J Nat Med (2014) 68:730–736
DOI 10.1007/s11418-014-0851-y

NOTE

Cytotoxicity of compounds from the fruits of *Derris indica* against cholangiocarcinoma and HepG2 cell lines

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Received: 26 April 2014 / Accepted: 25 May 2014 / Published online: 10 June 2014

Abstract Two new compounds, derrivanone (**1**) and derrischalcone (**2**), were isolated from the crude hexane extract of the fruits of *Derris indica.* In addition, 14 known compounds were isolated from the fruits of this plant. Chalcones **2–4** showed strong cytotoxicity against cholangiocarcinoma cell line (M156) and human hepatoma HepG2 cells. In addition, flavanones **15** and **16** exhibited potent and high cytotoxic efficacy.

Keywords *Derris indica* · Cholangiocarcinoma · HepG2 · Flavanone · Chalcone

Introduction

Derris indica (synonyms *Pongamia pinnata*, *P. glabra* and *Cytisus pinnaus)* is popularly known as Karanj or Karanja in Hindi and as Yi-Nam in Thai, and is a mangrove plant belonging to the Leguminosae family [1]. It is a medium-sized, fast-growing tree which is widely distributed in Southeast Asia and the Pacific Islands. In Thailand, it is widely found in the southern part [2]. The leaves of the plant consist of five or seven leaflets, the flowers are pinkish-white in color and the bark is thin gray to grayish-brown. It has been used in folk medicine for bronchitis, whooping cough, rheumatic arthritis, ulcers and diabetes [3]. Chemical investigation of various parts of the plant has led to the isolation of flavonoids, chalcones and rotenoids [4]. In addition, the seeds contain about 27–39 % oil which may be used for tanning leather, for making soap, as a liniment to treat scabies, for curing rheumatism and as lighting oil [5]. Nowadays, the oil is used as fuel in diesel engines, showing a good thermal efficiency. It has been reported that the oil from the seed is composed of saturated and unsaturated fatty acids such as oleic (42.44 %), stearic (29.64 %) and palmitic (18.58 %) acids [6]. In this study, the purification of the crude hexane extract led to two new compounds together with fourteen known compounds. Their cytotoxicity against cholangiocarcinoma (CCA) M156 cell line and human hepatoma HepG2 cell line were evaluated.

Results and discussion

The fruits of *D. indica,* collected in Krabi Province in August 2012, were air-dried, pulverized and sequentially extracted with hexane, EtOAc and MeOH. The hexane extract was subjected to silica gel column chromatography and further purified by chromatographic methods to obtain 16 pure compounds (**1–16**) (Fig. 1). The structures of all compounds were determined through the analysis of spectroscopic data, 1D- and 2D-NMR, and also MS analysis. It was found that **1** and **2** were new compounds.

Compound **1** was obtained as colourless needles, m.p. 174–176 °C. It was assigned the molecular formula $C_{25}H_{26}O_8$ as determined from its pseudo-molecular ion peak at *m/z* 477.1528 $[M + Na]^+$ in the HRESIMS,

Electronic supplementary material The online version of this article (doi:10.1007/s11418-014-0851-y) contains supplementary material, which is available to authorized users.

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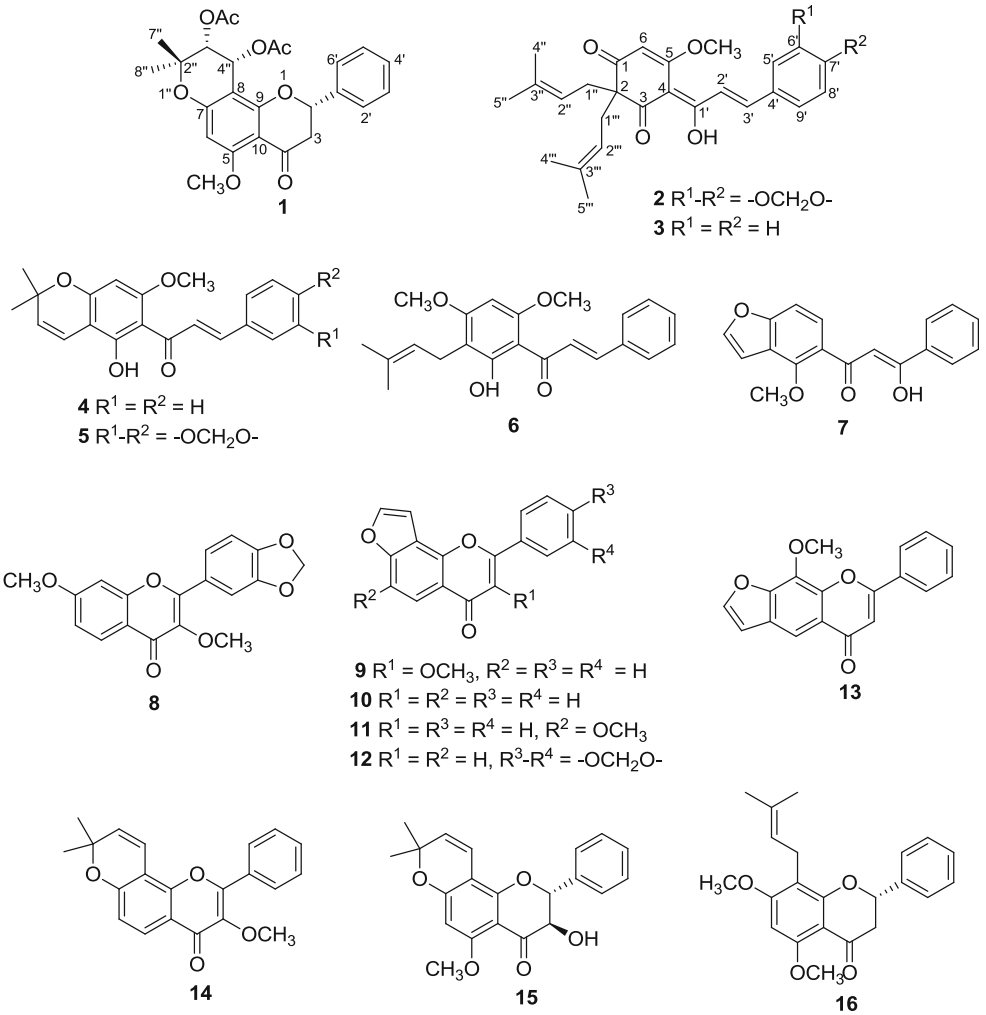

Fig. 1 The structure of all compounds isolated from *Derris indica*

corresponding to 13 degrees of unsaturation. The IR spectrum showed absorption bands of carbonyl groups at 1752, 1734 and 1681 cm^{-1}. In addition, the ^{13}C-NMR spectrum also showed three signals at δ 188.7, 170.0 and 169.8 which indicated the presence of three carbonyl groups. The ^{1}H-NMR spectrum displayed doublet of doublets signals at δ 5.39 (J = 13.2, 2.8 Hz), δ 2.96 (J = 16.4, 13.2 Hz) and δ 2.81 (J = 16.4, 2.8 Hz) which were assigned as H-2, H-3a and H-3b, respectively (Table 1). The coupling constant (J = 13.2 Hz) between H-2 and H-3a indicated the axial–axial orientation of these two protons and confirmed the α-position of the phenyl group. Five aromatic protons displayed signals at δ 7.28–7.37, indicating that this compound is a flavanone possessing an unsubstituted B ring. The methoxy group at δ 3.86 was connected to an aromatic carbon at δ 162.7 in the HMBC spectrum. A methine proton at δ 6.05 which was connected to carbon at δ 93.8 in the HMQC spectrum was assigned as H-6 by the HMBC correlations with C-5 (δ 162.7), C-7 (δ 159.9), C-8 (δ 99.9) and C-10 (δ 106.0) in the spectrum. The ^{1}H-NMR spectrum displayed two doublet signals at δ 6.23 (d, J = 4.8, H-4″) and δ 5.14 (d, J = 4.8, H-3″) which connected to carbons at δ 61.3 and 71.3, respectively. The ^{13}C-NMR and DEPT spectra displayed two acetyl groups at δ_C 169.8/20.6, OAc-3″ and 170.0/20.6, OAc-4″. The HMBC spectrum showed correlations between H-4″ and carbons at δ 163.1 (C-9), 159.9 (C-7), 99.9 (C-8), 77.3 (C-2″) and 71.3 (C-3″) (Fig. 2). The correlations of H-3″ to C-2″ and two geminal methyl groups at δ 26.2 and 21.6 were seen in this spectrum. In addition, the HMBC experiment showed the correlations of two methyl groups at δ 1.44 and 1.38 with an oxygenated quaternary carbon at δ 77.3 (C-2″). The relative stereochemistry at C-3″ and C-4″ was suggested as *cis* from the J value (4.8 Hz), which was similar to pongamone B [4] and 3-methoxy-(3″,4″-dihydro-3″,4″-diacetoxy)-2″,2″-dimethylpyrano-(7,8:5″,6″)-flavone [2] which was isolated from *D. indica*. The NOESY experiment showed correlations between H-3″/H-4″, H-3″/H-7″ and H-4″/H-7″; in addition, these protons (H-3″ and H-4″) showed no NOESY correlation with H-8″. The comparison of the ^{1}H-, ^{13}C- and also 2D-NMR spectra of

Table 1 ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectral data of compounds **1** and **2** (δ in ppm)

Position	**1** ($CDCl_3$)		**2** ($CDCl_3$)	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1			197.7 s	
2	79.1 d[a]	5.39 dd (13.2, 2.8)	61.8 s	
3	45.1 t	2.96 dd (16.4, 13.2)	205.6 s	
		2.81 dd (16.4, 2.8)		
4	188.7 s		105.6 s	
5	162.7 s		171.2 s	
6	93.8 d	6.05 s	98.9 d	5.51 s
7	159.9 s			
8	99.9 s			
9	163.1 s			
10	106.0 s			
1′	138.4 s		179.3 s	
2′	125.8 d	7.33 m	121.1 d	7.55 d (15.6)
3′	128.6 d	7.33 m	143.3 d	7.78 d (15.6)
4′	128.6 d	7.33 m	130.1 s	
5′	128.6 d	7.33 m	106.7 d	7.08 s
6′	125.8 d	7.33 m	148.6 s	
7′			150.1 s	
8′			108.9 d	6.84 d (7.2)
9′			125.4 d	7.07 d (7.2)
1″, 1‴[b]			38.4 t	2.64 d (7.2)
2″, 2‴	77.3 s		118.3 d	4.85 t (7.2)
3″, 3‴	71.3 d	5.14 d (4.8)	135.1 s	
4″, 4‴	61.3 d	6.23 d (4.8)	18.0 q	1.56 s
5″, 5‴			25.9 q	1.56 s
7″	26.2 q	1.38 s		
8″	21.6 q	1.44 s		
OCH_3-5	56.2 q	3.86 s	56.2 q	3.92 s
OCH_3-7				
$\underline{CH_3}CO$-3″	20.6 q	2.03 s		
$\underline{CH_3}CO$-4″	20.6 q	1.95 s		
$CH_3\underline{C}O$-3″	169.8 s			
$CH_3\underline{C}O$-4″	170.0 s			
-OCH_2O-			101.8 t	6.03 s

[a] Multiplicities were deduced from DEPT and HMQC experiments

[b] For compound **2**

this compound and pongamone B suggested that both compounds should have the same substructure of a pyrano ring in which H-3″ and H-4″ have β-configuration. The CD spectrum of this compound was similar to pongamone B, which showed a positive value at 334 nm and a negative value at 282 nm [4]. Thus, the absolute stereochemistry at C-2 was assumed as *2S*. From the results, the structure of **1** was elucidated as (2*S*)-5-methoxy-(3″,4″-dihydro-3″,4″-diacetoxy)-2″,2″-dimethylpyrano-(7,8:5″,6″)-flavanone, and it was named derrivanone (Fig. 1).

Compound **2** was obtained as a dark yellow solid, m.p. 110–112 °C. It was assigned the molecular formula $C_{27}H_{30}O_6$ as determined from its pseudo-molecular ion peak at *m/z* 473.1939 $[M + Na]^+$ in the HRESIMS, corresponding to 13 degrees of unsaturation. The IR spectrum showed absorption bands at 3451, 1640 and 1575 cm^{-1}. The ^{1}H-NMR spectrum showed two doublets signals at δ 7.78 ($J = 15.6$, H-3′) and 7.55 ($J = 15.6$, H-2′) indicating the *trans* geometry double bond (Table 1). In the HMQC experiment, these protons connected to carbons at δ_C 143.3(C-3′) and 121.1 (C-2′), respectively, which indicated the presence of an α,β-unsaturated double bond. The correlations of H-3′ to C-5′ (δ 106.7), C-9′ (δ 125.4) and C-1′ (δ 179.3), and of H-2′ to C-4′ (δ 130.1) and C-1′ (δ 179.3) were seen in the HMBC spectrum. Two doublet signals at δ 7.07 ($J = 7.2$, H-9′) and δ 6.84 ($J = 7.2$, H-8′) were located on carbons at δ_C 125.4 and 108.9, respectively. The correlations of H-9′ with C-7′ (δ 150.1), C-5′ (106.7) and C-8′ (108.9), and of H-8′ with C-6′ (δ 148.6) and C-4′ (δ 130.1) were seen in the HMBC spectrum. The methylenedioxy group showed signals at δ_H 6.03 (s, 2H) and at δ_C 101.8. The ^{13}C-NMR spectrum displayed four down-field signals at δ 205.6, 197.7, 179.3 and 171.2, which were assigned as C-3, C-1, C-1′ and C-5, respectively. The methoxy proton (δ_H 3.92/δ_C 56.2) correlated to C-5 in the HMBC spectrum. The proton H-6 showed a singlet signal at δ 5.51 and correlated with C-1, C-4 and C-5 in the HMBC experiment. The ^{1}H-NMR spectrum exhibited dimethylallyl groups at δ 2.64 (4H, d, $J = 7.2$, H-1″ and H-1‴), δ 4.85 (2H, t, $J = 7.2$, H-2″ and H-2‴) and δ 1.56 (12H, s, H-4″, H-4‴, H-5″ and H-5‴). The methylene groups at H-1″ and H-1‴ both correlated with C-1 (δ 197.7), C-2 (δ 61.8) and C-3 (δ 205.6), confirming that dimethylallyl groups connected at the C-2 position. The HMBC correlation between H-2′/C-1′ (δ 179.3) and the correlation between H-1″/C-3 (δ 205.6) and C-1 (δ 197.7) confirmed the enolic hydroxy at C-1′ and ketone group at C-1 and C-3. From the results, the structure of **2** was identified as 4-[1′-hydroxy-2′-ene-3′-(6′,7′-methylenedioxyphenyl)-propenylene]-5-methoxy-2,2-bisdimethylallyl-5-cyclohexene-1,3-diketone, and it was named derrischalcone (Fig. 1).

In addition, 14 known compounds, tunicatachalcone (**3**) [7], obovatachalcone (**4**) [8], glabrachromene (**5**) [9], ovalichalcone (**6**) [10], pongamol (**7**) [11], desmethoxykanugin (**8**) [12], karanjin (**9**) [13], lanceolatin B (**10**) [14], kanjone (**11**) [15], pongaglabrone (**12**) [16], 8-methoxyfurano(6,7:4″,5″)-flavone (**13**) [17], pongaflavone (**14**) [18], (2*R*,3*R*)-3-hydroxy-5-methoxy-2″,2″-dimethylpyrano [7,8:5″,6″]-flavanone (**15**) [19] and candidone (**16**) [20],

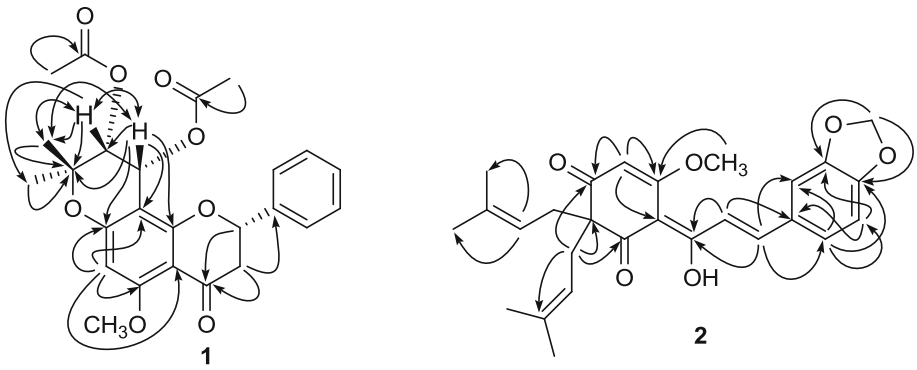

Fig. 2 The key HMBC () and NOESY () correlations of compounds **1** and **2**

Table 2 Cytotoxicity of a series of compounds against M156 and HepG2 cells

Compounds	M156 cells		HepG2 cells	
	E_{max} (%)[a]	IC_{50} (μg/mL)	E_{max} (%)	IC_{50} (μg/mL)
1	100	26.9 ± 20.1[b]	inactive	inactive
2	87.2 ± 8.9	7.0 ± 2.5	74.4 ± 9.0	5.3 ± 2.5
3	68.1 ± 4.5	0.73 ± 0.26	100	3.6 ± 0.9
4	82.8 ± 2.2	0.59 ± 0.09	91.6 ± 6.3	2.6 ± 0.9
5	Inactive	Inactive	97.7 ± 10.9	19.8 ± 7.4
6	100	20.4 ± 7.8	52.0 ± 15.3	13.1 ± 10.7
7	100	16.1 ± 6.6	99.4 ± 8.9	11.1 ± 2.9
8	100	30.2 ± 10.3	87.9 ± 7.9	17.6 ± 5.9
9	nd	nd	nd	nd
10	100	24.0 ± 17.1	Inactive	Inactive
11	97.0 ± 18.2	20.8 ± 13.1	74.2 ± 8.2	20.4 ± 7.6
12	48.6 ± 50.0	29.37 ± 54.7	Inactive	Inactive
13	100	43.0 ± 30.4	74.5 ± 18.9	8.7 ± 8.4
14	34.4 ± 8.2	0.59 ± 0.08	66.9 ± 8.5	0.86 ± 0.58
15	94.6 ± 12.7	7.8 ± 3.5	100	11.2 ± 3.6
16	100	2.4 ± 1.0	100	3.0 ± 1.4
Cisplatin	74.5 ± 14.2	3.36 ± 1.44	66.8 ± 3.7	0.66 ± 0.21

nd not determined due to the low solubility

[a] E_{max}: percent of maximal cancer cell killing effect

[b] Each value is represented the mean ± SEM, each from three experiments

were isolated and their structures elucidated by comparing their spectroscopic data with those reported in the literature.

Cytotoxicity of tested compounds [21]

The anticancer activity of all compounds (**1**–**16**) was tested against human CCA M156 cells and human hepatoma HepG2 cells. Almost all compounds were active toward both cancer cell types (Table 2). Compound **4** showed the most potent and highest anticancer efficacy against M156 and HepG2 cells. Most compounds were active against both cell types at micromolar concentrations. In addition, compounds **2**, **3**, **4**, **7**, **15** and **16** showed strong cytotoxicity against these two cell lines. Comparing the cytotoxicity between **2** and **3**, it seems that **3** was more efficacious than **2**. The results show convincingly that the methylenedioxy group appears to be detrimental for the activity. This effect was shown in the case of cytotoxicity against the HepG2 cell line. Compound **4** ($IC_{50} = 2.6 \pm 0.9$) exhibited stronger activity than **5** ($IC_{50} = 19.8 \pm 7.4$). Compound **14** showed cytotoxicity against M156 and HepG2 cell lines with IC_{50} values of 0.59 ± 0.08 and 0.86 ± 0.58, respectively, but the E_{max} values of this compound were 34.4 ± 8.2 and 66.9 ± 8.5 %. In contrast, flavanone **15** exhibited high values of E_{max} (94.6 ± 12.7 and 100 %) and showed IC_{50} values of 7.8 ± 3.5 and 11.2 ± 3.6 μg/mL, respectively. The cytotoxicity results show that flavone derivatives (**8–14**) exhibited weak activity while flavanone derivatives (**15** and **16**) showed good activity. These results show convincingly that flavanone may play an important role in cytotoxicity. It should be noted that compounds **1**, **10** and **12** were active only against M156 cells but not HepG2 cells. On the other hand, compound **5** was active against HepG2 cells but not M156 cells.

Experimental

General experimental procedures

Melting points were determined on a Sanyo Gallenkamp (UK) melting point apparatus and are uncorrected. UV spectra were measured on an Agilent 8453 UV–Visible spectrophotometer (Germany). IR spectra were recorded as KBr disks or thin films, using a Perkin Elmer Spectrum One FT-IR spectrophotometer (UK). The NMR spectra were recorded on a Varian Mercury plus spectrometer (UK) operating at 400 MHz (^{1}H) and at 100 MHz (^{13}C). Mass spectra were determined on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer with a Z-spray ES source (Micromass, Manchester, UK). Optical rotation was obtained using a Jasco DIP-1000 digital polarimeter. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} TLC aluminum sheets. 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 fruits of *Derris indica* were collected in August 2012 from Krabi Province, Thailand. The plant was identified by Prof. Dr. Pranom Chantaranothai, Faculty of Science, Khon Kaen University. A botanically identified voucher specimen (KKU0042012) was deposited at the herbarium of the Department of Chemistry, Faculty of Science, Khon Kaen University, Thailand.

Extraction and isolation

Air-dried fruits (5.8 kg) of *D. indica* were ground into powder and then extracted successively at room temperature with hexane (2 × 12 L), EtOAc (2 × 12 L) and MeOH (2 × 12 L). The filtered samples were combined, and the solvents were evaporated in vacuo to yield crude hexane (336 g), EtOAc (150 g) and MeOH (220 g) extracts. The crude hexane extract (336 g) was subjected to silica gel flash column chromatography and subsequently eluted with a gradient system of three solvents (hexane, EtOAc and MeOH) by gradually increasing the polarity of the elution solvent system. On the basis of their TLC characteristics, the fractions which contained the same major compounds were combined to give nine fractions, F_1–F_9. The first fraction was identified as the natural oil (262.4 g, 4.52 % yield). Purification of F_2 was carried out on silica gel and eluted with the three gradient systems above to give compound **4** (72.4 mg, 0.0012 %). The solid in fraction F_5 was recrystallized from CH_2Cl_2:EtOAc to afford a colorless solid of **9** (5.02 g, 0.0866 %). The filtrate of F_5 was evaporated to yield a viscous yellow oil, which was subjected to silica gel column chromatography using gradient elution of hexane:EtOAc mixtures to give six subfractions, $F_{5.1}$–$F_{5.6}$. Crystallization of subfraction $F_{5.3}$ from EtOAc:CH_2Cl_2 afforded a white solid of **14** (147 mg, 0.0025 %). Subfraction $F_{5.5}$ was rechromatographed on silica gel column chromatography and eluted with an isocratic system of 10 % EtOAc:hexane to give four subfractions, $F_{5.5.1}$–$F_{5.5.4}$. Subfraction $F_{5.5.2}$ was purified by reversed-phase silica gel column chromatography using 25 % water:MeOH as eluting solvent to give **3** (121.6 mg, 0.0021 %). Crystallization of subfraction $F_{5.5.3}$ using EtOAc:CH_2Cl_2 afforded a colorless solid of **8** (47.8 mg, 0.0008 %). The filtrate of $F_{5.5.3}$ was purified by reverse-phase silica gel chromatography using 20 % water:MeOH as eluting solvent to give **2** (25.9 mg, 0.0004 %) and **15** (4.3 mg, 0.0013 %). Subfraction $F_{5.6}$ was rechromatographed on reverse-phase silica gel column chromatography and eluted with an isocratic system of 40 % water:MeOH to give five subfractions, $F_{5.6.1}$–$F_{5.6.5}$. Crystallizations of subfractions $F_{5.6.4}$ using EtOAc:CH_2Cl_2 afforded a colorless solid of **10** (24.4 mg, 0.0004 %). Purification of F_8 by silica gel column chromatography and elution with hexane:EtOAc mixtures of increasing polarity gave six subfractions, $F_{8.1}$–$F_{8.6}$. Subfraction $F_{8.1}$ was further purified by preparative thin-layer chromatography using 2 % EtOAc:hexane as an eluting solvent to give **7** (35.3 mg, 0.0006 %) and **5** (4.4 mg, 0.00008 %). Subfraction $F_{8.2}$ was further purified by preparative thin-layer chromatography using 15 % EtOAc:hexane as eluting solvent to give **1** (26.8 mg, 0.0005 %). Subfraction $F_{8.4}$ was rechromatographed on silica gel column chromatography and eluted with an isocratic system of 75 % CH_2Cl_2:hexane to give **11** (11.4 mg, 0.0002 %) and **12** (3.2 mg, 0.00006 %). Subfraction $F_{8.5}$ was subjected to silica gel column chromatography with 10 % acetone:hexane to give seven subfractions, $F_{8.5.1}$–$F_{8.5.7}$. Subfraction $F_{8.5.1}$ was further purified by silica gel column chromatography using 10 % EtOAc:hexane as eluting solvent to give **6** (40.9 mg, 0.0007 %) and **16** (90.6 mg, 0.0016 %). The colorless solid in subfraction $F_{8.5.4}$ was recrystallized with EtOAc:CH_2Cl_2 to give **13** (43.0 mg, 0.0007 %).

Cytotoxicity; cell culture

The human CCA cell line KKU-M156, kindly provided by Prof. Banchob Sripa, Faculty of Medicine, Khon Kaen University, was routinely cultured in Ham's F12, supplemented with 10 % fetal bovine serum, 12.5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES, pH 7.3), 100 U/ml penicillin G and 100 μg/ml gentamicin. Human hepatoma HepG2 cells, purchased from the

American Type Culture Collection (ATCC HB 8065), were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum (1 %), MEM nonessential amino acids (Gibco), 12.5 mM HEPES, pH 7.3, 100 U/ml penicillin and 100 μg/ml gentamicin. Cultured cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2. The cells were subcultured every 2–3 days before cultured cell confluence using 0.25 % trypsin–EDTA, and medium was changed after an overnight incubation.

KKU-M156 and HepG2 cells were seeded onto 96-well plates at a density of 7.5×10^3 and 1.5×10^4 cells/well, respectively. After an overnight incubation, cultured media were changed to serum-free media. Test compounds, dissolved in DMSO and diluted with medium to various concentrations, were added into cultured cells and incubated for 24 h. The cytotoxicity was assessed by the sulforhodamine B (SRB) assay as previously described [22]. In brief, cultured cells were fixed with 10 % trichloroacetic acid and stained with 0.4 % SRB. The protein-bound dye was solubilized with 10 mM Tris-base solution for determination of the absorbance at 540 nm with a microplate reader. The cytotoxicity was calculated as percent absorbance of controls. The IC_{50} value was calculated by a nonlinear curve-fitting program.

Derrivanone (**1**)

Colorless solid; m.p. 174–176 °C; $[\alpha]_D^{24}$ −240.0 (*c* 0.001, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 242 (4.04), 283 (4.25) nm; IR (KBr) ν_{max} 2923, 1752, 1734, 1681, 1603, 1583, 1419, 1369, 1292, 1236, 1137, 1106, 1049, 884, 771 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) spectroscopic data, see Table 1; HRESIMS *m/z* 477.1528 $[M + Na]^+$ (calcd. for $C_{25}H_{26}O_8Na$ 477.1525).

Derrischalcone (**2**)

Dark yellow solid; m.p. 110.0–112.0 °C; UV ($CHCl_3$) λ_{max} (log ε) 264 (4.15), 321 (4.20), 411 (4.66) nm; IR (KBr) ν_{max} 3451, 2916, 1640, 1575, 1446, 1406, 1358, 1243, 1035 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) spectroscopic data, see Table 1; HRESIMS *m/z* 473.1939 $[M + Na]^+$ (calcd. for $C_{27}H_{30}O_6Na$ 473.1940).

Acknowledgments We thank Khon Kaen University, Royal Golden Jubilee Scholarship (PHD/0020/2556) and The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education for financial support. The National Research University Project of Thailand through the Advanced Functional Materials Cluster of Khon Kaen University is gratefully acknowledged.

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