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Antioxidant activity and cytotoxicity against cholangiocarcinoma of carbazoles and coumarins from *Clausena harmandiana*

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ABSTRACT: Nine carbazoles and three coumarins were isolated from *C. harmandiana*. Crude extracts and all isolates were evaluated for antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and lipid peroxidation assay and cytotoxicity against cholangiocarcinoma, KKU-OCA17 and KKU-214 cell lines. 7-Hydroxyheptaphylline and nordentatin showed strong antioxidant activity on lipid peroxidation with IC_{50} values of 2.95 and 2.90 μM respectively, while the activity on a DPPH assay showed IC_{50} values of 56.82 and 29.3 μM, respectively. The two compounds exhibited strong cytotoxicity against KKU-OCA17 (IC_{50}=88.7 and 46.1 μM, respectively) and KKU-214 (IC_{50}=43.7 and 39.1 μM, respectively) cell lines. The results show convincingly that these compounds may be the promising leads for the development of cytotoxic agents.

KEYWORDS: lipid peroxidation, antioxidants, free radicals, natural products, drug development

INTRODUCTION

Free radicals including hydroxyl radical, superoxide anion, hydrogen peroxide, and peroxynitric species play an important role in biochemical processes of the immune system, cell differentiation, and signal transduction[1]. Generally, free radicals are maintained at a constant levels in the body by the antioxidant defence mechanism. Small molecule antioxidants such as ascorbic acid (vitamin C), uric acid, and glutathione also play significant roles as cellular antioxidants[2]. However, many situations such as an unhealthy physical conditions, ageing, or a stressful environment perturb the free radical balance resulting in several diseases including inflammation, neurodegenerative disorders, cardiovascular diseases, and cancer[3].

Cholangiocarcinoma (CCA) is the primary cancer of the bile ducts. It is a serious disease with high incidence in the northeast of Thailand where the prevalence of *Opisthorchis viverrini* (OV) infection is high[4]. CCA responds poorly to chemotherapy and radiotherapy[5]. At present, there is no effective chemotherapy regimen for treating patients with advanced cholangiocarcinoma and complete resection is the only way to cure the disease. Moreover, because cholangiocarcinoma is difficult to diagnose at early stages and extends diffusely, most patients have unresectable disease at clinical presentation, and the prognosis is very poor. In addition, it has been reported that the trend of CCA incidence is increasing in several countries in the world[6].

Clausena harmandiana, known as "Song Fa" in Thai, belongs to the Rutaceae family widely distributed in Southeast Asia. In Thailand, especially in the northeastern part, the young leaves are used as a vegetable in traditional dishes and as fodder for cattle and buffalo. This plant has shown some therapeutic activities in stomachache, headache, sickness, and is considered as a health promoting herb[7]. During previous investigations of this plant, carbazoles and coumarins have been isolated and evaluated for antimalarial, antifungal, anti TB, and cytotoxicity[7,8]. Moreover, carbazole alkaloids exhibit many activities such as topoisomerase II inhibition[9], antiplatelet aggregating, and vasorelaxing activities[10], antimycobacterial, anti-inflammatory[11], anti HIV-1 agents[12], and cytotoxicity against the human leukaemia cell line HL-60[13]. Biological activities of coumarins include antibacterial and antitumor promoting activity, as well as an inhibitory effect on iNOS protein expression[11].

As part of our continued screening program on bioactive substances from natural sources[14–18], it is expected that *C. harmandiana* may be the source of many antioxidant and cancer chemopreventive agents with novel structures. Crude hexane, EtOAc and MeOH extracts from the roots of this plant were investigated and 12 compounds were isolated. This paper describes the isolation, characterization, and evaluation of their antioxidant activity including DPPH and lipidperoxidation activity as well as cytotoxicity against cholangiocarcinoma cell lines in vitro in order to discover novel cytotoxic compounds.

MATERIALS AND METHODS

General

The NMR spectra were recorded on a Varian Mercury plus spectrometer operating at 400 MHz (^{1}H) and at 100 MHz (^{13}C). IR spectra were recorded as KBr disks or thin films, using a Perkin Elmer Spectrum One FT-IR spectrophotometer. Mass spectra were determined on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight mass spectrometer with a Z-spray ES source (Micromass, Manchester, UK). Melting points were determined on a SANYO Gallenkamp melting point apparatus and were uncorrected. Thin layer chromatography (TLC) was carried out on a MERCK silica gel 60 F_{254} TLC aluminium sheet. Column chromatography was done with silica gel 0.063–0.200 mm or less than 0.063 mm. Preparative layer chromatography (PLC) was carried out on glass supported silica gel plates using silica gel 60 PF_{254} for PLC. All solvents were routinely distilled prior to use.

Plant material

The roots of *C. harmandiana* were collected in April 2008 from Khon Kaen province. The plant was identified by Dr Pranom Chantaranothai, Faculty of Science, Khon Kaen University. A botanically identified voucher specimen (KKU255003) was deposited at the herbarium of the Department of Chemistry, Faculty of Science, Khon Kaen University, Thailand.

Extraction and isolation

The air-dried roots of *C. harmandiana* (1.3 kg) were sequentially extracted with hexane (2×2 l), ethyl acetate (2×2 l), and methanol (2×2 l) at room temperature for 3 days. The extracts were concentrated under reduced pressure at 40 °C yielding 22 g, 41 g, and 147 g of dry extracts, respectively. The crude hexane extract was fractionated through a silica gel chromatographic column eluting with EtOAc-hexane mixtures of increasing polarities yielding 13 groups of eluting fractions which were designated as F1 to F13. Purification of F7 was carried out on silica gel column (120 g) and eluted with the two gradient systems above to give **1** (2.86 g). Further separation of F8 on a silica gel column (200 g) eluted with hexane and a gradient of EtOAc afforded **2** (4.29 g) and **3** (3.25 g). Separation of F9 through silica gel column and eluted with a gradient of two solvents (hexane-EtOAc) gave compounds **4** (1.17 g) and **5** (1.56 g). Purification of fraction F11 on silica gel column chromatography (60 g) using gradient elution of hexane-EtOAc mixtures yielded compounds **6** (55 mg) and **7** (19 mg). The crude ethyl acetate extract was fractionated through silica gel chromatographic column eluting with EtOAc-MeOH mixtures of increasing polarities, yield 10 groups of eluting fractions designated as FE1 to FE10. Purification of FE4 on a silica gel column and eluted with hexane and a gradient of EtOAc gave **8** (90 mg). Fraction FE5 was subjected to silica gel column chromatography using gradient elution of EtOAc-MeOH mixtures to yield compound **9** (17 mg). Fraction FE6 was further purified by silica gel column chromatography and eluted with EtOAc and a gradient of MeOH to afford **10** (12 mg). Further rechromatography of FE7 by silica gel column chromatography, eluted with EtOAc-MeOH mixtures, gave compounds **11** (4.8 g) and **12** (2.2 g).

Heptaphylline (1): yellow crystals; mp 168–170 °C, IR (KBr) $\nu_{\max}$ cm^{-1}: 3292, 2926, 1614, 1474, 1449, 1329, 1237, 1183, 739; ^{1}H NMR ($CDCl_3$) δ 11.65 (1H, s, OH), 9.91 (1H, s, CHO), 8.23 (1H, br s, NH), 8.04 (1H, s, H-4), 7.97 (1H, d, $J = 7.8$ Hz, H-5), 7.37–7.41 (2H, m, H-7 and H-8), 7.24–7.28 (1H, m, H-6), 5.31 (1H, t, $J = 6.9$ Hz, H-2′), 3.64 (2H, d, $J = 6.9$ Hz, H-1′), 1.90 (3H, s, H-5′), 1.77 (3H, s, H-4′); ^{13}C NMR ($CDCl_3$) δ 195.4 (CHO), 157.8 (C-2), 145.0 (C-1a), 140.1 (C-8a), 134.2 (C-3′), 125.9 (C-4), 125.3 (C-7), 123.6 (C-5a), 121.2 (C-2′), 120.8 (C-6), 119.8 (C-5), 117.3 (C-4a), 115.4 (C-3), 110.9 (C-8), 109.0 (C-1), 25.7 (C-4′), 22.8 (C-1′), 18.1 (C-5′); HRMS m/z 280.1338 $[M + H]^+$ (calcd. for $C_{18}H_{17}NO_2 + H$, 280.1337).

7-Methoxyheptaphylline (2): yellow crystals; mp 150–152 °C, IR (KBr) $\nu_{\max}$ cm^{-1}: 3326, 2958, 1612, 1465, 1324, 1188, 1161, 1028; ^{1}H NMR ($CDCl_3$) δ 11.62 (1H, s, OH), 9.86 (1H, s, CHO), 8.16 (1H, br s, NH), 7.88 (1H, s, H-4), 7.81 (1H, d, $J = 8.5$ Hz, H-5), 6.89 (1H, br d, $J = 1.6$ Hz, H-8), 6.87 (1H, dd, $J = 8.5$, 1.8 Hz, H-6), 5.30 (1H, t, $J = 6.8$ Hz, H-2′), 3.90 (3H, s, OCH_3), 3.60 (2H, d, $J = 6.8$ Hz, H-1′), 1.88 (3H, s, H-5′), 1.76 (3H, s, H-4′); ^{13}C NMR ($CDCl_3$) δ 195.3 (CHO), 159.0 (C-7), 157.3 (C-

2), 145.2 (C-1a), 141.5 (C-8a), 134.1 (C-3′), 124.0 (C-4), 121.3 (C-2′), 120.5 (C-5), 117.5 (C-4a), 117.2 (C-5a), 115.3 (C-3), 109.0 (C-1), 108.9 (C-6), 95.6 (C-8), 55.7 (OCH_3-7), 25.7 (C-4′), 22.8 (C-1′), 18.1 (C-5′); HRMS m/z 310.1440 $[M + H]^+$ (calcd. for $C_{19}H_{19}NO_3 + H$, 310.1443).

Dentatin (3): colourless plates; mp 93–94 °C, IR (KBr) ν_{max} cm^{-1}: 3437, 2966, 1728, 1612, 1588, 1463, 1381, 1328, 1193, 1139, 1086, 986. 970; ^1H NMR ($CDCl_3$) δ 7.86 (1H, d, $J = 9.6$ Hz, H-4), 6.56 (1H, d, $J = 9.9$ Hz, H-6), 6.30 (1H, dd, $J = 17.4$, 10.6 Hz, H-2′), 6.18 (1H, d, $J = 9.6$ Hz, H-3), 5.69 (1H, d, $J = 9.9$ Hz, H-7), 4.94 (1H, d, $J = 17.4$ Hz, H-3′b), 4.87 (1H, d, $J = 10.6$ Hz, H-3′a), 3.82 (3H, s, OCH_3), 1.66 (6H, s, C-4′ and C-5′), 1.45 (6H, s, $2CH_3$-8); ^{13}C NMR ($CDCl_3$) δ 160.7 (C-2), 156.0 (C-9a), 154.0 (C-1a), 151.2 (C-5), 149.8 (C-2′), 138.9 (C-4), 130.4 (C-7), 119.2 (C-10), 116.3 (C-6), 111.7 (C-3), 108.3 (C-5a), 108.2 (C-3′), 107.5 (C-4a), 77.4 (C-8), 63.4 (OCH_3), 41.2 (C-1′), 29.4 (C-4′ and C-5′), 27.5 ($2CH_3$-8)**;** HRMS m/z 327.1597 $[M + H]^+$ (calcd. for $C_{20}H_{22}O_4 + H$, 327.1596).

Xanthoxyletin (4): colourless plates; mp 131–133 °C, IR (KBr) ν_{max} cm^{-1}: 3434, 2971, 1725, 1616, 1600, 1140, 1083; ^1H NMR ($CDCl_3$) δ 7.83 (1H, d, $J = 9.6$ Hz, H-4), 6.56 (1H, d, $J = 10.1$ Hz, H-6), 6.54 (1H, s, H-10), 6.19 (1H, d, $J = 9.6$ Hz, H-3), 5.69 (1H, d, $J = 10.1$ Hz, H-7), 3.85 (3H, s, OCH_3), 1.45 (6H, s, $2CH_3$-8); ^{13}C NMR ($CDCl_3$) δ 161.0 (C-2), 157.5 (C-9a), 155.5 (C-1a), 152.8 (C-5), 138.5 (C-4), 130.6 (C-7), 115.8 (C-6), 112.3 (C-3), 111.3 (C-5a), 107.3 (C-4a), 100.8 (C-10), 77.5 (C-8), 63.6 (OCH_3), 28.1 ($2CH_3$-8); HRMS m/z 259.0992 $[M + H]^+$ (calcd. for $C_{15}H_{14}O_4 + H$, 259.0970).

7-Methoxymukonal (5): yellow amorphous solid; mp 208–209 °C, IR (KBr) ν_{max} cm^{-1}: 3264, 2952, 1615, 1469, 1353, 1315, 1247, 1183, 1157, 1028, 815; ^1H NMR ($CDCl_3$) δ 11.42 (1H, s, OH), 9.92 (1H, s, CHO), 8.15 (1H, br s, NH), 8.04 (1H, s, H-4), 7.84 (1H, d, $J = 8.1$ Hz, H-5), 6.89 (1H, s, H-8), 6.87 (1H, d, $J = 8.1$, 2.1 Hz, H-6), 6.83 (1H, s, H-1), 3.89 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$) δ 195.2 (CHO), 160.0 (C-2), 159.1 (C-7), 146.6 (C-1a), 142.4 (C-8a), 125.6 (C-4), 120.1 (C-5), 118.0 (C-4a), 116.7 (C-5a), 114.9 (C-3), 108.3 (C-6), 96.1 (C-1), 95.2 (C-8), 55.0 (OCH_3-7); HRMS m/z 242.0816 $[M+H]^+$ (calcd. for $C_{14}H_{11}NO_3 + H$, 242.0817).

7-Hydroxyheptaphylline (6): yellow amorphous solid; mp 193–195 °C, IR (KBr) ν_{max} cm^{-1}: 3257, 2920, 1615, 1454, 1327, 1221, 1156; ^1H NMR ($CDCl_3 + CD_3OD$: 1:0.1) δ 9.82 (1H, s, CHO), 7.86 (1H, s, H-4), 7.72 (1H, d, $J = 8.4$ Hz, H-5), 6.82 (1H, br d, $J = 2.2$ Hz, H-8), 6.72 (1H, dd, $J = 8.4$, 2.2 Hz, H-6), 5.27 (1H, t, $J = 6.8$ Hz, H-2′), 3.56 (2H, d, $J = 6.8$ Hz, H-1′), 1.83 (3H, s, H-5′), 1.70 (3H, s, H-4′); ^{13}C NMR ($CDCl_3 + CD_3OD$) δ 195.3 (CHO), 156.9 (C-2), 155.8 (C-7), 145.2 (C-1a), 141.9 (C-8a), 133.7 (C-3′), 123.8 (C-4), 121.2 (C-2′), 120.4 (C-5), 117.7 (C-4a), 116.6 (C-5a), 115.0 (C-3), 109.4 (C-6), 109.0 (C-1), 97.3 (C-8), 25.6 (C-4′), 22.8 (C-1′), 17.9 (C-5′); HRMS m/z 318.1108 $[M+Na]^+$ (calcd. for $C_{18}H_{17}NO_3 + Na$, 318.1106).

Clausine-C (7)[19]: yellow amorphous solid; mp 193–195 °C, IR (KBr) ν_{max} cm^{-1}: 3285, 2918, 2453, 1698, 1600, 1440, 1315, 1261, 1206, 1165, 1097, 1030; ^1H NMR ($CDCl_3$) δ 8.70 (1H, s, H-4), 8.23 (1H, br s, NH), 8.06 (1H, d, $J = 8.5$ Hz, H-2), 7.98 (1H, d, $J = 8.5$ Hz, H-5), 7.38 (1H, d, $J = 8.5$ Hz, H-1), 6.93 (1H, br d, $J = 1.5$ Hz, H-8), 6.90 (1H, dd, $J = 8.5$, 1.9 Hz, H-6), 3.96 (3H, s, OCH_3-ester), 3.90 (3H, s, OCH_3-7); ^{13}C NMR ($CDCl_3$) δ 168.0 (C=O), 159.6 (C-7), 142.3 (C-1a), 141.3 (C-8a), 126.3 (C-2), 123.3 (C-4a), 121.9 (C-4), 121.5 (C-3), 121.4 (C-5), 117.1 (C-5a), 109.8 (C-1), 109.0 (C-6), 95.1 (C-8), 55.6 (OCH_3-7), 51.9 (OCH_3-ester); HRMS m/z 256.0958 $[M + H]^+$ (calcd. for $C_{15}H_{13}NO_3 + H$, 256.0968).

Nordentatin (8): pale yellow amorphous solid; mp 179–181 °C, IR (KBr) ν_{max} cm^{-1}: 3248, 2974, 1685, 1605, 1588, 1559, 1318, 1178, 1138; ^1H NMR ($CDCl_3$) δ 8.04 (1H, d, $J = 9.6$ Hz, H-4), 6.55 (1H, d, $J = 9.9$ Hz, H-6), 6.38 (1H, s, OH), 6.27 (1H, dd, $J = 17.4$, 10.6 Hz, H-2′), 6.13 (1H, d, $J = 9.6$ Hz, H-3), 5.67 (1H, d, $J = 9.9$ Hz, H-7), 4.91 (1H, d, $J = 17.4$ Hz, H-3′b), 4.84 (1H, d, $J = 10.6$ Hz, H-3′a), 1.63 (6H, s, C-4′ and C-5′), 1.43 (6H, s, $2CH_3$-8); ^{13}C NMR ($CDCl_3$) δ 161.7 (C-2), 156.0 (C-9a), 154.1 (C-1a), 150.0 (C-2′), 146.8 (C-5), 139.5 (C-4), 129.8 (C-7), 116.0 (C-10), 115.2 (C-6), 110.0 (C-3), 108.0 (C-3′), 106.2 (C-5a), 104.0 (C-4a), 77.1 (C-8), 41.0 (C-1′), 29.5 (C-4′ and C-5′), 27.3 ($2CH_3$-8)**;** HRMS m/z 313.1436 $[M + H]^+$ (calcd. for $C_{19}H_{20}O_4 + H$, 313.1440).

7-Methoxymurrayacine (9)[20]: yellow solid; mp 220–222 °C, IR (KBr) ν_{max} cm^{-1}: 3186, 2985, 1633, 1576, 1425, 1316, 1234, 1201, 1156, 813; ^1H NMR ($CDCl_3 + CD_3OD$: 1:0.1) δ 10.34 (1H, s, CHO), 8.20 (1H, s, H-6), 7.77 (1H, d, $J = 8.5$ Hz, H-7), 6.86 (1H, br d, $J = 2.1$ Hz, H-10), 6.76 (1H, dd, $J = 8.5$, 2.1 Hz, H-8), 6.68 (1H, d, $J = 9.9$ Hz, H-1), 5.70 (1H, d, $J = 9.9$ Hz, H-2), 3.83 (3H, s, OCH_3), 1.47 (6H, s, C-1′ and C-2′); ^{13}C NMR ($CDCl_3 + CD_3OD$)

δ 189.9 (CHO), 158.8 (C-9), 154.2 (C-4a), 142.2 (C-10a), 141.4 (C-11a), 129.5 (C-2), 120.6 (C-7), 118.3 (C-6), 117.7 (C-5), 117.6 (C-6a), 117.5 (C-7a), 116.6 (C-1), 108.1 (C-8), 104.1 (C-1a), 95.7 (C-10), 77.3 (C-3), 55.6 (OCH_3), 27.5 (C-1′ and C-2′); HRMS m/z 308.1294 $[M + H]^+$ (calcd. for $C_{19}H_{17}NO_3 + H$, 308.1286).

Clausine-E (10): deep brown amorphous solid; mp 193–194 °C, IR (KBr) ν_{max} cm^{-1}: 3330, 2949, 1698, 1663, 1594, 1500, 1436, 1355, 1318, 1263, 1088, 1005, 759; ^1H NMR ($CDCl_3 + CD_3OD$: 1:0.1) δ 8.30 (1H, s, H-4), 7.99 (1H, d, $J = 7.8$ Hz, H-5), 7.44 (1H, s, H-2), 7.42 (1H, d, $J = 8.2$ Hz, H-8), 7.35 (1H, t, $J = 8.1$ Hz, H-7), 7.16 (1H, t, $J = 7.5$ Hz, H-6), 3.87 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3 + CD_3OD$) δ 168.8 (C=O), 142.0 (C-1), 139.9 (C-8a), 132.8 (C-1a), 126.1 (C-7), 124.1 (C-4a), 123.6 (C-5a), 121.0 (C-3), 120.4 (C-5), 119.6 (C-6), 115.0 (C-4), 111.3 (C-8), 110.9 (C-2), 51.9 (OCH_3); HRMS m/z 242.0805 $[M + H]^+$ (calcd. for $C_{14}H_{11}NO_3 + H$, 242.0817).

Lansine (11)[21]: dark yellow amorphous solid; mp 210–212 °C (dec.), IR (KBr) ν_{max} cm^{-1}: 3249, 2858, 1665, 1604, 1471, 1430, 1274, 1251, 1157, 1036, 819; ^1H NMR ($CDCl_3 + CD_3OD$: 1:0.2) δ 10.32 (1H, s, CHO), 8.33 (1H, s, H-4), 7.81 (1H, d, $J = 8.5$ Hz, H-5), 6.92 (2H, s, H-1 and H-8), 6.79 (1H, dd, $J = 8.5, 2.1$ Hz, H-6), 3.97 (OCH_3-7), 3.86 (OCH_3-2); ^{13}C NMR ($CDCl_3 + CD_3OD$) δ 190.3 (CHO), 161.1 (C-2), 158.9 (C-7), 146.4 (C-1a), 142.1 (C-8a), 120.24 (C-5), 120.2 (C-4), 117.8 (C-3), 117.6 (C-4a), 117.0 (C-5a), 108.3 (C-6), 95.2 (C-8), 92.3 (C-1); HRMS m/z 278.0709 $[M+Na]^+$ (calcd. for $C_{15}H_{13}NO_3 + Na$, 278.0785).

Clausine-K (12)[22]: grey powder; mp 239–240 °C (dec.), IR (KBr) ν_{max} cm^{-1}: 3446, 3315, 2927, 1664, 1618, 1578, 1200, 1163, 1084; ^1H NMR (DMSO-d_6) δ 11.24 (1H, s, COOH), 8.32 (1H, s, H-4), 7.88 (1H, d, $J = 8.5$ Hz, H-5), 7.02 (1H, s, H-1), 6.96 (1H, br d, $J = 2.1$ Hz, H-8), 6.74 (1H, dd, $J = 8.5, 2.1$ Hz, H-6), 3.84 (6H, s, OCH_3-2 and OCH_3-7); ^{13}C NMR (DMSO-d_6) δ 168.3 (COOH), 158.5 (C-7), 157.7 (C-2), 143.8 (C-1a), 142.0 (C-8a), 123.4 (C-4), 120.8 (C-5), 116.6 (C-3), 116.1 (C-4a), 112.9 (C-5a), 108.6 (C-6), 95.5 (C-8), 94.3 (C-1), 56.3 (OCH_3-2), 55.7 (OCH_3-7); HRMS m/z 272.0930 $[M + H]^+$ (calcd. for $C_{15}H_{13}NO_4 + H$, 272.0923).

BIOASSAY

2,2-Diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay

The DPPH radical scavenging method was used to determine the ability of the test compounds to scavenge DPPH radical. The DPPH assay measures hydrogen atom (or one electron) donating activity and hence provides a measure of free radical scavenging antioxidant activity. DPPH is a purple coloured stable free radical. It is reduced to yellow coloured diphenylpicrylhydrazine. Fifty µl of various concentrations of the test compounds and 50 µl of 200 µM DPPH were mixed in a 96 well-plate. The reaction was carried out at room temperature for 30 min protecting it from light, and the disappearance of DPPH radical colour upon radical reduction was monitored by measuring the absorbance at 490 nm against the blank solution.

Lipid peroxidation assay

The ability of the test compounds to inhibit lipid peroxidation in pig brain homogenate was determined using thiobarbituric acid method. The lipid in the diluted brain homogenate was auto-oxidized to generate lipid radical and finally malondialdehyde (MDA). Therefore, lipid peroxidation was measured via the amount of liberated MDA in the system as assayed by measuring the pink product of the reaction between MDA and TBA. Pig brains were obtained from freshly slaughtered animals and conveyed to the laboratory packed in ice. The meninges and all blood clots were stripped off and brains were washed and homogenized. The supernatant fraction of pig brain homogenate was transferred to a series of test tubes containing 4 mM phosphate buffer pH 7.4, 467 mM KCl and various concentrations of the test compounds. Each test tube was vortexed and incubated in a shaking water bath at 37 °C for 30 min. The lipid peroxidation reaction was stopped by the addition of 0.4 ml of 35% perchloric acid. Precipitated protein was removed by centrifugation at 3000 rpm for 5 min and 1.5 ml of supernatant fluid were transferred to the test tube and then 0.5 ml of 1.0% w/v of TBA in 50% glacial acetic acid was added. The reaction mixture was heated at 100 °C for 15 min and the fluorescence was measured by spectrofluorometer. The percent inhibitions were determined at 4–5 concentrations (n=3).

Cell culture and cytotoxicity assay

Cancer cell lines tested in the study included CCA cell lines established in our institute, KKU-OCA17, and KKU-M214 cells derived from hu-

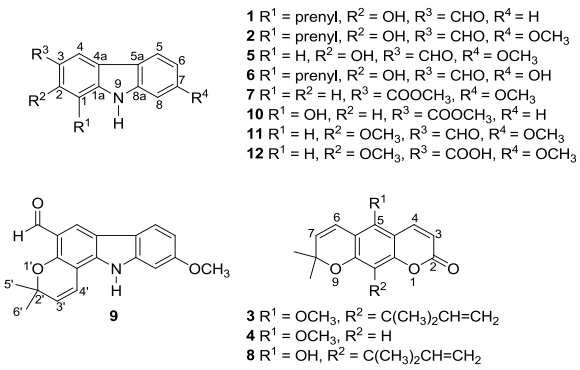

Fig. 1 Chemical structures of all (**1**–**12**) isolated compounds.

man intrahepatic cholangiocarcinoma tissues with histological types of well differentiated adenocarcinoma and moderately differentiated adenocarcinoma, respectively[23]. The CCA cells were routinely cultured in Ham's F12 media supplemented with 4 mM L-glutamine, 1 mM sodium pyruvate, 12.5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.3), 100 U/ml penicillin, 100 µg/ml streptomycin sulphate, and 10% foetal calf serum. The media was renewed every 3 days, trypsinized with 0.25% trypsin-EDTA, and subcultured in the same media.

CCA cells were seeded onto 96-well culture plates at a density of 5×10^3 cells/well for both cell lines. After overnight culture, the media was renewed with fresh media supplemented with test compounds. The test compounds were dissolved in DMSO and further diluted in cultured media. The final concentration of DMSO present in cultured cells was 0.5%, which did not appear to adversely affect cell viability or cell growth. The cells were cultured for further 48 h. Cytotoxicity was determined by sulphorhodamine B (SRB) dye binding assay[24]. In brief, cultured cells fixed with trichloroacetic acid were stained for 30 min. with 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM Tris base for determination of optical density in a plate reader with filter wavelength of 560 nm.

RESULTS AND DISCUSSION

Isolation and structural determination

Based on cytotoxicity, the crude EtOAc extract was the most potent part, followed by MeOH and hexane extracts. Activity guided fractionation of the crude hexane extract led to the isolation of 5 carbazoles: heptaphylline (**1**), 7-methoxyheptaphylline (**2**), 7-methoxymukonal (**5**), 7-hydroxyheptaphylline (**6**), and clausine C (**7**), as well as 2 coumarins: dentatin (**3**) and xanthoxyletin (**4**). In addition, the purification of the crude EtOAc extract afforded a coumarin: nordentatin (**8**), and 4 carbazoles: 7-methoxymurrayacine (**9**), clausine E (**10**), lansine (**11**), and clausine K (**12**). The structures of all compounds were identified by spectroscopic methods (IR, 1D and 2D NMR, HRMS) together with the comparison of their spectroscopic data with literature values. The chemical structures of all isolated compounds are shown in Fig. 1.

Table 1 Antioxidant activity of all compounds.

Compound	% inhibition (µM)	
	DPPH	Lipid peroxidation
Crude Hexane	1.67 mg/ml	0.47 mg/ml
Crude EtOAc	0.27 mg/ml	0.95 mg/ml
Crude MeOH	0.49 mg/ml	0.82 mg/ml
1	inactive[a]	14.1
2	843.42	3.61
3	inactive[a]	78.4
4	inactive[a]	inactive[a]
5	649.13	1.42
6	56.82	2.95
7	inactive[a]	539.7
8	29.3	2.90
9	inactive[a]	inactive[a]
10	57.54	1.69
11	inactive[a]	inactive[a]
12	inactive[a]	20.93
Vitamin E	21.7	59.3

[a] inactive at $>$ 1000 µM

Antioxidant activity

For lipid peroxidation inhibitory action assessed by the formation of thiobarbituric acid-reactive substances, eight out of twelve tested compounds inhibited lipid peroxidation with IC_{50} values ranging 1.4–78 µM (Table 1). Compounds **2** and **5** showed weak antioxidant activity on DPPH assay but **2** showed lipid peroxidation inhibitory activity with an IC_{50} value of 3.61 µM while **5** showed an IC_{50} value of 1.42 µM which was about 2.5-fold stronger than **2**. The results suggest that a prenyl group might decrease the antioxidant activity. Compound **6** showed stronger ability to DPPH assay than **2** which might be due to **6** containing a hydroxyl group at the C7 position, however both compounds showed nearly equal IC_{50} values on lipid peroxidation assay. The comparison of the lipid peroxidation data of esters **7** and **10** showed that the hydroxyl group on C1 might be responsi-

ble for lipid peroxidation inhibitory activity. Lipid peroxidation inhibitory data of **5** (IC_{50} = 1.42 μM) and **11** (IC_{50} = inactive) confirmed that the hydroxyl group may play an important role for this activity. In addition, coumarin derivatives **3** and **8** inhibited lipid peroxidation with IC_{50} values of 78.4 and 2.9 μM, respectively. The results convincingly demonstrate that a hydroxyl group is essential for a strong antioxidant activity.

Cytotoxicity

The crude hexane, ethyl acetate, and methanol extracts were evaluated for cytotoxicity against cholangiocarcinoma cell lines and the results are shown in Table 2. Considering from the IC_{50} and E_{max} (maximal cytotoxic effect) values, the most active fraction was the ethyl acetate extract. The two cell types exhibited different sensitivity and potency towards the standard compound (5-fluorouracil; 5-FU). In addition, the two cell lines showed similar sensitivity patterns to the three extracts. However, it is apparent that the KKU-OCA17 cells were generally more resistance to the extracts than KKU-M214 cells. Crude hexane extract and 4 compounds (**4**, **7**, **10**, and **11**) were almost completely inactive to this cell type. Compound **5** showed cytotoxicity against KKU-OCA17 with an IC_{50} value of 178.6 μM and an E_{max} value of 100% while **2** showed weak cytotoxicity with an IC_{50} value of 450.3 μM. On the other hand, **5** showed cytotoxicity against KKU-214 with an IC_{50} value of 138.5 μM (E_{max} = 100%) while **2** showed higher cytotoxicity with an IC_{50} value of 69.2 μM (E_{max} = 71%). The results indicate that these two cell lines, which were of different histological type, respond differently to different compounds. Compound **6** showed cytotoxicity against KKU-OCA17 and KKU-214 with IC_{50} values of 88.7 (E_{max} = 100%) and 43.7 (E_{max} = 100%), respectively. The result demonstrates that the hydroxyl group at C7 seems to be an important factor for cholangiocarcinoma cell lines. Compounds **11** and **12** exhibit cytotoxicity against KKU-214 with IC_{50} values of 156.3 (E_{max} = 57.7%) and 88.5 (E_{max} = 100%), respectively. The results suggest that the cytotoxicity against KKU-214 cell lines might be due to the presence of the carboxylic group at the C3 position. Among carbazole derivatives, compound **6** was a potent compound for cholangiocarcinoma cell lines.

Considering the cytotoxicity among 3 coumarins (**3**, **4** and **8**), **4** was inactive against KKU-OCA17 cells while **3** and **8** exhibited strong cytotoxicity with IC_{50} values of 43.5 and 46.1 μM, respectively. It is suggested that the 1,1-dimethylallyl group at the C10 position is favourable for the activity. In addition, E_{max} values of **3** and **8** were 26% and 97%, respectively. The data indicate that the hydroxyl group at the C5 position increases cytotoxicity. Moreover, compound **8** showed strong cytotoxicity against KKU-214 with an IC_{50} value of 39.1 μM and an E_{max} value of 100%. Among coumarin derivatives, **8** was a potent anticancer compound.

Among 12 compounds, it is clear that **8** and **6** were the most active for both cell lines, considering from the IC_{50} and E_{max} values. In addition, these two compounds displayed more potency and showed maximal cytotoxicity higher than the 5-FU standard, the backbone of chemotherapeutic agents for cancer chemotherapy of colorectal cancer and cholangiocarcinoma. It is noted that 5-FU has maximal efficacy of the cytotoxic effect lower than compounds **5**, **6**, and **8**.

Table 2 Cytotoxicity of all compounds.

Compound	KKU-OCA17		KKU-214	
	IC_{50} (μM)	E_{max} (%)	IC_{50} (μM)	E_{max} (%)
Crude Hexane	inactive[a]	inactive[a]	inactive[a]	80.9
Crude EtOAc	39.2 μg/ml	100	22.9 μg/ml	100
Crude MeOH	23 μg/ml	37.4	19.7 μg/ml	53.1
1	64.8	67.0	42.96	99.1
2	450.3	46.0	69.2	71.7
3	43.5	26.1	80.6	70.4
4	inactive[b]	inactive[b]	705.1	100
5	178.6	100	138.5	100
6	88.7	100	43.7	100
7	inactive[b]	inactive[b]	27.8	36.1
8	46.1	97	39.1	100
9	375.1	38.7	303.2	42
10	inactive[b]	inactive[b]	150.5	62.9
11	inactive[b]	inactive[b]	156.3	57.7
12	230.8	100	88.5	100
5-Fluorouracil	210.6	84.8	19.98	58.4

[a] inactive at > 100 μg/ml
[b] inactive at > 500 μM

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