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Keywords
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Antibacterial tetraoxygenated xanthones from the immature fruits of *Garcinia cowa*

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Abstract: A phytochemical investigation of the acetone extract from the immature fruits of *Garcinia cowa* led to the isolation of two novel tetraoxygenated xanthones, garcicowanones A (**1**) and B (**2**), together with eight known tetraoxygeanted xanthones. Their structures were determined by spectroscopic analysis. All isolated compounds were evaluated for their antibacterial activity against *Bacillus cereus* TISTR 688, *Bacillus subtilis* TISTR 008, *Micrococcus luteus* TISTR 884, *Staphylococcus aureus* TISTR 1466, *Escherichia coli* TISTR 780, *Pseudomonas aeruginosa* TISTR 781, *Salmonella typhimurium* TISTR 292 and *Staphylococcus epidermidis* ATCC 12228. α-Mangostin showed potent activity (MIC 0.25-1 μg/mL) against three Gram-positive strains and garcicowanone A and β-mangostin exhibited strong antibacterial activity against *B. cereus* with the same MIC values of 0.25 μg/mL.

Keywords: *Garcinia cowa*, Clusiaceae, tetraoxygenated xanthone, antibacterial activity

1. Introduction

Garcinia cowa, belonging to the Clusiaceae family, has been an abundant source of secondary metabolites, especially xanthones [1-5], phloroglucinols [5-9], flavonoids [2,10-11], and terpenoids [11-12]. This plant is widely distributed throughout Malaysia, Thailand and Burma. Xanthones are well recognized as chemotaxonomic markers for plants of the *Garcinia* species [1-5,13-17]. Prior to this study, seventy-eight unique compounds have been isolated and identified from *G. cowa*, forty six of there are xanthones [17], many of which have interesting pharmacological activities, including antimicrobial [1,5], anti-inflammatory [10], antimalarial [18], antioxidant [1,3,10], and cytotoxic activities [4,19]. *G. cowa* has been used in Thai folk medicine for its antipyretic properties of the latex and fruit [20], for its improvement in blood circulation, and as an expectorant in the treatment of coughs and indigestion from the fruit and leaves, while the bark, latex and root have been used to treat fever [21]. *G. cowa* is an edible plant and is used in Thai curries. Our previous phytochemical study of its inflorescences led to the isolation of one new benzophenone derivative, cowanone, and seven known xanthones [5]. From our ongoing search for bioactive compounds from Thai medicinal plants, we report here the isolation and structural elucidation of two new xanthones, garcicowanones A (**1**) and B (**2**), together with eight known xanthones (**3-10**) from the immature fruits of *G. cowa*. The antibacterial activity of the all isolated compounds is also reported. Compound **8** was significantly active against three Gram-positive bacteria strains with MIC values 0.25-1 μg/mL and compounds **1** and **4** also had strong antibacterial activities against *B. cereus* with the same MIC value of 0.25 μg/mL. Interestingly, an earlier study on the ripe fruit of *G. cowa* resulted in the isolation of benzoylphloroglucinols [8].

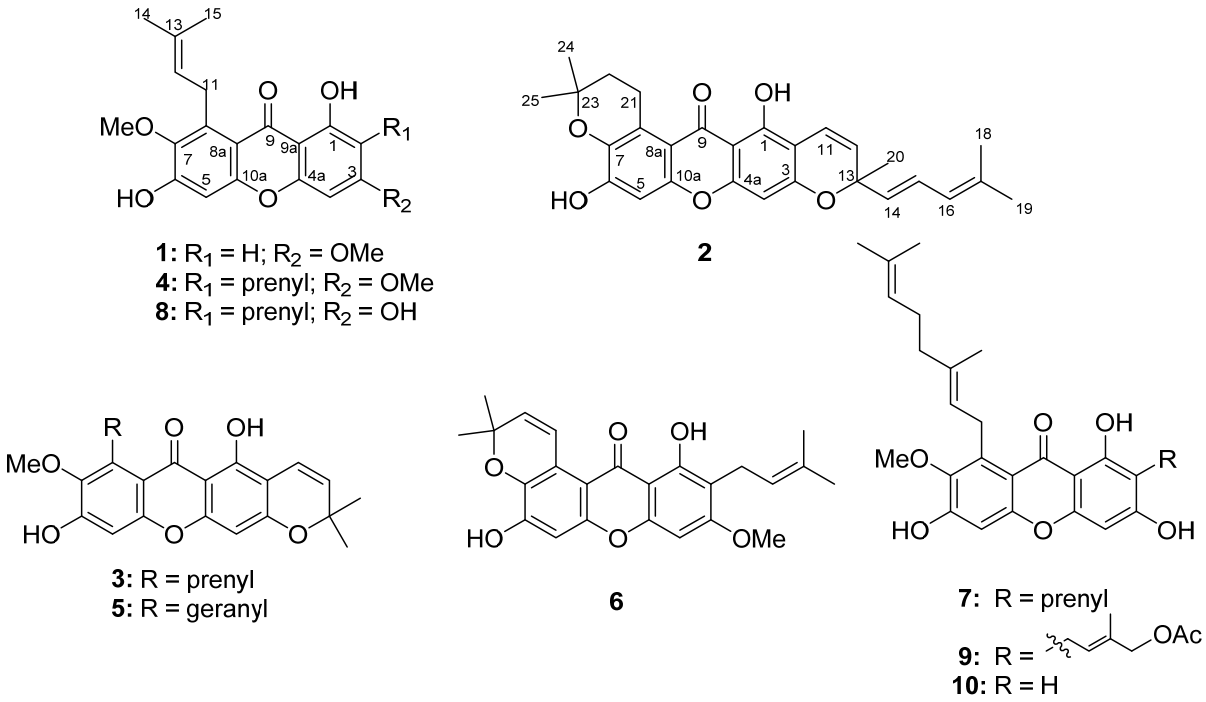

Fig. 1. Tetraoxygenated xanthones isolated from the immature fruits of *G. cowa*.

Table 1 NMR data (400 MHz in $CDCl_3$) for garcicowanones A (**1**) and B (**2**)

	1				**2**			
Position	δ_H (J in Hz)	δ_C		HMBC	δ_H (J in Hz)	δ_C		HMBC
1		163.7				157.9		
2	6.30, d (2.3)	97.0	CH	1, 3, 4, 9a		104.4		
3		166.0				159.9		
4	6.33, d (2.3)	92.0	CH	2, 3, 4a, 9a	6.25, s	94.3	CH	3, 4a, 9a
4a		157.0				156.6		
5	6.85, s	101.8	CH	6, 7, 8a, 10a	6.82, s	100.7	CH	6, 7, 8a, 10a
6		156.0				153.4		
7		142.9				138.1		
8		137.3				122.2		
8a		112.4				111.4		
9		182.1				182.2		
9a		104.1				104.0		
10a		154.9				151.5		
11	4.09, d (5.9)	26.7	CH_2	7, 8, 8a, 12, 13	6.72, d (10.0)	115.9	CH	1, 2, 3, 13
12	5.27, t (5.9)	123.2	CH	14, 15	5.56, d (10.0)	132.4	CH	2, 13, 20
13		132.4				78.0		
14	1.70, s	25.9	CH_3	12, 13, 15	5.60, d (15.4)	127.2	CH	13, 16, 20
15	1.84, s	18.4	CH_3	12, 13, 14	6.30, dd (15.4, 10.4)	125.9	CH	13, 16, 17
16					5.77, d (10.4)	124.2	CH	14, 18, 19
17						136.8		
18					1.74, s	26.1	CH_3	16, 17, 19
19					1.68, s	18.5	CH_3	16, 17, 18
20					1.46, s	26.9	CH_3	12, 13, 14
21					3.61, m and 3.30, m	22.5	CH_2	7, 8, 8a, 22, 23
22					2.06, m and 1.91, m	32.5	CH_2	8, 21, 23, 24, 2
23						77.8		
24					1.50, s	28.5	CH_3	22, 23, 25
25					1.50, s	28.5	CH_3	22, 23, 24
1-OH	13.37, s			1, 2, 9a	13.17, s			1, 2, 9a
6-OH					6.48, s			5, 6, 7
3-OMe	3.87, s	55.9	CH_3	3				
7-OMe	3.81, s	62.2	CH_3	7				

Table 2 Antibacterial activity of the isolated compounds from the immature fruits of *G. cowa*

Compounds	MIC (μg/mL)							
	Gram-positive				Gram-negative			
	B. cereus	*B. subtilis*	*M. luteus*	*S. aureus*	*E. coli*	*Ps. aeruginosa*	*S. typhimuriun*	*S. epidermidis*
1	0.25	2	4	64	64	128	64	4
2	Inactive[b]	128	64	Inactive[b]	Inactive[b]	128	64	64
3	8	2	4	64	Inactive[b]	128	64	2
4	0.25	4	16	64	Inactive[b]	128	64	2
5	128	128	64	Inactive[b]	Inactive[b]	128	64	64
6	64	128	64	Inactive[b]	Inactive[b]	128	64	32
7	32	4	4	Inactive[b]	Inactive[b]	128	64	2
8	0.5	0.25	1	64	64	128	64	0.5
9	4	4	8	Inactive[b]	Inactive[b]	64	64	4
10	2	1	2	Inactive[b]	64	128	64	4
Vancomycin[a]	0.25	0.25	0.25	0.25	–	–	–	–
Gentamycin[a]	–	–	–	–	0.25	2	0.125	0.25

[a] *Standard compound for antibacterial assays* [b] *Inactive at 200 μg/mL*

2. Experimental

2.1. General experimental procedures

Melting points were measured with a SANYO Gallenkamp melting point apparatus. Optical rotations were measured in MeOH at the sodium D-line on a Bellingham & Stanley ADP220 polarimeter. UV-vis absorption spectra were determined in MeOH with a Perkin-Elmer UV-vis spectrophotometer. The infrared (IR) spectra were recorded neat using a Perkin-Elmer FTS FT-IR spectrophotometer. The NMR spectra were recorded using 400 MHz Bruker FTNMR Ultra Shield spectrometer. Chemical shifts are expressed in δ (ppm) with referencing to the tetramethylsilane (TMS) peak. The MicroTOF data was obtained from a Bruker Daltonics mass spectrometer. Thin-layer chromatography (TLC) was performed on silica gel 60 GF_{254} (Merck). Column chromatography (CC) was carried out on Sephadex LH-20 and silica gel (Merck) type 100 (63-200 μm) and type 60 (5-40 μm) for Quick column chromatography (QCC). All solvents for extraction and chromatography were routinely distilled prior to use.

2.2 Plant Material

The immature fruits of *G. cowa* were collected in Nong khai Province of Thailand in August 2011. The plant was identified by Mr. James Maxwell, Herbarium of Chiang Mai University and the specimen (MFU-NPR0014) was deposited at the Natural Products Research Laboratory, School of Science, Mae Fah Luang University, Chiang Rai, Thailand.

2.3 Extraction and isolation

The dried immature fruits of *G. cowa* (3.24 kg) were extracted with acetone over a period of 3 days at room temperature. Removal of the solvent under reduced pressure provided the crude acetone extract (229.30 g) as a dark brown gum. The crude extract was separated by QCC over silica gel and eluted with a gradient of hexanes-EtOAc to provide six fractions (A-F). Fraction B (6.64 g) was separated by QCC with a gradient of hexanes-EtOAc to give five subfracrions (B1-B5). Subfraction B2 (564.8 mg) was further isolated by CC over silica gel with acetone/hexanes (1:9) to provide three subfractions. The second subfraction (105.8 mg) was purified by Sephadex LH-20 CC with 100% MeOH to afford compound **3** (63.2 mg). Subfraction B4 (1.22 g) was separated by QCC with a gradient of hexanes-CH_2Cl_2 to give four subfractions (B4A-B4D). The first subfraction contained compound **4** (298.3 mg). Compound **1** (25.8 mg) was obtained from the subfraction B4C (42.1 mg) after purification by CC over silica gel eluted with CH_2Cl_2/hexanes (2:3). Fraction C (24.72 g) was fractionated by QCC with a gradient of hexanes-acetone to provide five subfractions (C1-C5). Subfraction C2 (4.60 g) was separated by QCC with EtOAc/hexanes (1.5:8.5) to give six subfractions (C2A-C2F). Compound **5** (21.7 mg) was achieved from subfraction C2B (225.9 mg) after purification by Sephadex LH-20 CC with 100% MeOH. Compound **6** (3.1 mg) was obtained from subfraction C2C (557.6 mg) after purification by CC over silica gel with EtOAc/hexanes (1.5:8.5). Subfraction C2E (1.05 g) was subjected to QCC eluted with a

gradient of hexanes-acetone to give three subfractions, the second subfraction (142.2 mg) was isolated by Sephadex LH-20 CC with 100% MeOH to afford compound **7** (12.4 mg). Subfraction C4 (2.26 g) was isolated by QCC eluted with EtOAc/hexanes (1:4) to afford three subfractions (C4A-C4C). Compound **8** (37.7 mg) was obtained from the second subfraction (367.8 mg) after purification by CC over silica gel with acetone/hexanes (1:4). Fraction E (8.29 g) was further purified by QCC with a gradient of hexanes-acetone to provide four subfractions (E1-E4). Compound **2** (8.3 mg) was obtained from the subfraction E1 (25.8 mg) after separation with CC over silica gel with acetone/hexanes (1:9). Subfraction E3 (2.26 g) was further separated by Sephadex LH-20 CC with 100% MeOH to give four subfractions (E3A-E3D). Compounds **9** (13.7 mg) was obtained from subfraction E3A (499.5 mg) after separation by CC over silica gel with EtOAc/hexanes (3:7). Subfraction E3B (554.6 mg) was separated by CC over silica gel with EtOAc/hexanes (3:7) to provide four subfractions (E3B1-E3B4), the second subfraction (152.8 mg) was purified by CC over silica gel with 100% CH_2Cl_2 to give compound **10** (12.0 mg).

*Garcicowanone A (**1**)*: Yellow viscous oil; UV (MeOH) λ_{max}: 247 (4.19), 253, (4.22), 308 (3.98), 348 (3.57) nm; IR (neat) ν_{max}: 3424, 2919, 2851, 1653 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1; HRESI-TOFMS *m/z* 357.1349 $[M+H]^+$ (calcd. for $C_{20}H_{21}O_6$, 357.1338).

*Garcicowanone B (**2**)*: Yellow viscous oil; $[\alpha]_D^{29}$ -0.57° (c 0.034, MeOH); UV (MeOH) λ_{max}(log ε) 246 (4.19), 291 (4.30), 334 (3.78), 385 (3.20) nm; IR (neat) ν_{max}: 3450, 2918, 2850, 1653 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1; HRESI-TOFMS *m/z* 461.1984 $[M+H]^+$ (calcd. for $C_{28}H_{29}O_6$, 461.1964).

2.4 Antibacterial Assay

Bacillus cereus TISTR 688, *B. subtilis* TISTR 008, *Micrococcus luteus* TISTR 884, *Staphylococcus aureus* TISTR 1466, *S. epidermidis* ATCC 12228 *Escherichia coli* TISTR 780, *Pseudomonas aeruginosa* TISTR 781 and *Salmonella typhimurium* TISTR 292 were derived from the Microbiological Resources Center of the Thailand Institute of Scientific and Technological Research. The minimum inhibitory concentrations (MICs) were determined by a two-fold serial dilution method using Mueller Hinton broth according to the Clinical and Laboratory Standards Institute recommendations [22]. The test substances were dissolved in DMSO. The standard drugs, vancomycin and gentamycin, were used as reference substances.

3. Results and Discussion

The dried immature fruits of *G. cowa* were extracted with acetone and the crude plant extract was separated by column chromatography which led to the isolation of two new tetraoxygenated xanthones, garcicowanones A (**1**) and B (**2**), together with eight known tetraoxygenated xanthones; 9-hydroxycalabaxanthone (**3**) [5], *β*-mangostin (**4**) [5], fuscaxanthone A (**5**) [5], cowaxanthone D (**6**) [1], cowanin (**7**) [5], *α*-mangostin (**8**) [5], cowagarcinone E (**9**) [3] and rubraxanthone (**10**) [23]. The structures of these isolated compounds were elucidated using spectroscopic methods especially 1D and 2D NMR spectroscopy. The structures of the known compounds were determined and confirmed by comparison of their ^{1}H and/or ^{13}C NMR spectroscopic data with those previously published data.

Garcicowanone A (**1**) was obtained as a yellow viscous oil. Its molecular formula, $C_{20}H_{21}O_6$, was deduced from its HRESI-TOFMS. The UV-vis spectrum displayed absorption bands at λ_{max} 247, 253, 308 and 348 nm, suggesting the presence of a xanthone chromophore [24]. The IR spectrum showed hydroxy and pyrone carbonyl stretching bands at 3424 and 1653 cm^{-1}, respectively. The ^{1}H NMR spectrum (Table 1) showed resonances similar to those

of *β*-mangostin (**4**) [5]; these indicated a chelated hydroxy group, *δ* 13.37 (1H, *s*), a singlet aromatic proton (*δ* 6.85, 1H, *s*), two *meta*-coupled aromatic protons [*δ* 6.33 and 6.30 (each 1H, *d*, *J* = 2.3 Hz)], two methoxy groups [*δ* 3.87 and 3.81 (each 3H, *s*)] and a prenyl unit [*δ* 5.27 (1H, *t*, *J* = 5.9 Hz), 4.09 (2H, *d*, *J* = 5.9 Hz), 1.84 and 1.70 (each 3H, *s*)]. The chelated hydroxy group was attached at C-1 (*δ* 163.7) from the HMBC correlations between 1-OH (*δ* 13.37) and the ^{13}C NMR resonances for C-1, C-2 (*δ* 97.0) and C-9a (*δ* 104.1). The *meta*-coupled aromatic proton resonating at *δ* 6.30 was assigned to H-2 on the basis of its HMBC correlations to the ^{13}C NMR resonances for C-1, C-3 (*δ* 166.0), C-4 (*δ* 94.2) and C-9a. Thus the remaining *meta*-coupled aromatic proton at *δ* 6.33 was attributed to H-4. The methoxy group, resonating at *δ* 3.87, was attached to C-3 according to its HMBC correlation (Table 1). The highly deshielded position of the methylene proton resonance of the prenyl unit (*δ* 4.09, H-11) suggested that it was located at C-8 (*δ* 137.3), in a *peri* position to the carbonyl group. This assignment was confirmed by the HMBC correlation of H-11 of the prenyl unit with C-7 (*δ* 142.9), C-8 (*δ* 137.3) and C-8a (*δ* 112.4). The remaining methoxy group (*δ* 3.81) was located at C-7 based on the HMBC cross peak with C-7. The singlet aromatic proton resonating at *δ* 6.85 was assigned to C-5 (*δ* 101.8), on the basis of the HMQC data and the HMBC correlations with C-6 (*δ* 156.0), C-7, C-8a, and C-10a (*δ* 154.9). The substituent at C-6 was identified as a hydroxy group according to its ^{13}C NMR chemical shift (*δ* 156.0). Thus, garcicowanone A was assigned as 1,6-dihydroxy-3,7-dimethoxy-8-(3-methyl-2-butenyl)xanthone (**1**).

Garcicowanone B (**2**) was obtained as a yellow viscous oil. Its molecular formula, $C_{28}H_{29}O_6$, was deduced from its HRESI-TOFMS. The UV and IR data of **2** were similar to that of **1**. The ^{1}H NMR spectrum (Table 1) showed characteristics for a chelated hydroxy group, *δ* 13.17 (1H, *s*), a chroman ring [*δ* 3.61 and 3.30 (each 1H, *m*), 2.06 and 1.91 (each 1H, *m*) and 1.50 (6H, *s*)], a 2-methylpyran ring (*δ* 6.72 and 5.56, each 1H, *d*, *J* = 10.0 Hz and

1.46, 3H, *s*), a (1*E,* 3*E*)-4-methylpenta-1,3-diene unit [δ 6.30 (1H, *dd*, *J* = 15.4 and 10.4 Hz), 5.77 (1H, *d*, *J* = 10.4 Hz), 5.60 (1H, *d*, *J* = 15.4 Hz) and 1.74 and 1.68 (each 3H, *s*)], two singlet aromatic protons (δ 6.82 and 6.25, each 1H, *s*) and a hydroxy group at δ 6.48 (OH, *s*). Compound **2** showed fourteen quaternary (δ 182.2, 159.9, 157.9, 156.6, 153.4, 151.5, 138.1, 136.8, 122.2, 111.4, 104.4, 104.0, 78.0 and 77.8), seven methine (δ 132.4, 127.2, 125.9, 124.2, 115.9, 100.7 and 94.3), two methylene (δ 32.5 and 22.5) and five methyl (δ 28.5 (2C), 26.9, 26.1 and 18.5) carbons in the ^{13}C NMR and DEPT 135 spectra (Table 1). A chelated hydroxy group with a resonance at δ 13.17 was located at C-1 (δ 157.9) from the HMBC correlations between 1-OH and C-1, C-2 (δ 104.4) and C-9a (δ 104.0). The 2-methylpyran ring was located at C-2 and an ether linkage at C-3 (δ 159.9) on the basis that the lower-field olefinic proton resonance (δ 6.72) of the 2-methylpyran ring showed HMBC correlations with C-1, C-2 and C-3, together with the relative deshielded chemical shift of C-3. Moreover, the singlet aromatic proton resonance at δ 6.25 was assigned to that of H-4 (C-4, δ 94.3) according to its HMBC correlations with C-3, C-4a (δ 26.9) and C-9a. The presence of the (1*E,* 3*E*)-4-methylpenta-1,3-diene unit was confirmed by the ^{1}H-^{1}H COSY correlations between H-15 with H-14 and H-16, and the HMBC correlations of the methyl group resonances, H_3-18 and H_3-19, with C-16 (δ 124.2) and C-17 (δ 136.8). The attachment of this unit at C-13 (δ 78.0) was supported by the HMBC cross peaks between the resonances for H-14 and those for C-13 and C-20. The deshielded methylene protons of the chroman ring, resonating at δ 3.30 and 3.61, were attributed to H_2-21 due to the anisotropic effect of the carbonyl group. The HMBC correlations of H_2-21 with C-7 (δ 138.1), C-8 (δ 122.2) and C-8a (δ 111.4), together with the chemical shift of C-7, indicated that C-21 of the chroman ring was attached at C-8 and the ether linkage at C-7. The hydroxy group proton resonance at δ 6.48 indicated that this group was attached at C-6 (δ 153.4) on the basis of its HMBC correlations with C-5 (δ 100.7), C-6 and C-7. The singlet aromatic proton resonance at δ 6.82

was assigned to that of H-5 on the basis of its HMQC spectrum and its HMBC correlations with C-6, C-7, C-8a and C-10a (δ 151.5). Therefore, garcicowanone B was determined to be as 1,6-dihydroxy-(4-methyl-penta-1,3-dienyl)-6'-methylpyrano[2',3':3,2]-6",6"-dimethyldihydropyrano(2",3":7,8)xanthone (**2**). The absolute configuration of **2** was not determined.

All of the isolated compounds were examined for their antibacterial activity against Gram-positive bacteria; *Bacillus cereus* TISTR 688, *B. subtilis* TISTR 008, *Micrococcus luteus* TISTR 884 and *Staphylococcus aureus* TISTR 1466 and Gram-negative bacteria; *Escherichia coli* TISTR 780, *Pseudomonas aeruginosa* TISTR 781, *Salmonella typhimurium* TISTR 292 and *Staphylococcus epidermidis* ATCC 12228 (Table 2). In general these compounds showed better antibacterial activities again the Gram-positive strains than the Gram-negative strains (Table 2). All compounds were either weakly active (MIC 64 μg/mL) or inactive (MIC ≥ 128 μg/mL against *S. aureus* and *E. coli*. The three tricyclic xanthones, **1**, **4** and **8**, were generally the most active compounds and showed significantly high activities against the Gram-positive strains, *B. cereus* (MICs 0.25-0.5 μg/mL), *B. subtilis* (MICs 0.25-4 μg/mL), and *M. luteus* (MICs 1-16 μg/mL). In some cases these activities were the same as the positive control, vancomycin (MIC 0.25 μg/mL against all of these three strains). Further, these three compounds also showed significant activities against one Gram-negative strain, *S. epidermidis* (MICs 0.5-4 μg/mL), with compound **8** being the most active (MIC 0.5 μg/mL) and having similar activity to the positive control, gentamycin (MIC 0.25 μg/mL).The tricyclic compounds **7**, **9** and **10**, having a C-8 geranyl substituent, rather than the C-8 prenyl substituent found in the most active compounds **1**, **4** and **8**, showed slightly reduced activities against the aforementioned Gram-positive strains. With **9** and **10** having MICs in the range of 1-8 μg/mL while **7**, had MICs in the range 4-32 μg/mL. These three compounds were also active against *S. epidermidis* (MICs 2-4 μg/mL). The tetracyclic C-8 prenylated compound **3** showed slightly better antibacterial activities than compound **7** while its C-8 geranyl analogue **5** was weakly active or inactive on all bacterial strains (MICs ≥ 64 μg/mL). The pentacyclic and tetracyclic compounds **2** and **6** were also weakly active or inactive on all bacterial strains (MICs ≥

64 μg/mL). Clearly the compounds **1**, **4**, **7**−**10**, having a common tricyclic xanthone scaffold, showed the best antibacterial activities. Compound **8**, having a C-2 prenyl substituent and a C-3 hydroxy group, was the most active. Of the group of compounds **7**, **9** and **10**, compound **10**, also with a C-3 hydroxy group, was the most active. The tetracyclic compound **3** shows good activity, however it C-8 geranyl analogue is not active, most likely due to the longer side chain at C-8. Compounds **2** and **6** having a similar pyran-fused structure at C-7 and C-8 are both inactive, perhaps for unfavorable steric reasons.

Thus we have identified the structures of two new xanthones and identified eight compounds from the unripe fruits of *G. cowa*. These compounds have potent to good antibacterial activities against the Gram-positive strains, *B. cereus*, *B. subtilis* and *M. luteus* and the Gram-negative strain *S. epidermidis*. Further, these compounds are different to those isolated in an earlier study from the ripe fruits of *G. cowa* which were benzoylphloroglucinols [8].

Conflict of interest

The authors declare no conflicts of interest.

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