

Xanthenes from *Calophyllum gracilipes* and Their Cytotoxic Activity (Zanton daripada *Calofilum gracilipes* dan Aktiviti Ketoksikannya)

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ABSTRACT

Extraction and chromatographic isolation of the hexane, chloroform and methanol extracts of stem bark of Calophyllum gracilipes has led to the isolation of a new xanthone, gracixanthone (1) and the known zeyloxanthanone (2) and trapezifolixanthone (3) together with three common sterols, namely stigmaterol, friedelin and lupeol. The structures of the compounds were elucidated and established by spectroscopic analysis and compared with the spectral data from literature. The cytotoxicity of the compounds was evaluated and zeyloxanthanone (2) exhibited strong activity towards five cell lines with IC₅₀ values ranging at 8.00–26.00 μM.

Keywords: Calophyllum gracilipes; gracixanthone; sterols; trapezifolixanthone; zeyloxanthanone

ABSTRAK

Pengekstrakan dan pemisahan kromatografi terhadap ekstrak heksana, klorofom dan metanol kulit batang Calofilum gracilipes telah berjaya memisahkan satu zanton baru, graciksanton (1) dan dua zanton kenali, zeyloksantanon (2) dan trapezifoliksanton (3) bersama dengan tiga sterol biasa, stigmaterol, friedelin dan lupeol. Struktur sebatian ini telah dikenal pasti dengan kaedah analisis spektroskopi dan dibandingkan dengan data spektrum yang terdapat dalam rujukan. Ujian ketoksikan sebatian ini telah ditentukan dan zeyloksantanon (2) menunjukkan aktiviti yang kuat terhadap lima sel dengan nilai IC₅₀ dalam julat 8.00–26.00 μM.

Kata kunci: Calofilum gracilipes; graciksanton; sterol; trapezifoliksanton; zeyloksantanon

INTRODUCTION

The Guttiferae family is a well-known and important group of trees in tropical Asia and Africa. It is made up of four genera and one of the important and prominent genera is *Calophyllum*, locally known as ‘bitangor’ (Corner 1988; Whitmore 1973). There are about 150 species in this medium sized to large evergreen trees which can grow up to 30 m in height. Previous reports indicated the genus is widely used in traditional medicine as antiseptics, astringents, diuretics and purgatives (Ali et al. 1999). *Calophyllum* is a rich source of various types of phenolic compounds such as xanthenes, coumarins, flavonoids, benzophenones, steroids and triterpenes (Hay et al. 2004; Inuma et al. 1997; Ishikawa 2000; Kijjoa et al. 2000). A number of this compound particularly coumarins and xanthenes have been reported to exhibit various biological activities such as calanolide A and calanolide B isolated *Calophyllum lanigerum* for the treatment of HIV (Kostova 2006; Laure et al. 2008). Dimethylcalabaxanthone isolated from *Calophyllum caledonicum* has also been reported to exhibit very strong activity against chloroquine-resistant strains of *Plasmodium falciparum* (Hay et al. 2004). In a continuation of our work on *Calophyllum* species, we wish to report the isolation and structural determination of a new and two known xanthenes together with three

common sterols from the bark of *Calophyllum gracilipes* (Ee et al. 2011; Nasir et al. 2011). Preliminary cytotoxic test results of the three xanthenes against five cell lines are also reported.

EXPERIMENTAL DETAILS

GENERAL

The melting points were determined using a Leica Galen III apparatus. UV spectra were determined in EtOH using a Shimadzu UV-160A spectrophotometer. NMR spectra were obtained with either JEOL JNM CRX 400 or 500 MHz FT-NMR spectrometer in CDCl₃ or acetone-*d*₆ as solvent and tetramethylsilane as internal standard. IR spectra were obtained using a Perkin Elmer FTIR model 1725X spectrometer. EIMS were recorded on a Shimadzu GCMS-QP5050A spectrometer. HREIMS was analysed by ToF mass spectrometry at Chemistry Department, Universiti Kebangsaan Malaysia, Selangor. Silica gel 60H 1.07736 Merck and 60 (0.063–0.200 mm) 1.07734 Merck were used for column chromatography. Precoated sheets of silica gel 60F₂₅₄ Merck were used for TLC analysis and the spots were visualized either with a UV lamp (254 nm and 356 nm) or by iodine vapour.

PLANT MATERIAL

The stem bark of *Calophyllum gracilipes* was collected from Sandakan Sabah, East Malaysia in 2002. A voucher specimen (FRCSE 556) has been deposited at the Herbarium, Department of Forestry in Sepilok, Sandakan, Sabah, Malaysia.

EXTRACTION AND CHROMATOGRAPHIC SEPARATION

The finely ground air-dried stem bark of *Calophyllum gracilipes* (2.1 kg) was sequentially extracted at room temperature with n-hexane, chloroform and methanol to give 12.6, 27.8 and 56.1 g of dark viscous materials on solvent removal, respectively. Repeated silica gel chromatographic separation of the hexane extract eluted with system solvent of increasing polarity made up of hexane, chloroform and ethyl acetate led to the isolation of the common sterols stigmaterol, friedelin and lupeol. Similarly, the chloroform extract was separated by column chromatography eluted with the above solvent system to give 42 fractions of 200 mL each. The combined fractions 30-35 were further purified by column chromatography to give 35 fractions of 150 mL each. The yellowish solid obtained from fractions 8-15 were combined and recrystallised with chloroform to give zeyloxanthone (2) (0.30 g) as yellow powder with m.p. 151-152°C. Silica gel chromatographic separation of fractions 19-27 gave another yellow solid and recrystallised with chloroform to give trapezifolixanthone (3, 0.32 g) as yellow prisms, m.p. 145-146°C. Similar treatment on the methanol extract gave gracixanthone (1, 0.22 g.) as light orange needle-shaped crystals with m.p. 241-242°C.

ANALYTICAL DATA OF COMPOUNDS (1-3)

Gracixanthone (1) The compound was obtained as light orange needle-shaped crystals with m.p. 241-242°C; UV (EtOH) λ_{\max} (log ϵ) nm: 243 (3.91), 288 (0.95), 310 (1.02); IR ν_{\max} cm^{-1} (KBr): 3372, 2947, 2847, 1647, 1459, 1160; EIMS m/z (%): 274 [M^+] (80), 259 (60), 231 (100), 137 (12); HR-EIMS m/z 274.0445 ($\text{C}_{14}\text{H}_{10}\text{O}_6$ calc. For 274.0477); $^1\text{H-NMR}$ (400 MHz, acetone- d_6) and $^{13}\text{C-NMR}$ (100MHz, acetone- d_6) (Table 1 & Figure 1).

Zeyloxanthone (2) Zeyloxanthone was obtained as yellow powder with m.p. 151-152°C; UV (EtOH) λ_{\max} (log ϵ) nm: 234, 259 (2.63), 294 (2.12); IR ν_{\max} cm^{-1} (CHCl_3): 3363, 2921, 1705, 1452, 1376, 1307, 829; EIMS m/z (%): 450 [M^+] (15), 381 (20), 325 (100) and 165 (15); $^1\text{H-NMR}$ (500 MHz, CDCl_3) and $^{13}\text{C-NMR}$ (125MHz, CDCl_3) (Table 1 & Figure 1).

Trapezifolixanthone (3) The compound was recrystallised as yellow prisms with m.p. 145-146°C; UV (EtOH) λ_{\max} (log ϵ) nm: 250 (2.48), 257 (2.06), 269 (2.61); IR ν_{\max} cm^{-1} (CHCl_3): 3207, 1647, 1614, 1497, 1433, 1124, 757; EIMS m/z (%): 378 [M^+] (35), 363 (100), 335 (20), 154 (10); $^1\text{H-NMR}$ (500 MHz, CDCl_3) and $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) (Table 1 & Figure 1).

CYTOTOXIC ASSAY

The test was carried out by using the normal MTT assay and the five cell lines used were human breast adenocarcinoma (MCF-7), colon carcinoma (HTC-116), prostrate carcinoma (PC3), African Green Monkey kidney (VERO) and mouse macrophages (RAW 264.7) cells. The cells were obtained from American Tissue Culture Collection (Virginia, USA). The cells were maintained in RPMI 1640 culture medium except RAW 264.7 cell line which was maintained in DMEM media. Briefly, exponentially growing cells were seeded into 96-ell plate and allowed to adhere overnight. Treatments in the final concentration ranged between 0.1 and 100.00 μM were introduced. The control wells were treated with 0.1% of DMSO equivalent to the amount of DMSO used as a vehicle in the compound treated wells. After 96 h of incubation, 50 μL of MTT solution was added and incubated for additional 4 h. Medium and excessive MTT were aspirated and formazan formed are solubilised by addition of 100 μL DMSO. Absorbance, as a measure of viable cell number, was read at 550 nm with Versa Max microplate reader. Using the absorbance value on 0-day as initial optical density, the dose response growth curves were constructed, the growth percentages were determined by using the standard formula and IC_{50} values were determined from the curves.

RESULTS AND DISCUSSION

Compound (1) was obtained as light yellow needle-shaped crystals after recrystallisation with chloroform with m.p. 241-242°C. The compound is suspected to have xanthone skeleton based on UV spectrum with the occurrence of absorption at 243 (3.91), 288 (0.95) and 310 (1.02) typical characteristic of xanthone skeleton (Inuma et al. 1996). The prominent and broad absorption at 3372 cm^{-1} in the IR spectrum indicated the presence of hydroxyl group together with prominent band at 1647 cm^{-1} for the existence of chelated carbonyl group. The molecular formula of the compound was calculated as $\text{C}_{14}\text{H}_{10}\text{O}_6$ based on EIMS spectrum which exhibited molecular ion peak at m/z 274 and the base peak at m/z 231. HR-EIMS gave molecular ion peak at m/z 274.0545 ($\text{C}_{14}\text{H}_{10}\text{O}_6$ calc. for 274.0477). The presence of ten protons in the molecular formula is further substantiated by the integration of the $^1\text{H-NMR}$ spectra which made up of a methoxyl, four aromatic protons and three hydroxyl groups. Three of the four aromatic protons occurred as an ABC system with the existence of three doublet of doublet resonances at δ 7.24 (1H, *dd*, 7.3, 7.8 Hz, H-2), 7.30 (1H, *dd*, 7.8, 1.8 Hz, H-3) and δ 7.60 (1H, *dd*, 7.3, 1.8 Hz, H-1). The correlations of these three aromatic protons could be further seen in the COSY spectrum. The fourth and isolated aromatic proton appeared as sharp singlet at δ 6.48 (1H, *s*, H-5). The three proton singlet at the upfield region of δ 3.83 is assigned to the methoxy group.

The existence of fourteen carbon atoms is further supported by the $^{13}\text{C-NMR}$ and DEPT spectra made up of nine

TABLE 1. ^1H -NMR and ^{13}C -NMR spectral data of gracixanthone (1), zeyloxanthone (2) and trapezifolixanthone A (3)

| H/C | (1) in acetone- d_6 δ_{H} | δ_{C} | HMBC | (2) in CDCl_3 δ_{H} | δ_{C} | (2) [#] δ_{C} | (3) in CDCl_3 δ_{H} | δ_{C} | (3) [#] δ_{C} |
|----------|----------------------------------------------|---------------------|-------------------|-----------------------------------------------|---------------------|-----------------------------------------|-----------------------------------------------|---------------------|-----------------------------------------|
| 1 | 7.60 (<i>dd</i> , 7.3, 1.8 Hz) | 116.1 | C2, C3, C9, C9a | 13.46 (OH) | 159.5 | 160.5 | 13.03 (OH) | 159.0 | 156.0 |
| 2 | 7.24 (<i>dd</i> , 7.3, 7.8 Hz) | 124.7 | C1, C3, C4, C9a | - | 111.8 | 111.3 | - | 104.1 | 123.9 |
| 3 | 7.30 (<i>dd</i> , 7.8, 1.8 Hz) | 121.7 | C1, C2, C4, C4a | 6.20 (OH) | 162.1 | 162.0 | - | 158.2 | 158.3 |
| 4 | - | 146.8 | | 6.30 (<i>s</i> , 1H) | 93.6 | 94.0 | - | 107.0 | 107.0 |
| 4a | - | 146.0 | | - | 155.7 | 156.0 | - | 153.6 | 153.7 |
| 5 | 6.48 (<i>s</i> , 1H) | 94.6 | C6, C7, C8a, C10a | 2.81 (2H, <i>t</i> , 8.0 Hz) | 26.7 | 27.6 | 5.76 (<i>s</i> , OH) | 144.6 | 144.4 |
| 6-OMe | 3.83 (<i>s</i> , 3H) | 60.6 | C6 | - | - | - | - | - | - |
| 6 | - | 131.5 | | 2.52 (2H, <i>t</i> , 8.0 Hz) | 38.4 | 38.8 | 7.27 (1H, <i>dd</i> , 7.4, 1.8 Hz) | 119.7 | 119.7 |
| 7 | - | 155.3 | | - | 212.7 | 213.4 | 7.19 (1H, <i>dd</i> , 7.4, 7.7 Hz) | 123.8 | 123.9 |
| 8 | 13.09 (OH) | 159.1 | C6, C8, C8a | - | 55.5 | 55.7 | 7.72 (1H, <i>dd</i> , 7.7, 1.8 Hz) | 116.9 | 116.8 |
| 8a | - | 121.6 | | - | 117.3 | 117.8 | - | 120.8 | 120.9 |
| 9 | - | 182.0 | | - | 181.9 | 182.7 | - | 180.9 | 181.0 |
| 9a | - | 103.9 | | - | 105.9 | 105.6 | - | 103.3 | 103.3 |
| 10a | - | 153.7 | | - | 164.8 | 165.0 | - | 144.1 | 144.2 |
| 1' | | | | 3.43 (2H, <i>br d</i> , 6.8 Hz) | 21.9 | 22.3 | 6.70 (1H, <i>d</i> , 10.0 Hz) | 115.6 | 115.7 |
| 2' | | | | 5.27 (1H, <i>t</i> , 6.8 Hz) | 122.1 | 122.3 | 5.58 (1H, <i>d</i> , 10.0 Hz) | 127.5 | 127.4 |
| 3' | | | | - | 135.2 | 135.4 | - | 78.3 | 78.3 |
| 4' | | | | 1.75 (3H, <i>s</i>) | 25.8 | 26.6 | 1.45 (3H, <i>s</i>) | 28.3 | 28.3 |
| 5' | | | | 1.82 (3H, <i>s</i>) | 18.9 | 18.7 | 1.45 (3H, <i>s</i>) | 28.3 | 28.3 |
| 1''/1''' | | | | 2.66 (2H, <i>dd</i> , 8.0, 8.5 Hz) | 35.8 | 36.3 | 3.45 (2H, <i>d</i> , 7.36 Hz) | 21.6 | 21.7 |
| | | | | 3.12 (2H, <i>dd</i> , 9.2, 8.0 Hz) | | | | | |
| 2''/2''' | | | | 4.77 (1H, <i>dd</i> , 8.5, 9.2 Hz) | 119.8 | 120.7 | 5.18 (1H, <i>t</i> , 7.36 Hz) | 122.6 | 122.7 |
| 3''/3''' | | | | - | 134.6 | 135.6 | - | 131.6 | 131.6 |
| 4''/4''' | | | | 1.53 (3H, <i>s</i>) | 26.2 | 26.7 | 1.68 (3H, <i>s</i>) | 25.6 | 25.5 |
| 5''/5''' | | | | 1.45 (3H, <i>s</i>) | 18.4 | 18.6 | 1.83 (3H, <i>s</i>) | 17.9 | 17.9 |

[#] Iinuma et al. 1997; ^{*} Seo et al. 1999

quaternary carbon atoms at δ 159.1, 153.7, 131.5, 146.8, 146.0, 103.9, 182.0, 121.6 and 155.3 ppm and assigned to C-8, C-10a, C-6, C-4, C-4a, C-9a, C-8a and C-7, respectively, while the methine carbon atoms at C-1, C-3, C-2 and C-5 occurred at δ 116.1, 121.7, 124.7 and 94.6 ppm, respectively. The upfield signal at δ 60.6 indicated the presence of a methoxy group. The assignment of the carbon signals were further supported by HMQC spectrum. The two-bond and three-bond correlations in the HMBC spectrum revealed cross peaks of H-1 with C-2 (124.7), C-3 (121.7), C-9 (182.0) and C-9a (103.9); H-2 with C-1 (116.1), C-3 (121.7), C-4 (146.8) and C-9a (121.6); and H-3 with C-1 (116.1), C-2 (124.7) and C-4 (146.8)

confirmed the presence of ABC system (Figure 2). Further HMBC correlations of H-5 with C-6 (131.5), C-7 (155.3), C-8a (121.6) and C-10a (153.7) support the occurrence of isolated aromatic proton. Based on these evidences, the compound is considered as new and given a trivial name gracixanthone (1).

Compound (2) was obtained as yellow powder after recrystallisation with m.p. 151-152°C. The IR spectrum indicated the presence of hydroxyl group with the occurrence of a broad and strong absorption at 3363 cm^{-1} and carbonyl functionality with prominent band at 1705 cm^{-1} . The mass spectrum showed the presence of molecular ion peak at m/z 450 which correspond to molecular formula

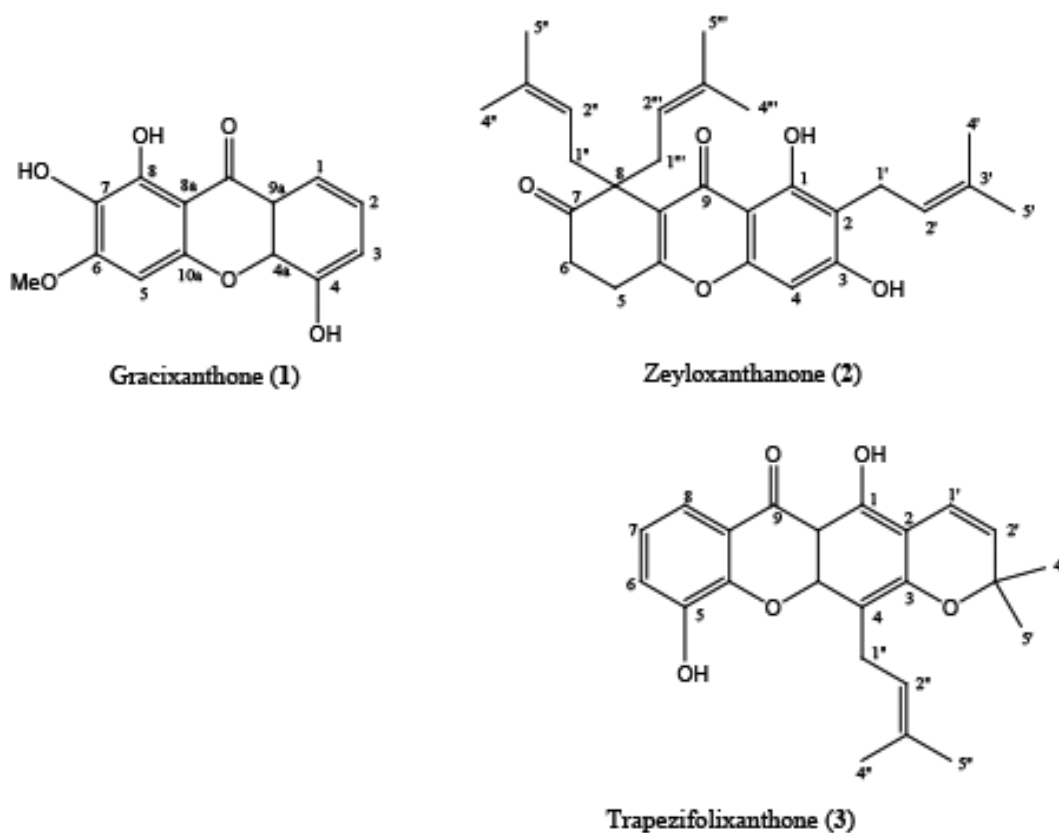


FIGURE 1. Structure of compounds (1), (2) and (3)

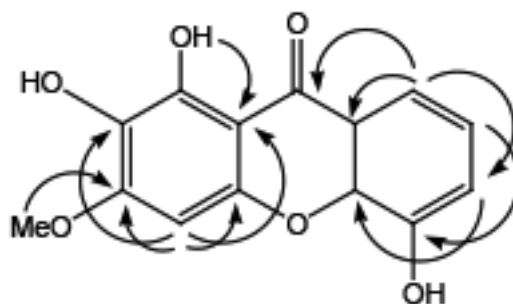


FIGURE 2. Selected HMBC correlations of gracixanthone, (1)

$C_{28}H_{34}O_5$ with the base peak at m/z 325. The presence of thirty four protons was supported by the integration in the 1H -NMR spectrum which consists of six methyls, five methylene, four methines and two hydroxyls groups. One of the hydroxyl groups is chelated and occurred at very field region of δ 13.46 and the other at δ 6.20 as a broad singlet (Table 1). The occurrence of an isolated aromatic proton at δ 6.30 could be seen clearly and the other three methines protons are parts of the three prenyl substituents attached at C-2 and C-8. The various NMR spectra of the compound corresponded to reported data of zeyloxanthanone (2) (Table 1) previously isolated and identified from *Calophyllum apetalum* (Iinuma et al. 1997).

Isolation work on the chloroform extract afforded compound (3) as yellow prism crystals with m.p 145-146°C and the IR spectrum indicated the presence of broad and strong absorptions for OH and carbonyl group at 3207 cm^{-1} and 1647 cm^{-1} , respectively. The molecular formula of $C_{23}H_{22}O_5$ was established on the basis of the mass spectrum with molecular ion peak observed at m/z 378 and the base peak at m/z 363 resulted from the loss of one methyl group. The integration of the 1H -NMR spectrum displayed the existence of twenty two protons. Three methyl singlets resonances were observed, one of which integrated for six protons assigned to the two methyl groups of the chromene ring at δ 1.45 (3H, s, H-4', H-5') and the other two singlets belongs to the isopentenyl side chain at δ 1.68 (3H, s, H-4'') and δ 1.83 (3H, s, H-5''). A pair of doublets at δ 6.70 (1H, d, H-1') and δ 5.58 (1H, d, H-2'') with common coupling constant 10.0 Hz attributed to the two methine protons of the chromene ring system. The three doublet of doublets at δ 7.27 (1H, dd, 7.4, 1.8 Hz, H-6), δ 7.19 (1H, dd 7.4, 7.7 Hz, H-7) and δ 7.72 (1H, dd, 7.7, 1.8 Hz, H-8) were due to the three aromatic protons arranged in an ABC system. The methylene protons of the isopentenyl side chain occurred at δ 3.45 as doublet. The chelated hydroxyl group resonance at the low field region δ 13.03 (OH, s) and the second hydroxyl group at C-5 occurred as broad singlet at δ 5.67 (OH, s). Further evidences to support the proposed structure were obtained from COSY, ^{13}C -NMR, DEPT, HMQC and HMBC spectra. Based on these data and comparison with literature reports, the compound was identified as trapezifolixanthone (3) previously reported to occur in roots of *Tovomita brevistaminea* (Seo et al. 1999).

The three xanthenes were tested for cytotoxicity against five cell lines, human breast adenocarcinoma (MCF-7), colon carcinoma (HCT-116), prostrate carcinoma (PC3), African green monkey kidney (VERO) and mouse macrophages (RAW 264.7) by using MTT assay. The cells were obtained from American Tissue Culture Collection (Virginia, USA). Only zeyloxanthanone (2) exhibited potent cytotoxicity with IC_{50} values of 10.00, 9.56, 8.00, 26.00 8.22 μM , respectively. The compound displayed no significant selectivity towards a particular cell line. The IC_{50} values for all cell lines were in a close range compared with one another, except for VERO cell line which had a value of 2 to 3 folds higher than the other cell lines. In

comparison, the IC_{50} values obtained for standard drug doxorubicin against the five cell lines were 3.68, 0.37, 3.31, 12.34 and $>184.16 \mu M$, respectively. The other two xanthenes were inactive against the five cell lines.

CONCLUSION

Chromatographic separation of hexane, chloroform and methanol extracts of stem bark of *Calophyllum gracilipes* yielded a new xanthone identified as gracixanthone and two known xanthenes, zeyloxanthanone and trapezifolixanthone together three common sterols, stigmasterol, friedelin and lupeol. The structures of the compounds were established by detail spectral analysis and in comparison with literature values. The three xanthenes were assayed for cytotoxicity against five cell lines and zeyloxanthanone was found to be strongly active with IC_{50} values ranging at 3.6-11.7 mg/mL.

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