



Xanthenes from *Calophyllum inophyllum*

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ABSTRACT

Repeated separation and purification of fractions from the crude chloroform extract of *Calophyllum inophyllum* via column chromatography afforded five known xanthone caloxanthenes A, B, C, macluraxanthone and pyranojacareubin. Structural elucidations and determination of the isolated compounds were supported by spectral analyses obtained by subjecting the compounds to various spectroscopic techniques. The chloroform extract, when tested against RAW264.7 cells for anti-inflammatory potentials, exhibited the most promising activity with an IC₅₀ value of 14.81±0.04 µg/mL compared to moderate activities shown by the ethyl acetate and n-hexane extracts. Antimicrobial tests showed that the n-hexane and chloroform extracts acted moderately against *Staphylococcus epidermidis* S273 and *Bacillus subtilis* B145.

Keywords: anti-inflammatory, antimicrobial, *Calophyllum inophyllum*, xanthenes

INTRODUCTION

Calophyllum is one of the 47 genera of the Clusiaceae (Guttiferae) family. This family comprises a stunning 1350 different species across the world (Daud et al., 2014). Habitat distribution of plants from this family includes tropical areas such as the Indo Pacific region. *Calophyllum* is known locally as ‘bintangor’ or ‘tamanu’ (Mah et al. 2012). Secondary metabolites from this genus are main contributors towards various biological activities reported from previous work (Mah et al. 2015). *Calophyllum* species have been reported to be rich in secondary metabolites such as xanthenes, coumarins, terpenoids and chromene acids (Dharmaratne et al.,

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2009). This genus has been vastly utilised as folk or traditional medicine to treat ailments. It has also been reported that this genus possesses compounds with antifungal, anti-microbial, anti-cancer and anti-HIV properties (Alkhamaiseh et al., 2011). A greenish oil extracted from the fruit kernel of *Calophyllum inophyllum* was reported to relieve joint pain, bruises and even rheumatism (Zakaria et al., 2014). Recent studies on the leaf extract of this species also exposed its anti-inflammatory potential, suggesting that it can be used in applications for inflammatory-related illnesses (Tsai et al., 2012). This paper focusses on the isolation and structural elucidation of xanthenes. In addition, cell viability and toxicity of LPS stimulated RAW264.7 cells and anti-microbial activities of crude extracts will also be discussed.

MATERIALS AND METHODS

Plant Material

The stem bark sample of *Calophyllum inophyllum* was acquired from the campus grounds of Universiti Putra Malaysia (UPM), Serdang, Selangor. Sample identification was carried out by Associate Professor Dr Rusea Go, a biologist from the Biology Department, UPM. A voucher specimen (RG 5016) was deposited in the Herbarium, Biology Department, UPM.

Analysis Instrumentation

NMR spectra were generated from a JEOL FT-NMR 500 MHz spectrophotometer with tetramethylsilane (TMS) as the internal

standard. EIMS data were recorded on a Shimadzu GC-MS model QP2010 Plus spectrophotometer whereas ultraviolet spectra were recorded in EtOH on a Shimadzu UV-160A, UV Visible Recording Spectrophotometer. Infrared spectra were accomplished using a universal attenuated total reflection (UATR) technique on a Perkin-Elmer 100 Series FT-IR spectrometer. A Leica Galen III microscope equipped with a Testo 720 temperature recorder was used to determine the melting points of compounds isolated.

Extraction and Isolation

A mass of 3.8 kg of *Calophyllum inophyllum* stem bark was primarily air dried and subsequently ground into fine powder. Successive extractions of the powder in a selection of organic solvents with increasing polarity, namely, n-hexane, chloroform, ethyl acetate and methanol were achieved. The filtered liquid extracts underwent the drying process under reduced pressure by rotary evaporator to afford 106.0 g, 80.3 g, 63.5 g and 67.4 g of n-hexane, chloroform, ethyl acetate and methanol dry extracts. A designated portion of the dried chloroform extract was subjected to vacuum column chromatography over silica gel 60 PF₂₅₄ for isolation and separation process. Thirty different fractions were acquired from the elution of the sample-packed silica gel with a stepwise gradient of n-hexane-chloroform and chloroform-ethyl acetate mixtures. A series of meticulous steps in the purification process of fractions 3-8 afforded yellow needles of pyranojacareubin

(5). Caloxanthenes B (2) and C (3) were also isolated from further separation of fractions 10-16 in the appearance of yellow crystals. A similar chromatographic technique was used to separate fractions 20-23 and fractions 26-28, respectively, which resulted in the isolation of two additional xanthenes, namely caloxanthone A (1) and macluraxanthone (4) after the recrystallisation process.

Cell Viability and Cytotoxicity Test

The seeding process of raw cells (2×10^6 cells/mL) was carried out in a 96-well plate and subsequently subjected to a 24-hour incubation stage. These cells were then treated and induced with $10 \mu\text{g/mL}$ lipopolysaccharide (LPS) in the presence of the crude n-hexane extract and made up to a final volume of $100 \mu\text{L}$ and further incubated for 24 hours. Then, Griess reagent ($50 \mu\text{L}$) was added to react with $50 \mu\text{L}$ of cell-free culture supernatant, after which, incubation was carried out for 10 minutes at room temperature. These cells were introduced into the microplate reader and readings at $\text{OD}=550\text{nm}$ were taken. A fresh culture medium was used as a blank. The results were expressed as mean \pm SEM. Repetition of the following steps was carried out on the remaining crude extracts and isolated compounds.

Anti-Microbial Test

This test was carried out by placing a 6-mm diameter of paper disc containing antibiotics

onto a plate in which microbes were growing. The microbe culture was standardised to 0.5 McFarland standard, which was approximately 10^8 cells. Streptomycin standard was used for bacteria, while nystatin standard was used for yeast. The plates were inverted and incubated at 30°C to 37°C for 18 to 24 h or until sufficient growth had occurred. Each plate was examined after incubation. The diameter of the zone of complete inhibition was measured, including the diameter of the disc. Zones were measured to the nearest whole millimetre (mm).

RESULTS AND DISCUSSIONS

Column chromatographic separation and purification of the fractions of the crude chloroform extracts gave rise to a total of five known xanthenes. They were caloxanthone A (1), caloxanthone B (2), caloxanthone C (3), macluraxanthone (4) and pyranojacareubin (5). Their structures and spectral data are as shown below.

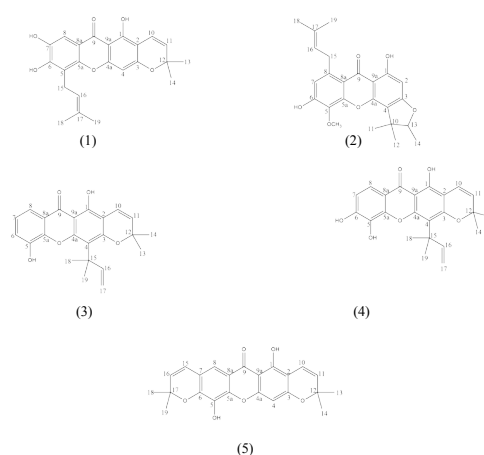


Figure 1. Structures of compounds 1-5

Caloxanthone A (1): Yellow needles; m.p. 237-240°C (Lit 238-240°C) (Iinuma et al., 1994); EIMS (m/z) : 394[M⁺], C₂₃H₂₂O₆; UV (EtOH) λ_{max} : 388, 494nm; IR ν_{max} cm⁻¹ : 3393, 2941, 1611, 1457; ¹H NMR (CDCl₃, 500MHz) δ_H : 6.32(1H, s, H-4), 7.43(1H, s, H-8), 6.63(1H, d, J=10.3Hz, H-10), 5.69(1H, d, J=10.3Hz, H-11), 1.43(2x3H, s, H-13&H-14), 3.57(2H, d, J=8Hz, H-15), 5.27(1H, t, J=7.4Hz, H-16), 1.86(3H, s, H-18), 1.62(3H, s, H-19), 13.60(1H, s, 1-OH); ¹³C NMR (CDCl₃, 125MHz) δ_C : 157.5(C-1), 104.1(C-2), 159.9(C-3), 94.5(C-4), 157.2(C-4a), 151.4(C-5a), 115.7(C-5), 142.8(C-6), 149.9(C-7), 105.5(C-8), 112.5(C-8a), 180.1(C-9), 102.7(C-9a), 115.0(C-10), 127.8(C-11), 78.0(C-12), 27.7(C-13&C-14), 22.2(C-15), 121.6(C-16), 131.7(C-17), 17.3(C-18), 25.1(C-19)

Caloxanthone B (2): Yellow needles; m.p. 157-158°C (Lit 160.5°C) (Iinuma et al., 1994); EIMS (m/z) : 410 [M⁺], C₂₄H₂₆O₆; UV (EtOH) λ_{max} : 317, 247nm; IR ν_{max} cm⁻¹ : 3288, 2929, 1641; ¹H NMR (CDCl₃, 500MHz) δ_H : 6.21(1H, s, H-2), 6.79(1H, s, H-7), 1.58(3H, s, H-11), 1.29(3H, s, H-12), 4.51(1H, q, J=6.9Hz, H-13), 1.39(3H, d, J=6.9Hz, H-14), 3.98(2H, d, J=8Hz, H-15), 5.34(1H, t, J=8Hz, H-16), 1.73(3H, s, H-18), 1.70(3H, s, H-19), 13.75(1H, s, 1-OH), 3.98(3H, s, 5-OCH₃); ¹³C NMR (CDCl₃, 125MHz) δ_C : 164.5(C-1), 94.1(C-2), 165.6(C-3), 112.2(C-4), 151.8(C-4a), 151.2(C-5a), 132.4(C-5), 153.6(C-6), 113.5(C-7), 142.0(C-8), 112.7(C-8a), 182.2(C-9), 104.0(C-9a), 43.7(C-10), 25.6(C-11), 21.7(C-12), 90.8(C-13), 14.2(C-

14), 33.6(C-15), 122.4(C-16), 133.5(C-17), 26.0(C-18), 18.0(C-19), 61.9(5-OCH₃).

Caloxanthone C (3): Yellow needles; m.p. 212-213°C (Lit 217°C) (Iinuma et al., 1994); EIMS (m/z) : 378 [M⁺], C₂₃H₂₂O₅; UV (EtOH) λ_{max} : 382, 290, 271, 240nm; IR ν_{max} cm⁻¹ : 3428, 2929, 1589, 1711; ¹H NMR (CDCl₃, 500MHz) δ_H : 7.34(1H, d, 8Hz, H-6), 7.24(1H, t, J=8Hz, H-7), 7.63(1H, d, J=8Hz, H-8), 6.68(1H, d, J=10.3Hz, H-10), 5.72(1H, d, J=10.3Hz, H-11), 1.48(2x3H, s, H-13&H-14), 6.48(1H, dd, J=10.3Hz, 17.2Hz, H-16), 4.85(1H, d, J=10.3Hz, H-17a), 5.00(1H, d, J=17.2Hz, H-17b), 1.71(2x3H, s, H-18&H-19), 13.67(1H, s, 1-OH), 8.79(1H, s, 5-OH); ¹³C NMR (CDCl₃, 125MHz) δ_C : 156.4(C-1), 105.0(C-2), 159.3(C-3), 113.7(C-4), 155.1(C-4a), 145.1(C-5a), 146.4(C-5), 120.1(C-6), 124.1(C-7), 115.2(C-8), 120.9(C-8a), 181.6(C-9), 103.6(C-9a), 115.4(C-10), 127.8(C-11), 78.5(C-12), 27.2(C-13&C-14), 41.0(C-15), 151.8(C-16), 106.7(C-17), 29.2(C-18&C-19).

Macluraxanthone (4) Yellow needles; m.p. 170-172°C (Lit 170-172°C) (Iinuma et al., 1994); EIMS (m/z) : 394 [M⁺], C₂₃H₂₂O₆; UV (EtOH) λ_{max} : 337, 270, 242nm; IR ν_{max} cm⁻¹ : 3348, 2965, 1589, 1434; ¹H NMR (CDCl₃, 500MHz) δ_H : 6.93(1H, d, J=8Hz, H-7), 7.67(1H, d, J=8Hz, H-8), 6.76(1H, d, J=9.2Hz, H-10), 5.60(1H, d, J=10.3Hz, H-11), 1.50(2x3H, s, H-13&H-14), 6.71(1H, dd, J=10.8Hz, 18.3Hz, H-16), 5.21(1H, d, J=18.3Hz, H-17a), 5.04(1H, d, J=10.3Hz, H-17b), 1.64(2x3H, s, H-18&H-19),

13.52(1H, *s*, 1-OH), 6.26(1H, *s*, 5-OH), 5.93(1H, *s*, 6-OH); ¹³C NMR (CDCl₃, 125MHz) δ_C : 156.8(C-1), 105.7(C-2), 159.0(C-3), 113.1(C-4), 154.2(C-4a), 144.6(C-5a), 131.1(C-5), 149.1(C-6), 112.8(C-7), 117.6(C-8), 113.8(C-8a), 180.9(C-9), 103.1(C-9a), 116.2(C-10), 127.3(C-11), 78.3(C-12), 28.0(C-13&C-14), 41.5(C-15), 156.9(C-16), 103.4(C-17), 28.3(C-18&C-19).

Pyranojacareubin (5): Yellow needles; m.p. 259-260°C (Lit 259-261°C) (Harrison et al., 1993); EIMS (*m/z*) : 392 [M⁺], C₂₃H₂₀O₆; UV (EtOH) λ_{max} : 347, 300, 291, 264nm; IR ν_{max} cm⁻¹ : 3627, 2924, 1612, 1458; ¹H NMR (CDCl₃, 500MHz) δ_H : 6.32(1H, *s*, H-4), 7.56(1H, *s*, H-8), 6.72(1H, *d*, *J*=9.1Hz, H-10), 5.58(1H, *d*, *J*=10.3Hz, H-11), 1.46(2x3H, *s*, H-13&H-14), 6.87(1H, *d*, *J*=10.3Hz, H-15), 5.74(1H, *d*, *J*=10.3Hz, H-16), 1.54(2x3H, *s*, H-18&H-19), 13.30(1H, *s*, 1-OH); ¹³C NMR (CDCl₃, 125MHz) δ_C : 157.7(C-1), 104.6(C-2), 160.2(C-3), 94.9(C-4), 157.0(C-4a), 145.8(C-5a), 141.9(C-5), 146.5(C-6), 109.3(C-7), 108.8(C-8), 114.1(C-8a), 180.1(C-9), 103.3(C-9a), 115.7(C-10), 127.5(C-11), 78.2(C-12), 28.4(C-13&C-14), 115.53(C-15), 130.0(C-16), 79.4(C-17), 28.4(C-18&C-19).

The cell viability and cytotoxicity test of the plant crude extracts on the RAW264.7 cells found that the chloroform extract exhibited significant activity whereas the n-hexane and ethyl acetate extracts indicated moderate activity. This showed that the chloroform extract had anti-inflammatory

potential. Moreover, the n-hexane and chloroform extract showed favourable activity against *Staphylococcus epidermidis* S273 and *Bacillus Subtilis* B145.

Table 1
Cell Viability and Cytotoxicity in LPS Stimulated RAW264.7 Cells Treated with Crude Extracts and Compounds

Crude Extracts and Compounds	IC50 (µg/mL)
n-hexane	32.50±0.06
Chloroform	14.81±0.04
Ethyl acetate	35.65±0.09
Methanol	>100

Note: Each value of IC50 represented mean ±S.E.M.

Compound 1 was acquired as yellow needles with a melting point of 237-240°C. It had a molecular formula of C₂₃H₂₂O₆, which corresponded with the molecular ion peak of *m/z* 394 as displayed in the EIMS spectrum. The fragment ion peak due to the loss of a methyl group could be observed at *m/z* 379 in the spectrum, indicating the presence of a methyl side chain in the compound. Absorption bands at 3393 cm⁻¹ (OH), 2941 cm⁻¹ (sp³ CH), 1611 cm⁻¹ (C=O) and 1457 cm⁻¹ (C-O) shown from the FTIR spectrum were consistent with that of the xanthenone skeleton. The ¹H NMR spectrum indicated a one-proton triplet at δ 5.27 (*J*=7.4Hz) and a two-proton doublet at δ 3.57 (*J*=8.0Hz), which were represented by H-16 and H-15, respectively. The COSY NMR spectrum further suggested that H-15 and H-16 were coupled with one another. Hence, the presence of a prenyl side chain was deduced.

Table 2
Anti-Microbial Activities of Crude Extracts via Disc Diffusion Method Against Gram Positive Bacteria and Yeast

Crude Extracts	Target Microbes (Bacteria & Yeast)						
	I	II	III	IV	V	VI	VII
n-hexane	-	-	16	13	-	-	-
Chloroform	-	-	16	13	-	-	-
Ethyl acetate	-	-	7	6	-	-	-
Methanol	-	-	-	-	-	-	-

Note: Each value is in millimetres (mm)

I: Methicillin Resistant *S. aureus* (MRSA)

II: *Streptococcus* sp

III: *Staphylococcus epidermidis* S273

IV: *Bacillus Subtilis* B145

V: *Staphylococcus aureus* S276

VI: *Candida albicans* C244

VII: *Candida tropicalis* A3

-: no activity recorded

In addition, a pyrano ring was also found to be present in this compound and it was supported by a 2J and 3J coupling exhibited by H-10 and H-11 with C-2, C-3, and C-12. Through comparison with previous reports, compound 1 was established to be caloxanthone A (Iinuma et al., 1994).

Compound 2 was isolated as yellow needles with a melting point of 157-158°C. A molecular formula of $C_{24}H_{26}O_6$ was supported by the molecular ion peak in the EIMS spectrum at m/z 410. The FTIR spectrum displayed absorptions at 3288 cm^{-1} (OH), 2929 cm^{-1} (sp^3 CH) and 1641 cm^{-1} (C=O), which fundamentally fulfilled the characteristics of a xanthone. A 2J correlation between H-15 and C-8 observed from the HMBC spectrum suggested the attachment of the prenyl side chain to C-8. This suggestion was further strengthened by a triplet at δ 5.34 ($J=8Hz$), which had a 3J correlation with C-18 and C-19. A

sharp and strong singlet peak at δ 3.98 representing a methoxyl (OCH₃) side group was attributed to C-5 via a 3J correlation between the methoxyl protons and C-5. A dihydrofuran ring was suggested from a $3J$ correlation between H-13 with C-11 and C-12. The attachment of the ring to C-3 and C-4 was further evidenced by 3J correlation between H-12 with C-4, C-11 and C-13. By associating the spectral data with literature data, it was concluded that compound 2 was caloxanthone B (Iinuma et al., 1994).

Compound 3 was isolated as yellow needles with a melting point of 212-213°C. The molecular formula, $C_{23}H_{22}O_5$, tallied with the molecular ion peak at m/z 378 as displayed in the EIMS spectrum. Absorptions at 3428 cm^{-1} (OH), 2929 cm^{-1} (sp^3 CH), 1711 cm^{-1} (C=O), and 1589 cm^{-1} (C=C) evidently showed the presence of a xanthone skeleton. Compound 3 and compound 4 differed from each other by an additional hydroxyl (OH)

group attached to C-6 in compound 4. The presence of a pyrano ring was justified by two doublet signals at δ 6.68 ($J=10.3\text{Hz}$) and δ 5.72 ($J=10.3\text{Hz}$), which were cis-coupled. The attachment of the ring to C-2 and C-3 was supported by the 3J correlations of H-10 with C-1 and C-3. Apart from that, a prenyl moiety was proposed due to a doublet of doublet signal observed at δ 6.48 ($J=10.3, 17.2\text{Hz}$), a singlet at δ 1.71 and a pair of doublets at δ 4.85 ($J=10.3\text{Hz}$) and δ 5.00 ($J=17.2\text{Hz}$), respectively. The splitting pattern for the peak at δ 6.48 was due to two non-equivalent protons at δ 4.85 (H-17a) and δ 5.00 (H-17b). 3J correlations of H-18 and H-19 with C-4 proved that the prenyl moiety was attached to C-4. Hence, it was concluded that compound 3 was caloxanthone C (Iinuma et al., 1994).

Compound 4 appeared as yellow needles with a melting point of 170-172°C. Analysis of the EIMS spectrum revealed the molecular ion peak at m/z 394, which consequently supported the molecular formula, $\text{C}_{23}\text{H}_{22}\text{O}_6$. Absorptions of the FTIR spectrum at 3348 cm^{-1} (OH), 2965 cm^{-1} (sp^2 CH), 1589 cm^{-1} (C=O) and 1434 cm^{-1} (C=C) presumed the presence of a xanthone skeleton. A prenyl moiety was attached to C-4 and its presence was evidenced by the presence of a doublet of doublet at δ 6.71 ($J=10.8\text{Hz}, 18.3\text{Hz}$) and a pair of doublets at δ 5.21 ($J=18.3\text{Hz}$) and δ 5.04 ($J=10.3\text{Hz}$). This position of this moiety was established via two 3J correlations between H-18 and H-19 with C-4. Apart from that, 2J and 3J correlations of H-10 and H-11 with C-2 proved that there was

an attachment of the pyrano ring to C-2 and C-3. Comparison with provided literature data enabled us to conclude that compound 4 was macluraxanthone (Iinuma et al., 1994).

Compound 5 was isolated as fine yellow needles with a melting point of 259-260°C. The molecular ion peak at m/z 392 as observed from the EIMS spectrum matched with the molecular formula of $\text{C}_{23}\text{H}_{20}\text{O}_6$. The presence of the functional groups present in the xanthenes was displayed in the IR spectrum with absorptions at 3627 cm^{-1} (OH), 2924 cm^{-1} (sp^2 CH), 1612 cm^{-1} (C=O), and 1458 cm^{-1} (C=C). From the ^1H NMR spectrum, a pair of doublets at δ 6.72 ($J=9.1\text{Hz}$) and δ 5.58 ($J=10.3\text{Hz}$) along with another pair at δ 6.87 ($J=10.3\text{Hz}$) and δ 5.74 ($J=10.3\text{Hz}$) pointed out the presence of two pyrano rings. The position of the first pyrano ring was determined via 3J coupling between H-10 and C-3 and between H-11 and C-2. The second pyrano ring position was supported by 3J correlation between H-16 and C-7 and between H-15 and C-6. Therefore, it was concluded that compound 5 was pyranojacareubin after comparing with previous literature data (Harrison et al., 1993).

CONCLUSION

Five known xanthenes, namely, caloxanthone A, caloxanthone B, caloxanthone C, macluraxanthone and pyranojacareubin were obtained from the chloroform extract of *Calophyllum inophyllum* stem bark. In addition, the chloroform extract also showed promising activity in the cell viability and cytotoxicity test of LPS-stimulated

RAW264.7 cells and acted moderately against *Staphylococcus epidermidis* S273 and *Bacillus Subtilis* B145. Hence, there is a possibility for the stem bark of *Calophyllum inophyllum* to provide potential cytotoxic lead compounds that can be developed into drugs for use in cancer therapy.

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