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Cytotoxic and antioxidant constituents from *Garcinia subelliptica*

Kai-Wei Lin [a], A-Mei Huang [b], Shyh-Chyun Yang [a,*], Jing-Ru Weng [c], Tzyh-Chyuan Hour [b], Yeong-Shiau Pu [d], Chun-Nan Lin [c,e,*]

[a] Faculty of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[b] Institute of Biochemistry, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[c] Department of Biological Science and Technology, School of Medicine, China Medical University, Taichung 40402, Taiwan
[d] Department of Urology, College of Medicine, National Taiwan University, Taipei 10002, Taiwan
[e] Faculty of Fragrance and Cosmetics, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

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ABSTRACT

Two new triterpenoids, garcinielliptones Q (**1**) and S (**3**), and a new phloroglucinol, garcinielliptone R (**2**), were isolated from the seed of *Garcinia subelliptica*. Their structures were established by analysis of their spectroscopic data. Phloroglucinol, garcinielliptone FC (**4**) from this plant exhibited a significant increase of antiproliferative effect, while **4** combined with cisplatin significantly caused decrease of cell inhibition induced by cisplatin in NTUB1. Exposure of NTUB1 cells to **4** cotreated with cisplatin for significantly decreased the amount of reactive oxygen species (ROS) than that of the total amount generated by **4** and cisplatin. These results suggested that **4** could protect the cisplatin toxicity through reduction of ROS in NTUB1. Phloroglucinols, garcinielliptones, A (**5**) and F (**7**), and garsubelline A (**6**), from this plant, revealed ABTS radical cation scavenging activity and **5** displayed an inhibitory effect on xanthine oxidase. These finding showed that **5**–**7** may be used as antioxidants.

1. Introduction

Traditionally used herbs have greater attention in the food industry due to their antioxidant and antitumor properties. Now in most countries there are some limitations on using synthetic antioxidants in the food products because of their side effects, therefore natural sources have became more important in order to find proper and safe food antioxidants.

Garcinia subelliptica Merr. (Guttiferae) is a tree that serves as a dye source in tropical and subtropical areas. The seeds of this plant did not report that it may serve as folk medicine. Previously the isolation and characterization of various phloroglucinols and terpenoids from the seeds of this plant have been reported (Weng, Lin, Tsao, & Wang, 2003a, 2003b; Weng, Tsao, Wang, Wu, & Lin, 2004). Several phloroglucinols and terpenoids isolated from the seed of this plant exhibited significant anti-inflammatory activity. These active phloroglucinols or terpenoids may be of value in the therapeutic treatment or prevention of central as well as peripheral inflammatory diseases associated with the increase of chemical mediators in inflammatory cells.

Recently, two phloroglucinols, garcinielliptones C and P from the seeds of this plant were identified as xanthine oxidase (XO) inhibitors (Lin et al., 2009, 2011a). The XO inhibitor, β-amyrin from the fruit of this plant indicated that cell cycle arrest and apoptosis induced by β-amyrin or β-amyrin combined with cisplatin-treated NTUB1 for 24 h was mediated through an increased amount of ROS in cells exposed to β-amyrin or β-amyrin cotreated with cisplatin (Lin et al., 2011a). In addition, the isolation and characterization of nine cytotoxic prenylphloroglucinols from the fruit of this plant also have been reported (Zhang et al., 2010). These results revealed that the active constituents from the seeds or fruits of this plant can be further evaluated and developed into therapeutic agents or various appropriate crude fractions may be used as a crude drug for ROS-mediated diseases.

Therefore, the continual investigation on natural triterpenoids or phloroglucinols with cytotoxic and antioxidative activities from the seed of *Garcinia subelliptica* led to the isolation of two new triterpenoids, garcinielliptones Q (**1**) and S (**3**), and a new phloroglucinol, garcinielliptone R (**2**). In the present paper, the structure elucidation of these three new compounds, the cytotoxic activity of **3** and **4**, previously reported compound with prooxidant activity (Wu et al., 2008), and the antioxidant activity of **1** and **3**, and previously reported compounds, **5**–**7** (Weng et al., 2003a, 2003b), isolated from this plant, are reported. An insufficient amount of compound **2** was obtained for biological testing.

* Corresponding authors. Tel.: +886 7 3121101; fax: +886 7 5562365 (C.-N. Lin), tel.: +886 7 3121101; fax: +886 7 3210683 (S.-C. Yang).
E-mail addresses: scyang@kmu.edu.tw (S.-C. Yang), lincna@cc.kmu.edu.tw (C.-N. Lin).

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2. Material and methods

2.1. Instruments and apparatus

Melting point (uncorrected) was determined with a Yanaco Micro-Melting Point apparatus (Yanaco Seisakusho Co. Ltd., Kyoto, Japan). Optical rotations were recorded on a DIP-370 polarimeter (JASCO, Oklahoma, USA) using acetone as solvent. UV spectra were obtained in MeOH on a JASCO UV–Vis spectrophotometer (JASCO, Oklahoma, USA). IR spectra were measured on a Hitachi 260–30 spectrophotometer using KBr pellets or film on NaCl. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra and ^{1}H–^{1}H COSY, NOESY, HMQC, and HMBC experiments were recorded on a Bruker AMX-400 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany). MS were obtained on a JMS-HX100 mass spectrometer (JEOL Ltd., Tokyo, Japan).

2.2. Reagents

Silica gel (Merck), particle size 15–40 μM, was used for column chromatography. Silica gel 60 F_{254} precoated aluminum sheets (0.2 mm, Merck) were employed for TLC. All solvents were HPLC grade. Xanthine (XA), xanthine oxidase (XO), allopurinol, tocopherol (TOC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cisplatin was obtained from Pharmacia & Upjohn, Milan, Italy. All culture reagents were obtained from Gibco BRL (San Francisco, USA).

2.3. Plant material

The seeds of *G. subelliptica* were collected at Kaohsiung, Taiwan, in July 2001. A voucher specimen (2001–3) has been deposited at the Department of Medicinal Chemistry, School of Pharmacy, Kaohsiung Medical University.

2.4. Extraction and isolation

The seed (7.5 kg), obtained from the fresh fruit (22.8 kg) of *G. subelliptica*, were extracted with chloroform (10 L) at room temperature. The $CHCl_3$ extract was dried under reduced pressure to afford a brown residue (130 g). This residue was fractionated by chromatography over silica gel and eluted with a gradient of *n*-hexane-EtOAc-MeOH (4:4:1) to *n*-hexane-EtOAc-MeOH (1:2:1) to yield two fractions. Fraction 1 was subjected to repeated chromatography on silica gel and eluted with *n*-hexane-acetone (3:1) to yield three fractions. Fraction 2 was repeated chromatography on silica gel and eluted with $CHCl_3$-EtOAc (9:1) yield **1** (10 mg) and $CHCl_3$-EtOAc (6:1) yield **2** (5 mg) and **3** (10 mg).

2.5. Spectral measurements

Garcinielliptone Q (**1**): colorless oil; $[\alpha]^{25}{}_D$44 (*c* 0.1, MeOH); IR $_{max}$ (KBr): 3424 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EIMS (70 eV) *m/z* 444 $[M]^+$ (1), 426 $[M - H_2O]^+$ (100), 411 $[426 - Me]^+$ (10), 357 $[411-(-CH=C(CH_3)_2) + H]^+$ (39), 300 [426 – side chan + H]$^+$ (33), 207 (64), 109 (100); HREIMS *m/z* 444.3962 $[M]^+$, $C_{30}H_{52}O_2$; calc. 444.3967.

Garcinielliptone R (**2**): colorless oil; $[\alpha]^{25}{}_D - 38$ (*c* 0.13, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 215 (4.80) nm; IR $_{max}$ (film on NaCl): 3461, 1771, 1724 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EIMS (70 eV) *m/z* 482 $[M - 2H_2O]^+$ (10), 464 [*m/z* 482 $- H_2O]^+$ (21), 346 [*m/z* 482-isogeranyl]$^+$ (93); HREIMS *m/z* 482.2930 $[M - 2H_2O]^+$, $C_{30}H_{42}O_5$; calc.482.2940).

Garcinielliptone S (**3**): colorless powder; $[\alpha]^{25}{}_D5$ (*c* 0.64, $CHCl_3$); IR $_{max}$ (KBr): 3424, 1694 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EIMS (70 eV) *m/z* 426 $[M]^+$ (81), 408 $[M - H_2O]^+$ (25), 357 $[M - (-CH_2CH(CH_3)_2)]^+$ (47), 339 $[357 - H_2O]^+$ (27), 218 (28), 203 $[218 - CH_3]^+$ (59), 189 (100); HREIMS *m/z* 426.3872 $[M]^+$, $C_{30}H_{50}O$; calc. 426.3862.

2.6. Cell culture and MTT assay for cell viability

NTUB1 human bladder carcinoma cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin-G, 100 μg/ml streptomycin, and 2 mM L-glutamine. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. For evaluating the cytotoxic effect of **3** and **4**, cisplatin, and **4**/cisplatin combination, a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) assays were performed (Hour et al., 2000). Briefly, the cells were plated at a density of 1800 cells/well in 96-well plates and incubated at 37 °C overnight before drug exposure. Cells were then cultured in the presence of graded concentrations of **3** and **4** with or without 10 μM cisplatin (Pharmacia & Upjohn, Milan, Italy) at 37 °C for 24 or 72 h. At the end of the culture period, 50 ml of MTT (2 mg/ml in PB) was added to each well and allowed to react for 3 h. Following centrifugation of plates at 1000 × g for 10 min, media were removed and 150 ml DMSO were added to each well. The proportions of surviving cells were determined by absorbance spectrometry at 540 nm using MRX (DYNEX-CO) microplate reader. The cell viability was expressed as a percentage to the viable cells of control culture condition. The IC_{50} values of each group were calculated by the median-effect analysis and presented as the mean ± standard deviation (SD).

2.7. Quantitative analysis of intracellular ROS

Production of ROS was analyzed by flow cytometry as described previously (Pu et al., 2002). Briefly, cells were plated and treated as indicated: 10 μM dichlorofluorescein diacetate (H_2DCFDA; Molecular Probes, Eugene, OR) was added to the treated cells 30 min prior harvest. The cells were collected by trypsinization and washed with PBS. The green fluorescence of intracellular DCF (2′,7′-dichlorofluorescein) was then analyzed immediately by FACScan flow cytometer with a 525 nm band pass filter (Becton Dickinson).

2.8. Flow cytometry analysis

DNA content was determined following propidium iodide (PI) staining of cells as previously described (Huang et al., 2004). Briefly, 8×10^5 cells were plated and treated with 20 μM cisplatin and various concentrations of **4** for 24 h. These cells were harvested by trypsinization, washed with 1 × PBS, and fixed in ice-cold MeOH at −20 °C. After overnight incubation, the cells were washed with PBS and incubated with 50 μg/ml propidium iodide (Sigma, Co.) and 50 μg/ml RNase A (Sigma, Co.) in PBS at room temperature for 30 min. The fractions of cells in each phase of cell cycle were analyzed using FACScan flow cytometer and Cell Quest software (Becton Dickinson).

2.9. Assay of xanthine oxidase

The xanthine oxidase activity with xanthine as the substrate was measured at 25 °C, according to the protocol of Kong and others (Kong, Zhang, Pan, Tan, & Cheng, 2000) with modification. The assay mixture consisting of 50 μl of test solution, 60 μl of 70 mM phosphate buffer (pH 7.5) and 30 μl of enzyme solution [0.1 units/ml in 70 mM phosphate buffer (pH 7.5)] was prepared immediately before use. After preincubation at 25 °C for 15 min,