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XANTHONES DERIVATIVES FROM *Cratoxylum cochinchinense* BLUME. AND THEIR CYTOTOXICITIES

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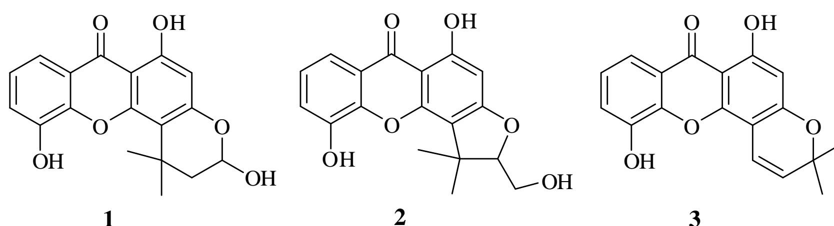
Abstract

Three cytotoxic xanthones (**1-3**), together with other ten known compounds, have been isolated from the barks of *Cratoxylum cochinchinense* Blume. (Mei-thanyo), collected from Kachin State, Northern Myanmar. These structures were established on the basis of extensive spectroscopic methods. Among the isolated compounds, six xanthones and one anthraquinone were evaluated for their cytotoxicities by using the MTT assay. Compound **1** showed a significant cytotoxicity against all tested human cancer cell lines with IC_{50} values in the range 3-9 μM, on average lower than the anticancer drug cisplatin. Compounds **2** and **3** exhibited, instead, high cytotoxicity only against some cell lines. In striking contrast, other compounds showed from moderate to no activity.



Keywords: cytotoxic xanthones, *Cratoxylum cochinchinense* Blume., human cancer cell lines, MTT assay, cisplatin

Introduction

Natural products and their derivatives have been recognized for many years as a source of therapeutic agents and of structural diversity. The relationships of natural products, traditional medicine, and health care seem to be the contemporary situation. Recent reviews have highlighted the continuing

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role that natural products and structures derived from or related to natural products continue to play in the development of new drugs (Ji *et al*., 2009; Newman and Cragg, 2007). Promising sources of novel bioactive compounds include plants growing in several third world countries where the local flora, is still largely uninvestigated. This is the case of Myanmar, especially in Kachin state.

Cratoxylum cochinchinense Blume. is a small genus belonging to the Clusiaceae (Guttiferae) family, which is found mainly in Southeast Asia. This plant is known as "Mei-thanyo" locally in Myanmar. The bark, roots, and leaves of this plant have been used in traditional medicine to treat fevers, coughs, diarrhoea, itches, ulcers, and abdominal complaints (Vo, 1997). In previous phytochemical studies this plant has been reported to be a rich source of xanthones, triterpenoids, tocotrienols, benzophenones, and bisanthraquinone, among which xanthones are the most abundant metabolites (Boonnak *et al*., 2009; Duan *et al*., 2012; Laphookhieo *et al*., 2008; Mahabusarakam *et al*., 2006; Sia *et al*., 1995; Udomchotphruet *et al*., 2012; Nguyen and Harrison, 1998; Yu *et al*., 2009; Rattanaburi *et al*., 2014). It has also been reported that some of these xanthones possessed significant pharmacological properties, including antimalarial, cytotoxicity, and antibacterial activity (Boonnak *et al*., 2009; Laphookhieo *et al*., 2006; Mahabusarakam *et al*., 2008). The use of this plant as a traditional medicine, and the earlier interesting results stimulated us to search bioactive compounds from a sample of the plant collected in Northern Myanmar, for which no phytochemical study exists. We have now analysed the MeOH extract of the bark of *C. cochinchinense*, collected from Kachin State, Myanmar. In this investigation, eight known xanthones (**1-8**) and one anthraquinone (**9**), as well as four common triterpenes were isolated. The structures were established by interpretation of their spectroscopic data (^{1}H, ^{13}C, COSY, HSQC and HMBC) analysis as well as comparison with those reported in the literature and some structures were also confirmed by X-ray crystallography. This report also describes the *in vitro* cytotoxic effects of some isolates.

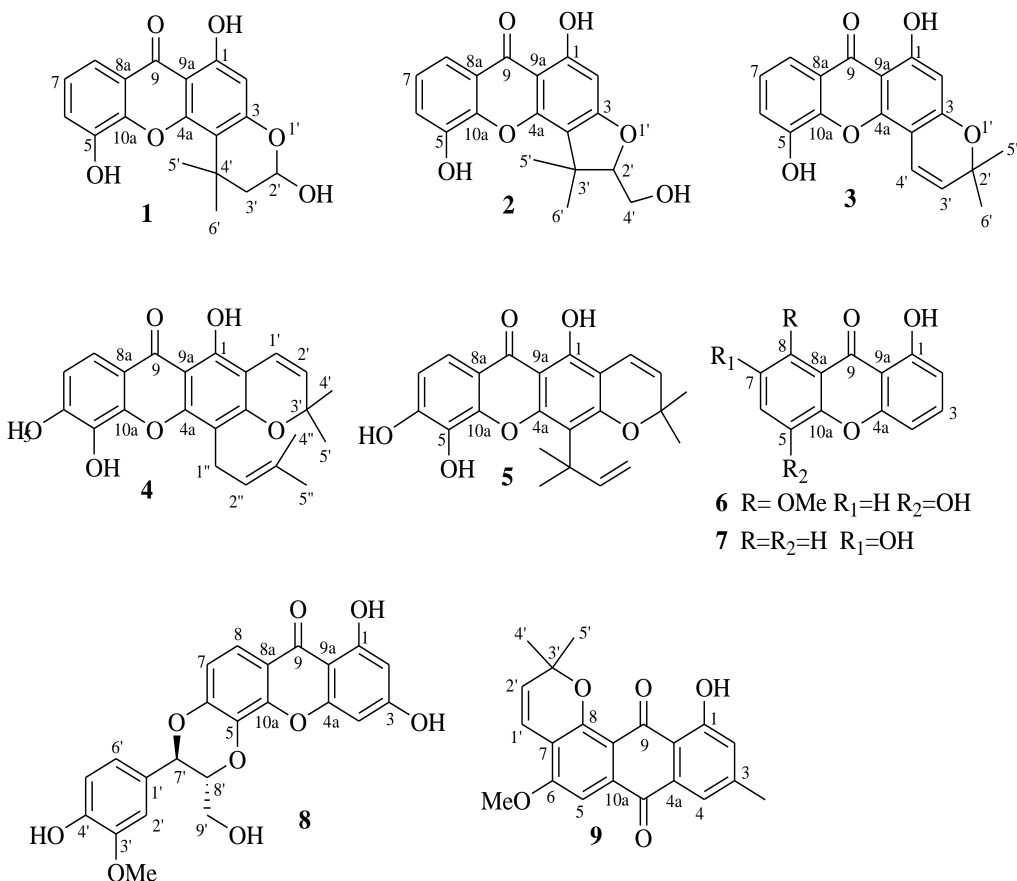

Figure 1: Structures of Compounds (**1-9**) from *C. cochinchinense*

Materials and Methods

Optical rotation was measured with a Perkin-Elmer 241 polarimeter. Infrared spectra were recorded on NaCl disks on an FT IR Perkin Elmer Paragon 100 PC spectrometer; ν in cm^{-1}. NMR experiments were performed on a Bruker AV 300 spectrometer, at 300 MHz (^{1}H) and 75.47 MHz (^{13}C) without TMS or a Bruker 600 MHz (^{1}H) and 150 MHz (^{13}C) with TMS. NMR chemical shifts are reported in ppm and solvent peaks were used as internal standards. The abbreviations s = singlet, d = doublet, dd = double doublet and t = triplet are used throughout; coupling constants (*J*) are reported in Hz. The multiplicity of each carbon atom was determined by DEPT and APT experiments. COSY, DEPT, HSQC, HMBC spectra were recorded using

standard pulse sequences. NMR spectra were recorded in in $CDCl_3$ and MeOH-d_4 (99.8 % deuterium atoms), purchased from Sigma-Aldrich. ESIMS data were recorded on a Thermo TSQ mass spectrometer, by flow injection analysis (FIA), with electron spray ionization source (ESI). High Resolution mass spectra were measured on a FT-ICR Bruker Daltonics Apex II mass spectrometer. For silica gel and reversed phase column chromatography, Merck Kieselgel 60 (40-63 μ m) and Merck LiChroprep RP-18 (25-40 μ m) were employed, respectively; for direct phase and reversed phase TLC, 0.25 mm silica gel 60 (GF_{254}, Merck) or RP-18 (F_{254}s, Merck), aluminium-supported plates were used. Compounds were visualized under UV light (254 and 366 nm) and, additionally, they were stained by exposure to a 0.5% solution of vanillin in H_2SO_4-EtOH (4:1) or dipping in $KMnO_4$ in acetone, followed by gentle heating at 100 °C. Preparative MPLC separations were performed on a *Isolera* instrument equipped with home-made silica gel and RP-18 filled cartridges and a UV detector. Reagent grade solvents from Aldrich were used for extraction and chromatographic separations.

Plants Materials

The barks of *C. cochinchinense* (Mei-thanyo) were collected near Nam-Pha Lake, about 2.5 km east of the Nam-Pha village, Banmaw Township, Kachin State, Myanmar in December 2012. The plant was analyzed and identified by Professor Dr Htar Htar Lwin, Department of Botany, Banmaw University, Myanmar.

Extraction and Isolation

Chopped, dried barks of *C. cochinchinense* (1 kg) were exhaustively extracted with methanol. Evaporation of solvent under vacuum produced a MeOH extract (29 g) which was partitioned between water and ethyl acetate to give an EtOAc soluble fraction (8.19 g). Subsequently, the EtOAc fraction (8.19 g) was partitioned between hexane and MeCN. Removal of solvents in vacuo (< 40 °C) produced a yellow-brown, viscous hexane fraction (3.24 g) and a dark-brown MeCN fraction (3.61 g), respectively. A sample of the MeCN fraction (1.12 g) was fractionated by column chromatography on RP-18. Elution with a gradient of MeCN in water (from 33 to 100 % MeCN) afforded 10 fractions (A1-A10). Fraction A3 (133 mg) was subjected to

further fractionation, using a silica gel CC. Elution with a gradient of hexane-EtOAc (from 0 to 100 % EtOAc) afforded twelve subfractions (A3.1 - A3.12). CC of fraction A3.2 (4.76 mg) over silica gel yielded 6-deoxyisojacareubin (**3**, 2.4 mg), upon elution with hexane-EtOAc, 9:1. CC of fraction A3.3 (6.4 mg) over silica gel gave pruniflorone N (**1**, 3.3 mg), upon elution with hexane-EtOAc, 4:1. CC of fraction A3.9 (5 mg) on silica gel yielded pruniflorone M (**2**, 2.5 mg), upon elution with hexane-EtOAc, 7:3. In addition, multiple chromatographic separations of the MeCN (2.40 g) and hexane (3.24 g) fractions gave xanthone V_1 (**4**, 3.4 mg), macluraxanthone (**5**, 16 mg), 1,5-dihydroxy-8-methoxyxanthone (**6**, 2.9 mg), 1,7-dihydroxyxanthone (**7**, 3.1 mg), 5'-demethoxycadesin G (**8**, 4.3 mg), vismiaquinone D (**9**, 2.8 mg), α-amyrin (10.7 mg), α-amyrenone (28.7 mg), lupeol (70.5 mg), and lupenone (47.5 mg).

MTT assay

Cell cultures

MCF7 breast and HepG2 hepatocellular cancer cells were maintained in DMEM/F-12 and DMEM, respectively, supplemented with 10 % fetal bovine serum (FBS), 100 mg/mL penicillin/streptomycin and 2 mM L-glutamine (*Life Technologies*, Milan, Italy). SkBr3 breast and BG-1 ovarian cancer cells were cultured in RPMI-1640 and DMEM medium respectively, without phenol red supplemented with 10 % FBS, 100 mg/mL penicillin/streptomycin and 2 mM L-glutamine (*Life Technologies*, Milan, Italy). Ishikawa endometrial cancer cells were maintained in MEM supplemented with 10 % FBS, 100 mg/ml penicillin/streptomycin, 2 mM L-glutamine and 1 % Non-Essential Amino Acids Solution (*Life Technologies*, Milan, Italy). Mesothelioma cancer cells IST-MES1 were maintained in Nutrient Mixture F-10 Ham (*Sigma-Aldrich*, Milan, Italy) supplemented with 20 % FBS, 100 mg/mL penicillin/streptomycin. All cell lines were obtained by ATCC and used less than 6 months after resuscitation, except IST-MES1 cells which were kindly provided by "*Istituto Nazionale per la Ricerca sul Cancro,* Genova, Italy".

Cell proliferation

The effects of each compound on cell viability were determined by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme. 24 Cells were seeded in quadruplicate in 96-well plates in regular growth medium and grown until 70 % confluence. Cells were washed once they had attached and then treated with increasing concentrations of each compound for 48 h in regular medium supplemented with 1 % FBS. The relative cell viability was determined by using the MTT assay according to the manufacturer's protocol (*Sigma-Aldrich*, Milan, Italy). The mean absorbance for each drug dose was expressed as percentage of the cells treated with vehicle absorbance and plotted versus drug concentration. Cisplatin was used as the positive control.

Results and Discussion

Extensive preparative chromatographic separation of the methanol extract of the bark of *C. cochinchinense* on silica gel and reverse phase columns, afforded the xanthones pruniflorone N (**1**), pruniflorone M (**2**) (Boonnak *et al*., 2010), 6-deoxyisojacareubin (**3**) (Sabphon *et al*., 2012), xanthone V_1 (**4**) (Botta *et al*., 1986), macluraxanthone (**5**) (Laphookhieo *et al*., 2009; Botta *et al*., 1986), 1,5-dihydroxy-8-methoxyxanthone (**6**), 1, 7-dihydroxy xanthone (**7**) (Nagem and de Oliveira, 1997), 5'-demethoxycadesin G (**8**) (Sia *et al*., 1995), and the anthraquinone vismiaquinone D (**9**) (Reyes-Chilpa *et al*., 2014). In addition, four common triterpenoids, α-amyrin (Chaturvedula and Prakash, 2013), α-amyrenone (de Oliveira *et al*., 2012), lupeol (Jash *et al*., 2013), and lupenone (Ahn and Oh, 2013) were isolated. 1,7-Dihydroxyxanthone (**7**), 1,5-dihydroxy-8-methoxyxanthone (**6**), pruniflorone N (**1**), pruniflorone M (**2**), α-amyrin, α-amyrenone, lupeol and lupenone were isolated for the first time from this plant, while 6-deoxyisojacareubin (**3**) and vismiaquinone D (**9**) were isolated from the genus *Cratoxylum* for the first time.

Structural Elucidation of Isolated Compounds (1-3)

Compound 1 (pruniflorone N)

Compound **1** was isolated as yellow small crystals and was assigned as the molecular formula $C_{18}H_{16}O_6$ on the basis of its ^{13}C NMR data (Table 1) and an ion at *m/z* 327.15 [M-H]$^-$ in the ESIMS spectrum (negative mode), indicating 11 degrees of unsaturation. The FT IR spectrum exhibited absorption bands for hydroxyl (3394 cm^{-1}), chelated aromatic carbonyl (1648 cm^{-1}) and aromatic ring (1578 cm^{-1}).

The presence of a hydroxyl-chelated carbonyl group in a xanthone nucleus was indicated by the quaternary carbon signal at δ_C 182.8 ppm in the ^{13}C NMR spectrum and the singlet at δ_H 13.0 ppm in the ^{1}H NMR spectrum in Me_2CO-d_6 (Boonnak *et al*., 2010), which was attributed to a phenolic OH group attached to the aromatic quaternary carbon C-1, resonating at δ_C 162.2 ppm in the ^{13}C NMR spectrum. The ^{1}H NMR spectrum showed a sharp singlet of an isolated aromatic proton at δ_H 6.20 ppm [1H, s (H-2)] and an ABM splitting pattern, attributed to a 1,2,3-trisubstituted benzene ring, at δ_H 7.63 ppm [1H, dd, *J* = 7.7 and 2.0 Hz (H-8)], δ_H 7.27 ppm [1H, dd, *J* = 7.7 and 2.0 Hz (H-6)] and δ_H 7.22 ppm [1H, t, *J* = 7.7 Hz (H-7)]. Characteristic protons resonances of a *gem*-dimethyl-substituted δ-lactol ring were evident at δ_H 5.43 ppm [1H, dd, *J* = 8.5 and 2.5 Hz (H-2')], 1.97 ppm [1H, dd, *J* = 11.2 and 2.5 Hz (H_b-3')], 1.91 ppm [1H, dd, *J* = 11.2 and 8.5 Hz (H_a-3')], 1.60 ppm [3H, s (H_3-5')], and 1.72 ppm [3H, s (H_3-6')]. These ^{1}H NMR (Table 1) data as well as COSY correlations (Figure 2) suggested for compound **1** a trioxygenated xanthone structure, sharing an oxygen atom with a fused δ-lactol ring.

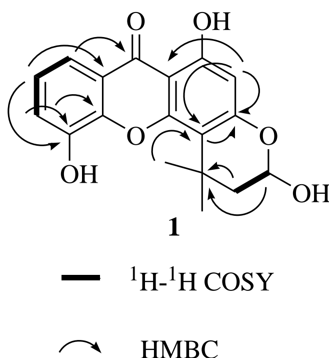

Figure 2: Selected COSY (bold bond) and key HMBC correlations of compound 1

In the HMBC spectrum (Figure 2), the most deshielded aromatic proton (δ_H 7.63) showed a correlation to the carbonyl carbon (δ_C 182.8), revealing its attachment to C-8. On the other hand, in the ^{1}H NMR spectrum, H-8 appeared as a double-doublet with *ortho*- and *meta*- coupling constants (J = 7.7 and 2.0 Hz), indicating that its *ortho*-coupled partner (δ_H 7.22, t, J = 7.7 Hz) and *meta*-coupled partner (δ_H 7.27, dd, J = 7.7 and 2.0 Hz) had to be positioned at C-7 and C-6, respectively. In addition to HMBC correlations with the *ortho*-protonated aromatic carbon (δ_C 115.8, C-8) and one oxygenated aromatic carbon (δ_C 148.3, C-5), the H-7 proton was correlated with a quaternary aromatic carbon (δ_C 122.5), which was then identified as C-8a. This pattern was confirmed by HMBC correlations of H-6 with C-8, C-5 and another oxygenated quaternary aromatic carbon resonating at δ_C 146.7, which was assigned to C-10a. These data firmly identified the carbon and hydrogen atoms of the C-ring of the xanthone nucleus, including an OH substituent at C-5.

The upfield shifted isolated aromatic proton, resonating at δ_H 6.20 in the ^{1}H NMR spectrum of **1**, was identified as H-2, on the basis of the resonance at δ_C 100.2 of C-2, identified in the HSQC spectrum. This value nicely corresponded to the chemical shifts determined for the C-2 carbons of 1,3-dioxygenated xanthones (Boonnak *et al*., 2010). Accordingly to this assignment, H-2 correlated to two oxygenated aromatic carbons, occurring at δ_C 162.2 and 161.9, respectively, which were identified as C-1 and C-3, and to two quaternary aromatic carbons, occurring at δ_C 105.3 and 111.0, respectively, which were identified as C-9a and C-4, respectively.

The 6-hydroxy-4,4-dimethyldihydropyran ring and its angular fusion to the C-3 and C-4 of the xanthone nucleus were established on the basis of HMBC correlations (Figure 2) of the hemiacetal methine proton (δ_H 5.43) to C-4' (δ_C 33.4) and C-3' (δ_C 47.2), the H_b-3' proton (δ_H 1.97) to C-4' (δ_C 33.4), and the methyl protons H_3-5' (δ_H 1.60) and H_3-6' (δ_H 1.72) to C-4 (δ_C 111.0), C-4' (δ_C 33.4), and C-3' (δ_C 47.2). The remaining oxygenated aromatic carbon (δ_C 157.1) was thus assigned to C-4a. The compound was therefore assigned the structure of 3,6,11-trihydroxy-1,1-dimethyl-2,3-dihydropyrano[2,3-*c*] xanthen-7(1*H*)-one, known in the literature with the trivial name of pruniflorone N (Boonnak *et al*., 2010).

Interestingly, although the configuration at C-2' could easily racemize, compound **1** showed a small but not-null positive optical rotation power. The vicinal coupling constants of H-2', *J* = 8.5 and 2.5 Hz, clearly indicated that this proton was axially oriented; however, the absolute configuration was not established for lack of material. Compound **1** showed potent nitric oxide inhibitory activity *in vitro*, with an IC_{50} value of 4.4 μ M, better than that of the positive control indomethacin, which is a well-known non-steroidal anti-inflammatory drug (IC_{50} = 20.1 μ M) (Boonnak *et al*., 2010).

Compound 2 (pruniflorone M)

Compound **2** was obtained as a yellow amorphous powder. The molecular formula $C_{18}H_{16}O_6$, was deduced from the NMR data (Table 1) and an ion peak at *m/z* 327.15 $[M-H]^-$ in the ESIMS spectrum (negative mode), indicating 11 degrees of unsaturation. The FT-IR spectrum showed hydroxyl, conjugated carbonyl and aromatic functionalities at 3448 cm^{-1}, 1648 cm^{-1} and 1581 cm^{-1}, respectively. Thus compounds **1** and **2** were isomeric.

Indeed, the NMR data of the protons and carbons of rings B and C and the oxygenation pattern of ring A of compound **2** were almost coincident with those of **1**, while the signals of the heterocyclic rings differ significantly. In fact, compound **2** contained a dihydrofuran instead of a dihydropyran ring. Accordingly, the ^1H NMR spectrum of **2** showed a triplet at δ_H 3.89, integrating for 2H, that was attributed to a hydroxymethyl group (H_2-4'), a doublet at δ_H 4.49, integrating for 1H, coupled (COSY) with a *J* = 6.0 Hz to the hydroxymethyl group and thus assigned to H-2', and two germinal methyl groups resonating at δ_H 1.44 (H_3-5') and 1.69 (H_3-6'), respectively. Moreover, the two methyl groups showed HMBC correlations (Figure 3) with the oxygenated methine carbon C-2' (δ_C 96.0) and with a quaternary aromatic carbon at δ_C 114.2 (C-4), that also showed a HMBC cross-peak with an isolated aromatic proton resonating at δ_H 6.23 (H-2). The last proton also showed HMBC cross- peaks with another quaternary aromatic carbon at δ_C 104.7, attributed to C-9a, and to oxygenated quaternary aromatic carbons at δ_C 167.6 and 165.5, assignable to C-1 and C-3.

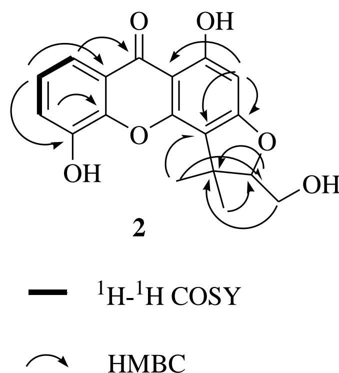

Figure 3: Selected COSY (bold bond) and key HMBC correlations of compound **2**

Consequently, the compound **2** was identified as 5,10-dihydroxy-2-(hydroxymethyl)-1,1-dimethyl-1*H*-furo[2,3-*c*]xanthen-6(2*H*)-one, known in the literature with the trivial name of pruniflorone M (Boonnak *et al*., 2010). Interestingly, the sign of the optical rotatory power of our sample of pruniflorone M was opposite to that reported in the literature (Boonnak *et al*., 2010). Compounds **1** and **2** are very rare in nature and, to the best of our knowledge, they have been isolated before only from another *Cratoxylum* species, namely *C. formosum* ssp *pruniflorum* (Boonnak *et al*., 2010).

Compound 3 (6-deoxyisojacareubin)

Compound **3** was a bright yellow amorphous solid, showed the molecular formula $C_{18}H_{14}O_5$ as determined by the ion peak at *m/z* 309 $[M - H]^-$ in the ESIMS spectrum (negative mode) and the ^{13}C NMR data, requiring 12 degrees of unsaturation. The FT IR spectrum showed the presence of hydroxyl (3417 cm^{-1}), conjugated carbonyl (1651 cm^{-1}) and aromatic ring (1574 cm^{-1}).

The presence of a chelated carbonyl group in a xanthone nucleus was indicated by the quaternary carbon signal at δ_C 182.5 ppm in the ^{13}C NMR spectrum and the singlet at δ_H 13.0 ppm in the ^1H NMR spectrum in Me_2CO-d_6 (Sabphon *et al*., 2012). The ^1H NMR spectrum in MeOH-d_4 revealed the signals of an isolated aromatic proton at δ_H 6.19 ppm [1H, s (H-2)] and the ABM splitting pattern of a 1,2,3-trisubstituted benzene ring at δ_H 7.66 ppm [1H, dd, J = 7.7 and 2.0 Hz (H-8)], 7.28 ppm [1H, dd, J = 7.7 and 2.0 Hz

(H-6)] and 7.23 ppm [1H, t, J = 7.7 Hz (H-7)]. In addition, the presence of a dimethyl-substituted chromene ring was indicated by two doublets, each assigned to an olefinic proton, at δ_H 7.03 ppm [1H, d, J = 10.1 Hz (H-4')] and 5.72 ppm [1H, d, J = 10.1 Hz (H-3')], respectively, and one singlet at δ_H 1.48 ppm, integrating for 6H of two coincident methyl groups (H_3-5' and H_3-6'). The remaining atoms of the chromene ring were identified in the ^{13}C NMR spectrum as one oxygenated quaternary sp^3 carbon at δ_C 79.5 ppm (C-2'), carrying the geminal methyl groups H_3-5' and H_3-6', and two quaternary sp^2 carbons at δ_C 102.7 ppm (C-4) and 162.3 ppm (C-3), the latter bonded to the oxygen of the pyran ring. HMBC correlations (Figure 4) were fully consistent with these assignments.

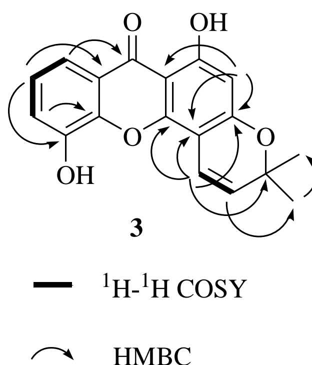

Figure 4: Selected COSY (bold bond) and key HMBC correlations of compound **3**

In the HMBC spectrum (Figure 4), the most deshielded aromatic proton (δ_H 7.66) correlated to the carbonyl carbon (δ_C 182.5), revealing its attachment to C-8. Its *ortho*-coupled proton (δ_H 7.23), bonded at C-7, showed correlations through three bonds with a phenolic carbon (δ_C 147.7, C-5) and a quaternary aromatic carbons (δ_C 122.6) which was assigned to C-8a. On the other hand, the H-6 proton gave a cross-peak with another oxygenated aromatic carbon (δ_C 146.9) which was assigned to C-10a. These data firmly identified the carbon and hydrogen atoms of the C-ring of the xanthone nucleus, including an OH substituent at C-5. The angular fusion of the pyran ring at C-3 (δ_C 162.3) and C-4 (δ_C 102.7) of the xanthone nucleus was clearly indicated by the HMBC correlation of H-4' (δ_H 7.03) with the oxygenated quaternary sp^2 carbon at δ_C 153.0, that was assignable to C-4a. As a further evidence of this attribution, the carbon bearing the most shielded

aromatic proton (δ_H 6.19) resonated at δ_C 99.9, consistent with the chemical shift expected for the C-2 of 1, 3-dihydroxyxanthones (Sabphon *et al.*, 2012), while the attached proton H-2 showed HMBC correlations with two oxygenated aromatic carbons (δ_C 162.3 and 164.2) which were C-3 and C-1. Compound **3** was thus assigned the structure of 6,11-dihydroxy-3,3-dimethylpyrano[2,2-*c*] xanthen-7(3H)-one, known in the literature with the trivial name of 6-deoxyisojacareubin (Sabphon *et al.*, 2012).

Table 1: ^{1}H NMR (300 MHz) and ^{13}C NMR (75.47 MHz) Spectroscopic Data for Compound 1-3 (in MeOH-d$_4$, □ in ppm, *J* in Hz)

Position	1		2		3	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	-	162.2, C[a]	-	167.6, C[a]		164.2, C
2	6.20, s	100.2, CH	6.23, s	94.4, CH	6.19, s	99.9, CH
3	-	161.9, C[a]	-	165.5, C[a]		162.3, C
4	-	111.0, C	-	114.2, C		102.7, C
5	-	148.3, C	-	148.0, C		147.7, C
6	7.27, dd (7.7, 2.0)	120.7, CH	7.26, dd (7.7, 2.0)	121.2, CH	7.28, dd (7.7, 2.0)	121.9, CH
7	7.22, t (7.7)	125.0, CH	7.20, t (7.7)	125.0, CH	7.23, t (7.7)	125.2, CH
8	7.63, dd (7.7, 2.0)	115.8, CH	7.64, dd (7.7, 2.0)	116.2, CH	7.66, dd (7.7, 2.0)	116.5, CH
9	-	182.8, CO	-	182.3, CO	-	182.5, CO
4a	-	157.1, C	-	154.0, C	-	153.0, C
10a	-	146.7, C	-	147.8, C[c]	-	146.9, C[c]
8a	-	122.5, C	-	122.7, C	-	122.6, C
9a	-	105.3, C	-	104.7, C[c]	-	104.3, C[c]
1'	-	-	-		-	-
2'	5.43, dd (8.5, 2.5)	94.2, CH	4.49, t (6.0)	96.0, CH	-	79.5, C
3'	1.97, dd (11.2, 2.5) 1.91, dd (11.2, 8.5)	47.2, CH_2		44.4, C	5.72, d (10.1) -	128.4, CH
4'	-	33.4	3.89, d (6.0)	61.7, CH_2	7.03, d (10.1)	164.2, C
5'	1.60, s[a]	28.9,CH_3[b]	1.44, s[a]	21.1, CH_3[b]	1.48, s	28.6, CH_3
6'	1.72, s[a]	29.1,CH_3[b]	1.69, s[a]	27.1, CH_3[b]	1.48, s	28.6, CH_3
1-OH	-	-	-	-	-	-
3-OH	-	-	-	-	-	-
5-OH	-	-	-	-	-	-

[a,b]Assignments in the same vertical column can be interchanged; [c]Chemical shift assigned from HMBC spectra.

Pruniflorone N (**1**): yellow crystals (MeOH); mp >300°C with decomposition, $[\alpha]_D^{20}$ = +2.14 (c = 0.0015, MeOH), Lit. (Boonnak *et al*., 2010): +5.2 (*c* 0.42, Me_2CO); ESIMS *m/z* 327 $[M - H]^-$ for $C_{18}H_{16}O_6$; FT IR (NaCl) ν_{max} 3394, 2958, 1648, 1578, 1420, 1171, 1058 cm^{-1}; ^1H NMR (300 MHz, MeOH-d_4) see Table 1; ^{13}C NMR (75.47 MHz, MeOH-d_4) see Table 1.

Pruniflorone M (**2**): yellow amorphous powder; $[\alpha]_D^{20}$ = -42.8 (c = 0.0012, MeOH), Lit. (Boonnak *et al*., 2010): +64.6 (*c* 0.04, $CHCl_3$); ESIMS *m/z* 327 $[M - H]^-$ for $C_{18}H_{16}O_6$; FT IR (NaCl) ν_{max} 3448, 2974, 2867, 1648, 1581, 1460, 1069, 911 cm^{-1}; ^1H NMR (300 MHz, MeOH-d_4) see Table 1; ^{13}C NMR (75.47 MHz, MeOH-d_4) see Table 1.

6-Deoxyisojacareubin (**3**): bright yellow amorphous powder; ESIMS *m/z* 309 $[M - H]^-$ for $C_{18}H_{14}O_5$; FT IR (NaCl) ν_{max} 3417, 2958, 1651, 1574, 1462, 1062, 761 cm^{-1}; ^1H NMR (300 MHz, MeOH-d_4) see Table 1; ^{13}C NMR (75.47 MHz, MeOH-d_4) see Table 1.

Xanthone V_1 (4): Yellow amorphous; ESIMS $[M-H]^-$ *m/z* 393 for $C_{23}H_{22}O_6$; FT IR (NaCl) ν_{max} 3350, 3054, 2970, 2929, 1651, 1589, 1463, 1265, 1188, 1133, 741 cm^{-1}; ^1H NMR Spectral Data (600 MHz, methanol-d_4): δ 13.18 (1H, s, 1-OH), 7.74 (1H, d, *J* = 8.5 Hz, H-8), 6.96 (1H, d, *J* = 8.5 Hz, H-7), 6.75 (1H, d, *J* = 10 Hz, H-1'), 5.60 (1H, d, *J* = 10 Hz, H-2'), 5.25 (1H, d, *J* = 6.7 Hz, H-2''), 3.50 (2H, d, *J* = 6.7 Hz, H-1''), 1.88 (3H, s, H-4''), 1.73 (3H, s, H-5''), 1.48 (6H, s, H-4' and H-5'); ^{13}C NMR Spectral Data (150 MHz, methanol-d_4): δ 180.9 (C-9), 158.3 (C-3), 156.6 (C-1), 154.3 (C-4a), 149.5 (C-6), 145.5 (C-4b), 130.8 (C-5), 127.7 (C-2'), 123.3 (C-2''), 118.6 (C-8), 116.5 (C-1'), 114.7 (C-8a), 112.9 (C-7), 107.4 (C-4), 105.3 (C-2), 103.3 (C-8b), 78.6 (C-3'), 28.7 (C-4' and 5'), 26.0 (C-4''), 22.1 (C-1''), 18.3 (C-5'').

Macluraxanthone (5): Yellow crystal ($CDCl_3$); mp. 181-182; ESIMS $[M-H]^-$ *m/z* 393.25 for $C_{23}H_{22}O_6$; ^1H NMR Spectral Data (300 MHz, $CDCl_3$): δ 7.69 (1H, d, *J* = 8.8 Hz, H-8), 6.95 (1H, d, *J* = 8.8 Hz, H-7), 6.77 (1H, d, *J* = 10.2 Hz, H-2'), 6.76 (1H, dd, *J* = 17.7 and 10.6 Hz, H-2''), 5.62 (1H, d, *J* = 10.2 Hz, H-1'), 5.22 (1H, d, *J* = 17.7 Hz, H-3a''), 5.05 (1H, d, *J* = 10.6 Hz, H-3b''), 1.65 (6H, s, H-4'' and H-5''), 1.52 (6H, s, H-4' and H-5'); ^{13}C NMR Spectral Data (75 MHz, $CDCl_3$): δ 181.1 (C-9), 159.3 (C-3), 157.2 (C-2''), 157.1 (C-4a), 154.4 (C-1), 149.3 (C-6), 144.9 (C-5a), 131.4 (C-5), 127.5 (C-2'), 117.8 (C-8),

116.4 (C-1'), 114.1 (C-4), 113.4 (C-9a), 113.1 (C-7), 105.9 (C-2), 103.6 (C-3''), 103.4 (C-8a) 78.6 (C-3'), 41.8 (C-1''), 28.3 (C-4' and 5'), 28.5 (C-4'' and C-5'').

1,5-dihydroxy-8-methoxyxanthone (6): Yellow powder ($CDCl_3$); ESIMS $[M+Na]^+$ *m/z* 258 for $C_{14}H_{10}O_5$; FT IR (NaCl) ν_{max} 3359, 2926, 2854, 1651, 1593, 1264, 1070, 739 cm^{-1}; ^1H NMR Spectral Data (600 MHz, $CDCl_3$): δ 12.98 (s, 1-OH), 7.56 (1H, t, *J* = 8.3 H-3), 7.32 (1H, d, *J* = 8.9 Hz, H-6), 6.74 (1H, d, *J* = 8.9 Hz, H-7), 3.99 (3H, s, OCH_3), 6.90 (1H, dd, *J* = 8.3 and 1.1 Hz, H-4), 6.81 (1H, dd, *J* = 8.3 and 1.1 Hz, H-2); ^{13}C NMR Spectral Data (150 MHz, $CDCl_3$): δ 182.9 (C-9), 162.8 (C-1), 155.0 (C-4a), 154.1 (C-8), 145.6 (C-5a), 138.2 (C-5), 136.8 (C-3), 121.3 (C-6), 111.6 (C-8a), 109.6 (C-9a), 111.8 (C-2), 106.3 (C-4), 105.8 (C-7), 56.9 (8-OMe).

1,7-dihydroxyxanthone (7): Yellow needle (MeOH); mp. 150-151°C (Lin *et al*., 1996); ESIMS $[M-H]^-$ *m/z* 257 for $C_{14}H_{10}O_5$; ^1H NMR Spectral Data (300 MHz, methanol-d_4): δ 7.65 (1H, t, *J* = 8.4, H-3), 7.55 (1H, d, *J* = 3.0 H-8), 7.47 (1H, d, *J* = 9.05, H-5), 7.34 (1H, dd, *J* = 9.05 and 3.0 Hz, H-6), 6.98 (1H, dd, *J* = 8.4 and 0.8 Hz, H-4), 6.76 (1H, dd, *J* = 8.4 and 0.8 Hz, H-2); ^{13}C NMR Spectral Data (75 MHz, methanol-d_4): δ 163.2 (C-1), 158.2 (C-4a), 155.9 (C-7), 151.8 (C-5a), 138.2 (C-3), 126.7 (C-6), 122.5 (C-8a), 120.6 (C-5), 110.9 (C-2), 109.5 (C-4), 108.4 (C-8).

5'-demethoxycadesin G (8): White amorphous; ESI-MS $[M-H]^-$ *m/z* 437 for $C_{23}H_{18}O_9$; FT IR (NaCl) ν_{max} 3350, 3054, 2928, 1651, 1574, 1455, 1265, 1103, 738 cm^{-1}; ^1H NMR Spectral Data (600 MHz, methanol-d_4): δ 7.68 (1H, d, *J* = 8.9 Hz, H-7), 7.06 (1H, d, *J* = 1.9 Hz, H-2'), 6.97 (1H, d, *J* = 8.9 Hz, H-8), 6.95 (1H, dd, *J* = 8.1 and 1.9 Hz, H-6'), 6.87 (1H, d, *J* = 8.1 Hz, H-5'), 6.39 (1H, d, *J* = 2.1 Hz, H-4), 6.18 (1H, d, *J* = 2.1 Hz, H-2), 5.10 (1H, d, *J* = 8.1 Hz, H-7'), 4.20 (1H, ddd, *J* = 8.1, 3.8 and 2.3 Hz, H-8'), 3.99 (3H, s, 3'-OCH_3), 3.91 (1H, dd, *J* = 12.7 and 2.3 Hz, H-9'), 3.57 (1H, dd, *J* = 12.7 and 3.8 Hz, H-9'); ^{13}C NMR Spectral Data (150 MHz, methanol-d_4): δ 181.3 (C-9), 167.4 (C-3), 164.7 (C-1), 159.3 (C-4a), 150.7 (C-6), 149.3, (C-3'), 148.7 (C-4'), 147.6 (C-5a), 133.2 (C-5), 128.1 (C-1'), 121.9 (C-6'), 118.1 (C-8), 116.4 (C-5'), 116.1 (C-8a), 114.7 (C-7), 103.4 (C-9a), 112.2 (C-2'), 99.4 (C-2), 95.3 (C-4), 80.1 (C-8') and 78.4 (C-7'), 61.8 (C-9'), 56.5 (3'-OMe).

Vismiaquinone D (9): Yellow powder; ESI-MS $[M+Na]^+$ *m/z* 373.17 for $C_{21}H_{18}O_5$; FT IR (NaCl) ν_{max} 3054, 2978, 1636, 1560, 1266, 1126, 738 cm^{-1}; ^1H NMR Spectral Data (300 MHz, $CDCl_3$): δ 13.20 (1H, s, 1-OH), 7.57 (1H, d, *J* = 1.1 Hz, H-4), 7.44 (1H, s, H-5), 7.07 (1H, d, *J* = 1.1 Hz, H-2), 6.73 (1H, d, *J* = 10 Hz, H-1'), 5.84 (1H, d, *J* = 10 Hz, H-2'), 4.02 (3H, s, 6'-OCH_3), 2.43 (3H, s, H-3), 1.57 (6H, s, H-4' and H-5'); ^{13}C NMR Spectral Data (75 MHz, $CDCl_3$): δ 187.4 (C-9), 182.9 (C-10), 162.8 (C-1), 158.9 (C-6), 156.5 (C-8), 146.9 (C-3), 135.6 (C-4a), 132.8 (C-5a), 132.3 (C-2'), 124.7 (C-2), 119.9 (C-4), 116.5 (C-7), 116.3 (C-1'), 115.6 (C-8a) and 114.9 (C-9a), 103.0 (C-5), 77.9 (C-3'), 56.4 (6-OMe), 28.2 (C-4' and 5'), 22.2 (C-3).

Cytotoxic Activity

The effects of compounds **1-5**, **7**, and **9** on the proliferation of tumor cells were evaluated in comparison with the well-known antitumor drug cis-diamminedichloroplatinum (II) (cisplatin) by using MTT assays. In particular, MCF7 and SkBr3 breast, endometrial Ishikawa, ovarian BG-1, mesothelioma IST-MES1 and hepatocellular HepG2 cancer cells were treated for 48 h with increasing concentrations of tested compounds. Macluraxanthone **(5)**, 1, 7-dihydroxyxanthone **(7)** and vismiaquinone D **(9)** were inactive on all cell lines, whereas xanthone V_1 **(4)** exhibited moderate effects on all cells (Table 2). The most active xanthones of the series were **1-3**. In particular, pruniflorone N (**1**) showed a stronger inhibitory activity than cisplatin on five of the six cell lines, while pruniflorone M (**2**) and 6-deoxyisojacareubin (**3**) were more active on four (MCF7, HepG2, Ishikawa, and BG-1) and two (MCF7 and BG1) types of cells, respectively (Table 2).

Table 2: Cytotoxic Activity of Tested Compounds on Breast MCF7 and SkBr3, Endometrial Ishikawa, Ovarian BG-1, Mesothelioma IST-MES1 and Hepatocellular HepG2 Cancer Cells, after 48 h Treatment, as Determined by Using the MTT Assay. IC_{50} values were calculated by probit analysis (P<0.05, □2 test).

Compound	IC_{50} (μ M)±S.D					
	MCF7	SKBR3	Ishikawa	BG-1	IST-MES1	HepG2
Pruniflorone N (**1**)	7 (±3.1)	9 (±2.4)	7 (±3.7)	3 (±1.6)	5 (±2.8)	6 (±2.4)
Pruniflorone M (**2**)	10 (±2.3)	12 (±2.8)	7 (±1.6)	6 (±1.7)	25 (±4.9)	7 (±1.3)
6-Deoxyisojacareubin (**3**)	6 (±2.2)	7 (±2.3)	>50	7 (±3.1)	>50	>50
Xanthone V_1 (**4**)	20 (±2.2)	19 (±1.9)	21 (±2.3)	25 (±1.7)	20 (±3.3)	18 (±1.6)
Macluraxanthone (**5**)	>100	>100	>100	>100	>100	>100
1,7-Dihydroxyxanthone (**7**)	>50	>50	>50	>50	>50	>50
Vismiaquinone D (**9**)	>50	>50	>50	>50	>50	>50
Cisplatin	19 (±3.2)	4 (±2.8)	8 (±1.4)	15 (±4.3)	7 (±2.7)	13 (±2.3)

Structure-Activity Relationship Study

Interestingly, compared to the other xanthones, compounds **1-3** have an additional oxygenated heterocyclic ring fused to the xanthone nucleus at C-3/C-4, that may play an important role in the cytotoxicity. Instead, an isoprenyl moiety, such as that occurring in compounds **4** and **5**, seems to reduce cytotoxicity.

Conclusion

In conclusion, the results confirmed that *C. cochinchinense* is a rich source of bioactive secondary metabolites and that xanthones are the most abundant and characteristic ones. Of the thirteen compounds isolated from this plant, nine were known aromatic substances while four were common triterpenoids. The structures of all metabolites were elucidated by spectroscopic data analysis and seven compounds (**1-5**, **7**, and **9**) were selected for cytotoxic activity. Their cytotoxic activities were measured against six human cancer cell lines, by using MTT assay. Pruniflorone N (**1**) showed the highest activity, which was even higher than the very well-known chemotherapy agent cisplatin on five tumor cell lines. On the other hand,

pruniflorone M (**2**) and 6-deoxyisojacareubin (**3**) resulted to be more potent cytotoxic than cisplatin on four and two lines, respectively. Cell viability mostly affected by all three compounds **1-3** were the MCF-7 breast and the BG-1 ovarian cancers. Macluraxanthone **(5)**, 1,7-dihydroxyxanthone **(7)** and vismiaquinone D **(9)** showed no effect (IC_{50} > 50 μ M) on all cell lines, whereas xanthone V_1 **(4)** exhibited moderate effects on all cells. Vismiaquinone D (**9**) showed from low to no activity.

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