REPORT

Cytotoxic activity of phytochemicals from the stem bark of *Calophyllum castaneum*

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Abstract: Phytochemical investigation on the dichloromethane stem bark extract of *Calophyllum castaneum* resulted in the isolation of five compounds, namely isoblancoic acid (1), blancoic acid (2), euxanthone (3), friedelin (4) and friedelinol (5). All these compounds were isolated for the first time from this plant. Their chemical structures were elucidated based on the spectroscopic analyses. The cytotoxicity of compounds 1-5 was assessed on a panel of cancer cell lines including bone (Saos-2, mg63), colorectal (HT29, Caco-2, HCC2998, SW48, HCT116, KM12), liver (HepG2), lung (H1299, Calu-3), and brain (C6), using 5-fluorouracil as positive control. Pronounced antiproliferative activities were observed for compound 1 which exhibited a comparable activity with the positive control, against brain (C6) and colorectal (SW48, KM12, HCT116) cancer cell lines showing IC_{50} values in the range of 14 to 65µM. Meanwhile, compound 5 displayed a greater cytotoxic effect showing at least 2-fold more strongly than the positive control, against C6 brain cancer cells. The assay findings have unveiled the therapeutic value of phytochemicals from *Calophyllum castaneum* as anti-cancer agents.

Keywords: Chromanone acid, triterpene, xanthone, *Calophyllum castaneum*, cytotoxic activity.

INTRODUCTION

Calophyllum species were reported to be abundant in cytotoxic xanthones, coumarins, chromanones, triterpenoids and biflavonoids (Oliveira *et al*., 2014). In our continuous investigation on Malaysian *Calophyllum* species for new anticancer agents, our recent study on *Calophyllum castaneum* afforded isoblancoic acid (1), blancoic acid (2), euxanthone (3), friedelin (4) and friedelinol (5). Isoblancoic acid (1) and blancoic acid (2) were previously reported from *Calophyllum brasiliense* (Plattner *et al*., 1974). However, there was no report on the biological activity of compounds 1 and 2. Euxanthone (3) originated from *Calophyllum thwaitesii* (Dharmaratne *et al*., 2009), has been found to be HIV-1 reverse transcriptase inhibitor (Reutrakul *et al*., 2006) and to exhibit antiproliferative activity on human UACC-62 (melanoma), TK-10 (renal adenocarcinoma) and MCF-7 (breast adenocarcinoma) cancer cells (Pedro *et al*., 2002). Friedelin (4) and friedelinol (5), the two ubiquitous triterpenoids have been widely studied to show antifungal (Jain *et al*., 2001) and antibacterial (Viswanathan *et al*., 2012) activities. The present study was aimed to isolate and characterize cytotoxic compounds from the stem bark of *Calophyllum castaneum*.

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MATERIALS AND METHODS

General

Ultraviolet (UV) spectra were determined on a Perkin-Elmer Lambda 35 Ultraviolet/Visible spectrophotometer (Perkin-Elmer, Waltham, Massachusetts, United States), and infrared (IR) spectra were recorded as potassium bromide (KBr) discs on a Perkin-Elmer Spectrum RXI Fourier transform infrared (FTIR) spectrophotometer (Perkin-Elmer, Waltham, Massachusetts, United States). Mass spectra were obtained on an Agilent 5975C MSD mass spectrometer (Agilent Technologies, Santa Clara, California, United States). Nuclear magnetic resonance (NMR) spectra were acquired in either acetone-d_6 or $CDCl_3$, on a JEOL JNM-ECX 400MHz Fourier transform nuclear magnetic resonance (FTNMR) spectrometer (JEOL, Tokyo, Japan) and tetramethylsilane was used as an internal standard. Melting points were measured using a Stuart SMP10 electrothermal capillary melting point apparatus (Barloworld Scientific Limited, Staffordshire,

United Kingdom) and are uncorrected. Analytical grade solvents were used without further purification unless otherwise specified. Thin layer chromatography (silica gel 60 F_{254} plates, Merck, Darmstadt, Germany) was used to monitor fractions with visualization under ultraviolet (UV) light (254 and 365 nm). Column chromatography (CC) was carried out on Sephadex LH-20 (GE Healthcare, Sweden) and silica gel 60 (230-300 mesh, Merck, Darmstadt, Germany). 5-Fluorouracil (purity ≥ 99%) was purchased from Sigma-Aldrich.

Plant materials

The stem bark material of *C. castaneum* was collected from the forest in Landeh district of Sarawak, Malaysia in April 2013. A voucher specimen (UITM 3001) has been deposited at the herbarium of Universiti Teknologi MARA, Sarawak.

Extraction and isolation of compounds

The dried and powdered stem bark material of *C. castaneum* (2.0 kg) was extracted twice at room temperature with 5 L of dichloromethane for 72 hours. The dichloromethane solution was filtered and evaporated under vacuum at 40°C to give 41g of dried dichloromethane extract. About 35 g of dichloromethane extract was subjected to silica gel column chromatography (CC) using gradient mixture of *n*-hexane-dichloromethane in increasing polarity, followed by decreasing concentration of dichloromethane in ethyl acetate to give 20 fractions (CCA1-20; 200 ml each). After analyzed by thin-layer chromatography (TLC), fractions CCA8-9 which showed a similar TLC pattern were pooled and further chromatographed using Si gel and eluting with 0-100% *n*-hexane-ethyl acetate to give 20 subfractions (CCB1-20; 100 ml each). Subfractions CCB9 yielded friedelinol (5) as white needle-like crystals (74 mg). From subfraction CCB5-6, friedelin (4, 163 mg) was obtained. Fractions CCA14-15 were pooled and separated by a silica gel column (0-100% *n*-hexane-acetone) to give 20 subfractions (CCC1-20; 100 ml each). Subfractions CCC9-10 were pooled and rechromatographed over a silica gel column (0-100% *n*-hexane-acetone) to afford 20 subfractions (CCD1-20; 50 ml each). Subfractions CCD11-14 were combined and separated on a Sephadex LH-20 column eluting with a mixture of 90% MeOH and 10% dichloromethane to give euxanthone (3, 15 mg). Subfractions CCC12-15 were pooled and purified by silica gel column chromatography (CC) (0-100% *n*-hexane-acetone) to afford 20 subfractions (CCE1-20; 50 ml each). Subfractions CCE9-10 gave blancoic acid (2, 283 mg). Meanwhile, subfractions CCC17-18 were pooled and fractionated by Sephadex LH-20 column chromatography (CC) (90% MeOH: 10% dichloromethane) to give isoblancoic acid (1, 427 mg). The structural features of all isolated compounds were established via NMR, UV and IR spectroscopies, mass spectrometry, and comparison with previously reported spectral data (Plattner *et al*., 1974; Sousa *et al*., 2012; Dharmaratne *et al*., 2009). Their chemical structures are shown in fig. 1.

Cytotoxicity assay

Compounds 1-5 were tested on the brain glioma cells (C6), lung cancer cells (NCI-H1299 and Calu-3), human liver cancer cells (HepG2), human colorectal cancer cells (Caco-2, HCC2998, SW48, KM12, HT29 and HCT116) and human bone osteosarcoma cancer cells (mg63 and Saos-2) using the methyl thiazolyltetrazolium (MTT) cell viability assay as described previously by Mai *et al*. (2014). Briefly, all compounds were reconstituted using dimethylsulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA) to 100mM and further diluted to the desirable concentrations using ultra purified sterile water just prior to the assays. Cancerous cells (5×10^3 cells/well) were plated in sterile 96-well plates for 24 hour. Cells were treated with the isolated compounds in a dose dependent manner for 72 hours. The cell growth and anticancer effects were recorded at test wavelength of 570 nm and reference wavelength of 630 nm using the Tecan® Infinite F200 plate reader (Mannedorf, Switzerland). The results were compiled in a dose-response curve to enable the quantification of IC_{50}, or the concentration of the isolated compounds that inhibits cell proliferation by 50%.

RESULTS

The isolated compounds 1-5 were subjected to UV/Visible, NMR and mass analyses. Their physical and spectral data are given as under.

Isoblancoic acid (1)

Yellowish gum; UV λ_{max} nm ($CHCl_3$) (log ε): 206 (0.55), 275 (1.72), 312 (0.54); IR υ_{max} (KBr) cm^{-1} : 3419 (O-H stretch), 2925 (C-H stretch), 1706 (C=O stretch), 1645 (C=C stretch), 1626 (C=C stretch), 1442 (C-H bend), 1132 (C-O stetch), 993, 771; ^{1}H NMR (400 MHz, acetone-d_6): 12.38 (1H, s, 5-OH), 6.58 (1H, d, *J* = 9.8 Hz, H-6), 5.44 (1H, d, *J* = 9.8 Hz, H-7), 4.42 (1H, brs, H-2), 3.67 (1H, m, H-13), 2.81 (2H, dd, *J* = 14.6, 8.5 Hz, H-14), 2.63 (2H, dd, *J* = 14.6, 6.7 Hz, H-14), 2.51 (1H, m, H-3), 1.81 (1H, m, H-16), 1.52 (1H, m, H-16), 1.42 (3H, s, H-21), 1.35 (3H, s, H-22), 1.34 (3H, d, *J* = 6.7 Hz, H-11), 1.24 (2H, m, H-18), 1.21 (2H, m, H-19), 1.18 (2H, m, H-17), 1.13 (3H, d, *J* = 7.3 Hz, H-12), 0.81 (3H, t, *J* = 6.7 Hz, H-20); ^{13}C NMR (100 MHz, acetone-d_6): 201.4 (C-4), 179.6 (C-15), 160.1 (C-9a), 160.0 (C-10a), 157.3 (C-5), 125.8 (C-7), 115.7 (C-6), 108.8 (C-10), 102.6 (C-4a), 101.3 (C-5a), 78.1 (C-8), 76.3 (C-2), 44.3 (C-3), 38.7 (C-14), 33.2 (C-16), 31.8 (C-18), 30.8 (C-13), 28.5 (C-21), 28.2 (C-17), 27.5 (C-22), 22.7 (C-19), 16.4 (C-11), 14.2 (C-20), 9.4 (C-12); EIMS (70-eV) *m/z* (rel. int.): 416 ($[M]^{+\cdot}$ 9), 401 (100), 357 (4), 301 (6), 285 (12), 229 (8), 215 (8), 43 (4).

Table 1: *In vitro* cytotoxic activities of compounds 1-5 against a panel of cancer cell lines.

Cell Lines	$IC_{50}(\mu M)$[a]					
	1	2	3	4	5	5-Fluorouracil
C6	24.60±4.16	>100	53.84±5.20	>100	16.01± 3.48	45.50 ± 4.50
H1299	80.71±0.62	>100	68.86±0.73	59.90±2.84	77.56± 4.65	>100
Calu-3	>100	>100	>100	81.94±1.62	>100	>100
HepG2	69.00±0.72	>100	64.10±3.78	61.84±1.62	79.94±1.63	28.53±2.77
Caco-2	26.96±8.98	>100	84.51±1.85	>100	17.59±4.12	15.50±2.27
HCC2998	>100	>100	93.01±1.54	46.35±3.89	54.53±1.92	70.20±8.67
SW48	40.08±0.29	>100	>100	>100	>100	69.57±5.55
KM12	64.79±0.41	>100	>100	>100	>100	78.05±9.89
HT29	26.89±1.12	>100	>100	24.97±1.04	42.45±8.82	13.88±1.17
HCT116	14.57±0.11	>100	>100	>100	>100	14.96±2.86
mg63	>100	>100	>100	54.66±1.92	>100	>100
Saos-2	96.72±1.52	>100	80.80±3.62	61.97±0.81	>100	>100

[a] Data are reported as means ± SD for minimum three independent experiments.

Blancoic acid (2)

Greenish gum; UV λ_{max} nm ($CHCl_3$) (log ε): 209 (0.81), 274 (2.42), 311 (0.78); IR υ_{max} (KBr) cm^{-1} : 3430 (O-H stretch), 2852 (C-H stretch), 1628 (C=C stretch), 1445 (C-H bend), 1293 (C-O stetch), 994, 771, 669; ^{1}H NMR (400 MHz, $CDCl_3$): 12.45 (1H, s, 5-OH), 6.58 (1H, d, *J* = 10.0 Hz, H-6), 5.43 (1H, d, *J* = 10.0 Hz, H-7), 4.08 (1H, m, H-2), 3.65 (1H, m, H-13), 2.79 (1H, dd, *J* = 15.8, 8.5 Hz, H-14), 2.65 (1H, dd, *J* = 15.8, 6.7 Hz, H-14), 2.50 (1H, m, H-3), 1.81 (1H, m, H-16), 1.54 (1H, m, H-16), 1.45 (3H, d, *J* = 6.7 Hz, H-11), 1.41 (3H, s, H-21), 1.37 (3H, s, H-22), 1.29 (2H, m, H-18), 1.24 (2H, m, H-19), 1.20 (2H, m, H-17), 1.17 (3H, d, *J* = 6.7 Hz, H-12), 0.81 (3H, t, *J* = 6.7 Hz, H-20); ^{13}C NMR (100 MHz, $CDCl_3$): 199.5 (C-4), 179.7 (C-15), 160.0 (C-9a), 157.1 (C-5 & C-10a), 125.7 (C-7), 115.7 (C-6), 109.1 (C-10), 102.6 (C-5a), 101.9 (C-4a), 79.0 (C-2), 78.2 (C-8), 45.8 (C-3), 38.8 (C-14), 33.2 (C-16), 31.8 (C-18), 30.7 (C-13), 28.4 (C-21), 28.3 (C-22), 27.5 (C-17), 22.6 (C-19), 19.6 (C-11), 14.2 (C-20), 10.5 (C-12); EIMS (70-eV) *m/z* (rel. int.): 416 ($[M]^{+\cdot}$ 10), 401 (100), 357 (4), 301 (6), 285 (11), 229 (7), 215 (7), 43 (6).

Euxanthone (3)

Yellow needle-like crystals; mp, 236–238°C; UV λ_{max} nm ($CHCl_3$) (log ε): 206 (0.89), 235 (1.79), 260 (2.25), 387 (0.52); IR υ_{max} (KBr) cm^{-1} : 3133 (C-H stretch), 1479 (C-H bend), 1223 (C-O stetch), 990, 770, 669; ^{1}H NMR (400 MHz, acetone-d_6): 12.70 (1H, s, 1-OH), 9.04 (1H, brs, 7-OH), 7.68 (1H, t, *J*=7.3 Hz, H-3), 7.58 (1H, d, *J* = 3.0 Hz, H-8), 7.50 (1H, d, *J* = 9.2 Hz, H-5), 7.41 (1H, dd, *J* = 9.2, 3.0 Hz, H-6), 6.98 (1H, d, *J* = 7.3 Hz, H-4), 6.75 (1H, d, *J* = 7.3 Hz, H-2); ^{13}C NMR (100 MHz, acetone-d_6): 182.2 (C-9), 161.9 (C-1), 156.5 (C-4a), 154.2 (C-7), 150.2 (C-10a), 137.0 (C-3), 125.4 (C-6), 121.0 (C-8a), 119.4 (C-5), 109.7 (C-8), 108.3 (C-2 & C-9a), 107.0 (C-4); EIMS (70-eV) *m/z* (rel. int.): 228 ($[M]^{+\cdot}$ 100), 200 (14), 171 (5), 144 (6), 115 (10), 63 (5).

Friedelin (4)

White needle-like crystals; mp, 258-260°C; UV λ_{max} nm (EtOH) (log ε): 206 (0.40), 222 (0.27), 288 (0.06); IR υ_{max} (KBr) cm^{-1} : 2927 (C-H stretch), 2870 (C-H stetch), 1715 (C=O stetch), 1458 (C-H bend), 1389 (C-H bend), 1073; ^{1}H NMR (400 MHz, $CDCl_3$): 2.37 (1H, dd, *J* = 13.9, 5.4 Hz, H_a-2), 2.28 (1H, m, H_b-2), 2.26 (1H, m, H-4), 1.95 (1H, m, H_a-1), 1.16 (3H, s, H-28), 1.03 (3H, s, H-27), 0.98 (6H, s, H-26 & H-30), 0.93 (3H, s, H-29), 0.86 (3H, d, *J* = 6.6 Hz, H-23), 0.85 (3H, s, H-25), 0.70 (3H, s, H-24); ^{13}C NMR (100 MHz, $CDCl_3$): 213.4 (C-3), 59.5 (C-10), 58.3 (C-4), 53.2 (C-8), 42.8 (C-18), 42.2 (C-5), 41.6 (C-6), 41.4 (C-2), 39.8 (C-13), 39.3 (C-22), 38.4 (C-14), 37.5 (C-9), 36.1 (C-16), 35.7 (C-11), 35.4 (C-19), 35.1 (C-30), 32.8 (C-15), 32.5 (C-21), 32.2 (C-28), 31.9 (C-29), 30.6 (C-12), 30.1 (C-17), 28.3 (C-20), 22.4 (C-1), 20.4 (C-26), 18.8 (C-27), 18.3 (C-7), 18.0 (C-25), 14.7 (C-24), 6.9 (C-23); EIMS (70-eV) *m/z* (rel. int.): 426 ($[M]^{+\cdot}$ 9), 411 (7), 302 (15), 273 (31), 246 (23), 231 (28), 218 (28), 205 (41), 179 (34), 163 (42), 137 (39), 123 (85), 109 (91), 95 (100), 69 (97), 55 (63), 41 (31).

Friedelinol (5)

White needle-like crystals; mp, 279–280°C; UV λ_{max} nm (EtOH) (log ε): 206 (0.23); IR υ_{max} (KBr) cm^{-1} : 3429 (O-H stretch), 2924 (C-H stretch), 1635 (C=C stretch), 1446 (C-H bend), 1219 (C-O stretch), 772, 669; ^{1}H NMR (400 MHz, $CDCl_3$): 3.73 (1H, s, H-3), 1.89 (1H, dt, *J* = 10.4, 2.4 Hz, H_a-2), 1.73 (1H, (1H, dt, *J* = 12.8, 3.0 Hz, H-6), 1.59 (1H, m, H_b-2), 1.16 (3H, s, H-28), 1.00 (3H, s, H-29), 0.99 (3H, s, H-26), 0.98 (3H, s, H-27), 0.95 (3H, s, H-30), 0.94 (3H, s, H-24), 0.93 (3H, d, *J* = 6.7 Hz, H-23), 0.85 (3H, s, H-25); ^{13}C NMR (100 MHz, $CDCl_3$): 72.8 (C-3), 61.4 (C-10), 53.3 (C-8), 49.2 (C-4), 42.9 (C-18), 41.8 (C-6), 39.8 (C-22), 39.4 (C-14), 38.4 (C-13), 38.0 (C-9), 37.2 (C-5), 36.2 (C-16), 35.6 (C-19), 35.4 (C-2), 35.3 (C-29), 35.1 (C-11), 32.9 (C-15), 32.4 (C-28), 32.2 (C-21), 31.9 (C-30), 30.7 (C-12), 30.1 (C-17), 28.3 (C-20), 20.2 (C-27), 18.7 (C-26), 18.3 (C-25), 17.6 (C-7),

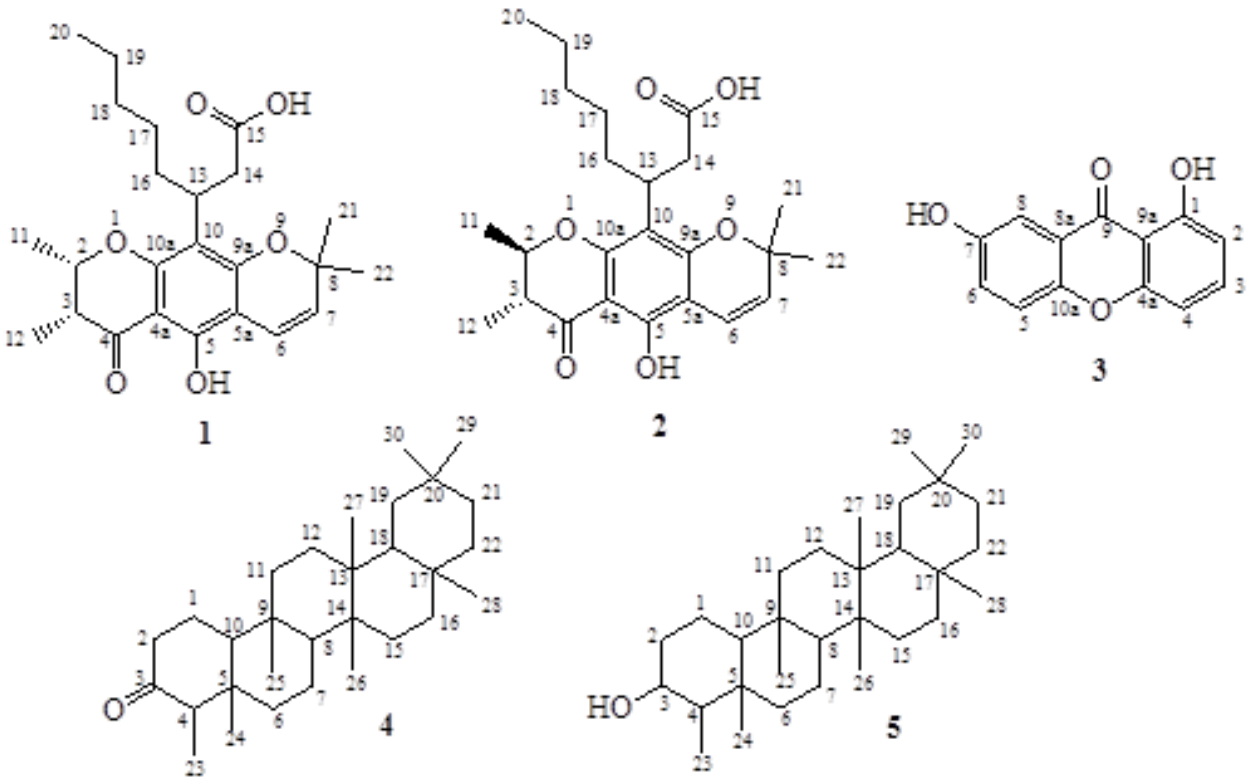

Fig. 1: Structures of compounds 1-5 isolated from stem bark of *C. castaneum*.

16.5 (C-24), 15.9 (C-1), 11.7 (C-23); EIMS (70-eV) *m/z* (rel. int.): 428 ([M]$^{+\cdot}$ 4), 413 (13), 275 (26), 257 (12), 231 (30), 205 (27), 191 (27), 177 (34), 165 (77), 137 (37), 123 (73), 109 (84), 95 (100), 69 (82), 55 (55), 41 (28).

The cytotoxic results of compounds 1-5 along with the positive control on brain (C6), lung (H1299, Calu-3), liver (HepG2,), colorectal (Caco-2, HCC2998, SW48, KM12, HT29, HCT116), and bone (mg63, Saos-2) cancer cell lines are presented in table 1.

DISCUSSION

In this assay, all the compounds except for compound 2 showed selective cytotoxic effect on the cancer cell lines tested. Among the test compounds, isoblancoic acid (1) showed pronounced inhibitory activity against C6, Caco-2, HT29, HCT116 cancer cell lines showing IC_{50} values below 27 µM, and a comparable activity with the positive control was observed for compound 1 against C6, SW48, KM12, HCT116 cancer cell lines. Conversely, blancoic acid (2) which is the stereoisomer of compound 1 was seen to show a total loss of cytotoxic activity against the same panel of cancer cell lines. Compound 1 is *cis*-2,3-dimethyl substituted while compound 2 is *trans*-2,3-dimethyl substituted on the chromanone ring. These two stereoisomers may vary in their interactions with chiral targets such as receptors, enzymes and proteins in cancer cells leading to differences in cytotoxic activity (Nguyen *et al*., 2006). The cancer cells can recognize the two stereoisomers as two different substances, and their interaction each other will therefore elicit different responses. Apart from that, compound 5, a friedelane triterpenoid demonstrated a greater cytotoxic activity with at least 2-fold more strongly than the positive control, against C6 brain cancer cell line. On the other hand, compound 4 differing from compound 5, by a hydrogen bond acceptor keto group replacing the hydrogen bond donor hydroxyl group at carbon C-3, was found to show totally devoid of inhibitory activity towards C6 cancer cells. This may be attributed to the failure of carbonyl group to form effective intermolecular hydrogen bonding at the receptor site of cancer cells. Meanwhile, compounds 3 exerted weak activities against the cancer cells tested.

CONCLUSION

Present study shows that isoblancoic acid (1) exhibited promising cytotoxicity against C6, and HCT116 cancer cell lines while friedelinol (5) showed good inhibitory activity against C6 cancer cell line. We are reporting for the first time the isolation of cytotoxic compounds from *Calophyllum castaneum*. The results evidenced the therapeutic potential of compounds 1 and 4 as lead compounds for developing new anti-cancer drugs against C6 brain and HCT116 colorectal cancers.

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