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Cytotoxicity of extractives from *Taiwania cryptomerioides* heartwood

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Abstract

The cytotoxicity of the dominant lignans and sesquiterpenoids from Taiwania (*Taiwania cryptomerioides* Hayata) was investigated. Three human tumor cells including A-549 lung carcinoma, MCF-7 breast adenocarcinoma and HT-29 colon adenocarcinoma were selected to illustrate the structure–cytotoxicity relationships of Taiwania's dominant compounds. Taiwanin A, taiwanin E and dimethylmatairesinol exhibited significant cytotoxicity against three human tumor cells. Among them, taiwanin A possesses the strongest cytotoxic activity. In addition, the morphology-based evaluation, flow cytometric analysis, and DNA fragmentation assays demonstrated that the tumor cell death induced by taiwanin A was due to apoptosis. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Taiwania cryptomerioides; Lignan; Sesquiterpenoid; Taiwanin A; Taiwanin E; Dimethylmatairesinol; Cytotoxicity; Apoptosis

1. Introduction

Many compounds isolated from woody plants are of great potential as medicines for disease control or as raw materials for synthesizing useful analogues in industry. Taxol and podophyllotoxin (Hartwell, 1976), for example, are well-known antitumor compounds isolated from conifers. In the search for natural antitumor agents, cytotoxicity-based isolation and purification processes are widely used. In addition, it is well known that the natural durability of a tree is often dependent on the type and quantity of the chemical constituents present.

Taiwania (*Taiwania cryptomerioides* Hayata) (Taxodiaceae) is an endemic tree that grows at elevations from 1800 to 2600 m in Taiwan's central mountains. The heartwood of Taiwania is yellowish-red with distinct purplish-pink streaks. Taiwania timbers are well-recognized for their decay resistance and excellent durability in Taiwan. With regard to the investigation of Taiwania extractives which have been reported by many researchers, nine lignans, eight flavones, twenty-one sesquiterpenoids, eighteen diterpenoids, three lipids, two cyclitols, and one steroid were isolated from Taiwania (Wang et al., 1997). Recently, we investigated the contribution of chemical constituents to the wood properties, including the photodiscoloration and antifungal activities in Taiwania heartwood (Wang et al., 1998; Su et al., 1998; Chang et al., 1999a,b,c, 2000). Our research has revealed that lignans and sesquiterpenoids are the dominant constituents in Taiwania. Lignans are distributed widely in the plant kingdom. The breadth of their biological activities has recently attracted great attention. Much interest has been focused on their effectiveness as antineoplastic agents and research in this area has revealed several modes of action by which they can regulate the growth of mammalian cells (MacRae and Towers, 1984; Ayres and Loike, 1990;

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Ward, 1993, 1995, 1997; Charlton, 1998). Taiwanin E methyl ether isolated from *Justicia ciliata* exhibited significant in vitro cytotoxicity against human cervical carcinoma (Day et al., 1999). In this paper, the cytotoxicity of lignans **1–8** and sesquiterpenoids **9–11** isolated from Taiwania heartwood and the possible mechanism for cell-growth suppression of cancer cells were investigated. Previously, α-cadinol **9** isolated from Taiwania showed selectivity for the human colon tumor cell lines (He et al., 1997).

2. Results and discussion

Although many chemical constituents have been isolated from Taiwania, their bioactivity has hardly been investigated. In our previous studies (Su et al., 1998; Wang et al., 1998; Chang et al., 1999a,b,c, 2000), eight lignans and three sesquiterpenoids, including taiwanin A **1**, savinin **2**, taiwanin C **3**, taiwanin E **4**, helioxanthin **5**, hinokinin **6**, arctigenin **7**, dimethylmatairesinol **8**, α-cadinol **9**, T-cadinol **10**, and T-muurolol **11**, were isolated; their structures are shown in Fig. 1.

To study the cytotoxicity of the major chemical ingredients of Taiwania, we first assessed the effects of eight lignans and three sesquiterpenoids on the viability of three human tumor cells including A-549 lung carcinoma, MCF-7 breast adenocarcinoma, and HT-29 colon adenocarcinoma. The results are summarized in Table 1. ED_{50} values of less than 4 μg/ml for pure compounds are considered to be active in the search of antitumor compounds (Suffness and Pezzuto, 1991). Although the cytotoxicity of lignans and/or sesquiterpenoids of Taiwania showed little differential cytotoxicity, some of them, including taiwanin A **1**, taiwanin E **4** and dimethylmatairesinol **8**, exhibited significant cytotoxicity against all human tumor cells tested. In addition, savinin **2** for MCF-7 and HT-29, α-cadinol **9** for HT-29, and T-muurolol **11** for A-549 and MCF-7, passed the meaningful dosage criterion ($ED_{50} < 4$ μg/ml). Overall, taiwanin A **1** is the most cytotoxic. The ED_{50} values of taiwanin A **1** against A-549, MCF-7, and HT-29 were 0.2, 0.2, and 0.1 μM, respectively.

Table 1
Cytotoxicity of lignans and sesquiterpenoids isolated from *Taiwania cryptomerioides* heartwood (ED_{50} values in μM)

Compounds	Cell-line		
	A-549	MCF-7	HT-29
1	0.2 (0.4)[a]	0.2 (0.5)	0.1 (0.3)
2	6.7 (19.1)	0.5 (1.5)	1.5 (4.3)
3	5.8 (16.7)	4.1 (11.7)	14.3 (41.1)
4	1.2 (3.4)	0.5 (1.4)	0.6 (1.5)
5	11.3 (32.4)	12.6 (36.1)	13.4 (38.6)
6	9.2 (26.1)	4.9 (13.8)	4.0 (11.4)
7	5.6 (15.1)	10.4 (27.9)	9.6 (26.0)
8	1.9 (5.0)	1.8 (4.7)	1.4 (3.5)
9	3.1 (14.4)	2.5 (11.1)	0.7 (3.0)
10	5.4 (24.5)	2.5 (11.2)	7.9 (35.7)
11	3.2 (14.7)	0.6 (2.7)	1.8 (8.0)
Adriamycin	0.01 (0.02)	0.1 (0.1)	0.1 (0.1)

[a] ( ): ED_{50} values in μg/ml.

There are two types of lignans, namely the dibenzyl-γ-butyrolactone type and arylnaphthalide type, that have been isolated from Taiwania heartwood. According to MacRae and Towers (1984), the following features of a lignan, (1) a five membered lactone ring; (2) a 3,4,5-trimethoxyphenyl group; (3) a methylenedioxy group; and (4) two substituted phenyl groups separated by a four-carbon linked chain (i.e. C6–C4–C6, 8,8-linked), might be important structural characteristics which contribute to the activity of lignans as antitumor agents. All of the lignans that were evaluated in this study possess the features mentioned by MacRae and Towers. For the dibenzyl-γ-butyrolactone type of lignans (taiwanin A **1**, savinin **2**, hinokinin **6**, arctigenin **7**, and dimethylmatairesinol **8**), an unsaturated double bond between C_7–C_8 and/or C_7'–C_8' (taiwanin A **1** and savinin **2**) is associated with the stronger cytotoxicity noted. Moreover, the number of double bonds is proportional to cytotoxic potency: taiwanin A **1** (two double bonds) was stronger than savinin **2** (one double bond). Although savinin **2** possesses modest cytotoxcity in this study, it showed no activity against the human nasopharyngeal carcinoma and murine lymphocytic leukemia (Badawi et al., 1981). This indicates that savinin **2** has different selectivity for different cell lines. The other three dibenzyl-γ-butyrolactone type lignans had lower cytotoxicity than taiwanin A **1** and savinin **2**. In addition, the presence of the two 3,4-dimethoxyphenyl groups in lignans may increase the cytotoxicity, as dimethylmatairesinol **8** (3,4-dimethoxy phenyl groups) was stronger than arctigenin **7** (one 3,4-dimethoxy phenyl group) and hinokinin **6** (two methylenedioxy groups).

With regard to the cytotoxicity of the arylnaphthalide type lignans isolated from Taiwania heartwood, including taiwanin C **3**, taiwanin E **4**, and helioxanthin **5**, taiwanin E **4** showed the strongest cytotoxicity in this group (Table 1). Taiwanin C **3** and helioxanthin **5** were inactive against the human tumor cell lines used in this study. It appears that the hydroxyl group at the C_7 position enhances cytotoxicity. A similar result was observed by Kelleher (Kelleher, 1978) in a study of the structure–activity relationships of podophyllotoxin analogues. This study suggested that the polarity of the C_7 substituent, rather than its size, is a predominant factor for antitumor activity.

The main cadinanes in Taiwania, including T-cadinol **10**, T-muurolol **11**, and α-cadinol **9**, were recently isolated and tested for their antifungal effectiveness (Chang et al., 2000). The order of antifungal index of the three

compounds for both *Coriolus versicolor* and *Laetiporus sulphureus* was α-cadinol **9** > T-cadinol **10** > T-muurolol **11**. α-cadinol **9** completely inhibited the growth of *C. versicolor* and *L. sulphureus* at the level as low as 100 ppm. Comparison of their configurations revealed that cadinane-type sesquiterpenoids with an equatorial hydroxyl group at C-9 and a *trans* ring junction, exhibited the strongest antifungal activity. However, the relationship between the configuration and the cytotoxicity of these cadinanes was less obvious (Table 1). Alpha-cadinol **9** was modestly active against HT-29. A similar observation was reported by He and coworkers (He et al., 1997). T-Muurolol **11** was modestly active against both the HT-29 and MCF-7 cell lines. No significant cytotoxicity against A-549 was found with the cadinanes.

Based on the distinct morphological and biochemical changes of the dying cells, two modes of cell death have been described, apoptosis and necrosis. A number of anticancer drugs have been shown to induce apoptosis in cancer cells (Park et al., 1997). Apoptosis is a genetically controlled response by which cells commit suicide and has become a major focus in the study of cancer biology (White, 1996). It is thus important to establish the mode of cell death induced by taiwanin A **1**. Fig. 2 shows the morphological changes in MCF-7 cells examined 24 h after the addition of 0 μM (control, Fig. 2A), 0.5 μM (Fig. 2B), and 2 μM (Fig. 2C), 4 μM (Fig. 2D) of taiwanin A **1**. With dosage above 2 μM, the tumor cells show that chromatin condensation and apoptotic bodies (such as the one indicated by a white arrow in Fig. 2D) were widespread throughout the entire population. These morphological changes suggested that taiwanin A **1** induced apoptotic cell death in MCF-7 cells (Kaufmann, 1997; Wang and Wang, 1999). In addition to the morphology-based identification, flow cytometric analyses (Fig. 3) indicated that 44.3% of cells (sub-G1 peak) experienced apoptosis when MCF-7 cells were treated for 24 h with 2 μM of taiwanin A **1**. In the control cells, only a minor fraction of the cell population (4.6%) experienced apoptosis. Finally, DNA fragmentation assay was used to confirm that the mode of cell death induced by taiwanin A **1** on MCF-7 was apoptosis. Fig. 4 shows the DNA fragmentation induced by various concentrations of taiwanin A **1**. On the basis of results mentioned above, it was determined that the tumor cell death induced by taiwanin A **1** was due to apoptosis. The mechanisms for inducing apoptosis in tumor cells require further elucidation in future studies.

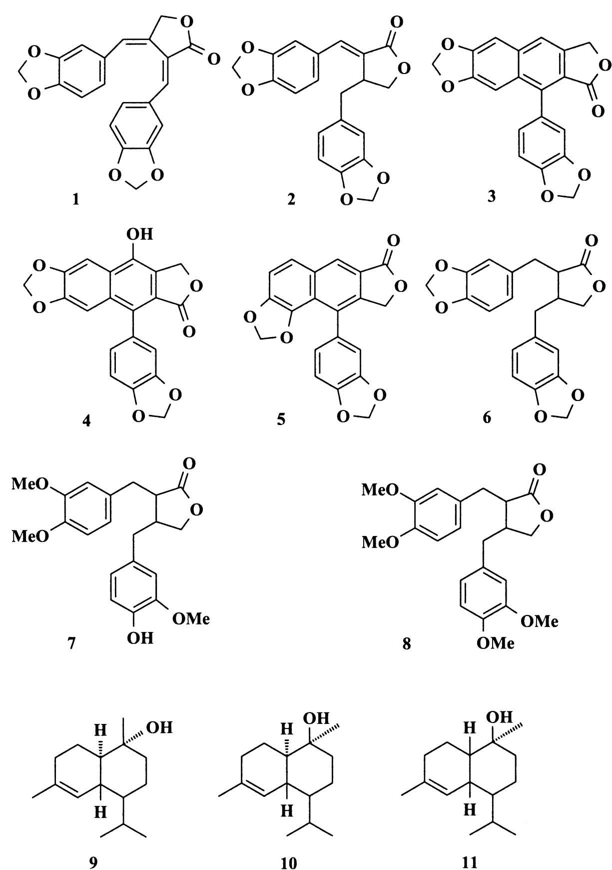

Fig. 1. Structures of eleven compounds from the heartwood of Taiwania.

3. Experimental

3.1. General

HPLC (high performance liquid chromatography) was performed with a Jasco model PU980 pump equipped with a Jasco RI-930 RI detector and Hibar Lichrosorb Si 60 (25×1 cm i.d.) column. FTIR spectra were recorded

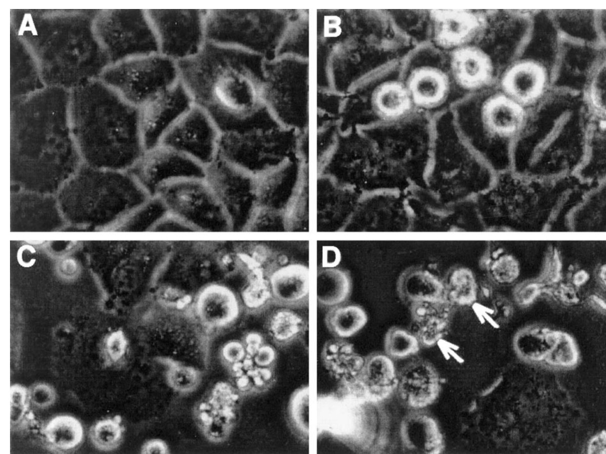

Fig. 2. Morphological changes in the MCF-7 cells examined 24 h after the addition of 0 μM (A), 0.5 μM (B), 2 μM (C), and 4 μM (D) of taiwanin A to the media. MCF-7 cells were plated in a 96-well plate and photographed under a phase contrast microscope (×150). White arrows indicate apoptotic bodies.

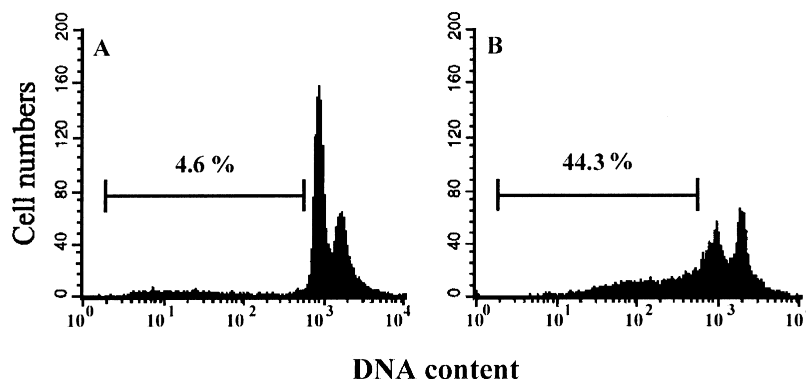

Fig. 3. Flow cytometric analysis of untreated MCF-7 cells (A) and 2 μM taiwanin A treated cells (B) after 24-h treatment.

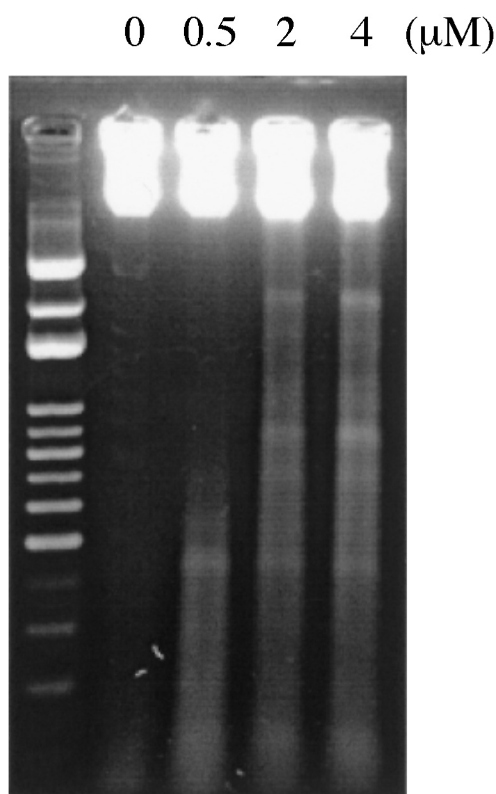

Fig. 4. DNA fragmentation of MCF-7 cells induced by various concentrations of taiwanin A after treating for 24 h.

on a Bio-Rad model FTS-40 spectrophotometer. MS were obtained on a Finnigan MAT-958 mass spectrometer. ^{13}C and ^{1}H NMR spectra were recorded on a Bruker Advance- 300 MHz FT-NMR spectrometer.

3.2. Extraction and isolation

The wood used was cut from a twenty-seven-year-old *Taiwania cryptomerioides* tree growing at the forest land in the Experimental Forest of National Taiwan University. Taiwania heartwood chips were prepared from freshly cut wood. Following our previous isolation and purification procedures (Wang et al., 1998; Su et al., 1998; Chang et al., 1999a,b,c, 2000), eight lignans and three sesquiterpenoids were isolated. Briefly, the air-dried heartwood chips (5.7 kg) were exhaustively extracted with methanol (MeOH). The extractives were condensed to ca. 286.4 g, and extracted successively with *n*-hexane (n-C_6H_{14}), chloroform ($CHCl_3$), ethyl acetate (EtOAc), and methanol (MeOH) to yield the n-C_6H_{14}, $CHCl_3$, EtOAc, MeOH soluble fractions, and MeOH insoluble fraction. Taiwanin A **1** was separated from the n-C_6H_{14} fraction during removal of the solvent (Chang et al., 1999b). Savinin **2**, taiwanin C **3**, taiwanin E **4**, helioxanthin **5**, hinokinin **6**, arctigenin **7**, and dimethylmatairesinol **8** were isolated and purified from the $CHCl_3$ soluble fraction by flash column chromatography and semi-preparative HPLC (Wang et al., 1998; Su et al., 1998; Chang et al., 1999a,c). Further chromatographic separation with Si-gel column and semi-preparative HPLC, gave α-cadinol **9**, T-cadinol **10**, and T-muurolol **11**, obtained from the n-C_6H_{14} soluble fraction (Chang et al., 2000). The structures of these compounds isolated from Taiwania were confirmed by FTIR, MS, and NMR analyses.

3.3. Tumour cell growth inhibition assay

A microassay for cytotoxicity was performed using a MTT (4,5-dimethylthiazol-2-yl-2,5,-diphenyltetrazolium bromide) assay (Alley et al., 1988; Song et al., 1994). Briefly, exponentially growing cells (human tumor cells including A-549 lung carcinoma, MCF-7 breast adenocarcinoma, and HT-29 colon adenocarcinoma) (1×10^5 cells/ml) were seeded into a 96 well plate in triplicate and preincubated for 12 h in order to perform cell attachment. The medium was then aspirated and 100 μl fresh medium containing various concentrations of test compound were added to the cultures. The cells were incubated with each compound at 37°C for 6 days under humidified air containing 5% CO_2. Cell survival was

evaluated by adding 10 μl tetrazolium salt solution (1 mg MTT/ml in PBS). After 4 h of incubation at 37°C, 100 μl DMSO were added to dissolve the precipitates of reduced MTT. Microplates were then shaken for 15 min, and the absorbance was determined at 550 nm in a multiwell scanning spectrophotometer.

3.4. Fluorescence microscopy

For fluorescence microscopic measurement, MCF-7 cells were collected and fixed in MeOH/Me_2CO (1/3, V/V) solution for 5 min and washed with PBS (phosphate-buffered saline). Then fixed cells were stained with 0.1 ng/ml Hoechst 33258 for 10 min in the dark. Cells were observed and photographed under a Nikon fluorescence microscope.

3.5. Flow cytometry analysis

Trypsinized or pelleted MCF-7 cells were washed with ice-cold PBS followed by fixing in 70% EtOH at −20°C for at least 1 h. After fixation, cells were washed twice and then incubated in 0.5 ml of 0.5% triton x−100/PBS at 37°C for 30 min with 1 mg/ml of RNase A, and followed by staining with 0.5 ml of 50 mg/ml propidium iodide for 10 min. Fluorescence emitted from the propidium iodide-DNA complex was quantitated after laser excitation of the fluorescent dye by a FACScan flow cytometry (Becton Dickinson, CA).

3.6. DNA fragmentation assay

Both floating and adherent cells were collected. After washing with PBS twice, cells were successively lysed in 100 μl lysis buffer (50 mM Tris (pH 8.0), 10 mM EDTA, 0.5% sodium lauryl sarkosinate and 1 mg/ml proteinase K) at 56°C for 3 h and then treated with 0.5 mg/ml of RNase A at 56°C for an additional 1 h. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1). The DNA sample in a loading buffer [50 mM Tris, 10 mM EDTA, 1% (W/V) low melting point agrarose, 0.25% (W/V) bromophenol] was loaded onto persolidified, 2% (W/V) agarose gel containing 0.1 μg/ml ethidium bromide. Agarose gels were run at 50 V for 90 min in TBE buffer (108 g Tris + 55 g boric acid + 40 ml of 0.5 M EDTA, made up to 1 l with water). Finally, gels were observed and photographed under UV light.

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