Isolation of Chamuangone, a Cytotoxic Compound against *Leishmania major* and Cancer Cells from *Garcinia cowa* Leaves and its HPLC Quantitative Determination Method

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Abstract: On the basis of a leishmanicidal assay-guided isolation, chamuangone was purified from *Garcinia cowa* leaves together with four inactive compounds; 5-hydroxymethylfurfural; D-glyceropentaric acid,2-deoxy-3-*C*-(methoxycarbonyl)-1,4-lactone,5-ethyl ester; isoorientin-6"-*O*-rhamnoside; and dulcinoside. Chamuangone possessed a cytotoxic activity against *Leishmania major* with an IC_{50} value of 10.7 µM, and also exhibited strong inhibitory activity against lung adenocarcinoma (SBC3 and A549) and leukemia (K562, and K562/ADM) cells with IC_{50} values of 6.5, 7.5, 3.8, and 2.2 µM, respectively. The HPLC method utilised a TSK-gel ODS-80Tm column with the mixture of acetonitrile and 2% phosphoric acid in water (97:3, v/v) as the mobile phase at a flow rate of 1 mL/min, and UV detection at 245 nm. The parameters of linearity, precision, accuracy, specificity and sensitivity of the method were evaluated. The recoveries of the method were 100.4-101.6% and good linearity ($r^2 \geq 0.9999$) was obtained. A high degree of specificity, sensitivity and precision were also achieved.

Keywords: 5-hydroxymethylfurfural, D-glyceropentaric acid,2-deoxy-3-*C*-(methoxycarbonyl)-1,4-lactone,5-ethyl ester, isoorientin-6"-*O*-rhamnoside, dulcinoside, method validation.

INTRODUCTION

Vegetables are rich in fiber, vitamins, minerals, antioxidants and phytochemicals, whose actions help to protect against cancer. It is most likely that the combination of these nutrients in whole foods can reduce the risk of certain cancers [1]. However, vegetables are thought to protect against cancer directly through specific anti-carcinogenic compounds. Many potential anti-cancer compounds have therefore been isolated from various vegetables [2].

Leishmaniasis is a zoonotic protozoan disease caused by *Leishmania*. Treatment of leishmaniasis remains problematic in developing countries, where it is most often found. Many of the available drugs against the disease are expensive and in certain cases parasite drug resistance has developed [3]. Searching for new naturally occurring anti-leishmanial compounds would be extremely beneficial for the control of the disease.

There is a huge biodiversity in Thai edible plants and it is possible that some could be great resources for detecting new anticancer and anti-leishmanial agents. We have previously isolated two new cytotoxic monoterpene coumarins from a Thai vegetable, *Micromelum minutum* [4].

Garcinia cowa Roxb. ex DC. is another Thai vegetable belonging to the family Clusiaceae. In Thailand, it is commonly known as ‘‘Chamuang’’. In our previous investigations we reported an antibacterial activity for *G. cowa* leaf extracts against gastrointestinal pathogenic bacteria. Purification of an ethyl acetate extract of *G. cowa* leaves using an antimicrobial assay-guided isolation afforded a polyprenylated benzophenone, chamuangone (**1**) (Figure **1**), that exhibited satisfactory antibacterial activity against *Streptococcus pyogenes* (MIC 7.8 µg/mL), *S. viridans* and *Helicobacter pylori* (MICs 15.6 µg/mL), and *Staphylococcus aureus*, *Bacillus subtilis* and *Enterococcus* sp. (MICs 31.2 µg/mL) [5].

In this study, we report a phytochemical study of *G. cowa* leaves with the aid of a leishmanicidal assay-guided isolation, and also report on the cytotoxic activity of the isolated compounds against lung adenocarcinoma (A549 and SBC3) and leukemia (K562, and K562/ADM) cell lines. Moreover, a rapid HPLC method for the quantitative analysis of

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ISSN: 1929-2260 / E-ISSN: 1929-2279/17

chamuangone in *G. cowa* leaf extracts has been described. The developed HPLC method was successfully applied for investigating the best extraction solvent, which yielded a high level of chamuangone in the leaf extracts.

EXPERIMENTAL

Plant Materials

G. cowa leaves were collected from the Hat-Yai District, Songkhla Province, Thailand, in December, 2010. The plant was authenticated at the Herbarium of the Southern Center of Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand, where herbarium specimen (Voucher No. SKP 083 07 03 01) is kept. The leaves were dried at 50 ºC for 24 h in a hot air oven and were then reduced to a coarse powder using a grinder, and the powder was passed through a sieve (No. 45).

General Experimental Procedures

^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM α-400 spectrometer at 400 MHz and 100 MHz, respectively. HR-ESI mass spectra (positive-ion mode) were obtained on an Applied Biosystems QSTAR XL System. IR spectra were recorded on a Jasco IR-810 infrared spectrometer (using a KBr disk). UV spectra were obtained in methanol on a Hewlett Packard 84520A diode array spectrophotometer. Silica gel column chromatography was performed on silica gel 60 (E. Merck, Darmstadt, Germany). Reversed-phase ODS column chromatography was performed on a Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan). HPLC was performed on a semi-preparative RP-C_{18} HPLC column (Inertsil; GL Science, Tokyo, Japan; 250 mm × 10 mm), using a refractive index detector.

Preparations of Plant Extracts

The dried leaf powder of *G. cowa* (1 kg) was extracted three times with ethyl acetate (3 L × 3) under reflux conditions for 1 h, to obtain (after solvent evaporation) a greenish brown extract (32 g). The marcs were subsequently extracted with methanol (3 L × 3) under the same conditions, to obtain (after solvent evaporation) a brown extract (56 g).

Anti-*Leishmanis major* Activity

The cytotoxic activity against *L. major* promastigotes was determined by the colorimetric cell viability 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [6]. The promastigotes obtained from a culture in its logarithmic growth phase in M199 medium supplemented with 10% heat-inactivated fetal bovine serum and 100 µg/mL of kanamycin were used for the assay. In a 96-well plate, 1 µL of the sample solutions at a concentration range from 0.5-100 µg/mL and the promastigotes (1×10^5 cells/well) in 100 µL of medium were added to each well, and then the plate was maintained at 27 °C in a 5% CO_2 atmosphere for 48 h. A solution of MTT (100 µL) was then added to each well and the incubation was continued for a further 24 h. The formazan product of the MTT reduction was then dissolved in DMSO and an absorbance was measured using a Molecular Devices Versamax tunable microplate reader. DMSO was used as a negative control and amphotericin B as a positive control. The experiment was performed in triplicate. The anti-*Leishmania major* activity was quantified as the percentage of the control absorbance of the reduced dye at 540 nm. The inhibitory activity was calculated as:

$$\% \text{ inhibition} = [1-(A_{test} - A_{blank})/(A_{control} - A_{blank})] \times 100$$

Where $A_{control}$ is the absorbance of the control (DMSO) well, A_{test} is the absorbance of the test wells and A_{blank} is the absorbance of the cell-free wells.

Bioassay Guided Isolation

The crude ethyl acetate extract was subjected to silica gel column chromatography. The extract, that had been pre-adsorbed on silica gel, was applied to the top of the silica gel column (7.5 cm in diameter and 45 cm in height), and the column was subsequently eluted with mixtures of hexane and ethyl acetate, using a stepwise-gradient elution starting from 100% hexane to 100% ethyl acetate. Based on TLC chromatograms of each fraction, 15 pooled fractions (fractions 1-15) were obtained. The fractions were then subjected to leishmanicidal assay. The leishmanicidal active fraction (fraction 4) was further purified by a reversed-phase ODS column eluted with mixtures of water and methanol, using a stepwise-gradient elution starting from 60% methanol to 100% methanol to give 12 pooled fractions (fractions 4.1-4.12). The fractions were then subjected to the leishmanicidal assay. Compound **1** (50 mg) was obtained as a yellowish oily liquid from the pooled leishmanicidal active fractions 4.10, after being purified using a semi-preparative RP-C18 HPLC column with 100% acetonitrile as an eluent, with a flow rate of 3.0 mL/min.

Isolation of Compound 2

The fraction 5 obtained from the silica gel column chromatography as described above was further purified by a reversed-phase ODS column eluted with mixtures of water and methanol, using a stepwise-gradient elution starting from 70% methanol to 100% methanol to give 15 pooled fractions (5.1-5.15). Compound **2** (43 mg) was obtained as a yellowish oily liquid from fraction 5.1 after being purified using a semi-preparative RP-C_{18} HPLC column with a mixture of water and methanol (60: 40 v/v) as an eluent, with a flow rate of 3.0 mL/min.

Isolation of Compounds 3-5

Compounds **3**-**5** were purified from the methanol extract of *G. cowa* leaves. The extract, that had been pre-adsorbed on silica gel, was applied to the top of the silica gel column (7.5 cm in diameter and 45 cm in height), and the column was subsequently eluted with mixtures of ethyl acetate and methanol, using a step-gradient elution starting from 100% ethyl acetate to 100% methanol. Based on TLC chromatograms of each fraction, 20 pooled fractions (fractions 1-20) were obtained. Fraction 6 was further purified by a reversed-phase ODS column eluted with mixtures of water and methanol, using a stepwise-gradient elution starting from 10% methanol to 100% methanol to give 19 pooled fractions (fractions 6.1-6.19). Compound **3** (43 mg) was obtained as a yellowish oily liquid from fraction 6.3, and compounds **4**-**5** (12 and 24 mg, respectively) were obtained as yellow powders from fractions 6.4 and 6.5, respectively after being purified using a semi-preparative RP-C_{18} HPLC column with a mixture of water and methanol (90: 10 v/v) as an eluent, with a flow rate of 3.0 mL/min.

Identification of Compounds 1-5

Compound 1

Yellow oil, HRESI-MS *m/z:* 503.3166 $[M+H]^+$; IR ν_{max} (KBr) cm^{-1}: 3350, 1730, 1670, 1600-1530, 1460-1380, 1240 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) 0.95 (1H, *s*, H-17), 1.36 (1H, *dt*, *J* = 3.7, 12.7 Hz, H-18a), 1.44 (1H, *t*, *J* = 13 Hz, H-6ax), 1.50 (3H, *overlapped*, H-28), 1.55 (1H, *m*, H-7), 1.60 (1H, *overlapped*, H-24a), 1.60 (3H, *overlapped*, H-27), 1.63 (3H, *overlapped*, H-32), 1.64 (1H, *overlapped*, H-18b), 1.64 (3H, *overlapped*, H-23), 1.69 (3H, *overlapped*, H-22), 1.73 (3H, *overlapped*, H-33), 1.95 (1H, *m*, H-19), 2.05 (1H, *dd*, *J* = 3.2, 13 Hz, H-6eq), 2.10 (1H, *overlapped*, H-24b), 2.34 (1H, *overlapped*, H-19b), 2.35 (1H, *overlapped*, H-29a), 2.52 (1H, *dd*, *J* = 9.5, 13.9 Hz, H-29b), 3.4 (1H, *s*, H-1), 4.87 (1H, *m*, H-25), 5.07 (1H, *m*, H-20), 5.17 (1H, *m*, H-30), 7.35 (1H, *t*, *J* = 7.8 Hz, H-13), 7.35 (1H, *t*, *J* = 7.8 Hz, H-15), 7.45 (1H, *d*, *J* = 7.3 Hz, H-12), 7.45 (1H, *d*, *J* = 7.3 Hz, H-16), 7.50 (1H, *t*, *J* = 7.3 Hz, H-14), ^{13}C NMR ($CDCl_3$, 125 MHz) 17.7 (CH_3, C-17), 17.8 (CH_3, C-22), 17.9 (CH_3, C-32), 18.1 (CH_3, C-27), 21.9 (CH_2, C-19), 25.7 (CH_3, C-23), 25.7 (CH_3, C-28), 26.0 (CH_3, C-33), 28.1 (CH_2, C-24), 30.4 (CH_2, C-29), 38.5 (CH_2, C-18), 40.9 (CH, C-7), 42.6 (CH2, C-6), 48.5 (C, C-8), 64.6 (CH, C-5), 64.8 (C, C-1), 115.6 (C, C-3), 119.6, (CH, C-30), 121.9 (CH, C-25), 123.8 (CH, C-20), 127.8 (CH, C-13), 127.8 (CH, C-15), 128.5 (CH, C-12), 128.5 (CH, C-16), 132.1 (C, C-21), 132.4 (CH, C-14), 133.4 (C, C-26), 134.8 (C, C-31), 137.1 (C, C-11), 191.4 (C, C-2), 194.6 (C, C-4), 198.0 (C, C-10), 206.5 (C, C-9).

Compound 2

Yellow oil; ESI-MS *m/z*: 127.2 $[M+H]^+$; IR ν_{max}(KBr) cm^{-1}: 3390, 2932, 2871, 1680, 1023; ^1H NMR ($CDCl_3$, 400 MHz) 4.72 (2H, *s*, H-1′′), 6.52 (1H, *d*, *J* = 3.5 Hz, H-4), 7.21 (1H, *d*, *J* = 3.5 Hz, H-3), 9.59 (1H, *s*, H-1′), ^{13}C NMR ($CDCl_3$, 100 MHz) 57.6 (CH2, C-1′′), 109.9 (CH, C-4), 122.5 (CH, C-3), 152.4 (C, C-2), 160.6 (C, C-5), 177.6 (CH, C-1′).

Compound 3

Yellow oil; ESI-MS *m/z* 233.2 $[M+H]^+$; IR ν_{max}(KBr) cm^{-1}: 3390, 1801 1751, 1467-1376; ^1H NMR (CD_3OD, 400 MHz) 1.28 (3H, *t*, *J* = 7.1 Hz, H-3′), 2.63 (1H, *dd*, *J* = 0.4, 17.6 Hz, H-3a), 3.21 (1H, *d*, *J* = 17.6 Hz, H-3b), 3.78 (3H, *s*, H-2′′), 4.22 (2H, *m*, C-2′), 4.91 (1H, *s*, H-5); ^{13}C NMR (CD_3OD, 100 MHz) 14.3 (CH_3, C-3′), 40.6 (CH_2, C-3), 53.6 (CH_3, C-2′′), 63.4 (CH_2, C-2′), 80.9 (C, C-4), 85.8 (CH, C-5), 168.4 (C, C-1′), 171.4 (C, C-1′′), 175.1 (C, C-2).

Compound 4

Yellow solid; ESI-MS *m/z* 579.3 $[M+H]^+$; ^1H NMR (DMSO-d_6, 400 MHz) 1.11 (3H, *d*, *J* = 6.2 Hz, H-6′′′), 3.10-3.56 (1H, *m*, H-2′′), 3.10-3.56 (1H, *m*, C-3′′), 3.10-3.56 (1H, *m*, H-4′′), 3.10-3.56 (1H, *m*, H-5′′), 3.10-3.56 (1H, *m*, H-3′′′), 3.10-3.56 (1H, *m*, H-4′′′), 3.43 (2H, *d*, *J* = 8.4 Hz, H-6′′), 3.83 (1H, *d*, *J* = 8.8 Hz, H-2′′′), 4.00 (1H, *d*, *J* = 9.2 Hz, H-5′′′), 4.52 (1H, *br*, H-1′′′), 4.68 (1H, *d*, *J* = 9.9 Hz, H-1′′), 6.26 (1H, *s*, H-8), 6.63 (1H, *s*, H-3), 6.87 (1H, *d*, *J* = 8.2 Hz, H-5′), 7.42 (1H, *d*, *J* = 1.8 Hz, H-2′), 7.44 (1H, *dd*, *J* = 1.8, 8.2 Hz, H-6′), 13.16 (OH, 5-OH); ^{13}C NMR (DMSO-d_6, 100 MHz) 17.8 (CH_3, C-6′′′), 68.0 (CH_2, C-6′′), 68.3 (CH, C-5′′′), 70.2-80.4 (CH, C-2′′), 70.2-80.4 (CH, C-3′′), 70.2-80.4 (CH, C-4′′), 70.2-80.4 (CH, C-5′′), 70.2-80.4 (CH, C-3′′′), 70.2-

80.4 (CH, C-4‴), 71.0 (CH, C-2‴), 73.3 (CH, C-1″), 98.1 (CH, C-8), 100.6 (CH, C-1‴), 102.4 (CH, C-3), 104.0 (C, C-4a), 104.3 (C, C-6), 114.0 (CH, C-6′), 115.5 (CH, C-3′), 118.9 (CH, C-2′), 122.0 (C, C-1′), 145.8 (C, C-5′), 149.6 (C, C-4′), 155.9 (C, C-7), 160.4 (C, C-8a), 162.5 (C, C-5), 164.0 (C, C-2), 182.0 (C, C-4).

Compound 5

Yellow solid; ESI-MS *m/z* 561.2 $[M+H]^+$; IR ν_{max}(KBr) cm^{-1}: 3402, 1650, 1600-1530; ^1H NMR (DMSO-d_6, 400 MHz) 1.11 (3H, *d*, *J* = 6.2 Hz, H-6‴), 3.09-4.04 (1H, *overlapped*, H-2‴), 3.09-4.04 (1H, *overlapped*, H-3‴), 3.09-4.04 (1H, *overlapped*, H-4‴), 3.09-4.04 (1H, *overlapped*, H-5‴), 3.29-3.86 (1H, *overlapped,* H-2″), 3.29-3.86 (1H, *overlapped*, H-3″), 3.29-3.86 (1H, *overlapped*, H-4″), 3.29-3.86 (1H, *overlapped*, H-5″), 3.29-3.86 (2H, *overlapped*, H-6″), 4.52 (1H, *brs*, H-1‴), 4.59 (1H, *overlapped*, H-1″), 6.50 (1H, *s*, H-8), 6.75 (1H, *s*, H-3), 6.92 (1H, *d*, *J* = 8.9 Hz, H-3′), 6.92 (1H, *d*, *J* = 8.9 Hz, H-5′), 7.91 (1H, *d*, *J* = 8.9 Hz, H-2′), 7.91 (1H, *d*, *J* = 8.9 Hz, H-6′); ^{13}C NMR (DMSO-d_6, 100 MHz) 17.8 (CH_3, C-6‴), 67.4 (CH_2, C-6″), 68.2 (CH, C-5‴), 70.1 (CH, C-3‴), 70.3 (CH, C-2″), 70.4 (CH, C-4‴), 70.6 (CH, C-4″), 71.6 (CH, C-2‴), 73.1 (CH, C-1″), 78.7 (CH, C-5″), 79.6 (CH, C-3″), 93.6 (CH, C-8), 100.5 (CH, C-1‴), 102.7 (CH, C-3), 104.0 (C, C-8a), 108.4 (C, C-5), 116.0 (CH, C-3′), 116.0 (CH, C-5′), 121.1 (C, C-1′), 128.4 (CH, C-2′), 128.4 (CH, C-6′), 160.4 (C, C-4a), 160.6 (C, C-4′), 161.1 (C, C-7), 163.1 (C, C-6), 163.3 (C, C-2), 181.9 (C, C-4).

Cytotoxic Activity against Lung Adenocarcinoma and Leukemia Cells

The cytotoxic activity against lung adenocarcinoma and leukemia cell lines was determined by the MTT colorimetric cell viability assay [6]. Four cancer cell lines, A549, SBC3, K562, and K562/ADM were kindly provided from the JCRB Cell Bank, Japan. SBC3 and A549 cells were cultured in DMEM medium supplemented with 10% heat inactivated FCS, kanamycin (100 µg/mL) and amphotericin B (5.6 µg/mL), while the K562 and K562/ADM cells were cultured in RPMI-1690 medium supplemented with 10% heat inactivated FCS, and kanamycin (100 µg/mL) and amphotericin B (5.6 µg/mL). In a 96-well plate, 1 µL of the sample solutions at a concentration range from 0.5-100 µg/mL and the cancer cells (5×10^3 cells/well) in 100 µL medium were added to each well, and then the plate was maintained at 37 °C in a 5% CO_2 atmosphere for 72 h. The MTT solution (100 µL) was then added to each well and incubation was continued for a further 1 h. An absorbance of each well was measured at 540 nm using a Molecular Devices Versamax tunable microplate reader. DMSO was used as a negative control and doxorubicin as a positive control. The cytotoxic activity was calculated as:

$$\% \text{ inhibition} = [1-(A_{test} - A_{blank})/(A_{control} - A_{blank})] \times 100$$

Where $A_{control}$ is the absorbance of the control (DMSO) well, A_{test} is the absorbance of the test wells and A_{blank} is the absorbance the cell-free wells.

HPLC Analysis

HPLC analysis was carried out using an Agilent 1100 series equipped with a photodiode-array detector (PDA) and an autosampler. Data analysis was performed using Agilent ChemStation software (Agilent, USA). Separation was achieved at 25 ºC on a TSK-gel ODS-80Tm column (5 µm, 150 mm × 4.6 mm i.d., Tosho Bioscience, Japan). The mobile phase consisted of acetonitrile and 2% phosphoric acid in water (97:3, v/v). The mobile phase flow rate was 1 mL/min. Sample injection volumes were 20 µL, and detection was by UV at a wavelength of 245 nm.

A stock solution of the reference standard, chamuangone was made in acetonitrile, and subsequently diluted to provide a series of the standard ranging from 0.01-0.5 mg/mL for use in constructing a calibration curve for chamuangone.

G. cowa leaf powder (100 mg) was extracted with hexane (20 mL) under reflux conditions for 1 h. The extract was then filtered, the solvent was evaporated to dryness under reduced pressure, and the residue reconstituted and adjusted to 10 mL with acetonitrile. Samples were filtered through a 0.45 µm membrane filter and analysed immediately after extraction in order to avoid possible chemical degradation. All assays of samples were performed in triplicate.

Validation of HPLC Method

For validation of the analytical method, the guidelines of the International Conference on Harmonization of Technical Requirement for the Registration of Pharmaceuticals for Human Use were followed [7]. Calculating data for the linearity, accuracy, intra-day and inter-day precision, specificity, limit of determination (LOD) and limit of quantitation (LOQ) were used to validate the HPLC method.

Determination of the Best Solvent for Extraction

G. cowa dried leaf powders (100 mg) were separately extracted with hexane, chloroform, ethyl acetate and methanol (30 mL × 3) under reflux conditions for 1 h. The extracts were then filtered, and the solvent subsequently evaporated to dryness under reduced pressure (40 °C). The residues were reconstituted, and the volume adjusted to 10 mL with acetonitrile. These samples were filtered through a 0.45 µm membrane filter and analysed immediately in order to avoid possible chemical degradation. All samples were analysed in triplicate.

Statistical Analysis

Values are expressed as a mean ± SD. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Tukey's test (P<0.05).

RESULTS AND DISCUSSION

Evaluation of the leishmanicidal activity of the ethyl acetate and methanol extracts of *G. cowa* leaves revealed that the ethyl acetate extract (100 µg/mL) possessed cytotoxic activity against *L. major* with 78% leishmanicidal activity, while the methanol extract showed much lower activity (16% leishmanicidal activity) than the ethyl acetate extract. Further purification of the ethyl acetate extract by leishmanicidal assay-guided purification, produced compound **1** and this was identified as a polyprenylated benzophenone, chamuangone (Figure **1**) by compared of ^{1}H NMR, ^{13}C NMR and EI-MS data with those reported in the literature [5]. Chamuangone exhibited a good leishmanicidal activity with an IC_{50} value of 10.7 µM compared to the positive control, amphotericin B (IC_{50} 0.14 µM). Chamuangone was showing a satisfactory security factor that could turn it into a potential drug for treatment of leishmaniasis.

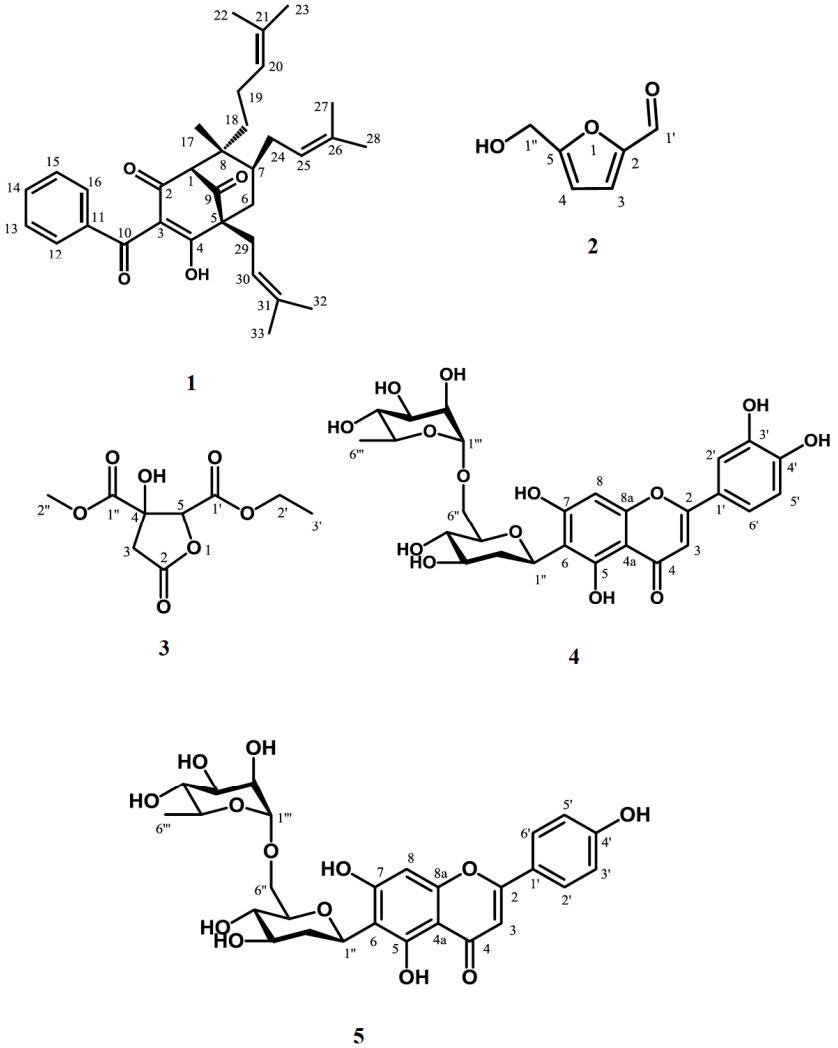

Figure 1: Chemical structures of compounds isolated from *G. cowa* leaves.

Compound **2** was also purified from the ethyl acetate extract and identified as 5-hydroxymethylfurfural (Figure **1**) by comparison of ^{1}H NMR, ^{13}C NMR and EI-MS data with those reported in the literature [8].

In addition, compounds **3-5** were purified from the methanol extract. Compounds **3** was identified as D-glycero-pentaric acid, 2-deoxy-3-*C*-(methoxycarbonyl)-1,4-lactone,5-ethyl ester (Figure **1**) by comparison of ^{1}H NMR, ^{13}C NMR and EI-MS data with those reported in the literature [9].

The molecular formula of compound **4** was assigned as $C_{27}H_{30}O_{14}$ on the basis of ESI-MS data analysis by the peak at *m*/*z* 595.2 $[M+H]^{+}$. The ^{1}H-NMR spectrum showed the characteristic resonances of a flavone proton at δ 6.63 (1H, *s*, H-3), a hydrogen-bonded hydroxyl proton at δ 13.16 (1H, *s*, 5-OH) and an aromatic proton at δ 6.26 (1H*, s*, H-8). The proton signal at δ 7.44 (1H, *dd*, *J* = 8.2, 1.8, H-6′), 6.87 (1H*, d*, *J* = 8.2, H-5′), and 7.42 (1H, *d*, *J* = 8.2, H-2′) was established as an *ortho*-hydroxylation in ring B at C-3′ and C-4′, typical for a hydroxylation at C-5 and C-7, hence representing H-8. In the ^{13}C-NMR spectrum, the shift of the C-6 to 104.3 ppm indicated the link to a sugar unit. The presence of a β-glucose moiety was suggested from the resonances at δ 4.68 (1H, *d*, *J* = 9.9 Hz, H-1′′), 3.10-3.56 (1H, *m*, H-5′′) and 3.43 (2H, *d*, *J* = 8.4 Hz, H-6′′). A rhamnose moiety was identified from the resonances at δ 4.52 (1H, *brs*, H-1′′′), 3.83 (1H, *d*, *J* = 8.8, H-2′′′), 3.10-3.56 (1H, *m*, H-3′′′), 3.10-3.56 (1H, *m*, H-4′′′), 4.00 (1H, *d*, *J*= 9.2, H-5′′′) and 1.11 (3H, *d*, *J* = 6.2 Hz, H-6′′′). The linkage of the glucose unit to the flavone nucleus was identified from the HMBC correlation of H-1′′ to C-5, C-6 and C-7. The chemical shift value of C-1′′ (δ 73.3 ppm) indicated that compound **4** is a C-glycoside. The (1→6) glycosidic bond, of rhamnose to glucose was characterized from the cross-peak of H-1′′′ to C-6′ and the down field shift of the C-6′′ of the glucose unit. Thus, compound **4** was identified as isoorientin-6′′-*O*-rhamnoside (Figure **1**).

Compounds **5** was identified as dulcinoside (Figure **1**) by ^{1}H NMR, ^{13}C NMR and ESI-MS and compared with data in the literature [10]. This is the first report of compounds **2**-**5** from *G. cowa* leaves.

The cytotoxic activity of **1**-**5** was determined against *L. major* and four cancer cell lines (A549, SCB3, K562, and K562/ADM). Only chamuangone exhibited a significant cytotoxic activity against *L. major* with an IC_{50} value of 10.7 μM, and strong cytotoxic activity against A549, SCB3, K562, and K562/ADM cells with IC_{50} values of 6.5, 7.5, 3.6, and 2.2 μM, respectively (Table **1**). The results agree with the previous report on the cytotoxic activity of other polyprenylated benzophenones isolated from the twigs of *G. cowa* [11]. On the basis of its potency for the cancer cell line used, chamuangone is a potential nutraceutical chemopreventive agent, and is worthy of further investigation for its *in vivo* activity and mechanistic effects.

Appropriate conditions for HPLC quantitative analysis of chamuangone in *G. cowa* leaf extracts were achieved using an isocratic reversed-phase HPLC. Mixtures of acetonitrile and 2% aqueous phosphoric acid were examined as the mobile phase, and the ratios were altered until the compound was satisfactorily resolved at the baseline in 7 minutes (Figure **2**). The most suitable ratio of acetonitrile and 2% aqueous phosphoric acid to obtain a good resolution of chamuangone was 97:3, v/v.

The linearity, accuracy, intraday- and interday-precision, specificity and limits of detection and quantitation were determined to validate the HPLC method. Linearity was evaluated using standard chamuangone over six calibration points (10 - 500 μg/mL) with six measurements for each calibration point. Chamuangone exhibited linearity over the evaluated ranges with a linear equation of Y = 21398X-4.1215 ($r^2 \geq 0.9999$). Intraday-precision was estimated by the relative standard deviation of six measurements for chamuangone. Analysis of three independently prepared samples of *G. cowa* leaf extracts determined the interday-precision. The relative standard deviation values for both intraday and interday analysis of chamuangone were less than 1% and 2%, respectively.

Table 1: IC_{50} Values of Compounds Isolated from *G. cowa* Leaves against Cancer Cell Lines

Compounds	Cancer cell lines/ IC_{50} ± SD (μM)			
	SCB3	A549	K562	K562/ADM
Chamuangone	6.5 ± 1.17	7.5 ± 0.28	3.8 ± 0.16	2.2 ± 0.46
Doxorubicin	< 0.4	0.7 ± 0.04	0.6 ± 0.06	> 5.5

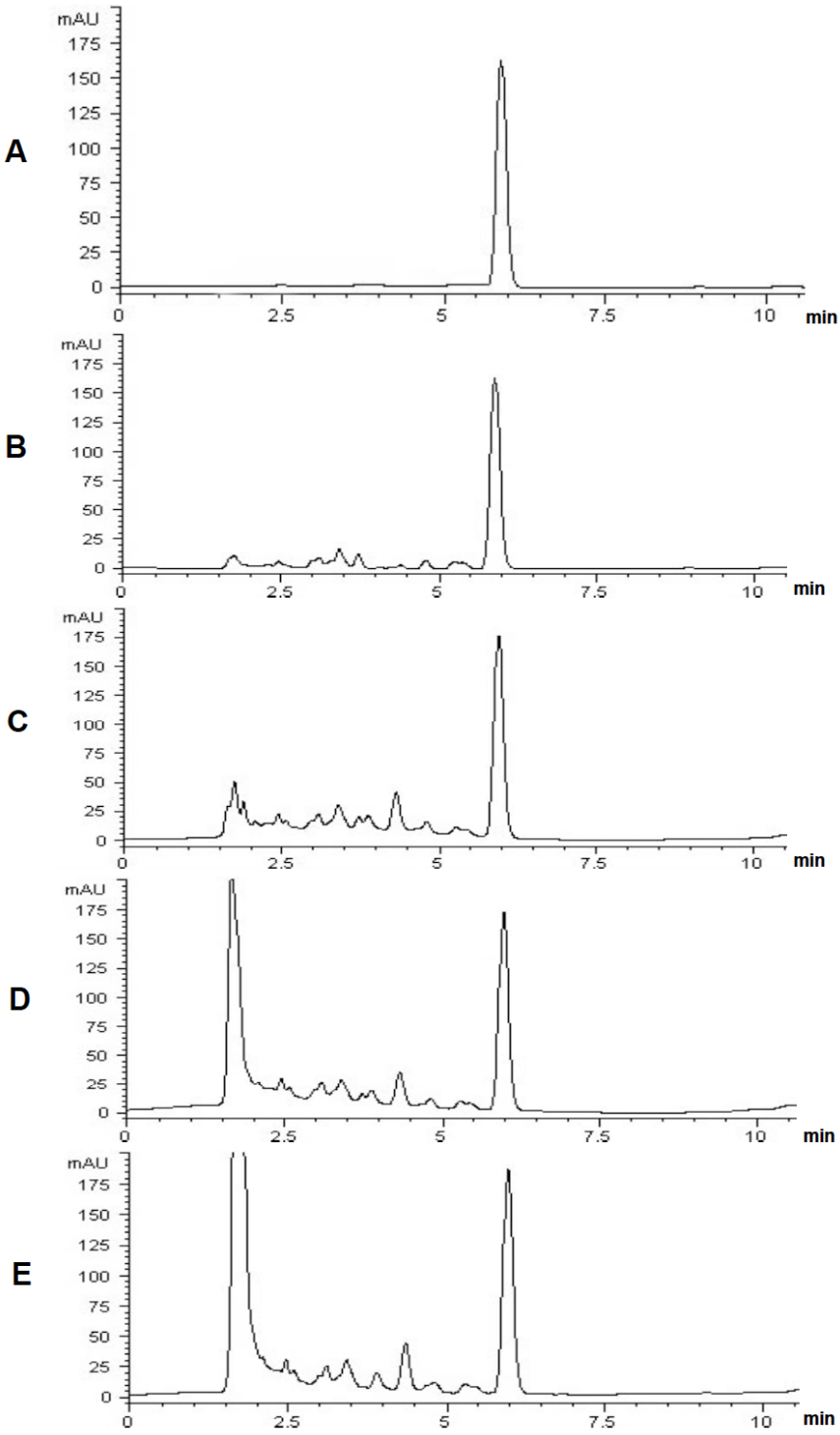

Figure 2: HPLC chromatograms of authentic chamuangone (**A**), hexane (**B**), chloroform (**C**), ethyl acetate (**D**), and methanol (**E**) extracts of *G. cowa* leaves.

The accuracy of the method was determined by analysing *G. cowa* leaf extracts fortified with known quantities of the standard analyte. Recoveries in the range of 100.4 - 101.6% were observed for chamuangone. Specificity of the method was evaluated using the UV-absorption spectra produced by the PDA detector. The spectra were taken at three points of the peak for chamuangone. When it was compared with the standard chamuangone, the spectra of the peak were observed to be homogenous. Finally, it was found that the HPLC method was very sensitive for detecting chamuangone with an LOD and LOQ of 0.93 and 1.87 µg/mL, respectively.

Table 2: Extraction Yield and Chamuangone Content of *G. cowa* Leaf Extracts Obtained from Various Extraction Solvents

Solvents	Extraction yield (% w/w of dried powders; Mean ± SD)	Chamuangone content (% w/w of dried extracts; Mean ± SD)
Hexane	3.29 ± 0.08	11.56 ± 0.37
Chloroform	8.43 ± 0.54	5.20 ± 0.35*
Ethyl acetate	19.55 ± 0.89	2.17 ± 0.09*
Methanol	38.89 ± 4.68	1.15 ± 0.12*

*Significant difference (P<0.05) when compared with hexane in the same column.

Different extraction solvents were used to determine the solvent that produced the maximum amount of chamuangone from the *G. cowa* leaves. Although hexane gave the lowest amount of soluble material, it produced the highest chamuangone content (11.6% w/w) (Table **2**). This indicates that hexane is a suitable solvent for selective extraction of chamuangone from the *G. cowa* leaves.

ACKNOWLEDGEMENTS

The authors wish to thank Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0207/2550) for a financial support in this research as well as the Analytical Center of Molecular Medicine and the Analysis Center of Life Science of the Graduate School of Biomedical Sciences, Hiroshima University for NMR and HR-ESI-MS instruments. Also thanks to Dr. Brian Hodgson for assistance with the English.

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Received on 28-02-2017 Accepted on 30-03-2017 Published on 14-06-2017

DOI: https://doi.org/10.6000/1929-2279.2017.06.02.3