Xanthones from *Calophyllum gracilipes* and Their Cytotoxic Activity
(Zanton daripada *Calophyllum 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 stigmasterol, 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 IC$_{50}$ values ranging at 8.00-26.00 µM.

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

**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 xanthones, coumarins, flavonoids, benzophenones, steroids and triterpenes (Hay et al. 2004; Iinuma et al. 1997; Ishikawa 2000; Kijjoa et al. 2000). A number of this compound particularly coumarins and xanthones 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 xanthones 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 CDCl$_3$ 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 60F$_{254}$ 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 (FRCS 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 stigmasterol, 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 15 fractions of 150 mL each. The yellowish solid obtained from fractions 8-15 were combined and recrystallised with chloroform to give zeyloxanthane (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): 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 (69), 231 (100), 137 (12); HR-EIMS m/z 274.0445 (C_{13}H_{16}O_{2} calc. For 274.0477); 1H-NMR (400 MHz, acetone-d_{6}) and 13C-NMR (100MHz, acetone-d_{6}) (Table 1 & Figure 1).  

Zeyloxanthane (2)  Zeyloxanthane was obtained as yellow powder with m.p. 151-152°C; UV (EtOH) λ_{max} (log ε): 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), 355 (30), 165 (15); 1H-NMR (500 MHz, CDCl_{3}) and 13C-NMR (125 MHz, 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): 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); 1H-NMR (500 MHz, CDCl_{3}) and 13C-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), prostate 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-well 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 (Inumia 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 C_{13}H_{16}O_{2} 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 274.0454 (C_{13}H_{16}O_{2}) calc. For 274.0477). The presence of ten protons in the molecular formula is further substantiated by the integration of the 13H-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 13C-NMR and DEPT spectra made up of nine
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<th>δ_C</th>
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* 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-9, 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)
and VERO 50. The value of 2 to 3 folds higher than the other cell lines. In
with one another, except for IC
were obtained from American Tissue Culture Collection (PC3), African green monkey kidney (COSY, HMQC and HMBE spectra. Based on these data and comparison with literature reports, the compound was identified as gracixanthone (3) previously reported to occur in roots of Tovomita brevistamina (See et al. 1999).

The three xanthones 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
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
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
values obtained for standard drug doxorubicin against the five cell lines were 3.68, 0.37, 3.31, 12.34 and >184.16 μM, respectively. The other two xanthones 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 xanthones, 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 xanthones were assayed for cytotoxicity against five cell lines and zeyloxanthanone was found to be strongly active with IC
values ranging at 3.6-11.7 mg/mL.

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