Cytotoxic Constituents from the Leaves of *Cinnamomum subavenium*

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Two new butanolides, subamolide D (1) and subamolide E (2), and a new secobutanolide, secosubamolide A (3), along with 21 known compounds were isolated from the leaves of *Cinnamomum subavenium*. The structures of 1–3 were determined by spectroscopic analysis. Propidium iodide staining and cytometry analysis were used to evaluate the cell cycle progression of the treated SW480 cells and it was found that 1 and 2 caused DNA damage in a dose- and time-dependent manner.

Key words *Cinnamomum subavenium*; subamolide D; subamolide E; secosubamolide A

Cinnamomum subavenium MiQ. (Lauraceae) is a medium-sized evergreen tree, found in central to southern mainland China, Burma, Cambodia, Taiwan, Malaysia, and Indonesia.¹⁾ The chemical constituents and the biological activity of the leaves of this plant have not yet been reported. Recently, we reported three new butanolides, subamolides A–C, a new secobutanolide, secosubamolide, along with 17 known compounds from the stems of *C. subavenium*.^{2,3)} We also reported the structural elucidation of the newly isolated butanolides and secobutanolide as well as their effects on DNA damage on a human colorectal cancer cell line, SW480.^{2,3)} In continuation of a program studying chemotaxonomy and biologically active components from Formosan Lauraceous plants,^{2–9)} two new butanolides, namely, subamolide D [(4*R*,3*Z*)-4-hydroxy-5-methylene-3-undecylidenedihydrofuran-2-one] (1), and subamolide E [(4*R*,3*E*)-4-hydroxy-5-methylene-3-undecylidenedihydrofuran-2-one] (2), and a new secobutanolide, secosubamolide A {methyl[(2*E*)-2-[(1*S*)-1-hydroxy-2-oxopropyl]-octadec-2-enoate]} (3), were isolated. Also confirmed were 21 known compounds, including the butanolides linderanolide B⁸⁾ and isolinderanolide B¹⁰⁾; one ionone, (+)-abscisic acid¹¹⁾; one sesquiterpenoid, caryophyllene oxide¹²⁾; one triterpenoid, squalene¹³⁾; six benzenoids, vanillin,¹⁴⁾ vanillic acid,¹⁴⁾ *p*-hydroxybenzoic acid,¹⁴⁾ *p*-hydroxybenzaldehyde,¹⁴⁾ syringic acid,¹⁴⁾ and eugenol¹⁵⁾; two polyprenols, ficaprenol-10,¹⁶⁾ and ficaprenol-11¹⁶⁾; two chlorophylls, pheophytin-a,¹⁷⁾ and aristophyll-C¹⁸⁾; four steroids, b-sitosterol,¹⁹⁾ stigmasterol,¹⁹⁾ b-sitosteryl-D-glucoside,²⁰⁾ and stigmasteryl-D-glucoside²⁰⁾; two aliphatic compounds, palmitic acid²¹⁾ and stearic acid²²⁾ were isolated from the leaves of *C. subanenum* by MeOH extraction. In this paper, we report the structural elucidation of 1–3 and explore the cytotoxic effect of 1 and 2 on human colon cancer cells.

Subamolide D was isolated as a pale yellowish liquid. Its molecular formula, C₁₆H₂₆O₃, was established by HR-FAB-MS. The UV absorption at 228 nm was similar to that of linderanolide B,¹⁰⁾ indicating the presence of β -hydroxy- γ -methylene- α,β -unsaturated γ -lactone.²³⁾ The IR spectrum showed absorption bands for a hydroxy group at 3450 cm⁻¹, and an α,β -unsaturated γ -lactone moiety at 1770 and 1680 cm⁻¹. The ¹H-NMR spectrum of 1 was similar to that of linderanolide B,¹⁰⁾ indicating that 1 has the same β -hy-

droxy- γ -methylene- α,β -unsaturated γ -lactone skeleton and the same *Z* geometry of the trisubstituted double bond [δ 6.69 (1H, td, *J*=8.0, 2.0 Hz, H-1')]. The presence of a broad singlet δ 1.26 (14H, br s, H-4'–10') was attributed to protons in an aliphatic chain in 1. The exocyclic olefinic protons appeared at δ 4.67, 4.89 (each 1H, dd, *J*=2.8, 1.5 Hz, H-6a, b), and one hydroxymethine proton was located at δ 5.11 (1H, br s, H-4). The position of these groups was established from the HMBC spectrum. The exocyclic olefinic signals at δ 4.67 and 4.89 were correlated with a quaternary carbon at δ 159.7 (C-5) and a methine carbon at δ 69.1 (C-4). The former carbon also correlated with the signal at δ 5.11 (H-4). The geometry of the alkylidene side chain of this compound was *cis* to the carbonyl group based on the chemical shifts of H-1' (δ 6.69) and H-2' (δ 2.76) in the ¹H-NMR spectrum.⁴⁾ This *cis* geometry was confirmed in the NOESY spectrum, which showed cross-peaks between H-4 and H-1'. The configuration at C-4 in 1 was determined as 4*R* on the basis of the correlations between the [α]_D value [+44.3° (*c*=0.55, CHCl₃)] and the configuration at C-4 for 3-alkylidene-4-hydroxyl-5-methylenebutanolide derivatives.^{24,25)} Thus, the structure of subamolide D was represented as 1 and elucidated as (4*R*,3*Z*)-4-hydroxy-5-methylene-3-undecylidenedihydrofuran-2-one.

Subamolide E, a pale yellowish liquid, also had the molecular formula C₁₆H₂₆O₃, as deduced from HR-FAB-MS. Its spectroscopic data (IR, UV, ¹H- and ¹³C-NMR) were similar to those of 1. A large difference involves H-1', δ 7.09 (td, *J*=8.0, 2.0 Hz) in 2 versus δ 6.69 in 1, suggesting an *E*-configuration for $\Delta^{3(1')}$ in 2. The ¹H-NMR spectrum of 2 was similar to that of isolinderanolide B,¹⁰⁾ indicating that 2 has the same β -hydroxy- γ -methylene- α,β -unsaturated γ -lactone skeleton and the same *E* geometry of the trisubstituted double bond [δ 7.09 (1H, td, *J*=8.0, 2.0 Hz, H-1')]. The presence of a broad singlet, δ 1.26 (14H, br s, H-4'–10') was attributed to protons in an aliphatic chain in 2. The exocyclic olefinic protons appeared at δ 4.72, 4.95 (each 1H, dd, *J*=2.8, 1.5 Hz, H-6a, b) and one hydroxymethine proton was located at δ 5.26 (1H, br s, H-4). The geometry of the alkylidene side chain was *trans* to the carbonyl group on the basis of the chemical shifts of H-1' (δ 7.09) and H-2' (δ 2.47) in the ¹H-NMR spectrum. The downfield shift of H-1' (δ 7.09), compared with that of H-1' (δ 6.69) in subamolide D, can be

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ascribed to the effect of the carbonyl group of a lactone ring. The position of these groups was established from the HMBC spectrum. Its *trans* geometry was also confirmed in the NOESY spectrum, which showed cross-peaks between H-4/H-2'. The stereochemistry of C-4 of **2** was similar to that of subamolide D (**1**).^{24,25} Thus, the structure of **2** was elucidated as (4*R*,3*E*)-4-hydroxy-5-methylene-3-undecylidenedihydrofuran-2-one. The ¹H- and ¹³C-NMR data of **2** were assigned by comparison with the data of **1** (Table 1).

Secosubamolide A (**3**), a faint yellowish liquid, also had a

Chart 1

Table 1. NMR Data of Subamolides D (**1**) and E (**2**) (400 MHz, δ in ppm, *J* in Hz, CDCl₃)

H No.	Subamolide D (1)		Subamolide E (2)	
	δ_C	δ_H	δ_C	δ_H
2	166.1 (s)		167.3 (s)	
3	128.7 (s)		129.3 (s)	
4	69.1 (d)	5.11 (1H, br s)	66.6 (d)	5.26 (1H, br s)
5	159.7 (s)		159.9 (s)	
6	88.9 (t)	4.67 (1H, dd, <i>J</i> =2.8, 1.5) 4.89 (1H, dd, <i>J</i> =2.8, 1.5)	90.0 (t)	4.72 (1H, dd, <i>J</i> =2.8, 1.5) 4.95 (1H, dd, <i>J</i> =2.8, 1.5)
1'	149.6 (d)	6.69 (1H, td, <i>J</i> =8.0, 2.0)	148.5 (d)	7.09 (1H, td, <i>J</i> =8.0, 2.0)
11'	14.3 (q)	0.88 (3H, t, <i>J</i> =7.0)	14.4 (q)	0.87 (3H, t, <i>J</i> =7.0)

molecular formula, C₂₂H₄₀O₄, as deduced from HR-FAB-MS. The UV absorption at 215 nm was similar to that of secokotomolide A,^{3–5} indicating the presence of a secobutanolide skeleton.^{3–5} The IR spectrum of **3** showed characteristic absorption bands due to the presence of hydroxyl (3450 cm⁻¹), ester (1735 cm⁻¹), and ketone (1710 cm⁻¹) groups. The ¹H-NMR spectrum of compound **3** was similar to that of secomahubanolide,²³ with a substitution for the *E* geometry of the trisubstituted double bond [δ 7.08 (1H, t, *J*=7.6 Hz, H-3)] in **3** as compared to the *Z* geometry [δ 6.34 (1H, t, *J*=7.6 Hz, H-3)] in secomahubanolide.²³ Secomahubanolide showed two more methylene units [δ 1.27 (28H, brs, H-6—19)] than **3** [δ 1.25 (24H, brs, H-6—17)] in the side chain. An acetyl and one *O*-methyl group were observed at δ 2.15 (3H, s, H-3') and 3.73 (3H, s, OMe-1), respectively. Compound **3** showed a positive specific rotation $\{[\alpha]_D^{25} + 33.5^\circ$ (*c*=0.15, CHCl₃)}, indicating the 1'*S* configuration similar to that of secoisolancifolide $\{[\alpha]_D^{25} + 102.7^\circ$ (*c*=0.49, CHCl₃)},²³ but contrary to that of secokotomolide A $\{[\alpha]_D^{25} - 52.1^\circ$ (*c*=0.15, CHCl₃)}.³ From the above data, **3** was defined structurally as methyl[(2*E*)-2-[(1*S*)-1-hydroxy-2-oxopropyl]-octadec-2-enoate].

Different natural products such as resveratrol, curcumin and diallyl trisulfide have been studied and were found to induce apoptosis in malignant cells.^{26,27} To elucidate the cyto-

Fig. 2. An Increase in the Number of Cells Arrested in the G₂/M Phase of the Cell Cycle

(A, B) The percentage distribution of colon cells in different phases of the cell cycle was quantified by a ModFit LT software. Mean values of three experiments with duplicate samples are shown. * Significantly different from control, *p*<0.05.

Fig. 1. Effect of Subamolide D and Subamolide E on SW480 Cell Cycle Progression

The cells were exposed to varying concentrations of subamolides for 24 h. The percentage of cells in G₁, S and G₂/M phase was measured by flow cytometry after propidium staining. The data represented the average values from three experiments.

Fig. 3. Time Dependence of Subamolide D and Subamolide E on Cell Cycle and Sub-G1 Fraction Generation

Cells were exposed to DMSO (untreated), 100 μ M subamolide D or 100 μ M subamolide E for different time period (4–24 h) and stained by propidium iodide to determine DNA content. The data represented the average values from three experiments.

Fig. 4. Subamolides (D and E)-Induced Apoptosis

(A, B) Cells were exposed to DMSO ($\circ$), 50 μ M (Δ), 75 μ M ($\square$), and 100 μ M ($\bullet$) subamolide D or subamolide E for different time period (4–24 h) and analyzed by flow cytometry as described in Experimental. Mean values of three experiments with duplicate samples are shown.

toxic mechanism of subamolides (**1**, **2**), the capacity of **1** and **2** to inhibit cell cycle phase distribution was analyzed by flow cytometry.^{28,29} The representative result depicting the effect of subamolides (**1**, **2**) treatment for 24 h on cell cycle progression in SW480 cells is shown in Fig. 1. The marked increase in sub-G1 apoptotic fraction appeared in cells treated with 25–100 μ M **1** or **2** in a concentration-dependent manner. Additionally, incubation with 25, 50 and 75 μ M **1** for 24 h resulted in the accumulation of cells in G2/M phase (25.4, 29.8, 23.0%, respectively) compared with that in control (15.4%) (Fig. 2A). Flow cytometric analysis was also examined under **2** treatment and similar results were obtained (Fig. 2B).

Subsequently, the time-dependent effect of both subamolides were evaluated in the human colorectal cancer cell line. As shown in Fig. 3, untreated cells expressed less than 2.2% of SubG₁. DNA damage did not significantly increase by treatment with 100 μ M of **1** or **2** within 4 h, and the SubG₁ expression was less than 10%. When cells were treated with 100 μ M **1** or **2**, >25%, >78%, and >85% sub-G1 fraction

was noted for treated cells after 8 h, 16 h, and 24 h exposure, respectively. From Fig. 4, **1** and **2** caused DNA damage in a dose- and time-dependent manner. This study is the first to investigate the effect of **1** and **2** on cell cycle phase distribution in human colorectal cancer cells.

Experimental

Melting points were determined using a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were obtained in MeCN using a JASCO V-530 spectrophotometer. The IR spectra were measured on a Hitachi 260-30 spectrophotometer. ¹H (400 MHz, using CDCl₃ as solvent for measurement), ¹³C (100 MHz), DEPT, HETCOR, COSY, NOESY, and HMBG NMR spectra were obtained on a Unity Plus Varian NMR spectrometer. LR-FAB-MS and LR-EI-MS were obtained with a JEOL JMS-SX/SX 102A mass spectrometer or a Quattro GC-MS spectrometer with a direct inlet system. HR-FAB-MS and HR-EI-MS were measured on a JEOL JMS-HX 110 mass spectrometer. Silica gel 60 (Merck, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.20 mm) were used for analytical TLC, and precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.50 mm) were used for preparative TLC. Spots were detected by spraying with 50% H₂SO₄ and then heating on a hot plate. Flow cytometry analysis was performed using a Becton-Dickinson FACS-Calibur flow cytometer. The DNA labeling dye, propidium iodide (PI), was used to determine DNA damage and the cell cycle progression.^{28,29} Data is presented as means $\pm$ standard deviation (S.D.) and analyzed using one-way ANOVA with Scheffe's test. A *p* value of less than 0.05 was considered as statistically significant.

Plant Material The leaves of *C. subavenium* were collected from Wulai Hsiang, Taipei County, Taiwan in May, 2005. A voucher specimen (Cin-namo. 5) was characterized by one of the authors (Y.-R.H.) and deposited in the Basic Medical Science Education Center, Fooyin University, Kaohsiung County, Taiwan.

Extraction and Isolation The air-dried leaves of *C. subavenium* (11.0 kg) were extracted with MeOH (501 $\times$ 6) at room temperature and a MeOH extract (326.5 g) was obtained upon concentration under reduced pressure. The MeOH extract, suspended in H₂O (1 l), was partitioned with CHCl₃ (21 $\times$ 5) to give fractions soluble in CHCl₃ (198.5 g) and H₂O (101.2 g). The CHCl₃-soluble fraction (198.5 g) was chromatographed over silica gel (800 g, 70–230 mesh) using *n*-hexane/EtOAc/MeOH mixtures as eluents to produce five fractions. Part of fraction 1 (9.33 g) was subjected to silica gel chromatography by eluting with *n*-hexane–EtOAc (20 : 1), enriched with EtOAc to furnish five fractions (1-1–1-5). Fraction 1-1 (7.02 g) was

subjected to further silica gel chromatography, eluting with *n*-hexane–EtOAc (100:1) and enriched gradually with EtOAc, to obtain three fractions (1-1-1—1-1-3). Fraction 1-1-1 (2.41 g) was further purified by passage over another silica gel column using *n*-hexane/EtOAc mixtures to obtain stearic acid (42 mg), palmitic acid (57 mg), squalene (23 mg), aristophyllin-C (25 mg), and pheophytin-a (10 mg). Fraction 1-1-2 (1.64 g), eluted with *n*-hexane–EtOAc (30:1), was further separated using silica gel column chromatography and preparative TLC (*n*-hexane–EtOAc (30:1) and gave linderanolide B (32 mg) and isolinderanolide B (45 mg). Fraction 1-1-3 (1.11 g) was subjected to silica gel column chromatography and purified by preparative TLC (thin layer chromatography) to yield *p*-hydroxybenzoic acid (15 mg), *p*-hydroxybenzaldehyde (19 mg), syringic acid (4 mg), vanillin (12 mg), and vanillic acid (19 mg). Fraction 1-2 (3.02 g) was subjected to silica gel chromatography, eluted with *n*-hexane–EtOAc (50:1) and enriched gradually with EtOAc, to obtain three fractions (1-2-1—1-2-3). Fraction 1-2-2 (2.26 g), eluted with *n*-hexane–EtOAc (40:1), was further separated using silica gel column chromatography and preparative TLC (*n*-hexane–EtOAc (30:1) and gave submolide D (1) (34 mg), submolide E (2) (51 mg) and secosubamolide A (3) (12 mg). Part of fraction 2 (8.11 g) was subjected to silica gel chromatography by eluting with *n*-hexane–EtOAc (10:1), then enriched gradually with EtOAc to furnish five fractions (2-1—2-5). Fraction 2-1 (2.02 g) was subjected to silica gel chromatography, eluting with CHCl₃–MeOH (100:1), and enriched gradually with MeOH, to obtain five fractions (2-1-1—2-1-5). Fractions 2-1-1 (0.31 g) and 2-1-2 (0.24 g) were subjected to further silica gel column chromatography and purified by preparative TLC (thin layer chromatography) to yield eugenol (24 mg), and a mixture of ficaprenol-10 and ficaprenol-11 (48 mg). Fraction 2-4 (1.21 g) was subjected to silica gel chromatography, eluting with *n*-hexane–EtOAc (40:1), and enriched gradually with EtOAc to obtain five fractions (2-4-1—2-4-5). Fraction 2-4-3 (1.31 g), eluted with *n*-hexane–EtOAc (10:1), was further separated using silica gel column chromatography and preparative TLC (*n*-hexane–EtOAc (20:1) and gave caryophyllene (57 mg). Fraction 2-5 (6.03 g, *n*-hexane–EtOAc (10:1) was purified by passage over a silica gel column (400 g, 230—400 mesh), using CHCl₃/MeOH, to obtain a mixture of b-sitosterol and stigmasterol (1.34 g). Part of fraction 3 (7.21 g) was subjected to silica gel chromatography by eluting with *n*-hexane–EtOAc (1:1) and enriched gradually with EtOAc, to furnish five fractions (3-1—3-5). Part of fraction 4 (2.42 g) was subjected to silica gel chromatography by eluting with *n*-hexane–EtOAc (1:5), enriched with EtOAc to furnish five further fractions (4-1—4-5). Fraction 4-2 (1.62 g) was purified on a silica gel column (200 g, 230—400 mesh) using CHCl₃/MeOH mixtures to obtain (+)-abscisic acid (21 mg). A mixture of b-sitosteryl-*D*-glucoside and stigmasteryl-*D*-glucoside (50 mg) was recrystallized (MeOH) from fraction 5. The known compounds have been characterized by comparison of their spectroscopic data with literature values.^{10–22)}

Subamolide D [(4*R*,3*Z*)-4-Hydroxy-5-methylene-3-undecylenedihydrofuran-2-one] (1): Pale yellowish liquid. [α]_D²⁵ +44.3° (*c*=0.55, CHCl₃); UV $\lambda_{\max}$ (MeCN) (log ϵ) 228 (4.12) nm; IR (neat) $\nu_{\max}$ 3450 (br, OH), 1770, 1680 (α,β -unsaturated γ -lactone), 1465, 1365, 1090 cm⁻¹; FAB-MS *m/z* 267 [M+H]⁺ (12), 239 (4), 215 (7), 211 (7), 167 (15), 149 (13), 140 (65), 135 (29), 126 (15), 121 (10), 112 (30), 97 (95), 83 (76), 69 (100); HR-FAB-MS *m/z* 267.1962 [M+H]⁺ (Calcd for C₁₆H₂₇O₃, 267.1960); ¹H- and ¹³C-NMR data, see Table 1.

Subamolide E [(4*R*,3*E*)-4-Hydroxy-5-methylene-3-undecylenedihydrofuran-2-one] (2): Pale yellowish liquid. [α]_D²⁵ +31.8° (*c*=0.55, CHCl₃); UV $\lambda_{\max}$ (MeCN) (log ϵ) 226 (4.11) nm; IR (neat) $\nu_{\max}$ 3450 (br, OH), 1770, 1680 (α,β -unsaturated γ -lactone), 1465, 1360, 1090 cm⁻¹; FAB-MS *m/z* 267 [M+H]⁺ (13), 239 (7), 215 (9), 211 (6), 167 (16), 149 (10), 140 (52), 135 (22), 127 (18), 125 (21), 121 (11), 111 (30), 97 (95), 83 (70), 69 (100); HR-FAB-MS *m/z* 267.1961 [M+H]⁺ (Calcd for C₁₆H₂₇O₃, 267.1960); ¹H- and ¹³C-NMR data, see Table 1.

Secosubamolide A {Methyl[(2*E*)-2-[(1*S*)-1-hydroxy-2-oxopropyl]-octadec-2-enolate]} (3): Faint yellowish liquid. [α]_D²⁵ +33.5° (*c*=0.15, CHCl₃); UV $\lambda_{\max}$ (MeCN) (log ϵ) 215 (3.80) nm; IR (neat) $\nu_{\max}$ 3450 (br, OH), 1735 (ester), 1710 (ketone) cm⁻¹; FAB-MS *m/z* 369 [M+H]⁺ (2), 337 (5), 323 (2), 309 (7), 297 (50), 265 (75), 247 (8), 237 (17), 219 (11), 191 (17), 167 (9), 155 (20), 149 (38), 125 (32), 115 (75), 97 (85), 83 (90), 69 (95), 55 (100); HR-FAB-MS *m/z* 369.3005 [M+H]⁺ (Calcd for C₂₂H₄₁O₄, 369.3006); ¹H-NMR (400 MHz, CDCl₃) δ 0.89 (3H, t, *J*=6.8 Hz, H-18), 1.25 (24H, brs, H-6—17), 1.63 (2H, m, H-5), 2.15 (3H, s, H-3'), 2.34 (2H, q, *J*=7.4 Hz, H-4), 3.73 (3H, s, OMe-1), 4.90 (1H, brs, H-1'), 7.08 (1H, t, *J*=7.6 Hz, H-3); ¹³C-NMR (100 MHz, CDCl₃) δ 14.1 (C-18), 22.6 (C-17), 24.4 (C-3'), 28.6 (C-4), 28.7 (C-5), 29.0—30.0 (C-6-15), 31.8 (C-16), 52.2 (OMe-1), 74.1 (C-1'), 129.5 (C-2), 149.4 (C-3), 166.5 (C-1), 206.3 (C-2').

Cell Culture and Drug Treatment The human colon cancer cell line (SW480) was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The SW480 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 100 μ g/ml streptomycin at 37 °C in a 5% CO₂ humidified atmosphere. Stock solutions of 1 and 2 (100 mM) were dissolved in DMSO and various concentrations were prepared in DMEM basal medium with a final DMSO concentration of 0.1%.

Cell Cycle Analysis For determination of cell cycle progression, the cells were analyzed by flow cytometry.^{28,29)} The colon cells were seeded at a density of 5×10⁵ per 60-mm tissue-culture dish. Culture medium was replenished when cells were 80% confluent. Monolayers were treated with various concentrations—of 1 and 2 for 24 h. At the end of treatment, adherent and floating cells were collected, washed twice with PBS, then fixed with PBS–methanol (1:2, volume/volume) solution, and stored at 4 °C for at least 18 h. After centrifugation, cell pellets were stained with PBS containing 50 μ g/ml propidium iodide (PI), and 50 μ g/ml DNase-free RNaseA for 30 min at room temperature in the dark. The samples were analyzed in a Becton-Dickinson system of FACS-Calibur flow cytometer by using CellQuest software. The percentage of cell cycle phases (G₀/G₁, S, and G₂/M) was quantified using a ModFit LT software (BD Biosciences).

Acknowledgements This investigation was supported by a grant from the National Science Council of the Republic of China NSC-94-2320-B-242-009 (C.-Y. Chen).

References

- 1) Liao J. C., "Lauraceae in Flora of Taiwan," 2nd. ed., Vol. 2, Editorial Committee of the Flora of Taiwan, Taipei, 1996, pp. 443—483.
- 2) Chen C. Y., Liu T. Z., Chen C. H., Wu C. C., Cheng J. T., Yiin S. J., Shih M. K., Wu M. J., Chern C. L., *Food Chem. Toxicol.*, **45**, 1268—1276 (2007).
- 3) Chen C. Y., Chen C. H., Wong C. H., Liu Y. W., Lin Y. S., Wang Y. D., Hsui Y. R., *J. Nat. Prod.*, **70**, 103—106 (2007).
- 4) Chen C. H., Lo W. L., Liu Y. C., Chen C. Y., *J. Nat. Prod.*, **69**, 927—933 (2006).
- 5) Chen C. Y., *Nat. Prod. Commun.*, **1**, 453—455 (2006).
- 6) Hsieh T. J., Chen C. H., Lo W. L., Chen C. Y., *Nat. Prod. Commun.*, **1**, 21—25 (2006).
- 7) Chen C. Y., Hsieh S. L., Hsieh M. M., Hsieh S. F., Hsieh T. J., *Chin. Pharm. J.*, **56**, 141—146 (2004).
- 8) Kuo P. L., Chen C. Y., Hsu Y. L., *Cancer Res.*, **67**, 7406—7420 (2007).
- 9) Chen C. Y., Hsu Y. L., Chen Y. Y., Hung J. Y., Huang M. S., Kuo P. L., *Eur. J. Pharmacol.*, **574**, 94—102 (2007).
- 10) Seki K., Sasaki T., Wano S., Haga K., Kaneko R., *Phytochemistry*, **40**, 1175—1181 (1995).
- 11) Sasaki K., Takahashi K., Nukano T., *Phytochemistry*, **48**, 8229—8238 (1992).
- 12) Thebtaranonth C., Thebtaranonth Y., Wanauppathamkul S., Yuthavong Y., *Phytochemistry*, **31**, 2645—2648 (1995).
- 13) Cheng M. J., Tsai I. L., Chen I. S., *J. Chin. Chem. Soc.*, **48**, 235—239 (2001).
- 14) Chen C. Y., Chang F. R., Teng C. M., Wu Y. C., *J. Chin. Chem. Soc.*, **46**, 77—86 (1999).
- 15) Chen L. H., Kuo Y. H., *J. Chin. Chem. Soc.*, **32**, 169—172 (1985).
- 16) Tsai I. L., Jeng Y. F., Jayaprakasam B., Chen I. S., *Chin. Pharm. J.*, **53**, 291—301 (2001).
- 17) Nakatani Y., Ourisson G., Beck J. P., *Chem. Pharm. Bull.*, **29**, 2261—2269 (1981).
- 18) Chan Y. Y., Leu Y. L., Wu T. S., *Chem. Pharm. Bull.*, **47**, 887—889 (1999).
- 19) Kojima H., Sato N., Hatana A., Ogura H., *Phytochemistry*, **29**, 2351—2355 (1990).
- 20) Li J., Kadota S., Kawada Y., Hattori M., Xu G. J., Namba T., *Chem. Pharm. Bull.*, **40**, 3133—3137 (1992).
- 21) Addae-Mensah I., Achenbach H., *Phytochemistry*, **24**, 1817—1819 (1985).
- 22) Dupont M. P., Llabres G., Delaude C., Tchissambou L., Gastmans J. P., *Planta Med.*, **63**, 282—284 (1997).
- 23) Cheng M. J., Tsai I. L., Lee S. J., Jayaprakasam B., Chen I. S., *Phytochemistry*, **66**, 1180—1185 (2005).
- 24) Martinez J. C., Yoshida M., Gottlieb O. R., *Phytochemistry*, **20**, 459—464 (1981).

- 25) Anderson J. E., Ma W., Smith D. L., Chang C. J., McLaughlin J. L., *J. Nat. Prod.*, **55**, 71—83 (1992).
- 26) Surh Y. J., Hurh Y. J., Kang J. Y., Lee. G., Kong G., Lee S. J., *Cancer Lett.*, **140**, 1—10 (1999).
- 27) Xiao D., Herman-Antosiewicz A., Antosiewicz J., Xiao H., Brisson M., Lazo J. S., Singh S. V., *Oncogene*, **24**, 6256—6268 (2005).
- 28) Jan C. R., Chen C. H., Wang S. C., Kuo S. Y., *Cell. Signal.*, **17**, 847—855 (2005).
- 29) Ho C. M., Kuo S. Y., Chen C. H., Huang J. K., Jan C. R., *Cell. Signal.*, **17**, 837—845 (2005).