 nm; IR ($CHCl_3$) ν_{max} 3567, 3386*br*, 1769, 1727, 1615 cm^{-1}; for ^{1}H and ^{13}C NMR spectra, see Table 1 EIMS *m*/*z* 360 (M^+, 20), 247 (6), 204 (100), 189 (15), 175 (40); HREIMS *m*/*z* 360.1194 (calcd for $C_{19}H_{20}O_7$, 360.1209).

3.7. In vitro EBV-EA activation experiments

The inhibition of EBV-EA activation was assayed using the same method described previously (Ito et al., 1999, 2000). In brief, Raji cells were grown to a density of 10^6 cells/mL, harvested by centrifugation and resuspended in RPMI 1640 medium (Sigma, St. Louis, MO, USA) with 10% FCS containing 4 mM *n*-butyric acid as inducer, 32 pmol of TPA (20 ng/mL in DMSO), and 32, 3.2 or 0.32 nmol of the test compound (DMSO solutions). The cells were incubated at 37 °C for 48 h. Cell number and viability were determined after 48 h by means of a hemocytometer (Trypan Blue staining method). Untreated cultures served as the controls. EBV-EA inhibitory activity of the test compounds was estimated on the basis of the percentage of the number of positive cells compared to that observed in the case of a control without the test product. In each assay, at least 500 cells were counted and the results were read blind.

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