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Phytochemistry and bioactive compounds from *Garcinia cowa* roxb.

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Abstract

Phytochemical investigation from the roots of *Garcinia cowa* Roxb. resulted in the isolation of forty-four compounds; Thirty-five xanthones, two anthraquinones, two flavonoids and five terpenes. Their structures were elucidated on the basis of spectroscopic method including UV, IR, 1D NMR, and 2D NMR. Thirty compounds were reported for the first time as metabolites of *G. cowa*. One active isolated compound, *β*-mangostin was found to show a strong antibacterial activity against *Bacillus cereus* TISTR 687 and methicillin resistant *Staphylococcus aureus* (MRSA)-SK1 with MIC value of 4 μg/mL. Whereas *α*-mangostin showed stronger activity against *B. cereus* TISTR 687 and MRSA-SK1 (MIC 0.5 μg/mL) than that of vancomycin (MIC 1 μg/mL). Two compounds, cowanin and cowanol exhibited strong antibacterial activities against *B. cereus* TISTR 687, MRSA-SK1, and *S. aureus* TISTR 1466 with MICs range of 2-4 μg/mL. Isocudraniaxanthone B, xanthone V_1, and kaempferol were expressed good antioxidative activity with IC_{50} values of 19.75 ± 0.39, 19.70 ± 0.39, and 11.67 ± 0.12 μM, respectively. In addition, the acetone extract showed cytotoxicity against Vero cells (African green monkey kidney) and anticancer activity against standard cell culture BC-Breast cancer with IC_{50} values of 36.13 and 40.89 μg/mL, respectively.

Keywords: *Garcinia cowa*, xanthones, antibacterial, antioxidation, cytotoxicity

1. Introduction

Garcinia cowa Roxb. known as Chamuang in Thailand belongs to the Guttiferae family. It grows widely in tropical rainforest area of Southeast Asia, West and East Afirica, and central and South America [1]. The plants in *Garcinia* genus are well known to be rich in a variety of oxygenated and prenylated xanthones [2] which were associated to the therapeutic potential. The various biological and phamacological activities of xanthones have been described such as antimicrobial, antioxidant, antitumor-promoting, cytotoxic, etc. [3-5]. Due to these properties, *Garcinia* genus has attracted attention as important sources for medicinal treatment. Many xanthones were recognized as a major compounds. Xanthones from the latex of *G. cowa* showed antimicrobial and free radical scavenging activities, xanthones from the stem bark showed antimalarial action, the fruit rind extracts have been reported antiaflatoxigenic and antioxidant activities [6]. *G. cowa* were reported the antioxidation activity of pure compounds [1, 6]. In addition, many xanthones used in folk medicine [3]. Moreover, natural products of plants origin are important sources of new chemical compounds leading to the future discovery of new drugs and more effective treatments. No phytochemical investigation on the root has been reported. Therefore, the present work deals with the chemical investigation of the roots of *G. cowa* and evaluated for cytotoxicity activity against Vero cells (African green monkey kidney), anticancer activity against KB-oral carvity cancer, BC-breast cancer, and NCI-H187 small cell cancer, and antimalarial activity *in vitro* against *Plasmodium falciparum* (KI strain) of acetone extract. In addition, the other activities such as antibacterial and antioxidation activities were evaluated for crude extracts and isolated compounds.

2. Materials and Methods

2.1 General

Melting points were determined by BÜCHI model B-540 melting point apparatus (BÜCHI, Switzerland), recorded in degree celsius (°C). Ultraviolet spectra (UV) were recorded using UV-Vis spectrometer (Perkin Elmer Lambda, United States of America). Principle bands (λ_{max}) were recorded as wavelengths (nm) and log ε in methanol solution. Infrared spectra (IR) were recorded on Perkin-Elmer FTS FTIR/Spectrum spectrometer (Perkin-Elmer, United States of America). Major bands (ν_{max}) were recorded in wavenumber (cm^{-1}). 1D and 2D NMR spectra were performed on a Brüker FTNMR Ultra Shield 300 MHz (Prince of Songkla University, Songkla), Brüker FTNMR Ultra Shield 400 MHz (Naresuan University, Phitsanulok). Spectra were recorded in acetone-d_6 solution. Column chromatography (CC) and quick column chromatography (QCC) were performed on silica gel 100 (0.063-0.200 mm, Merck, Germany) and silica gel 60 (0.063-0.230 mm, GF_{254}, Merck, Germany).

2.2 Plant materials

The roots of *G. cowa* were collected from Trang province, in the Southern part of Thailand, in November 2005. A specimen has been deposited in the herbarium of the Department of Biology, Faculty of Science, Prince of Songkla University, Thailand.

2.3 Extraction and isolation

The chopped dried roots of *G. cowa* (7.0 kg) were extracted by immersed at room temperature with acetone (8 liters) over a period of 7 days. The filtered solution was evaporated under reduce pressure to give the viscous acetone extract (RA, 111.45 g).

Crude acetone (RA, 111.45 g) was divided into three portions (A, B, and C). Portion A (25.730 g) was subjected to CC over silica gel eluted with hexane/Me_2CO (100:0 to Me_2CO 100%) to give sixteen fractions (A1-A16). Fraction A1 (0.102 g) was purified by CC over silica gel using hexane/CH_2Cl_2 (60:40 to CH_2Cl_2 100%) to give **1** (19.2 mg) and **2** (13.2 mg). Fraction A2 (1.220 g) was recrystallized from hexane/CH_2Cl_2 (80:20) to give **3** (25.4 mg) and **4** (59.9 mg). Fraction A3 (1.100 g) was purified by CC using hexane/CH_2Cl_2 (50:50 to CH_2Cl_2 100%) to give **5** (3.0 mg), **6** (20.0 mg), and **7** (7.3 mg). Fractions A4 and A5 were combined (0.180 g) and subjected to CC using hexane/CH_2Cl_2 (50:50 to CH_2Cl_2 90%) to give **8** (92.2 mg). Fraction A6 (5.230 g) was purified by CC using hexane/CH_2Cl_2 (50:50 to CH_2Cl_2 100%) to give **9** (230.6 mg), **10** (23.0 mg), **11** (4.5 mg), **12** (12.0 mg), and **13** (39.9 mg). Fraction A7 (0.840 g) was crystallized from hexane/CH_2Cl_2 (50:50 to CH_2Cl_2 90%) to give **10** (182.5 mg), **15** (10.9 mg), and **14** (73.1 mg). Fractions A8 (0.550 g) and A9 (6.810 g) were subjected by CC using hexane/CH_2Cl_2 (10:90) to give **16** (35.3 mg), and **17** (10.0 mg), **18** (22.6 mg), respectively. Fraction A11 (1.050 g) was purified by CC using CH_2Cl_2/Me_2CO (99:1) to give **19** (20.2 mg) and **20** (15.0 mg). Fraction A13 (0.895 g) was purified by CC using CH_2Cl_2/Me_2CO (70:30 to Me_2CO 60%) to give **21** (4.4 mg) and **22** (3.0 mg). Fraction A14 (103 mg) was purified by CC using CH_2Cl_2/EtOAc (90:10 to EtOAc 30%) to give **23** (4.0 mg), **24** (4.5 mg), **25** (5.2 mg), **26** (4.1 mg), and **27** (7.1 mg). Fraction A15 (0.581 g) was subjected to CC using CH_2Cl_2 100% to CH_2Cl_2/EtOAc (80:20) to give **28** (5.2 mg).

Portion B (35.730 g) was subjected to QCC over silica gel using hexane/Me_2CO (100:0 to Me_2CO 100%) in a polarity gradient manner to give twenty fractions (B1-B20). Fraction B2 (0.441 g) was fractionated by CC using hexane/CH_2Cl_2 (100:0 to CH_2Cl_2 50%) to give **29** (185.1 mg) and **30** (12.5 mg). Fraction B5 (0.215 mg) was purified by CC using hexane/CH_2Cl_2 (60:40 to CH_2Cl_2 60%) to give **31** (98.6 mg). Fraction B7 (3.210 g) was subjected to CC and gradient eluted with hexane/CH_2Cl_2 (50:50 to CH_2Cl_2 70%) to give **19** (32.0 mg), **32** (2.3 mg), **33** (12.3 mg), **34** (29.7 mg), and **35** (41.1 mg). Fraction B9 (4.230 g) was subjected to CC eluted with hexane/CH_2Cl_2 (10:90 to CH_2Cl_2 100%) to give **36** (111.0 mg). Fractions B13 and B14 were combined (1.200 g) and crystallized from the mixture of hexane/Me_2CO (75:25 to Me_2CO 50%) to yielded **37** (96.0 mg) and **38** (2.6 mg).

Portion C (36.250 g) was subjected to CC over silica gel using hexane/Me_2CO (100:0 to Me_2CO 100%) to give twenty-one fractions (C1-C21). Fraction C5 (1.047 g) was purified by CC and using hexane/CH_2Cl_2 (60:40 to CH_2Cl_2 50%) to give **39** (13.2 mg) and **40** (23.9 mg). Fraction C7 (1.355 g) was purified by CC eluted with hexane/CH_2Cl_2 (50:50 to CH_2Cl_2 100%) to give **41** (7.2 mg). Fraction C8 (3.810 g) was purified by CC using hexane/CH_2Cl_2 (10:90) to give **42** (2.0 mg), **43** (3.4 mg), and **44** (12.3 mg). All isolated compound structures were elucidated on the basis of UV, IR, and NMR data as well as by comparison of their NMR spectral data with reported values [3, 6-22].

2.4 Antibacterial assay

Antibacterial activity using broth microdilution method was used for screening and determining of minimum inhibition concentration (MICs) of crude extracts and isolated compounds. Five microorganism cultures (*Bacillus cereus* TISTR 687, *Escherichia coli* TISTR 780, *Pseudomonas aeruginosa* TISTR 781, *Salmonella typhimurium* TISTR 292, and *Staphylococcus aureus* TISTR 1466) were derived from the Microbiological Resources Centre of the Thailand Institute of Scientific and Technological Research whereas Methicillin resistant *Staphylococcus aureus* (MRSA)-SK1 was donated by the Department of Microbiology, Faculty of Science, Prince of Songkla University. The minimum inhibitory concentrations (MICs) were determined by a two-fold serial dilution method [23]. The test samples were dissolved in DMSO. Serial 2-fold dilutions of the test samples were mixed with melted Mueller Hinton broth (MHB) in microtiter plates. The final concentrations of the test crude sample and pure compounds in broth ranged from 2.5-1,280 µg/mL and 0.25-128 µg/mL, respectively. 50 µL of inoculum suspension was added to each well (final concentration of $1x10^4$ CFU/well). The inoculated plates were incubated at 35-37 ºC for 16-18 h. 10 µL of 0.18% resazurin was added to the microtiter plate and incubated at 35-37 ºC for 2-3 h. A blue color indicated that the sample was capable of inhibiting bacterial growth, while a pink color indicated that the sample was incapable of inhibiting bacterial growth. MICs were recorded by reading the lowest concentration capable of inhibiting visible growth. The tests were performed at least in triplicate. Vancomycin and gentamicin were used as positive control drugs.

2.5 Antioxidation assay

Antioxidation activities of the crude extracts and pure compounds isolated from *G. cowa* were assessed on the basis of scavenging activity of the stable DPPH free radical by flow injection analysis (FIA) method. This method was simple, good reproducibility, consumed less reagent, produced less waste, and rapid analysis of colorimetric method [24]. The following assay procedure was modified from those described in the previous report [24]. The DPPH 0.075 mM was flowed through the reaction coil (200 cm) with a flow rate of 1.0 mL/min. The absorbance (Abs) was measured at 520 nm. The sample (50 µL) was dispersed and reacted with DPPH streams, and the solution was flowed through the detector and the signal generated to FIA gram as shown in Figure 1. The measurements were performed at least in triplicate. The results expressed as percentage inhibition. The concentration needed to decrease % inhibition of DPPH solution to 50 (IC_{50}) was obtained by dose-response curve.

2.6 Cytotoxicity assay

Cytotoxicity assay of Vero cells (African green monkey kidney) was performed by the sulforhodamine B (SRB) assay. Ellipticine was used a positive control [25].

2.7 Anticancer activity assay

Two cell lines, including KB (oral cavity cancer, ATCCCCL-17) and BC (breast cancer) were determined by rezazurin microplate assay (REMA) which was a modified method of fluorescent dye for the mammalian cell cytotoxicity [26]. In brief, cells at a logarithmic growth phase are harvested and diluted to 2.2x104 cells/mL for KB, in fresh medium. The next step was to add the 5 µL of test sample diluted in 5% DMSO in to cell suspension 45 µL in the 384-well plates. Then incubated at 37ºC in 5% CO_2 incubator. After the incubation period (3 days for KB), 12.5 µL of 62.5 µg/mL resazurin solution is added to each well, and the plates are then incubated at 37ºC for 4 h. Fluorescence signal is measured using SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 and 590 nm. Dose response curves are plotted from 6 concentrations of 3-fold serially diluted test compounds and the sample concentrations that inhibit cell growth by 50% (IC_{50}) can be derived using the SOFTMax Pro software (Molecular Devices, USA). NCI-H187 (small cell lung cancer, ATCC CRT-5804) was a modified method of colorimetric, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromine (MTT) assay [27]. The MTT assay is a tetrazolium-dye based colorimetric microtitration assay. Metabolism-competent cells are able to metabolize the tetrazolium (yellow) to formazan (blue); this color change is measured spectrophotometrically with a plate reader. It isolated assumed cells that are metabolically deficient will not survive, thus the MTT assay is also an indirect measurement of cell viability. The cells were seeded in a 96-well plate at various densities of cell per well, and incubated for 48 h. The sample at various concentrations were added to the cells and incubated for 24 h. The test samples were removed from the cell cultures and the cells were reincubated for a further 24 h. in fresh medium and then tested with MTT assay. Briefly, 50 µL of MTT in PBS at 5 mg/mL was added to the medium in each well and the cells were incubated for 4 h. Medium and MTT were then aspirated from the wells, and formazan crystal was solubilised with 200 µL of DMSO and 25 µL of Sorensen's Glycine buffer, pH 10.5. The optical density was read with a microplate reader

(Molecular Devices) at a wavelength of 570 nm. The average of 4 wells was used to determine the mean of each point. A dose-response curve was derived from 4-8 concentrations in the test range using 4 wells per concentration. Results of toxic compounds are expressed as the concentration of sample required to kill 50% (IC_{50}) of the cells compared to controls. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the IC_{50} for each toxinsample. Ellipticine and doxorubicin were used as positive controls, and 0.5%DMSO and water are used as a negative control.

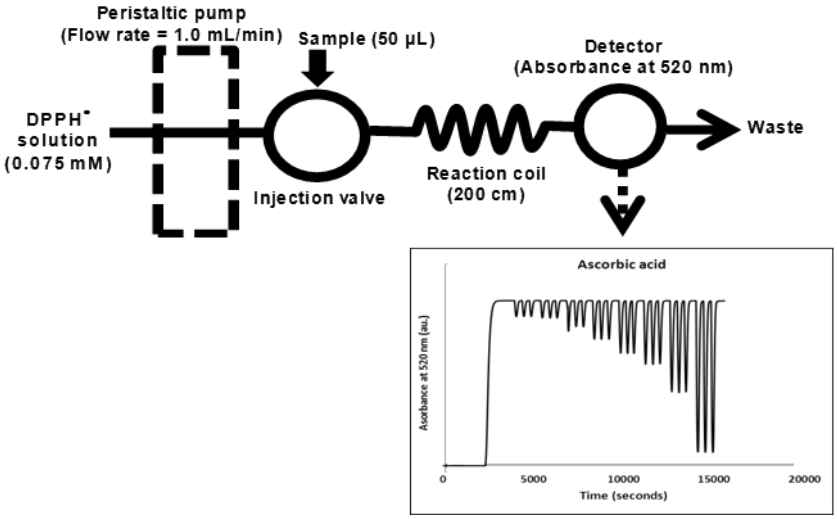

Figure 1. Diagram of flow injection-spectrophotometric system

3. Results and Discussion

Phytochemical investigation from the roots of *Garcinia cowa* resulted in the isolation of forty-four compounds (Figure 2), macluraxanthone, **1** [8], formoxanthone C, **2** [17], cochinchinone C, **3** [14], calophymembranol B, **4** [16], dulxanthone B, **5** [10], *β*-mangostin, **6** [14], cochinchinone G, **7** [15], 10-*O*-methylmacluraxanthone, **8** [17], cochinchinone A, **9** [14], cowaxanthone, **10** [6], euxanthone, **11** [8], 6-hydroxy-1,2,3,7-tetramethoxyxanthone, **12** [9], stigmasterol, **13** [12], *α*-mangostin, **14** [18], isocudraniaxanthone B, **15** [13], xanthone V_1, **16** [17], cowanin, **17** [3], cowanol, **18** [3], pyranojacareubin, **19** [6], 9,10-dihydroxy-5-methoxy-12-(1,1dimethyl-2-propenyl)-2*H*,6*H*-pyrano-[3,2-*b*]xanthen-6-one, **20** [8], 1,5,7-trihydroxy-3-methoxyxanthone, **21** [16], norathyriol, **22** [18], 7-geranyloxy-1,3-dihydroxyxanthone, **23** [13], gartanin, **24** [21], morusignin I, **25** [17], parvixanthone B, **26** [17], 1,3,7-trihydroxy-2-(3-methylbut-2-enyl)xanthone, **27** [14], 5-*O*-methylxanthone V_1, **28** [6], friedelin, **29** [20], lupenone, **30** [13], lupane, **31** [13], damnacanthal, **32** [6], 2,3-dihydroxy-1-methoxylanthraquinone, **33** [16], cochinchinone E, **34** [15], 1,3,6-trihydroxy-7- methoxy-2,5-*bis*(3-methyl-2-butenyl)xanthone, **35** [3], *β*-sitosterol, **36** [7], kaempferol, **37** [19], assiguxanthone B, **38** [10], 1,6-dihydroxy-5-methoxyxanthone, **39** [16], 3,4-dihydro-6,11-dihydroxy-2,2-dimethyl-pyrano-[3,2-*c*]-xanthene-7(2*H*)-one, **40** [16], morelloflavone, **41** [11], cowagarcinone B, **42** [6], 6-hydroxy-2,3-dimethoxyxanthone, **43** [22], and assiguxanthone A, **44** [10]. Thirty of the compounds (**1**-**5**, **7**-**9**, **12**, **15**, **16**, **19**-**21**, **23**-**28**, **30**-**34**, **38**-**40**, **43**, and **44**) were reported for the first time as metabolites of *G. cowa*.

The acetone extract (RA) and some of the isolated compounds of *G. cowa* were evaluated for antibacterial activity against Gram-positive (*B. cereus*, MRSA-SK1, *S. aureus*) and Gram-negative (*E. coli*, *Ps. aeruginosa*, *S. typhimurium*) by Broth Microdilution Method [23] and antioxidation activity using FIA method. Crude RA exhibited weak antibacterial activity against Gram-positive in the range of MICs 320-640 µg/mL. In table 1, Compound **14** was found to show a strong antibacterial activities against *B. cereus* and MRSA-SK1 with MIC value of 0.5 µg/mL. According to the previously reported antibacterial activity of *α*-mangostin against *B. cereus* TISTR 688 and MRSA-SK1 with MIC values of 0.5 and 2 µg/mL, respectively [28, 29]. Three compounds **6**, **17,** and **18** exhibited strong antibacterial activities against *B. cereus*, MRSA-SK1, and *S. aureus* with MICs range of 2-4 µg/mL. Compounds **7**, **15**, **20**, and **23** showed strong antibacterial activity against *E. coli* with MIC value of 8 µg/mL as shown in Table 1. It was noted that the activity of them based on the functional groups and their positions in the xanthone skeleton [30]. The methylated product compound **6** showed a strong antibacterial activity against *B. cereus* and MRSA-SK1 with MIC values of 4 and 4 µg/mL, respectively. Therefore, comparing of compound **14** results, one can assume that the absence of 3-OH group in **6** isolated the causative factor for the loss activity of **6**. Furthermore, compound **34** and **35** have shown no activity against *B. cereus* and MRSA-SK1. Therefore, it is clear that the presence of the C-3 and C-6 hydroxy groups and the prenyl side chain at C-2 and C-8 are essential for the antibacterial activity against *B. cereus* and MRSA-SK1. The acetone extract (RA) exhibited moderate IC_{50}

values of 35.86 ± 0.14 μg/mL. However, compounds **1** and **2** exhibited moderate antioxidative activity with IC_{50} values of 35.86 ± 0.15 and 35.55 ± 0.43, respectively. Compounds **15**, **16**, and **37** exhibited good antioxidative activity with IC_{50} values of 19.75 ± 0.39, 19.70 ± 0.39, and 11.67 ± 0.12 μM, respectively whereas ascorbic acid showed IC_{50} value of 2.10 ± 0.01 μM (Table 1). Kampferol has been reported antioxidant activity with IC_{50} value of 22.81 ± 1.47 μM [31]. From the results, **37** which contains four free hydroxyl groups at positions C-3, C-5, C-7, and C-4′ showed the reactive high inhibition with IC_{50} of 11.67 ± 0.12 μM. Crude RA extract of *G. cowa* showed cytotoxicity against Vero cell (African green monkey kidney) with IC_{50} value of 36.13 μg/mL (Ellipticine, IC_{50} 0.555 μg/mL). In addition, crude RA exhibited anticancer activity against BC-Breast cancer cell line with IC_{50} value of 40.89 μg/mL (Ellipticine IC_{50} 0.829 μg/mL and doxorubicine IC_{50} 0.362 μg/mL). Crude RA show no cytotoxicity against KB-oral carvity cancer and NCI-H187 small cell cancer.

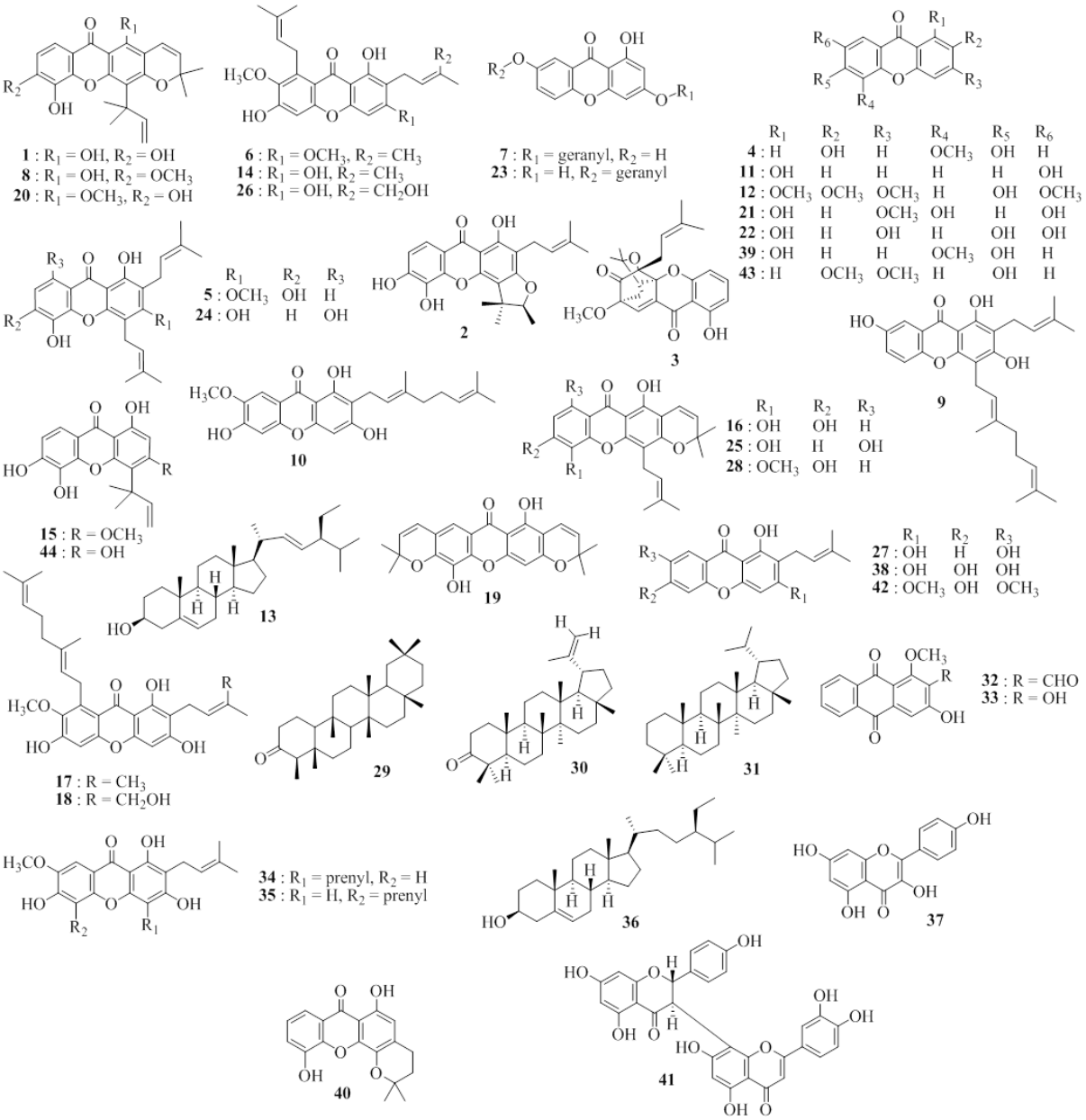

Figure 2. Structure of isolated compounds **1-44**

Table 1. Antibacterial activity of isolated compounds from *G. cowa*

Sample	MICs (μg/mL)					
	Gram-positive			Gram-negative		
	B.C	MRSA	S.A	E.C	Ps.A	S.T
RA	320	640	640	640	1280	1280
1	128	128	128	>128	>128	>128
2	128	128	64	128	64	128
3	16	16	128	128	128	128
4	128	>128	>128	128	128	128
6	4	4	4	128	128	128
7	128	128	128	8	128	128
8	128	128	128	128	>128	128
9	4	4	4	16	128	128
10	64	64	128	128	128	128
11	128	128	128	128	128	128
12	128	128	128	>128	>128	>128
14	0.5	0.5	2	128	128	128
15	128	128	16	8	128	128
16	16	16	128	128	128	128
17	2	2	2	128	128	128
18	4	4	2	128	128	128
19	128	128	128	128	128	128
20	128	128	128	8	128	128
21	128	>128	>128	128	128	128
23	128	128	128	8	128	128
26	128	128	128	>128	>128	>128
27	128	128	128	>128	>128	>128
32	128	128	128	>128	>128	>128
33	>128	>128	>128	>128	>128	128
34	128	128	128	128	128	128
35	128	128	128	128	128	128
37	128	128	128	128	128	128
38	16	128	32	128	128	128
39	128	128	128	>128	>128	>128
40	128	128	128	>128	>128	>128
42	128	128	128	>128	>128	>128
43	>128	>128	>128	128	128	128
44	>128	>128	>128	128	128	128
Vancomycin	1	1	0.5	-	-	-
Gentamicin	-	-	-	0.25	0.125	0.5

B.C = *Bacillus cereus*,
MRSA = Methicillin resistant *Staphylococcus aureus*,
S.A = *Staphylococcus aureus*,
E.C = *Escherichia coli*,
Ps.A = *Pseudomonas aeruginosa*,
S.T = *Salmonella typhimurium*

4. Conclusion

The phytochemical study from the roots of *G. cowa* had led to the isolation of forty-four compounds. *α*-mangostin (**14**) was found to show a strong antibacterial activities against *B. cereus* and MRSA-SK1 with MIC value of 0.5 μg/mL. Three compounds, *β*-mangostin (**6**), cowanin (**17**), and cowanol (**18**) exhibited strong antibacterial activities against *B. cereus*, MRSA-SK1, and *S. aureus* with MICs range of 2-4 μg/mL. Whereas, cochinchinone G (**7**), isocudraniaxanthone B (**15**), 9,10-dihydroxy-5-methoxy-12-(1,1dimethyl-2-propenyl)-2*H*,6*H*-pyrano-[3,2-*b*]xanthen-6-one (**20**), and 7-geranyloxy-1,3-dihydroxyxanthone (**23**) showed strong antibacterial activity against *E. coli* with MIC value of 8 μg/mL. Five compounds, macluraxanthone (**1**), formoxanthone C (**2**), isocudraniaxanthone B (**15**), xanthone V_1 (**16**), and kaempferol (**37**) exhibited antioxidative activity with IC_{50} values of 35.86 ± 0.15, 35.55 ± 0.43, 19.75 ± 0.39, 19.70 ± 0.39, and 11.67 ± 0.12 μM, respectively whereas ascorbic acid showed IC_{50} value of 2.10 ± 0.01 μM. In addition, the acetone extract (RA) showed cytotoxicity against Vero cells (African green monkey kidney) and anticancer activity against BC-Breast cancer with IC_{50} value of 36.13

and 40.89 μg/mL, respectively. Crude RA show no cytotoxicity against KB-oral carvity cancer and NCI-H187 small cell cancer. The combination of free hydroxy groups at C-3 and C-6 and prenyl side chain at C-2 and C-8 plays a prominent role in the antibacterial activity for *B.* cereus and MRSA of α-mangostin as well as the free hydroxyl group can be considered the most important structure feature for inhibitory activity against free radical.

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