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Cytotoxicity of Chromanone Acid from the Steam Bark of *Calophyllum incrassatum* against P-388 Leukemia Murine Cells

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Abstract: *Calophyllum incrassatum* is one of the plant species of the Calophylloceae family and is found endemic in Kalimantan and Sumatra. This study aims to isolate chromanone acid compounds from the stem bark of *C. incrassatum* and determine the relationship between structure and activity against murine Leukemia P-388 cells. The extraction method used was maceration using methanol solvent, partitioned with n-hexane and ethyl acetate solvents. Separation and purification of n-hexane and ethyl acetate extracts by vacuum liquid chromatography, pressed column, and radial. Two chromanone acids derivatives, isoapetalic acid (**1**), and methyl isoapetalic (**2**), have been successfully isolated from the stem bark of *Calophyllum incrassatum*. The structures of the two compounds were identified and elucidated using spectroscopic techniques such as UV, IR, and 1D and 2D NMR. The cytotoxic activity against murine leukemia P-388 cells of compounds **1-2** showed IC_{50} values of 8.51 and 1.47 µg/mL, respectively, and compound (**2**) exhibited high cytotoxic activity.

Keywords: *Calophyllum incrassatum*, chromanone acid, isoapetalic acid, methyl isoapetalic, cytotoxic.

Abstrak: *Calophyllum incrassatum merupakan salah satu spesies tumbuhan famili Calophylloceae dan ditemukan endemik di Kalimantan dan Sumatera. Penelitian ini bertujuan untuk mengisolasi senyawa asam kromanoat dari kulit batang C. incrassatum serta menentukan hubungan struktur dan aktiivitas terhadap sel murine Leukemia P-388. Metode ekstraksi yang digunakan yaitu maserasi menggunakan pelarut metanol, dipartisi dengan pelarut n-heksan dan etil asetat. Pemisahan dan pemurnian ekstrak n-heksan dan etil asetat dengan kromatografi cair vakum, kolom tekan, dan radial. Dua turunan asam kromanoat, asam isoapetaloat (**1**), dan metil isoapetaloat (**2**) telah diisolasi dari kulit batang Calophyllum incrassatum. Struktur kedua senyawa ditetapkan berdasarkan analisis UV, IR dan 1D dan 2D NMR. Uji aktivitas sitotoksik terhadap sel murin leukemia P-388 senyawa **1-2** masing-masing memperlihatkan nilai IC50 sebesar 8,51 dan 1,47 µg/mL dan senyawa **2** menunjukkan aktivitas yang sangat aktif.*

Kata kunci: *Calophyllum incrassatum, asam kromanoat, asam isoapetaloat, metil isoapetaloat, sitotoksik.*

INTRODUCTION

Calophyllum incrassatum (Calophyllaceae) known by the local name "bintangor" is an endemic plant of Southeast Asia found in Indonesia, Malaysia and Thailand. (Nasir *et al*. 2011). In Indonesia, *C. incrassatum* is found in Kalimantan and Sumatra, traditionally used as a medicine for wounds, fever, malaria and cancer. (Heyne 1987). One of the active compounds from *C. incrassatum* that has been isolated from the stem and leaves is a coumarin compound tested against cell line A-549 with an IC_{50} value of 87.71 µg/mL. (Aminudin *et al*. 2016). Metabolomics of *C. inophyllum* isolated from 95% ethanol extract of stem parts yields santone-derived compounds (Wei *et al*. 2011), 4-phenylkumarin was successfully isolated from *C. polyanthum* 95% ethanol extract from seed (Zhong *et al*. 2010), biflavonoids from *C. pulcherrimum* Wall leaf ethyl acetate extracts (Iraawan *et al*. 2009) and chromanone acid from the dichloromethanic extract of the stem bark of *C. teysmanni* (Lim *et al*. 2015). Calophyllum exhibits biological activities such as antibacterial, antioxidant (Aminudin *et al*. 2019), anti-inflammatory (Nguyen *et al*. 2017) and

anticancer (Zamakshshari *et al*. 2019). Compounds derivatives from 4-phenylkumarin and chromanone acid are only found in the plant Calophyllum (Su *et al*. 2008). The stereochemistry of chromanone acid has a minimum of three stereochemicals, two on the chroman ring and one on the alkyl chain of the carboxylic acid group. These stereochemicals have an important role in biological activity, especially cytotoxic activity against various cancer cells (Lim *et al*. 2015; Shen *et al*. 2004).

This study aimed to evaluate the cytotoxicity of two chromanone acid derivatives, namely isoapetalic acid (1) and methyl isoapetalic acid (2) obtained from the stem bark of *C. incrassatum*. The structures of compounds 1-2 were established based on UV, IR, and NMR analysis. The cytotoxic activity test of compound 1-2 was tested against murine leukemia P-388 cells which has never been published before.

MATERIALS AND METHOD

Materials

The tools used in this study were glassware, oven, simple distillation, rotary vacuum evaporator, UV lamp, micro pipette, gravity column chromatography, vacuum liquid chromatography, and radial chromatography. UV-Vis spectrophotometer Shimadzu 1800, IR spectra were determined with a Perkin Elmer spectrophotometer using KBr pellets. NMR spectra were determined with a JEOL ECA 400 NMR operating at 400 MHz (1H-NMR) and 100 MHz (13C-NMR). Materials used for gravity column chromatography used silica gel 60 Merck (article no. 1.0774931.1000) and radial chromatography used silica gel 60 PF254 Merck (article no. 1.07734.1000). Thin layer chromatography (TLC) analysis using silica gel 60 GF254 0.25 mm TLC plate (Merck) and stain spotting using cerium sulfate reagent and $CDCl_3$ solvent.

Plant material

The stem bark of *C. incrassatum* were obtained from Mandor District, Landak Regency, West Kalimantan, Indonesia Samples were identified by Dr. Ismail Rachman of Herbarium Bogoriense, Bogor, Center for Biological Research and Development, Cibinong, Indonesia. with specimen number CI-LMWK-IR1.

Extraction and Isolation

Extraction of dry powder of *C. incrassatum* stem bark (3.0 kg) was macerated using methanol for 48 hours at room temperature. The solvent was evaporated using a rotary vacuum evaporator to produce methanol extract of 280 g with a yield of 9.3%. The methanol extract was then partitioned with n-hexane and ethyl acetate. The n-hexane fraction (32 g) was subjected to vacum liquid chromatography over silica gel and eluted with n- mixture of n-hexane:ethyl acetate (9:1-8:2) to obtain fractions A (20 g) and B (2.12 g). Reseparation of fraction A 20 g with vacum liquid chromatography using the same eluent, n-hexan:ethyl acetate (9:1-8:2) produced two subfractions A1-A2. Separation of subfraction A1 (2.84 g) by radial chromatography using n-hexane:ether (9:1) eluent yielded 33 mg of compound 1. Purification of subfraction A2 (156 mg) with n-hexane:chloroform (9:1-7:3) eluent using radial chromatography yielded compound 2 (8.9 mg).

Cytotoxic Assay

Test of cytotoxic activity of compounds 1 and 2 against P-388 murine leukemia cells using the MTT method [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in vitro (Alley *et al*. 1988; Aldin *et al*. 2019; Tanjung *et al*. 2017). Test concentrations (0.1; 0.3; 1; 3; 10 and 30 ppm) were added to cell culture and incubated in 5% CO_2 for 24 hours at 37ºC. Repetition of each concentration test was done in triplicate. Cell growth activity was measured after treatment with MTT salt indicator, then incubated with stopper solution. Observation of inhibition of cytotoxic assay and determination of IC_{50} of isolated compounds using a microplate reader at 540 nm. The IC_{50} value was obtained by making a linear regression between concentration and percentage of living cells, by entering the value of y = 50%. The same treatment was applied to Artonin E compound which was used as a positive control.

RESULT AND DISSCUSION

Isoapetalic acid (1) with the molecular formula $C_{22}H_{28}O_6$ a yellow solid showing an optical angle of rotation $[\alpha]_D^{20}$ +16˚ (c= 0,0005, MeOH). UV spectrum of compound 1 in MeOH showed maximum absorption at λ maks (log ε): 226 (3.31), 274 (3.86) 300 (3.32), 304 (3.31), 312 (3.34) and 338 (2.55) absorption typical for chromanone acid compounds (Ha *et al*. 2012). The IR spectrum of compound 1 in KBr showed absorption bands at ν_{maks} (3435, 2926, 2864, 1707, 1579, 1533 and 1120) cm^{-1} which showed the presence of hydroxy, carbonyl carboxylic acid, aromatic and ether groups. Spektrum ^{1}H-NMR asam isoapetaloat terdiri dari cincin 2,3-dimetilkroman, cincin 2,2-dimetilpirano dan alkil heksanoat. The 1H-NMR spectrum of isoapetalic acid consists of a 2,3-dimethylchroman ring, a 2,2-dimethylpyrano ring and alkyl hexanoate. The ^{1}H-NMR spectrum of compound 1 shows the protons of the 2,3-dimethylchroman ring consisting of a pair of methine signals at chemical shifts δ_H 4,13 (1H, *dq*, *J* = 6,2 Hz dan 12,4 Hz, H-2), δ_H 2,52 (1H, *dq*, *J* = 7,0 Hz and 12,4 Hz, H-3), a pair of methyl proton signals at δ_H 1,45 (3H, *d*, *J* = 7,2 Hz, H-15), and δ_H 1,19 (3H, *d*, *J* = 7,0 Hz, H-16). The values of the coupling constants at H-2 and H-3 bound to C-2 and C-3 indicate compound 1 has stereochemical centers (2*e*, 3*a*) which are trans (Shen *et al*. 2004). The hydroxy proton signal at δ_H 12.48 (1H,*s*,5-OH) is a hydroxy proton bound to the aromatic core of the 2,3-dimethylchroman ring. The proton signal of the

2,2-dimethylpyrano ring linked to the aromatic of the 2,3-dimethylchromane ring consists of a pair of signals at cis vinylic at δ_H 6.59 (1H, *d*, *J*=10,0 Hz, H-6), δ_H 5,46 (1H, *d*, *J*=10,0 Hz, H-5) and two proton signals of the dimethyl gem at δ_H 1.47 (3H, *s*, H-17) and δ_H 1.46 (3H, *s*, H-18). The hexanoic acid alkyl proton signals consist of one methine proton signal at δ_H 3.69 (1H, *m*, H-19), two separate methylene proton signals at δ_H 2.69 (1H, *dd, J* = 6.9 and 14.9 Hz, respectively, H-20a), δ_H 2.81 (1H, *dd, J* = 8.2 and 14.9 Hz, H-20b), two methylene proton signals at δ_H 1.81 (2H, *m*, H-22), δ_H 1.13 (2H, *m*, H-23), and one methyl proton signal at δ_H 0.85 (3H*, t, J* = 7.3 Hz, H-24).

Analysis of the ^{13}C-NMR spectrum of compound 1 (Table 1) showed 22 carbon signals consisting of the 2,3-dimethylchroman ring [δ_C 201,2 (C-4), 160,4 (C-14), 157,4 (C-5/11), 108,9 (C-10), 102,6 (C-13), 101,3 (C-12), δ_C 76,1 (C-2), 44,3 (C-3), 16,3 (C-15), 9,4 (C-16)], 2,2-dimethylpyrano ring [δ_C 125,7 (C-7), 115,7 (C-6), 78,3 (C-8), 28,6 (C-17), 28,1 (C-18)] and alkyl hexanoate [δ_C 179,5 (C-21), 38,6 (C-20), 35,5 (C-22), 30,6 (C-19), 20,8 (C-23), 14,1 (C-24)]. The proton and carbon signal assignments of compound 1 were confirmed based on HMBC and HMQC spectra (direct correlation between H and C, in Table 1). Analyzing the HMBC spectrum, the methyl proton signal at δ_H 1.45 (H-15) showed the correlation of two methine carbons [C-2 and C-3] and the methyl proton signal at δ_H 1.19 (H-16) correlated with C-2, C-3 and the carbonyl carbon (C-4) of the 2,3 dimethylchroman ring. The signals of methine proton at δ_H 4.13 (H-2) and methine at δ_H 2.52 (H-3) showed correlation with C-4 and methyl carbon (C-16). The signal of the hydroxy proton attached to the aromatic of the 2,3 dimethylchroman ring at δ_H 12.48 (5-OH) showed correlation with the oxyaryl carbon at C-5 and two quaternary carbons at C-12 and C-13 (Figure 1). The methine proton signal of the alkyl

Table 1. ^{1}H and ^{13}C-NMR spectra data and HMBC of compounds (1) and (2) in $CDCl_3$

No.C	Isoapetalic acid (1)			Methyl isoapetalic (2)		
	δ_H (mult, *J* Hz)	**δ_C**	**HMBC**	**δ_H (mult, *J* Hz)**	**δ_C**	**HMBC**
2	4,13 (*dq*, 6,2; 12,4)	76,1	C-4; C-16	4,13 (*dq*, 6,2; 12,4)	78,9	C-4; C-15; C-16
3	2,52 (*dq*, 7,0; 12,4)	44,3	C-4; C-16	2,52 (*dq*, 7,0; 12,4)	45,8	C-4; C-15; C-16
4	-	201,2	-	-	199,4	-
5	-	157,4	-	-	157,1	-
6	6,59 (*d*, 10,0)	115,7	C-5;C-8;C-13; C-14	6,60 (*d*, 10,0)	115,8	C-5; C-8; C-13; C-14
7	5,46 (*d*, 10,0)	125,7	C-8; C-13; C-17	5,46 (*d*, 10,0)	125,8	C-8; C-13; C-17
8	-	78,3	-	-	78,1	-
9	-	-	-	-	-	-
10	-	108,9	-	-	109,3	-
11	-	157,4	-	-	157,4	-
12	-	101,3	-	-	101,4	-
13	-	102,6	-	-	102,8	-
14	-	160,4	-	-	159,9	-
15	1,45 (*d*, 7,2)	16,3	C-2; C-3	1,50 (*d*, 6,4)	19,7	C-2; C-3
16	1,19 (*d*, 7,0)	9,4	C-2; C-3; C-4	1,19 (*d*, 7,1)	10,4	C-2; C-3; C-4
17	1,47 (*s*)	28,6	C-7; C-8; C-18	1,41 (*s*)	28,4	C-7; C-8; C-18
18	1,46 (*s*)	28,1	C-7; C-8; C-17	1,41 (*s*)	28,3	C-7; C-8; C-17
19	3,69 (*m*)	30,6	C-10; C-14; C-20; C-21; C-22	3,69 (*m*)	29,8	C-10; C-14; C-20; C-21; C-22
20	2,69 (*dd*, 6,9; 14,9) 2,81 (*dd*, 8,2; 14,9)	38,6	C-10; C-19; C-21; C-22	2,73 (*m*)	38,8	C-10; C-19; C-21; C-22
21	-	179,5	-	-	172,8	-
22	1,81 (*m*)	35,5	C-19;C-23; C-24	1,81 (*m*)	35,6	C-23; C-24
23	1,13 (*m*)	20,8	C-19; C-22; C-24	1,14 (*m*)	20,9	C-19; C-22
24	0,85 (*t*, 7,3)	14,1	C-22; C-23	0,85 (*t*, 7,2)	14,1	C-22; C-23
5-OH	12,48 (*s*)	-	C-5; C-12; C-13	12,48 (*s*)	-	C-5; C-12; C-13
OCH_3	-	-	-	3,58 (*s*)	51,4	C-21

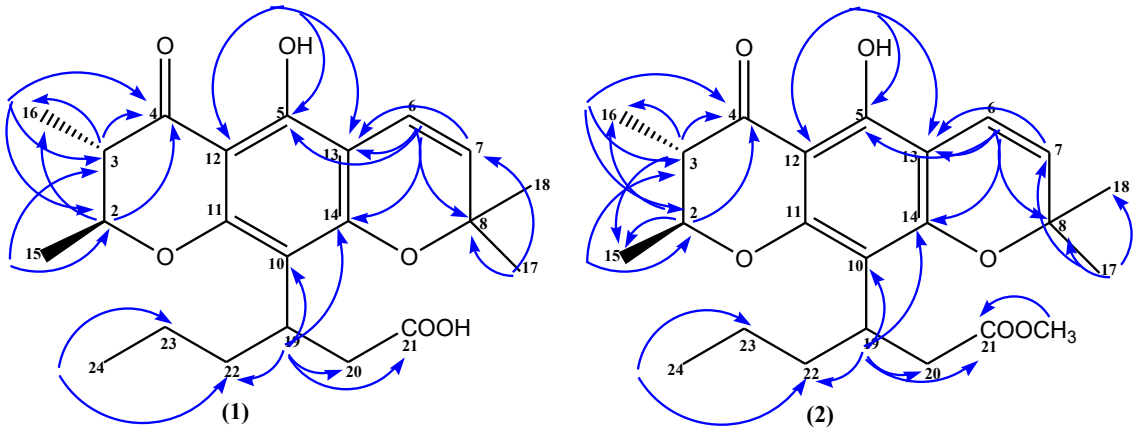

Figure 1. The main HMBC spectra of compounds 1 and 2

part of the carboxylic acid attached to the aromatic at δ_H 3.69 (H-19) correlates with the quaternary carbon at C-10, the oxyaryl carbon at C-14, the two methylene carbons at C-20, C-22 and the carboxylic acid carbonyl at C-21. These correlation results indicate the hexanoic acid alkyl is bound at C-10 of the aromatic core of the chroman ring (Figure 1). The vinylic proton signal of the 2,2-dimethyl pyrano at δ_H 6.59 (H-6) correlates with two oxyaryl carbons at C-5 and C-14 indicating the pyrano ring is connected to C-13 and C-14 (Figure 1). Based on the analysis of 1D and 2D NMR spectra, it is concluded that the chemical structure of compound 1 is an isoapetalic acid compound with the molecular formula $C_{22}H_{28}O_6$ (Aminudin *et al*. 2016; Shen *et al*. 2004). The main correlations that support structure 1 can be seen in Figure 1.

Methyl isoapetalic (2) with molecular formula $C_{23}H_{30}O_6$ is a yellow oil. UV-Vis spectrum analysis of compound 2 in MeOH solvent showed λmax (nm) (log ε): 219(3.32), 227(3.35), 274(3.92), 299(3.37), 311(3.38), and 336(2.59) which were similar to compound 1. IR spectrum analysis υmax (cm-1): 3423, 2929, 2872, 1737, 1631, 1577, 1539, and 1132 cm-1 indicating the presence of hydroxy, carbonyl carboxylic acid, conjugated carbonyl, aromatic and ether groups. The ^1H and ^{13}C NMR analysis of compound 2 also has similarities with the proton and carbon signals of compound 1, namely on the 2,3-dimethylchroman ring, and the 2,2-dimethyl pirano ring. The main difference is that compound 2 has a methoxy group at δ_H 3.58 at δ_C 51.4 (Shen *et al*. 2004), while compound 1 is a carboxylic acid indicated by the typical carboxylic acid carbonyl signal δ_C 179.6 (Shen *et al*. 2004) (Table 1). Based on the analysis of 1D and 2D NMR spectra have similarities, the difference is in HMBC additional proton methyl signal δ_H 3.58 methoxy group in compound 2 which correlates with carbonyl carbon of carboxylic acid at δ_C 172.8, it is concluded that the chemical structure of compound 2 is a methyl isoapetalic (Shen *et al*. 2004).

The cytotoxic assay of isoapetalic acid and methyl isoapetalic compounds against P-388 murine leukemia cells resulted in IC_{50} values of 8.51 and 1.47 µg/mL, respectively. The IC_{50} value was obtained from replacing y = 50% in the linear regression equation between concentration and percentage of live cells. Artonin E compound as a positive control has an IC_{50} value of 1.33 µg/mL. The activity of isoapetalic acid compound is categorized as inactive while methyl isoapetalic compound is categorized as very active. The cytotoxic properties of a test compound are categorized as strong if the IC_{50} value is < 2 ppm, moderate if IC_{50} > 2- 4 ppm, weak if IC_{50} > 4-8 ppm, and inactive if IC_{50} > 8 ppm (Alley et al, 1988; Ito *et al*. 2003). Compounds 1 and 2 have the same stereochemical center, namely (2*e*,3*a*). The presence of ester groups in compound 2 can increase anticancer activity compared to its acidic form in compound 1 (Shen *et al*. 2004).

CONCLUSION

Two chromanone acid compounds isoapetalic acid and methyl isoapetalic acid have been successfully isolated from C. incrassatum. The cytotoxic test of isoapetalic acid and methyl isoapetalic acid compounds against murine leukemia P-388 cells resulted in IC50 values of 8.51 µg/mL, respectively, which are inactive while compound 2 with IC50 of 1.47 µg/mL is highly active.

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