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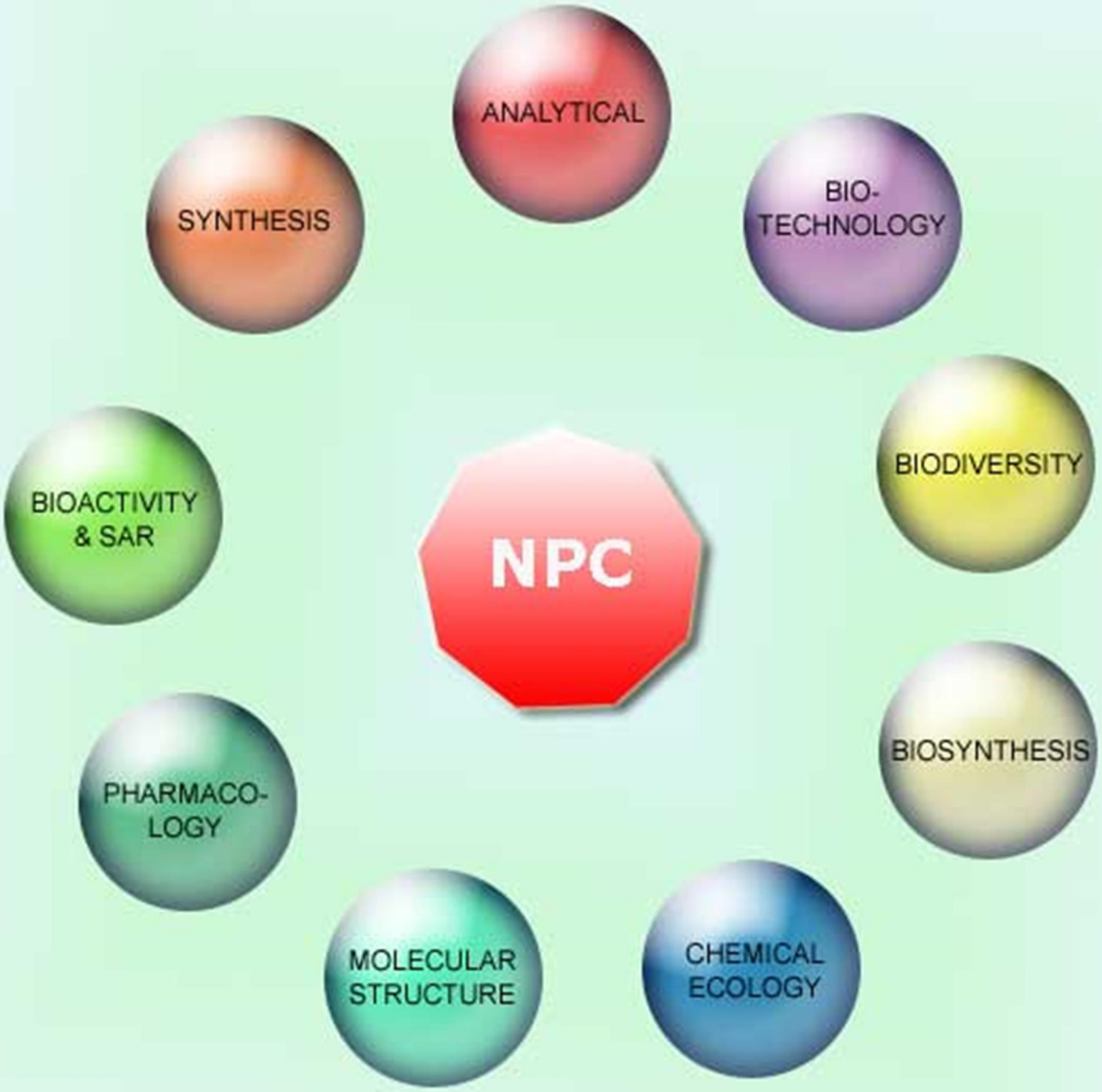

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Anti-proliferation Effect on Human Breast Cancer Cells *via* Inhibition of pRb Phosphorylation by Taiwanin E Isolated from *Eleutherococcus trifoliatus*

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Eleutherococcus trifoliatus has been used as a folk medicine since ancient times, especially as refreshing qi medicines. In our current study, taiwanin E, which possesses strong cytotoxicity, was isolated from the branches of *E. trifoliatus* by using a bioactivity guided fractionation procedure. Taiwanin E presented a potent anti-proliferation activity on the growth of a human breast adenocarcinoma cell line (MCF-7), with an IC_{50} value for cytotoxicity of 1.47 μM. Cell cycle analysis revealed that the proportion of cells in the G_0/G_1 phase increased in a dose-dependent manner (from 79.4% to 90.2%) after 48 h exposure to taiwanin E at a dosage range from 0.5 to 4μM. After treatment with taiwanin E, phosphorylation of retinoblastoma protein (pRb) in MCF-7 cells was inhibited, accompanied by a decrease in the levels of cyclin D_1, cyclin D_3 and cyclin-dependent kinase 4 (cdk4) and cdk6; in addition, there was an increase in the expression of cyclin-dependent kinase inhibitors $p21^{WAF-1/Cip1}$ and $p27^{Kip1}$. The results suggest that taiwanin E inhibits cell cycle progression of MCF-7 at the G_0/G_1 transition.

Keywords: *Eleutherococcus trifoliatus*, Araliaceae, Taiwanin E, Cytotoxicity, Cell cycle, G_1 arrest.

Eleutherococcus trifoliatus (L.) S.Y. Huvar. *trifoliatus* (Syn. *Acanthopanax trifoliatus*), adeciduous shrub or climber with prickles on branches and petioles, belongs to the Araliaceae family. The roots, bark, and leaves of this plant are used as a folk medicine foreither prevention or amelioration of tumors and aging, and for improving cardiovascular function [1-4]. A number of fatty acids, steroids, lupane-triterpene carboxylic acids, lupane-triterpene glycosides, kaurane-type diterpene glycosides, and phenyl-propanoid glycosides have been reported [5-13]. The essential oil of *E. trifoliatus* contained, as its main components, α-pinene, sabinene, terpinen-4-ol, β-pinene, and *p*-cymene [14]. Sithisarn and Jarikasem reported that the leaf aqueous extract of *E. trifoliatus* showed a high level of antioxidant activity and contained high contents of both phenolic and flavonoid compounds, but they did not identify any of them [15].

Recently, taiwanin E, a strong cytotoxic lignan, was isolated from the branches of *E. trifoliatus* by using a bioactivity guided fractionation procedure. The content of taiwanin E in the crude extract was determined by HPLC to be 23 mg/g. According to the MTT assay, when human breast adenocarcinoma cells (MCF-7) were treated with taiwanin E at dosages of 1.25 - 10 μM, a dose-dependent decrease of cell viability was observed. The IC_{50} value of taiwnin E was 1.47 μM, whereas that of plumbagin, which was used as a positive control, was 0.26 μM. To examine the mechanism responsible for taiwanin E mediated cell growth inhibition, cell cycle distribution was evaluated using flow cytometric analysis.

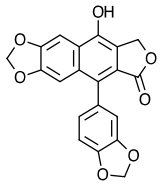

Figure 1: The cytotoxicity compound, taiwanin E, obtained from *E. trifoliatus*.

When MCF-7 cells were treated with 0, 0.5, 1, 2 and 4 μM of taiwanin E for 48 h, the percentage of G_1 cells was 76.8, 79.4, 82.4, 85.7 and 90.2, respectively (Table 1). Our results indicated that treating cells with taiwanin E caused a significant inhibition of cell cycle progression in the MCF-7 cell line, showing a clear increase in the percentage of cells in the G_1 phase along with a decrease in the S phase cells when compared with the control, reflecting that cell-cycle progression is impeded at the G_1 phase.

By contrast, protein levels of cdk2 and cyclin E were found to be unaltered by taiwanin E treatment. In addition, exposure of cells to taiwanin E resulted in a dose-dependent increase in the levels of tumor suppressorp$21^{Waf1/Cip1}$and $p27^{Kip1}$, which are the cyclin-dependent kinase inhibitors belonging to the Cip/Kip family ($p21^{Waf1/Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$). According to previous studies, increasing $p21^{Waf1/Cip1}$ and $p27^{Kip1}$expression causes cell cycle arrest at the G_1 phase, and, therefore, cell proliferation will be suppressed. In this situation, cells are efficiently exited from the *cell cycle* and

Table 1. Cell cycle distribution of MCF-7 cells treated with taiwanin E.

Taiwanin E (μM)	Percentage of cells in		
	G_1	G_2/M	S
0.0 (Control)	76.80	9.76	13.40
0.5	79.40	10.20	10.40
1.0	82.40*	9.51	8.13*
2.0	85.70*	7.89	6.45*
4.0	90.20*	5.74	4.10*

* $P < 0.05$ versus control by analysis of variance with Dunnett's post hoc test.

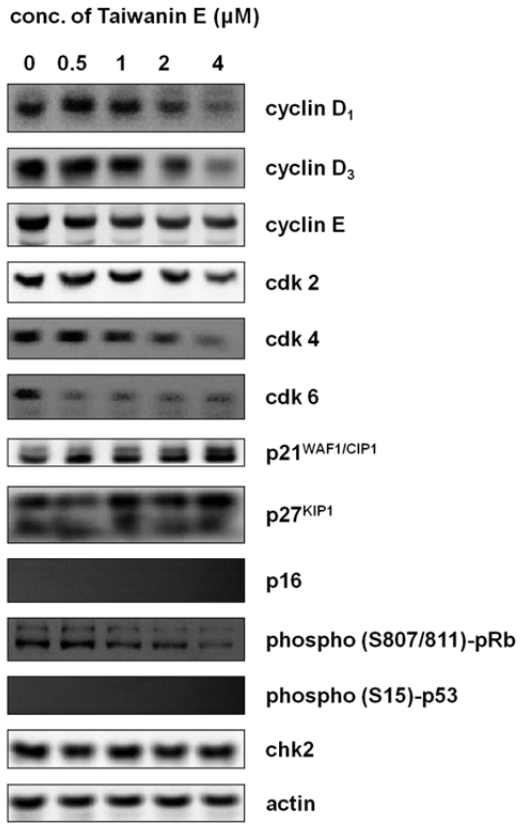

Figure 2: The expression of cell cycle-related proteins in MCF-7 cells treated with various concentrations of taiwanin E as determined by Western blotting.

enter a *quiescent* state, and cell division will be inhibited [16,17]. We did not find p16, a cdk4/cdk6 inhibitor, in MCF-7 cells, which is in agreement with the deletion detected of the MTS1 gene that encodes p16 in this cell line [18-19]. The level of pRb (phosphorylation at Ser807/811), which controls progression through G_0 within the G_1 phase of the cell cycle, was reduced in MCF-7 cells by taiwanin E treatment. pRb binds to and represses the transcription factor E2F during early and mid-G_1 phase [20]. During G_1/S transition, cdk4/6 release from their inhibitory proteins INK4 family and phosphorylate pRb [21-22]. Phosphorylated pRb releases E2F, permitting the transcription of the G_1/S transition genes [23-24]. In taiwanin E treated cells, inhibition of cdk activity by a combination of cdk4/6 and cyclin D_1/D_3 downregulation, and $p21^{Waf1/Cip1}$and $p27^{Kip1}$ upregulation, prevented pRb phosphorylation. It would, therefore, be expected to prevent G_1/S transition gene transcriptions by E2F. However, inactivated pRb in the quiescent cell promotes E2F1, inducing apoptosis. pRB regulated E2F1-induced apoptosis is actually distinguishable from its transcriptional control of other E2F proteins [25].

Expression of $p21^{WAF-1/Cip1}$ can be regulated by either p53-dependent or p53-independent mechanisms [26–29]. Previous studies indicated that G_1 arrest could also be achieved by p53, which is phosphorylated on Ser15 by ATM/ATR and on Ser20 by Chk2, through induction of $p21^{WAF-1/Cip1}$ transcription [30]. Taiwanin E did not induce p53 phosphorylation and change Chk2 expression in MCF-7 cells, which suggests that p21 is upregulated by a p53-independent mechanism. It could be concluded that taiwanin E possesses a potent cytotoxic activity against MCF-7 cells. The anti-proliferation effect of taiwanin E on MCF-7 cells occurs through downregulation of cyclin D_1/D_3, cdk4/6, and upregulation of $p21^{WAF-1/Cip1}$ by a p53-independent mechanism. Further study is needed to evaluate the antitumor activity.

Experimental

Materials: *E. trifoliatus* was collected in May 2012 from Nantou County, Taiwan, and was identified by Dr Yen-Hsueh Tseng (NCHU). The voucher specimen was deposited in the herbarium of the same university.

General: UV and IR spectra were recorded on Jasco V-550 and Bio-Rad FTS-40 spectrometers, respectively. Electrospray ionization-mass spectrometric (ESIMS)and high-resolution electron-impact mass spectrometric(HREIMS) data were collected with a Finnigan MAT-95S mass spectrometer, and NMR spectra were recorded with Bruker Avance 500 and 300 MHz FT-NMR spectrometers, at 500 MHz (^{1}H) and 75 MHz (^{13}C). $CDCl_3$ was used for NMR analysis. Taiwanin E was dissolved in MeOH and quantification was based on the measured integration area applying the calibration equation. The concentration of taiwanin E used for calibration was 0.025–1.0 mg/mL. The linear regression equation, y=25.249 x + 156.91, revealed a good linearity (R^2= 0.99812).

Extraction and purification: Air-dried branches of *E. trifoliatus* (350 g) were extracted with EtOH (10 L) at ambient temperature and concentrated under vacuum to yield the EtOH extract (40.13 g). This was partitioned between EtOAc-H_2O to give EtOAc-soluble (18.4 g) and H_2O-soluble fractions. The EtOAc-soluble fraction, which displayed potent cytotoxicity (IC_{50} = 30.3 μg/mL), was further chromatographed over silica gel (4 × 30 cm; 60–80 mesh; Merck) eluted with *n*-hexane and a gradient of *n*-hexane-EtOAc (100:0; 95:5; 90:10; 85:15; 80:20; 75:25; 70:30; 65:35; 60:40; 50:50; 40:60; 30:70; 20:80; 10:90; 0:100, each 2 L). The eluent was collected in constant volumes (each 500mL), and combined into 18 fractions based on TLC properties. Fraction 9 (obtained with *n*-hexane:EtOAc = 89:11, amount 5.3 g) displayed the strongest cytotoxicity (IC_{50} = 14.6μg/mL) and was further separated by HPLC using a normal-phase column (250 × 10 mm, 5 μm, Phenomenex Co.) with a mixture of *n*-hexane:EtOAc = 75:25 as eluent at a flow rate of 3 mL/ min to obtain taiwanin E(retention time 21.9 min).The structure of taiwanin E was elucidated and confirmed by spectroscopic analysis [31].

Cell culture: MCF-7 (human breast adenocarcinoma, BCRC 60436) was purchased from BCRC (Bioresource Collection and Research Center), Food Industry Research, and Development Institute, Taiwan. MCF-7 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, and 1 mM sodium pyruvate, and maintained at 37°C in 5% CO_2. All cells (1 × 103 per well) were seeded in 96-well plates and incubated for 24 h, and different dosages of taiwanin E were added to each well in triplicate for 5 days. The cell viability was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [32]. Plumbagin (Sigma) was used as positive control.

Cell cycle analysis: MCF-7 cells, seeded in a 60 mm dish (2×10^5 cells/dish), were treated with 0, 0.5, 1, 2, and 4μMtaiwanin E for 48 h. Subsequently, cells were trypsinized, collected with ice-cold PBS and resuspended in 1 mL PBS. Cells were then fixed by the addition of 3 mL ice-cold 95 % ethanol at -20°C overnight. The cell pellets were collected by centrifugation and rinsed with ice-cold PBS. Cells were stained with 1 mL of 50 μg/mL propidium iodide (PI) in RNase containing buffer (0.5% Triton X-100 in PBS and 0.5 mg/mL RNase A) for 30 min. Fluorescence emitted from the PI-DNA complex was quantified after excitation of the fluorescent dye by flow cytometry (Cytomics FC 500, Beckman Coulter).

Protein expression analysis: The protein expression after treatment with taiwanin E was determined by Western blotting assay. Briefly, MCF-7 cells incubated in 100 mm culture dishes (1×10^6 cells per dish) were treated with taiwanin E at dosages of 0, 0.5, 1, 2, and 4 μM for 48 h. 40 μg of total cell proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane. Detection was performed by immunostaining using specific primary antibodies and horseradish peroxidase-conjugated anti-IgG antibody. The proteins were detected by chemiluminescence (ECL, Pierce Biotechnology, Inc.). The following antibodies were used for the Western blots: rabbit polyclonal antibodies to $p27^{Kip1}$, cdk2, cdk4, phosphor (Ser807/811)-pRb, and Chk2; mouse polyclonal antibodies to cdk6, cdk4, cyclin D_1, cyclin D_3, cyclin E, $p21^{Waf1/Cip1}$, phosphor (Ser15)-p53, and β-actin; goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase-conjugate, and horse anti-mouse immunoglobulin G (IgG)-horseradish peroxidase-conjugate. Antibodies were used at working dilutions of 1:1000, with the exception of anti-bodies to β-actin, for which a working dilution of 1:10000 was employed.

Statistical analysis: Data are expressed as means ± SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences (* $p< 0.05$) between the control (untreated) and treated cells were analyzed by Dunnett's test.

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