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Original Article

Cytotoxicity of clerodane diterpenoids from fresh ripe fruits of *Casearia grewiifolia*

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Abstract

A new clerodane diterpene, Caseargrewiin M (**1**), and one known compound (**2**) were isolated from fresh ripe fruits of *Casearia grewiifolia*. The structures of all isolated compounds were evaluated on the basic 1D (^{1}H and ^{13}C) and 2D NMR (COSY, HSQC, HMBC and NOESY) spectral data. All compounds were examined cytotoxicity with five human cancer cell lines, Hep-G_2 (hepatocarcinoma), SW620 (colon adenocarcinoma), Chago-K1 (undifferentiated lung carcinoma), KATO-III (gastric carcinoma), and BT474 (breast ductal carcinoma). Isolated compounds showed broad cytotoxicity against five human cancer cell lines with IC_{50} values between 0.90–6.30 μg/mL.

Keywords: *Casearia grewiifolia*, clerodane diterpenoids, cytotoxicity

1. Introduction

Plants belonging to the Flacourtiaceae family have been found to be clerodane and kolovance diterpene ester (Mosaddik *et al*., 2004). Some of which displayed important roles in cytotoxicity (Kanokmedhakul, Kanokmedhakul, & Buayairaksa, 2007; Kanokmedhakul, Kanokmedhakul, Kanarsa, & Buayairaksa, 2005), insect antifeedant, and LEA-1/ICAM binding inhibitory activity (Beutler *et al*., 2000). *Casearia grewiifolia* Vent., one of Flacourtiaceae family, which is a shrubby tree widely distributed in northern and northeastern parts of Thailand. Several parts of this plant such as roots, barks, leaves, flowers, and seeds have been used for the fever therapy, the reliving of itch, and the diarrhea treatment (Smitinand, 2001). From the previous reports, the chemical constituents from several parts of this plant were clerodane diterpenes (Kanokmedhakul *et al*., 2005; Kanokmedhakul *et al*., 2007; Mosaddik, Forster, Booth, & Waterman, 2007) and phenolic compounds (Rayanil, Nimnoun, & Tuntiwachwuttikul, 2012) which displayed antimalarial, antibacterial (Kanokmedhakul *et al*., 2005; Mosaddik *et al*., 2004), and cytotoxicity (Kanokmedhakul *et al*., 2005; Kanokmedhakul *et al*., 2007; Mosaddik *et al*., 2004; Nguyen *et al*., 2015). In this research, chemical constituents and the cytotoxicity against five human cancer cell lines: Hep-G_2 (hepatocarcinoma), SW620 (colon adenocarcinoma), Chago-K1 (undifferentiated lung carcinoma), KATO-III (gastric carcinoma) and BT474 (breast ductal carcinoma) were investigated. The ethyl acetate soluble part of fresh ripe fruits of *C. grewiifolia* was separated to afford clerodane diterpenes which include a new compound, Caseargrewiin M (**1**), and a known compound, Caseargrewiin G (**2**). Both compounds showed the broad cytotoxicity against five human cancer cell lines. In this paper, we report the procedures of isolation,

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structure elucidation and cytotoxicity of isolated compounds.

2. Results and Discussion

Partitioning EtOAc of MeOH extract of fresh ripe fruits of *C. grewiifolia* were separated by silica gel column chromatography. The new clerodane diterpene (**1**) was isolated and elucidated its structure using basic 1D (^{1}H and ^{13}C) and 2D NMR (COSY, HSQC, HMBC, NOESY) techniques.

Compound **1** was obtained as colorless oil, $[\alpha]^{23}_D$ + 12.5 (c 0.05, $CHCl_3$). UV spectrum of **1** showed the absorption spectrum maximum wavelength at 242 nm in $CHCl_3$. IR spectrum displayed the hydroxyl group at 3,488 cm^{-1}, carbonyl groups at 1,750 and 1,731 cm^{-1}. The molecular formula of 1 was determined as $C_{28}H_{42}O_7$ from the HRESIMS ion at 513.2823 $[M+Na]^+$ (Calcd. for $C_{28}H_{42}NaO_7$, 513.2828), indicating eight degrees of unsaturation. The ^{1}H NMR spectrum of **1** (Table 1) displayed; five olefinic protons at δ_H 6.68 (1H, dd, J = 17.3, 11.9 Hz), 6.19 (1H, d, J = 3.5 Hz), 5.28 (1H, m), 5.19 (1H, d, J = 17.3 Hz) and 5.11 (1H, d, J = 10.9 Hz); four oxygenated methine protons at δ_H 6.61 (1H, s), 5.43 (1H, m), 5.33 (1H, brs) and 3.93 (1H, dd, J = 11.7, 4.4 Hz); one methoxy proton at δ_H 3.54 (3H, s); five methyl protons at δ_H 1.99 (3H, s), 1.80 (3H, s), 1.01 (6H, d, J = 6.6 Hz), 0.94 (3H, d, J = 6.6 Hz) and 0.78 (3H, s). The ^{13}C NMR and HSQC spectrum of **1** showed 28 carbon signals; two carbonyl carbons at δ_C 172.4 and 169.4; four quaternary carbons at δ_C 145.8, 133.5, 51.8 and 37.8; ten methine carbons at δ_C 133.5, 127.2, 124.5, 104.0, 98.0, 71.4, 66.4, 37.1, 35.6 and 26.0; four methylene carbons at δ_C 43.7, 35.8, 28.8 and 27.0; seven methyl carbons at δ_C 56.1, 25.1, 22.4x2, 21.5, 20.4 and 15.6. From the spectroscopic data above, the compound **1** is a clerodane

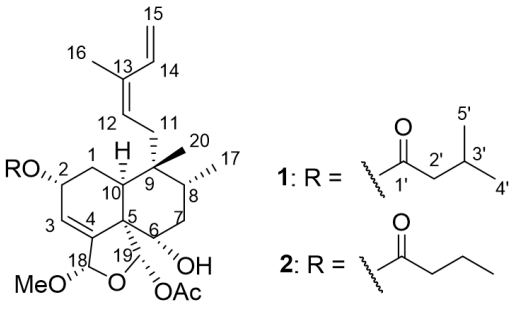

Figure 1. The chemical structures of compounds **1** and **2**.

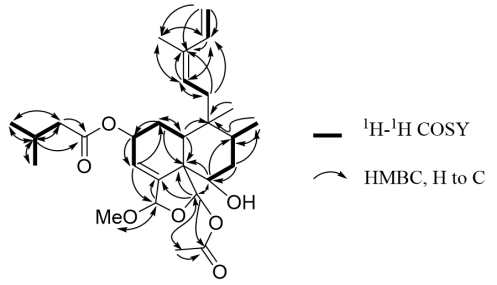

Figure 2. HMBC and ^{1}H-^{1}H COSY correlations of compound **1**.

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for **1** in $CDCl_3$ (J in Hz).

Position	**1**	
	δ_H (J in Hz)	δ_C
1	1.91 (2H, m)	27.0
2	5.43 (1H, m)	66.4
3	6.19 (1H, d, 3.5)	124.5
4		145.8
5		51.8
6	3.93 (1H, dd, 11.7, 4.4)	71.4
7	1.73 (1H, m)1.56 (1H, m)	35.8
8	1.78 (1H, m)	35.6
9		37.8
10	2.27 (1H, m)	37.1
11	2.41 (1H, m)1.69 (1H, m)	28.8
12	5.28 (1H, m)	127.2
13		133.5
14	6.68 (1H, dd, 17.3, 11.9)	133.5
15	5.19 (1H, d, 17.3)5.11 (1H, d, 10.9)	114.2
16	1.80 (3H, s)	20.4
17	0.94 (3H, d, 6.6)	15.6
18	5.33 (1H, brs)	104.0
19	6.61 (1H, s)	98.0
20	0.78 (3H, s)	25.1
1		172.4
2	2.35 (2H, m)	43.7
3	1.70 (1H, m)	26.0
4	1.01 (6H, d, 6.6)	22.4
5	1.01 (6H, d, 6.6)	22.4
O*Me*-18	3.54 (3H, s)	56.1
*Me*COO-19	1.99 (3H, s)	21.5
Me*C*OO-19		169.4

diterpenoid having two ester groups with an additional double bond at C-3 and C-4 [3-5, 7-9]. These ^{1}H and ^{13}C spectra were closely related to the previous report of caseargrewiin G (Kanokmedhakul *et al*., 2007). However, there were a few differences in chemical shift values corresponding to the attached group at C-2. To collaborate the above deductions and confirm this skeleton a HMBC experiment was performed (Figure 2). The HMBC correlation of four olefinic proton; H-15 at δ_H 5.19 (1H, d, J = 17.3 Hz) and δ_H 5.11 (1H, d, J = 10.9 Hz) with δ_C 133.5 (C-14), 133.5 (C-13) and 20.4 (C-16); H-14 at δ_H 6.70 (1H, dd, J = 19.4, 8.7 Hz) with δ_C 133.2 (C-13), 127.2 (C-12) and 20.4 (C-16); H-12 at δ_H 5.30 (1H, brd, J = 6.4 Hz) with δ_C 133.5 (C-14), 133.2 (C-13), 28.8 (C-11) and 20.4 (C-16) indicated two terminal methylene units in the six-carbon side chain (C-11 through C-16) of clerodane diterpenoid derivatives. Moreover, the HMBC correlation of the acetal proton; H-18 at δ_H 5.33 (1H, brs) with δ_C 145.8 (C-4), 56.1 (OMe-18) and 51.8 (C-5); H-19 at δ_H 6.61 (1H, s) with δ_C 169.4 (Me*C*OO-19), 145.8 (C-4), 71.4 (C-6) and 51.8 (C-5) displayed the presence of two acetal

protons at C-18 and C-19 which attached to a methoxy group at C-18 and an acetyl group at C-19. The HMBC correlation of the oxygenated methine proton (H-6) at δ_H 3.93 (1H, dd, J = 11.7, 4.4 Hz) to a carbon signal at δ_C 145.8 (C-4), 98.0 (C-19), 51.8 (C-5) and 35.8 (C-7), indicating that the hydroxyl group was located at C-6. Additionally, the 3-methyl-butanoyloxy group was deduced from the signals H-2′at δ_H 2.35 (2H, m), H-3′ at δ_H 1.70 (1H, m), H-4′and H-5′at δ_H 1.01 (6H, d, J = 6.6 Hz). The ^{1}H-^{1}H COSY correlations between H-2′/H-3′,H-3′/H-4′ and H-3′/H-5′ as well as the HMBC correlations of H-2′to δ_C 172.4 (C-1′), 26.0 (C-3′) and 22.4 (C-4′ and C-5′); H-3′to δ_C 172.4 (C-1′) and 22.4 (C-4′ and C-5′); H-4′ and H-5′ to δ_C 43.7 (C-2′) and 26.0 (C-3′) were also supported the presence of this group. The HMBC spectrum of H-2 at δ_H 5.43 (1H, m) to the carbonyl carbon at δ_C 169.4 (C-1′) of 3-methylbutanoyloxy group, which showed that this group was attached at C-2.

The relative configuration of compound **1** was established by the NOESY spectrum and coupling constant (Figure 3). NOESY correlations between H-1b/H-2, H-3/H-18, H-18/H-19, H-19/H-11b, H-12/H_3-17, H-17/H-6, and H_3-20/H-10 established the relative stereochemistry of 1. On the basis of the above observations, the structure of **1** was assigned as *rel*-(2*R*,5*S*,8*R*,9*R*,10*S*,18*R*,19*S*)-18-methoxy-19-acetoxy-18,19-epoxy-2-(3-methylbutanoyloxy)-6-hydroxy-cleroda-3,12(*Z*),14-triene. Compound **1** was named Caseargrewiin M.

Compound **2** was obtained as colorless oil, $[\alpha]^{23}_D$ + 19.5 (c 0.1, $CHCl_3$). The UV spectrum of 2 showed the maximum wavelength at 243 nm in $CHCl_3$. IR spectrum displayed a hydroxyl group at 3,461 cm^{-1}, carbonyl groups at 1,731 and 1,740 cm^{-1}. The molecular formula of **2** was determined as $C_{27}H_{40}O_7$ from the HRESIMS ion at 499.2686 $[M+Na]^+$ (Calcd for $C_{27}H_{40}NaO_7$, 499.2672), indicating eight degrees of unsaturation. The ^{1}H and ^{13}C NMR spectrum of **2** (Table 1) were similar to those of **1**. However, the 3-methylbutanoyloxy group at C-2 of **1** was replaced by the butanoyloxy group at δ_H 2.38 (H-2′, 2H, t, J = 7.2 Hz), 1.70 (H-3′, 2H, m) and 1.02 (H-4′, 3H, t, J = 7.8 Hz). The structure of 2 was elucidated by ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments. Thus compound **2** was identified as *rel*-(2*R*,5*S*,6*S*,8*R*,9*R*,10*S*,18*S*,19*S*)-18-methoxy,19-acetoxy-18,19-epoxy-2-butanoyloxy-6-hydroxycleroda-3,12(*Z*),14-triene. Compound **2** was previously isolated form *C. grewii-folia* and was named Caseargrewiin G (Kanokmedhakul *et al*., 2007).

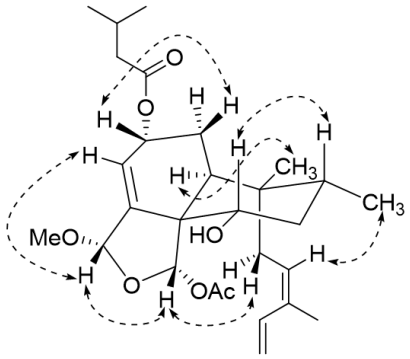

Figure 3. Selected NOESY correlations of **1**.

Cytotoxic activities of all isolated compounds are shown in Table 2. Caseargrewiin M and G (**1** and **2**) exhibited significant cytotoxicity against five human cancer cell lines (Hep-G_2, SW620, Chago-K1, KATO-III and BT474) with IC_{50} values between 0.90-6.30 μg/mL. Moreover, compound **2** showed higher potency than **1**, especially with Hep-G_2 cancer cell lines as IC_{50} value of 0.90 μg/mL. From the previous study, the cytotoxic activity of caseargrewiin G (**2**) against three cancer cell lines; epidermoid carcinoma in the mouth (KB), breast cancer cell (BC1), and small cell lung cancer, showed the IC_{50} values of 0.67, 3.97, and 5.57 μg/mL, respectively (Kanokmedhakul *et al*., 2007). In this research, the caseargrewiin G (**2**) displays cytotoxicity against five human cancer cell lines; BT474, Chago-K1, Hep-G_2, KATO-III, and SW620 at the IC_{50} values of 5.67, 6.10, 0.90, 5.46, and 3.85 μg/mL, respectively. These data suggested that caseargrewiin G (**2**) displayed specific cytotoxic activities against KB and Hep-G_2 rather than the other cancer cell lines.

3. Experimental Section

3.1 General experimental procedures

NMR spectra were recorded in $CDCl_3$ on Bruker AV400 spectrometers at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR using TMS (tetramethylsilane) as internal standard. HRESIMS spectra were obtained with a Bruker micrOTOF. UV and IR data were recorded on a CARY 50 Probe and Perkin-Elmer Model 1760X spectrophotometer, respectively. Optical rotations were measured on a JASCO P-1010 polarimeter at 589 nm. Merck's silica gel 60 No.7734

Table 2. Cytotoxic activities of compound **1** and **2**.

Compound	IC_{50} (μg/mL)				
	BT474	Chago-K1	Hep-G_2	KATO-III	SW620
1	6.30±0.56	6.10±1.25	4.64±0.66	5.50±0.83	5.50±1.98
2	5.67±0.77	6.10±0.34	0.90±0.29	5.46±1.27	3.85±0.44
doxorubicin	0.64±0.23	0.47±0.15	0.07±0.04	0.85±0.33	0.10±0.02

was used as adsorbents for open column chromatography. Merck's thin layer chromatography (TLC) aluminum, silica gel 60 F_{254} precoated, 20x20 cm, layer thickness 0.2 mm were used for TLC analysis. The TLC plate was visualized under ultraviolet light at 254 nm and dipped with $(NH_4)_6Mo_7O_{24}$ in 5% H_2SO_4 solution.

3.2 Plant material

The fresh ripe fruits of *C. grewiifolia* were collected in July 2014 from Nongkae, Huahin Prachuap Khiri Khan, Thailand. The voucher specimen (No. 190331) has been deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

3.3 Extraction and isolation

Fresh fruits of *C. grewiifolia* (1.0 kg) were ground and macerated with MeOH (3.0 L for 3 days) at ambient temperature. The MeOH extracted was dried in vacuo, and extract with EtOAc and water. The crude extract of EtOAc (2.26 g) were separated on a silica gel column chromatography (CC) eluted with a gradient of hexanes-EtOAc (10:0 to 0:10) to afford four fractions (I-IV). The fraction IV (0.50 g) was subjected to silica gel CC and eluted with hexanes-EtOAc (8:2), Sephadex LH-20 CC (CH_2Cl_2/MeOH; 1:9), and silica gel CC ($CHCl_3$/Hexanes; 1:9) to give Compound **1** (4.0 mg) and **2** (3.5 mg).

3.3.1 Caseargrewiin M (1)

Colorless oil (4.0 mg); $[\alpha]^{23}_D$ +12.5 (c 0.05, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε): 242 (3.79) nm, IR (KBr) ν_{max} 3488, 2965, 2987, 2878, 1750, 1731, 1463, 1373, 1227 cm^{-1}; ^1HNMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 1; HRESIMS ion at *m/z* 513.2823 $[M+Na]^+$ (Calcd for $C_{28}H_{42}NaO_7$, 513.2828)

3.4 Bioassays

The cytotoxicity assay was carried out at the institute of Biotechnology and Genetic Engineering, Chulalongkorn University. All isolated compounds were tested for their cytotoxic activity towards five human cancer cell lines including Hep-G_2 (hepatocarcinoma), SW620 (colon adeno-carcinoma), Chago-K1 (undifferentiated lung carcinoma), KATO-III (gastric carcinoma) and BT474 (breast ductal carcinoma) cancer cell lines. Herein, the *in vitro* cytotoxicity was determined by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphynyltrazolium bromide) colorimetric method. In principle, the viable cell number/well was directly proportional to the production of formazan, which could be measured spectrophotometrically.

The human cancer cell lines were harvested from exponential-phase maintenance cultures (T-25 cm^2 flask), counted by trypan blue exclusion, and seed cells in a 96-well culture plates at a density of $1x10^5$ cells/well in 200 μL of culture medium without compounds to be tested. Cells were cultured in a 5% CO_2 incubator at 37°C, 100% relative humidity for 24 h. Culture medium containing the sample was dispensed into the appropriate wells (control cells group, N=3; each sample treatment group, N=3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N=3) and medium/DMSO blank (N=3) for "background" determination. Culture plates were then incubated for 72 h prior to the addition of tetrazolium reagent. MTT stock solution (5 mg/mL in PBS) was sterilized by filtering through 0.45 μm filter units. MTT working solution was prepared just prior to culture application by dilution of MTT stock 1:5 (v/v) in pre-warmed standard culture medium. The freshly prepared MTT reagent (10 μL) was added into each well and mixed gently for 1 minute on an orbital shaker. The cells were further incubated for 4 h at 37°C in a 5% CO_2 incubator. After incubation, the formazan produced in the cells captured as dark crystals in the bottom of the wells. All of the culture medium supernatant was removed from wells and 150 μL of DMSO was added to dissolve the resulting formazan. Samples in the culture plate were mixed for 5 minutes on an orbital shaker. Subsequently, 25 μL of 0.1M glycine (pH 10.5) was added and the culture plate was shaken for five minutes. Following formazan solubilization, the absorbance was measured using a microplate reader at 540 nm (single wavelength, calibration factor = 1.00) (Carmichael, DeGraff, Gazdar, Minna, & Mitchell, 1987; Twentyman & Luscombe, 1987).

Acknowledgements

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