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Four new C-benzyl flavonoids from the fruit of Uvaria cherrevensis

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Abstract

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Four new C-benzyl flavonoids from the fruit of *Uvaria cherrevensis*

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Abstract: The phytochemical investigation of the fruit extracts of *Uvaria cherrevensis* led to the isolation and characterization of four new C-benzyl flavonoids; cherrevenones A-D (**1**-**4**) together with 11 known compounds. The isolated compounds were characterized using spectroscopic techniques. Compounds **1**, **3**, **5** and **11** showed moderate inhibitory activities against the *P. falciparum* strains TM4/8.2 and K1CB with IC_{50} values ranging from 21.0 ± 3.10 – 33.7 ± 7.69 and 21.0 ± 5.44 – 43.5 ± 11.9 µM, respectively. Compounds **1**, **2**, **5**, **10** and **11** exhibited strong cytotoxic activities against KB cells with IC_{50} values ranging from 0.60 ± 0.17 – 4.91 ± 2.69 µM which were similar to their cytotoxic activities found against Vero cells, except for compound **5**, which was non-toxic to Vero cells.

Keywords: *Uvaria cherrevensis*, Annonaceae, flavonoids, antiplasmodial activity, cytotoxic activity

1. Introduction

Uvaria cherrevensis, also known as "Nom Meaw Pa" in Thai, with the synonym of *Ellipeiopsis cherrrevensis*, is a monotypic species in the Annonaceae family [1-2]. This plant is a shrub which is distributed throughout the forests of Thailand [1]. The roots of this plant have been used in traditional medicine to treat urinary disorders [3]. Previous phytochemical studies of this plant reported the isolation of alkaloids, flavonoids, naphthalene derivatives, polyoxygenated cyclohexenes and terpenoids [3-6]. Some of these compounds were reported to have antimalarial, antimicrobial and cytotoxic activities [3,5,6]. In a previous report, we found 2-phenylnaphthalene derivatives, polyoxygenated cyclohexenes and flavonoids from the stems and roots extracts of this plant [5]. Herein, we report the results of a phytochemical investigation of the fruit extracts of *U. cherrevensis* which resulted in the isolation and identification of four new C-benzyl flavonoids (**1**-**4**) together with 11 known compounds. The antimalarial and cytotoxic activities of these compounds, against KB and Vero cells, are also reported.

2. Experimental

2.1. General experimental procedures

Melting points were determined on a Stuart SMP10 melting point apparatus and are uncorrected. Optical rotations were measured in acetone at the sodium D-line on a Rudolph Research Analytical Autopol I polarimeter. UV-vis absorption spectra were measured in MeOH with a Thermo Scientific Evolution 210 UV-vis spectrophotometer. The infrared (IR) spectra were recorded on a Bruker Tensor 27 FT-IR spectrophotometer. The NMR spectra were recorded on either a 400 MHz or a 500 MHz Bruker NMR spectrometer. Chemical shifts were recorded in parts per million (δ) in $CDCl_3$ (δ_H 7.26 and δ_C 77.0 ppm) and/or acetone-d_6 (δ_H 2.05 and δ_C (CO)

206.2 and (CH_3) 29.8 ppm) with TMS as internal standard. The HRESIMS data were obtained on a Bruker Daltonics and Thermo Fisher 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 or silica gel (Merck) type 100 (62-400 µm). Silica gel type 60 (5-40 µm) was used for quick column chromatography (QCC). Solvents for extraction and chromatography were distilled prior to use.

2.2 Plant Material

The fruit of *Uvaria cherrevensis* were collected from Doi Suthep National Park, Chiang Mai, Thailand in August 2015. This plant was identified by Dr. Tanawat Chaowasku from the CMUB Herbarium, Chiang Mai University, Thailand, where a voucher specimen has been deposited (specimen no. T. Ritthiwigrom 5).

2.3 Extraction and isolation

The air dried fruit of *U. cherrevensis* (306.6 g) was extracted with MeOH (3 L) at room temperature over a period of 3 d. Removal of the solvent under reduced pressure afforded a brown viscous oil (37.36 g). The oil was separated by QCC over silica gel. The column was eluted with a solvent gradient from hexane (100%) to acetone (100%) to MeOH (100%) to provide eight fractions (Fractions A-H, see the Supplementary Information for a flow chart of the isolation procedure). Fraction A (654.7 mg) was separated by CC over Sephadex LH-20 with MeOH elution to give four subfractions (A1-A4). Subfraction A2 (19.6 mg) was further purified by CC over silica gel by elution with EtOAc/hexane (15:85) to give compound **5** (10.0 mg) as a yellow viscous oil. Compound **1** (11.0 mg), as a yellow solid, mp 224-226 °C, was obtained from subfraction A4 after evaporation. Fraction B (394.2 mg) was purified by CC over Sephadex LH-

20 with MeOH elution to provide compound **6** (1.2 mg) as a yellow solid. Fractionation of Fraction C (1.85 g) by CC over Sephadex LH-20 with MeOH elution gave three subfractions (C1-C3). Subfraction C2 (48.3 mg) was purified by CC over silica gel by elution with EtOAc/hexane (1:4) to give compound **7** (12.7 mg) as a white solid, mp 195-197 °C, lit. 182-183 °C [3]. Fraction E (1.16 g) was separated by CC over silica gel by elution with MeOH/CH_2Cl_2 (1:99) to give ten fractions (E1-E10). Compound **8** (7.5 mg), as a yellow solid, mp 146-147 °C, lit. 179-181 °C [3], was obtained from subfraction E2 (10.4 mg) by CC over Sephadex LH-20 with MeOH elution. Subfraction E4 (285.7 mg) was separated by CC over silica gel by elution with MeOH/CH_2Cl_2 (1:99) to provide compounds **3** (11.5 mg) and **9** (23.9 mg) as a yellow viscous oil and a brown solid (mp 204-205 °C, lit. 203.5-204.5 °C [7]), respectively. Subfraction E6 (46.6 mg) was separated by CC over Sephadex LH-20 with MeOH elution to give four subfractions (E6A-E6D). Subfraction E6B (21.5 mg) was purified by CC over silica gel by elution with MeOH/CH_2Cl_2 (1:99) to provide compound **10** (6.5 mg) as a yellow viscous oil. Compound **11** (10.6 mg), as a yellow viscous oil, was obtained from subfraction E6C after evaporation. Subfraction E8 (46.2 mg) was separated by CC over Sephadex LH-20 with MeOH elution to give three subfractions (E8A-E8C). Subfraction E8B (25.5 mg) was purified by CC over silica gel by elution with MeOH/CH_2Cl_2 (1:99) to give compound **12** (7.4 mg) as a yellow solid, mp 99-100 °C (mp not reported in the literature). Subfraction E9 (47.1 mg) was separated by CC over Sephadex LH-20 with MeOH elution to give compound **13** (2.1 mg) as a brown viscous oil. Fraction F (1.11g) was separated by CC over Sephadex LH-20 with MeOH elution to give three subfractions (F1-F3). Subfraction F2 (34.3 mg) was separated by CC over silica gel by elution with EtOAc/CH_2Cl_2 (5:95) to provide compound **2** (2.5 mg) as a yellow viscous oil. Fraction H (530.0 mg) was separated by CC over Sephadex LH-20 with MeOH elution to

provide four subfractions (H1-H4). Compound **14** (13.5 mg), as a yellow solid, mp 225-227 °C (mp not reported in the literature), was isolated from subfraciton H2 (81.5 mg) by CC over silica gel by elution with MeOH/CH_2Cl_2 (2:98). Subfraction H3 (167.6 mg) was separated by CC over Sephadex LH-20 with MeOH elution to give five subfractions (H3A-H3E). Subfraction H3B (25.5 mg) was further purified by CC over silica gel by elution with MeOH/CH_2Cl_2 (2:98) to give compound **15** (3.5 mg) as a yellow viscous oil. Compound **4** (1.3 mg), as a yellow viscous oil, was obtained from subfraction H3D (13.1 mg) by CC over silica gel by elution with MeOH/CH_2Cl_2 (1:99).

Cherrevenone A (**1**): yellow solid; mp 224-226 °C; UV (MeOH) λ_{max} (log ε) 215 (4.54), 282 (4.20), 355 (4.27) nm; IR (neat) ν_{max} 3241, 1627, 1608, 1490 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) and ^{13}C NMR (100 MHz, acetone-d_6) see Table 1; HRESIMS *m/z* 473.1355 $[M + Na]^+$, calcd 473.1365, $C_{29}H_{22}O_5Na$.

Cherrevenone B (**2**): yellow viscous oil; UV (MeOH) λ_{max} (log ε) 220 (4.18), 281 (3.63), 370 (4.07) nm; IR (neat) ν_{max} 3243, 1619, 1604, 1513 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) see Table 1; HRESIMS *m/z* 391.1189 $[M - H]^-$, calcd 391.1182, $C_{23}H_{19}O_6$.

Cherrevenone C (**3**): brown viscous oil; UV (MeOH) λ_{max} (log ε) 211 (4.46), 281 (3.85), 400 (3.59) nm; IR (neat) ν_{max} 3231, 1628, 1595, 1489 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) see Table 1; HRESIMS *m/z* 505.16209 $[M + Na]^+$, calcd 505.16216, $C_{30}H_{26}O_6Na$.

Cherrevenone D (**4**): yellow viscous oil; $[\alpha]_D^{20}$ –52.2 (*c* 0.07, acetone); UV (MeOH) λ_{max} (log ε) 214 (4.37), 280 (3.93), 328 (3.87) nm; IR (neat) ν_{max} 3339, 1643, 1585, 1460 cm^{-1}; ^1H

NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) see Table 2; HRESIMS *m/z* 499.1743 $[M + H]^+$ calcd 499.1757, $C_{30}H_{27}O_7$.

2.4 Bioactivity Assays

The antimalarial activity testing against *Plasmodium falciparum* (TM4/8.2, a wild type sensitive strain and K1CB1, a multidrug resistant strain) was carried out using the Malaria SYBR Green I-base fluorescence (MSF) assay [8]. Briefly, following incubation of parasites (90 µL of 1% ring stages at 2% haematocrit) with serial dilution of compounds (10 µL in 0.1% DMSO containing RPMI media) in black 96-well plate for 48 h in a 3% CO_2 incubator at 37 °C, 100 µL SYBR Green I dye (0.2 µL of SYBR Green I/mL of in lysis buffer pH 7.5 (20 mM Tris containing 5 mM EDTA, 0.008% saponin, and 0.08%Triton X-100)) was added and mixed well. After 1 h incubation in the dark at 25 °C, the fluorescence signal of the treated and untreated control was measured with a fluorometer with excitation at 435 nm and emission at 535 nm. The 50% inhibition concentration (IC_{50}) was calculated from the sigmoidal dose-response curve. Cycloguanil and pyrimethamine were included as the reference compounds [9]. Cytotoxic assays against KB cells (human mouth epidermal carcinoma cells) and normal Vero cells (kidney epithelial cells of African green monkey, Cercopithecus aethiops) [10] were evaluated using the sulforhodamine B (SRB) assay [11]. Ellipticine was included as the reference compound.

3. Results and Discussion

The crude MeOH extract of the fruit of *U. cherrevensis* was separated by column chromatography resulting in the isolation of four new flavonoids, cherrevenones A-D (**1-4**), together with 11 known compounds; uvarindoles C, D and A (**5**, **10**, **11**) [12], cardamonin (**6**) [13], ferrudiol (**7**) [3], 2′,4′-dihydroxy-3′-(2-hydroxyphenyl)-6′-methoxychalcone (**8**) [3], 5-*O*-

methoxydichamanetine (**9**) [7], ellipeiopsol B (**12**) [4], zeylenol (**13**) [3], 7-*O*-methylisochamanetine (**14**) [14] and *epi*-methylphelligrin A (**15**) [15] (Figure 1). The structures of the isolated compounds were elucidated by spectroscopic techniques including UV, IR, NMR and MS analysis. Of these compounds, only compounds **8**, **9**, **12** and **13** were also isolated from the stem and root extracts of this plant [5].

Compound **1** was obtained as a yellow solid, mp 224-226 °C. The HRESIMS exhibited an ion peak at *m/z* 473.1355 ($[M + Na]^+$, calcd for $C_{29}H_{22}O_5Na$, 473.1365), indicating the molecular formula of $C_{29}H_{22}O_5$. The UV spectrum showed absorption bands at λ_{max} 215, 282 and 355 nm and the IR spectrum indicated stretching bands for a hydroxy group (3241 cm^{-1}), an α,β-unsaturated carbonyl functional group (1627 cm^{-1}) and an aromatic ring (1608 and 1490 cm^{-1}) that indicated a possible chalcone structure [16]. The ^1H NMR spectrum of **1** (Table 1) displayed resonances for a chelated hydroxy proton (δ_H 14.35, 1H, s), two olefinic protons of an (*E*)-α,β-unsaturated ketone [δ_H 8.16 and 7.83 (each 1H, d, *J* = 15.6 Hz)], a mono-substituted benzene [δ_H 7.82 (2H, d, *J* = 7.6 Hz), 7.50 (2H, d, *J* = 7.6 Hz), 7.48 (1H, t, *J* = 7.6 Hz)], a 2-hydroxybenzyl moiety [δ_H 7.44 (1H, d, *J* = 7.5 Hz), 7.07 (1H, t, *J* = 7.5 Hz), 6.93 (1H, d, *J* = 7.5 Hz), 6.84 (1H, t, *J* = 7.5 Hz), 3.98 (2H, s)] and a hydroxybenzyl-like moiety (C-1″ – C-6″ and C-8′) forming part of a xanthene skeleton [δ_H 7.30 (1H, d, *J* = 7.4 Hz), 7.25 (1H, t, *J* = 7.4 Hz), 7.11 (1H, d, *J* = 7.4 Hz), 7.05 (1H, t, *J* = 7.4 Hz), 3.91 (2H, s)] as found in isochamuvaritin [13]. The ^{13}C NMR spectrum of **1** (Table 1) showed resonances for 29 carbons including an α,β-unsaturated carbonyl carbon (δ_C 193.6), eleven quaternary aromatic carbons (δ_C 164.0, 160.6, 153.7, 152.7, 151.4, 136.4, 127.8, 121.2, 110.2, 105.6 and 101.2), thirteen aromatic methine (ArCH) carbons (δ_C 131.9, 131.2, 130.4, 130.0 (2C), 129.3 (2C), 128.8,128.3, 125.0, 121.7, 116.8 and 115.7), two

alkene methines (δ_C 142.9 and 128.7) and two methylene carbons (δ_C 22.8 and 22.7). The HMBC spectrum showed correlations between H-7 (δ_H 7.83/δ_C 142.9) and C-2/C-6 (δ_C 130.0) and C-9 (δ_C 193.6) and between the olefinic proton H-8 (δ_H 8.16/δ_C 128.7) and C-1 (δ_C 136.4) which confirmed that the mono-substituted benzene (ring B) was part of the chalcone core structure. The chelated hydroxy proton (δ_H 14.35) showed HMBC correlations to C-1′ (δ_C 105.6), C-2′ (δ_C 164.0) and C-3′ (δ_C 110.2). The methylene protons H-7′ (δ_H 3.98) of the 2-hydroxylbenzyl group displayed HMBC correlations to C-2′, C-3′, C-4′ (δ_C 160.6), C-1″ (δ_C 127.8), C-2″ (δ_C 153.7) and C-6″ (δ_C 131.9). The 2-hydroxybenzyl group-like structure, that formed part of the xanthene ring, was attached through a C-C bond between C-5′ (δ_C 101.2) and C-8′ (δ_C 22.8) and a C-O bond at C-6′ (δ_C 152.7) from the HMBC correlations of H-8′ (δ_H 3.91) with C-4′, C-5′, C-1‴ (δ_C 121.2), C-2‴ (δ_C 151.4) and C-6‴ (δ_C 130.4). The oxygenated aromatic carbons C-2′ and C-4′ were substituted with hydroxy groups due to their low field ^{13}C NMR chemical shifts. Thus, compound **1** (cherrevenone A) was identified as (*E*)-1-(1,3-dihydroxy-2-(2-hydroxybenzyl)-9*H*-xanthen-4-yl)-3-phenylprop-2-en-1-one, which is the 7,8-didehydro analogue of isochamuvaritin [17].

Compound **2** was isolated as a yellow viscous oil and its molecular formula was deduced to be $C_{23}H_{20}O_6$ based on the molecular ion peak at *m/z* 391.1189 $[M - H]^-$ (calcd for $C_{23}H_{19}O_6$, 391.1182) in the negative ion HRESIMS. The UV, IR and NMR spectroscopic data of **2** (Table 1) were similar to those of 2′,4′-dihydroxy-3′-(2-hydroxyphenyl)-6′-methoxychalcone (**8**) [3], except that the NMR resonances for the B-ring in **8** were replaced by resonances for a *p*-substituted benzene [δ_H 7.59 (2H, d, J = 8.6 Hz and 6.90 (2H, d, J = 8.6 Hz)]. The aromatic proton resonances for H-2/H-6 (δ_H 7.59/δ_C 131.3) showed HMBC cross peaks with C-4 (δ_C

160.8) and C-7 (δ_C 143.6). The aromatic carbon C-4 was substituent with a hydroxy group due to its low field ^{13}C NMR chemical shift. Thus, compound **2** (cherrevenone B) was determined to be 4,2′,4′-trihydroxy-3′-(2-hydroxybenzyl)-6′-methoxychalcone.

Compound **3** was obtained as a brown viscous oil and displayed a $[M + Na]^+$ ion peak at *m/z* 505.16209 (calcd for $C_{30}H_{26}O_6Na$, 505.16216) in the HRESIMS, corresponding to a molecular formula of $C_{30}H_{26}O_6$. The NMR spectroscopic data of **3** (Table 1) were similar to those 2′,4′-dihydroxy-3′-(2-hydroxyphenyl)-6′-methoxychalcone (**8**) except for the additional resonances for a second 2-hydroxybenzyl moiety [δ_H 7.08 (1H, dd, *J* = 7.9, 1.6 Hz), 7.02 (1H, td, *J* = 7.9, 1.6 Hz), 6.83 (1H, dd, *J* = 7.9, 1.6 Hz), 6.70 (1H, td, *J* = 7.9, 1.6 Hz), 3.96 (2H, s)] and the lack of a resonances for H-5′. The benzylic methylene protons CH_2-8′ (δ_H 3.96/δ_C 24.4), for this 2-hydroxybenzyl group showed HMBC correlations with C-4′ (δ_C 161.1), C-5′ (δ_C 115.1) and C-1‴ (δ_C 128.1) which confirmed placement of the 2-hydroxybenzyl group at C-5′. Therefore, compound **3** (cherrevenone C) was identified as 2′,4′-dihydroxy-3′,5′-di(2-hydroxybenzyl)-6′-methoxychalcone.

Compound **4** was obtained as a yellow viscous oil. Its molecular formula, $C_{30}H_{26}O_7$ was deduced from HRESIMS which showed a $[M + H]^+$ ion peak at *m/z* at 499.1743 (calcd $C_{30}H_{27}O_7$, for 499.1757). The UV spectrum showed maximum absorption bands at λ_{max} 214, 280 and 328 nm which were consistent with a flavanone structure [12]. The IR spectrum displayed stretching bands for a hydroxy group (3339 cm^{-1}), a carbonyl group (1643 cm^{-1}) and an aromatic ring (1585 and 1460 cm^{-1}). The NMR spectroscopic data of **4** (Table 2) were similar to those of 5-*O*-methoxydichamanetine (**9**) except for the presence of resonances for a *p*-disubstituted benzene [δ_H 7.43 (2H, d, *J* = 8.2 Hz), 6.92 (2H, d, *J* = 8.2 Hz)] instead of those for a mono-substituent

benzene in **9**. The aromatic proton resonance for H-2′/H-6′ (δ_H 7.43) showed HMBC correlations to C-2 (δ_C 79.7) and C-4′ (δ_C 158.6). The low field chemical shift of C-4′ suggested it was substituted with a hydroxy group. The methoxy group (δ_H 3.78) was located at C-5 (δ_C 159.3) from their mutual HMBC correlation. This was further supported from a 1D NOE difference experiment which showed an enhancement in the signal intensities of the resonances for H-6′′′ (δ_H 7.10) and CH_2-12 (δ_H 3.92) upon selective irradiation of the methoxy group. The configuration of **4** was assigned as 2*S* from a comparison of the sign of its specific rotation ($[\alpha]_D^{20}$ –52.2 (*c* 0.07, acetone)) with that of dichamanetin ($[\alpha]_D^{21}$ –9 (*c* 2.3, acetone)), the *O*-demethyl analogue of **4** [18]. Thus, compound **4** (cherrevenone D) was determined as 2*S*-7,4′-dihydroxy-6,8–di(2-hydroxybenzyl)-5-methoxyflavanone.

Compounds **1**-**3**, **5**, **7**, **10**, **11**, **14** and **15** were screened for their antimalarial activities against the *Plasmodium falciparum* strains, TM4/8.2 and K1CB1 (wildtype and multidrug resistant strains, respectively). Compounds **8**, **9**, **12** and **13** were tested earlier [5]. Compounds **1**, **3**, **5** and **11** showed moderate antimalarial activities with IC_{50} values ranging between 21.0 – 33.7 μM and 21.0 – 43.5 μM against the TM4/8.2 and K1CB1 strains, respectively (Table 3). Compounds **2** and **10**, were not active at 50 μM. By comparing the antimalarial activities and the cytotoxicities of these compounds against noncancerous Vero cell lines, representing normal mammalian cells, these compounds, except for **3** and **5**, are quite toxic with selectivity ratios (IC_{50} ratios of Vero cells: K1CB1 cells) much less than 1 (0.02 – 0.3). Derivatization of these compounds in the future may improve antimalarial activity with reduce cytotoxicities. These compounds were also tested for their cytotoxicity against KB cells (Table 3). Alkaloid **5** showed significant cytotoxicity against KB cells with an IC_{50} value 4.91 μM and had no cytotoxicity

against Vero cells at 50 μM with a selectivity ratio of greater than 10. The chalcone **1** exhibit strong cytotoxicity against KB cells but was equally toxic to Vero cell with IC_{50} values of *ca* 0.6 μM. Chalcone **2** and the alkaloids **10** and **11** were also toxic against KB and Vero cells with IC_{50} values in the ranges of 2.76 – 4.72 and 3.34 – 5.67 μM, respectively. In contrast, the compounds **3**, **7**, **14** and **15** were not cytotoxic against either cell line at 50 μM. Overall, most of these compounds were more cytotoxic than those previously isolated from the stem and root extract of this plant [5].

In conclusion, four new C-benzyl flavonoids (**1**-**4**) have been isolated, with compounds **1** and **2** showing significant cytotoxicities against KB and Vero cells. While C-benzyl flavonoids are known [3,5,7] these are a relatively rare subset of flavonoid natural products. Compared with our earlier study [5], on the stem and root extracts, only compounds **8**, **9**, **12** and **13** were found to be in common.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data (copies of 1D and 2D NMR spectra of compounds **1**-**4**) to this article can be found online at http://

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