Rapid communication

Muhammad Taher*, Wan Mohd Nuzul Hakimi Wan Salleh*, Suhaib Ibrahim Alkhamaiseh, Farediah Ahmad, Mohamad Fazlin Rezali, Deny Susanti and Choudhury Mahmood Hasan

A new xanthone dimer and cytotoxicity from the stem bark of *Calophyllum canum*

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Abstract: A phytochemical investigation of the stem bark of *Calophyllum canum* resulted in the isolation of a new xanthone dimer identified as biscaloxanthone (**1**), together with four compounds; trapezifoliaxanthone (**2**), trapezifolixanthone A (**3**), taraxerone (**4**) and taraxerol (**5**). The structures of these compounds were determined via spectroscopic methods of IR, UV, MS and NMR (1D and 2D). The cytotoxicity of compounds **1–3** were screened against A549, MCF-7, C33A and 3T3L1 cell lines, wherein weak cytotoxic activities were observed (IC_{50} > 50 µM).

Keywords: biscaloxanthone; *Calophyllum canum*; cytotoxicity; Guttiferae; xanthone dimer.

***Corresponding authors: Muhammad Taher**, Department of Pharmaceutical Technology, Faculty of Pharmacy, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, 25200, Kuantan, Pahang, Malaysia, E-mail: mtaher@iium.edu.my; and **Wan Mohd Nuzul Hakimi Wan Salleh**, Department of Chemistry, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris (UPSI), Tanjung Malim, 35900, Perak, Malaysia, E-mail: wmnhakimi@fsmt.upsi.edu.my. https://orcid.org/0000-0003-1408-229X
Suhaib Ibrahim Alkhamaiseh, Department of Pharmaceutical Technology, Faculty of Pharmacy, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, 25200, Kuantan, Pahang, Malaysia
Farediah Ahmad, Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310, Skudai, Johor, Malaysia
Mohamad Fazlin Rezali, SIRIM Berhad (National Metrology Laboratory), Lot PT 4803, Bandar Baru Salak Tinggi, 43900, Sepang, Selangor, Malaysia
Deny Susanti, Department of Chemistry, Faculty of Science, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, 25200, Kuantan, Pahang, Malaysia
Choudhury Mahmood Hasan, Departmnet of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, 1000, Dhaka, Bangladesh

1 Introduction

The genus *Calophyllum*, which belongs to family Guttiferae, is composed of about 200 species mainly found in the restrictive area of Southeast Asia. *Calophyllum* is a well-known species as a rich source of phenolic compounds, including xanthones, coumarins and chromanones, in which their biological activities (e.g. anti-cancer and anti-HIV) have been proven [1–4]. Xanthones refer to a group of flavonoid that is structurally diverse, biologically active, biosynthetically interesting and synthetically challenging natural products [5–8]. *Calophyllum canum* is locally known as '*bintangor merah*', which can be found in abundance across Peninsular Malaysia, Borneo and Sumatra. It is a medium-sized to fairly large tree with 35 m in length and grows in well-drained mixed dipterocarp forest on clay-rich soils and in peat swamps up to 1200 m altitude. The plant is an important source of timber in Sarawak, while the latex is used to stupefy fish [9]. Phytochemical investigation on the heartwood of *C. canum* revealed the isolation of three xanthones, namely, 2-(3,3-dimethylallyl)-1,3,5,6-tetrahydroxyxanthone, 2-(3,3-di methylallyl)-1,3,7-trihydroxyxanthone and osajaxanthone [10]. A preliminary study that the authors conducted revealed that *C. canum* extracts displayed significant activity against Gram-positive bacteria, as well as cytotoxicity towards A549 and MCF-7 cell lines [11]. As part of the authors' interest in Guttiferae family, phytochemicals from the stem bark of *C. canum* were assessed. As such, this paper reports the isolation of a new xanthone dimer, identified as biscaloxanthone (**1**), along with trapezifoliaxanthone (**2**), trapezifolixanthone A (**3**), taraxerone (**4**) and taraxerol (**5**), as well as their cytotoxic activity against several cell lines.

2 Results and discussion

Compound **1** was obtained as yellow powder. The HREIMS spectrum revealed a molecular ion peak at *m/z* 772.3315 (calculated 772.3319), which was ascribed to $C_{47}H_{48}O_{10}$. The calculated DBE value of 24 was linked with four aromatic rings, three pyron rings with two carbonyl and three

prenyl groups. The infrared (IR) spectrum displayed distinctive absorption bands that corresponded to hydroxyl group at 3349 cm^{-1} and carbonyl group at 1645 cm^{-1}. The bands at 1225 cm^{-1} for C-O and 1569 cm^{-1} for C=C aromatic (C=C) were observed as well.

The ^{1}H NMR spectra exemplified two downfield signals attributed to chelate hydroxyl groups at δ_H 13.34 (1-OH) and 13.27 (10-OH). Two multiplet signals, each resonated at δ_H 6.46 and 5.74, signified the presence of protons at pentenyl ring system located at C-1′′′′ and C-2′′′′, respectively. Three multiplet signals that resonated at δ_H 4.55, 4.65 and 2.91 indicated the existence of prenyl side chains found at C-1′, C-1″ and C-1‴, respectively. The signals of aromatic protons H-2 and H-4 were overlapped and resonated at δ_H 6.24 (s, 2H), while H-13 and H-14 were denoted by the signal at δ_H 7.48 (s, 2H) and the signal for H-5 resonated at δ_H 7.57 (s, 1H). The five singlet signals at δ_H 1.41, 1.43, 1.49, 1.50 and 1.60 were associated with nine methyl protons H-4″/H-5″, H-4‴/H-5‴, H-6′, H-4′/H-5′ and H-4′′′′/H-5′′′′, respectively. Both ^{13}C NMR and DEPT spectra reflected the presence of methyl protons of a pentenyl ring system which displayed a signal at δ_C 78.8 (C-3′′′′). Additionally, nine methines were observed at δ_C 121.4 (C-2′), 121.5 (C-2″), 132.3 (C-14), 90.9 (C-13), 129.5 (C-2‴), 93.8 (C-2), 93.5 (C-4), 116.0 (C-5), and 117.5 (C-1′′′′). Also, nine methyl groups were noted at δ_C 14.8 (C-4′/C-5′), 25.5 (C-4″/C-5″), 19.7 (C-4‴), 26.6 (C-5‴), 28.4 (C-4′′′′/C-5′′′′) and 21.1 (6′-CH_3). The HMBC spectrum displayed correlations of proton at C-2′′′′ (δ_H 5.74), while carbons at δ_C 146.2 (C-6), 117.5 (C-1′′′′), 78.8 (C-3′′′′) and 28.4 (C-4′′′′/C-5′′′′). Long-range correlations were noted for proton at C-1′′′′ (δ_H 6.46), whereas carbons at δ_C 146.2 (C-6), 117.5 (C-1′′′′), 78.8 (C-3′′′′) and 114.0 (C-7). This reflected that compound **1** had two xanthones connected by oxygen-bridge. The methylene protons at δ_H 4.55 (H-1′), 4.65 (H-1″) and 2.91 (H-1‴) were correlated with carbon signals at δ_C 112.8 (C-8), 121.4 (C-2′), 142.4 (C-16), 103.3 (C-18a), 121.5 (C-2″), 113.0 (C-15) and 129.5 (C-2‴), respectively. Methine protons at δ_H 5.71 (H-2′), 5.72 (H-2″) and 5.51 (H-2‴) were correlated with carbons at δ_C 21.7 (C-1′), 132.0 (C-3′); 21.8 (C-1″), 132.5 (C-3″) and 32.8 (C-1‴), 133.5 (C-3‴), respectively. This verified the attachment of three prenylated groups at C-8, C-15 and C-17 positions. Referring to the retrieved outcomes and detailed spectral analyses, the structure of compound **1** was established as 1,3,10,16-tetrahydroxy-7-methyl-8,15,17-tris(3-methyl-2-butenyl)-3′′′′,3′′′′--dimethyl-2H,6H-pyrano[11, 12] bisxanthen-9,18-dione, and given a trivial name of biscaloxanthone.

On the other hand, the known compounds, namely, trapezifolixanthone (**2**) [12], trapezifolixanthone A (**3**) [13], taraxerone (**4**) [14] and taraxerol (**5**) [15], were identified by comparing their spectroscopic data with published data. The chemical structures of the isolated xanthones **1–3** are

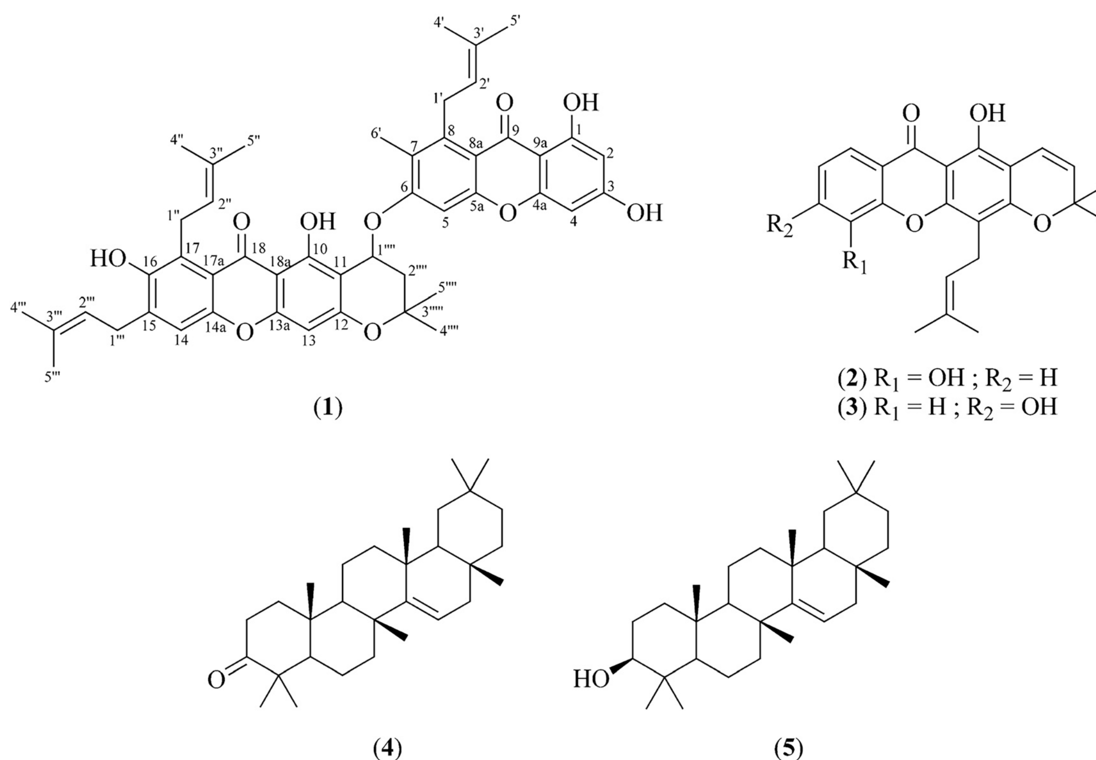

Figure 1: Chemical structures of isolated xanthones from *Calophyllum canum*.

illustrated in Figure 1. The cytotoxicity of compounds **1–3** against three cell lines, including human lung carcinoma (A-549), breast cancer (MCF-7), cervical cancer (C33A) and normal rat fibroblast (3T3L1), with taxol served as positive control. As a result, the compounds exemplified weak cytotoxic activities (IC_{50} > 50 μM). The results are shown in Table 2.

3 Experimental section

3.1 General experimental procedures

The melting point was measured on a Buchi B545 melting point apparatus and uncorrected. Optical rotation was measured on a Polax-2L polarimeter. The NMR (1D and 2D) spectra were recorded on a Bruker Avance 400 MHz in $CDCl_3$. EIMS were measured on Waters Acquity uPLC/SQD mass spectrometer. The IR spectra were obtained with a Perkin Elmer Spectrum RX Infrared spectrophotometer. Chemical shifts are reported in ppm and δ scale, while coupling constants are presented in Hz. Silica gel (70–230 and 230–400 Mesh, Merck, Germany) and Sephadex LH-20 (GE Healthcare Life Sciences) had been applied for chromatography. Paclitaxel was purchased from Sigma (St. Louis, MO).

3.2 Plant material

The stem barks of *C. canum* were collected in August 2008 from Pahang, as identified by Dr Shamsul Khamis from Universiti Kebangsaan Malaysia. The voucher specimen (MT-01) was deposited at the herbarium located in Faculty of Pharmacy, International Islamic University Malaysia.

3.3 Extraction and isolation

The stem barks of *C. canum* (1.0 kg) were air-dried, cold extracted with EtOH (3 × 2.5 L), and concentrated to yield 238.8 g (23.9%) of crude extract. The extract was fractionated via VLC technique and was eluted with mobile phases of different polarity (*n*-hexane:EtOAc:MeOH) to yield *n*-hexane (73.5 g, 7.35%), EtOAc (27.7 g, 2.8%) and MeOH (134.8 g, 13.5%) fractions. Further fractionation of *n*-hexane fraction was performed based on VLC technique eluted with *n*-hexane:EtOAc:MeOH to retrieve nine sub-fractions (I-IX). Sub-fraction II was chromatographed using SiO_2 and was eluted gradually with both *n*-hexane and CH_2Cl_2 to obtain 150 fractions. Fractions 134–139 were combined and purified with CC on Sephadex LH 20 eluted with acetone to yield compound **2** (24 mg, 0.0024%). The EtOAc fraction was purified by SiO_2 with a mixture of *n*-hexane, EtOAc, and MeOH to afford five sub-fractions (I-V). Sub-fraction I was subjected to CC and was eluted gradually with *n*-hexane: CH_2Cl_2 (1:7) to yield 75 fractions. Fractions 1–8

Table 1: NMR spectral data of compound (**1**)

C	^{1}H NMR	^{13}C NMR
1		164.2
2	6.24 (s, 1H)	93.8
3		165.8
4	6.24 (s, 1H)	93.5
4a		152.6
5a		144.5
5	7.57 (s, 1H)	116.0
6		146.2
7		114.0
8		112.8
8a		132.5
9		180.4
9a		114.7
10		164.1
11		103.3
12		165.9
13	7.48 (s, 1H)	90.9
13a		152.8
14a		144.8
14	7.48 (s, 1H)	132.3
15		113.0
16		142.4
17		117.9
17a		117.5
18		180.1
18a		103.3
1′	4.55 (m, 2H)	21.7
2′	5.71 (m, 1H)	121.4
3′		132.0
4′	1.50 (s, 3H)	14.8
5′	1.50 (s, 3H)	14.8
6′-CH_3	1.49 (s, 3H)	21.1
1″	4.65 (m, 2H)	21.8
2″	5.72 (m, 1H)	121.5
3″		132.5
4″	1.41 (s, 3H)	25.5
5″	1.41 (s, 3H)	25.5
1‴	2.91 (m, 2H)	32.8
2‴	5.51 (m, 1H)	129.5
3‴		133.5
4‴	1.43 (s, 3H)	19.7
5‴	1.43 (s, 3H)	26.6
1⁗	6.46 (m, 1H)	117.5
2⁗	5.74 (m, 2H)	32.6
3⁗		78.8
4⁗	1.60 (s, 3H)	28.4
5⁗	1.60 (s, 3H)	28.4
1-OH	13.34 (s, 1H)	
10-OH	13.27 (s, 1H)	

Table 2: Cytotoxicity assay of isolated xanthones (**1–3**)

Compounds	A549 (μM)	MCF-7 (μM)	C33A (μM)	3T3L1 (μM)
1	>100	>100	>100	>100
2	>100	88.70 ± 1.55	>100	47.83 ± 0.85
3	50.02 ± 0.35	>100	>100	n.a.
Taxol	0.80 ± 0.10	1.20 ± 0.10	1.90 ± 0.10	n.d

n.d: not determined; Statistical analysis of the data was carried out using Pearson correlation on SPSS 18 software. Significance was defined as $r > 0.7$ and $p < 0.05$.

were combined and triturated with MeOH to yield compound **4** (10 mg, 0.001%). Sub-fraction II was chromatographed with CC and was eluted gradually with both *n*-hexane and EtOAc to yield 188 fractions. Fractions 13–15 were combined and triturated with MeOH to gain compound **5** (18 mg, 0.0018%). Fractions 17–23 were combined and purified with CC packed with Sephadex LH-20 and were eluted with MeOH to yield compound **3** (12 mg, 0.0012%). Fractions 33–52 were combined, evaporated and subjected to preparative thin-layer chromatography (PTLC) which was eluted with PE:CH_2Cl_2 (1:2) to obtain compound **1** (7 mg, 0.0012%). Fractions 53–64 were combined, evaporated and subjected to PTLC with PE:CH_2Cl_2 (1:7) to yield compound **5** (7 mg, 0.0005%).

Biscaloxanthone (1): Yellow powder (7 mg, 0.0012%); IR ν_{max} (KBr) cm^{-1}: 3349, 2927, 1645, 1569, 1225; ^1H NMR (400 MHz, Acetone-d_6) and ^{13}C NMR (100 MHz, Acetone-d_6) as in Table 1. HREIMS: *m/z* 772.3315 (calculated 772.3319 for $C_{47}H_{48}O_{10}$). Supplementary data: the 1D and 2D-NMR spectra of compound **1** are available as Supplementary material.

3.4 Cytotoxic assay

Cytotoxicity assay was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described in [16]. The monolayer cells were cultured in a completed media until the cells were confluent. Next, the cells were seeded in a 96-microwell plate at 10^5 cells/well density and incubated at 37 °C in 5% CO_2 humidified incubator. After 24 h, a fresh medium was added and the cells were treated with a range of concentrations of compounds by serial dilution. After 24 h of incubation, the supernatant was discarded and the adherent cells were washed twice with phosphate buffer saline (PBS). After that, 20 μL of 5 mg/mL MTT stock solution was added to each well and the plate was incubated overnight at 37 °C. Next, 100 μL DMSO was added to each well to solubilise the water-insoluble purple formazan crystals, which were generated by viable cells. After completely dissolving the formazan blue, 100 μL of the solution was transferred to a new 96-microwell plate, in which the absorbance rate was measured at 570 nm and the reference wavelength was 690 nm by using a multi-detection microplate reader. All samples were assayed in triplicates. The percentage of cell viability was calculated based on the equation given below, while the concentrations required for inhibition of 50% cell viability (IC_{50}) were determined. % of cell viability = Absorbance of treated cells/Absorbance of control cells × 100%.

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