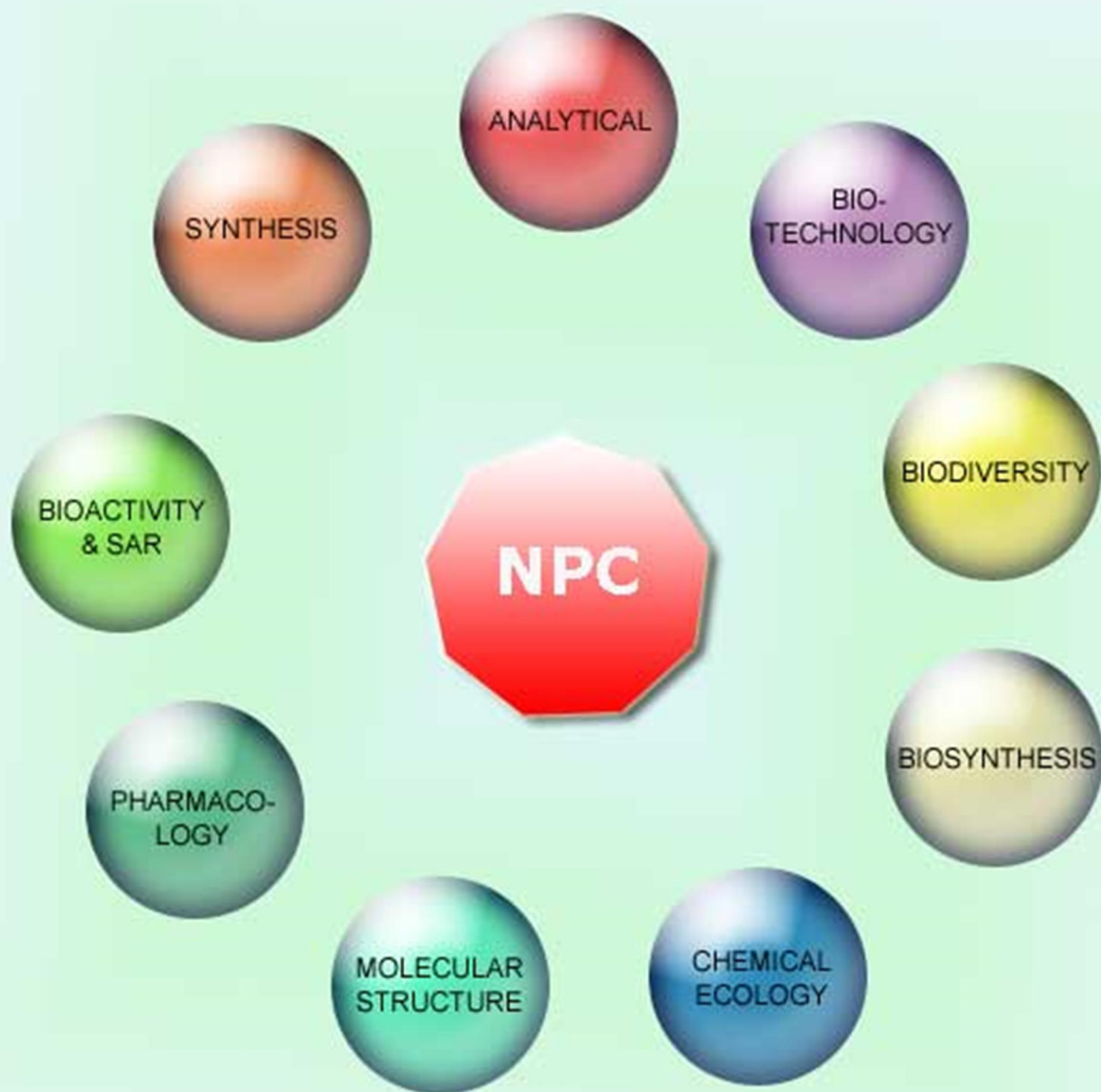


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## Anti-proliferation Effect on Human Breast Cancer Cells *via* Inhibition of pRb Phosphorylation by Taiwanin E Isolated from *Eleutherococcus trifoliatus*

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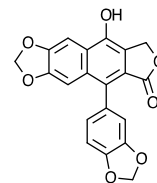
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*Eleutherococcus trifoliatus* has been used as a folk medicine since ancient times, especially as refreshing qi medicines. In our current study, taiwanin E, which possesses strong cytotoxicity, was isolated from the branches of *E. trifoliatus* by 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<sub>50</sub> value for cytotoxicity of 1.47 μM. Cell cycle analysis revealed that the proportion of cells in the G<sub>0</sub>/G<sub>1</sub> 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 4 μM. 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<sub>1</sub>, cyclin D<sub>3</sub> and cyclin-dependent kinase 4 (cdk4) and cdk6; in addition, there was an increase in the expression of cyclin-dependent kinase inhibitors p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup>. The results suggest that taiwanin E inhibits cell cycle progression of MCF-7 at the G<sub>0</sub>/G<sub>1</sub> transition.

**Keywords:** *Eleutherococcus trifoliatus*, Araliaceae, Taiwanin E, Cytotoxicity, Cell cycle, G<sub>1</sub> arrest.

*Eleutherococcus trifoliatus* (L.) S.Y. Huvar. *trifoliatus* (Syn. *Acanthopanax trifoliatus*), a deciduous 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 for either 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 phenylpropanoid 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<sub>50</sub> value of taiwanin 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.



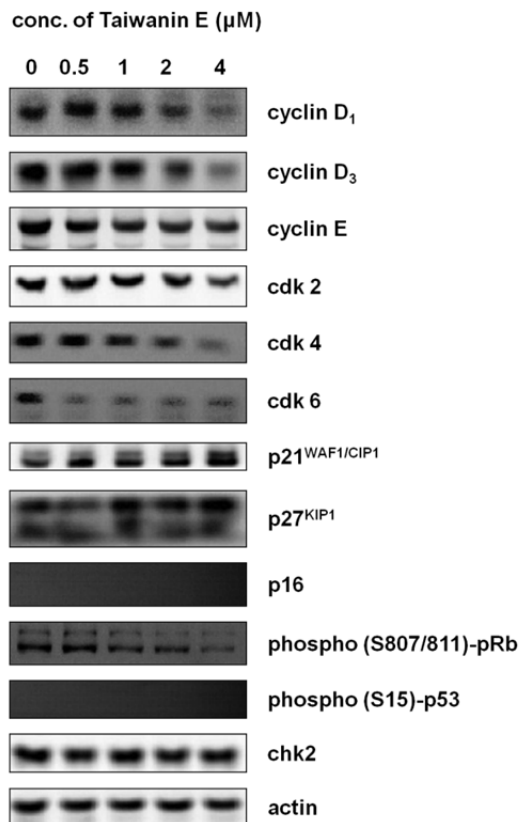
**Figure 1:** The cytotoxicity compound, taiwanin E, obtained from *E. trifoliatus*.

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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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 suppressor p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup>, which are the cyclin-dependent kinase inhibitors belonging to the Cip/Kip family (p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup>, and p57<sup>Kip2</sup>). According to previous studies, increasing p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> expression causes cell cycle arrest at the G<sub>1</sub> 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 ( $\mu\text{M}$ )	Percentage of cells in		
	G <sub>1</sub>	G <sub>2</sub> /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<sub>0</sub> within the G<sub>1</sub> 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<sub>1</sub> phase [20]. During G<sub>1</sub>/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<sub>1</sub>/S transition genes [23-24]. In taiwanin E treated cells, inhibition of cdk activity by a combination of cdk4/6 and cyclin D<sub>1</sub>/D<sub>3</sub> downregulation, and p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> upregulation, prevented pRb phosphorylation. It would, therefore, be expected to prevent G<sub>1</sub>/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<sup>WAF-1/Cip1</sup> can be regulated by either p53-dependent or p53-independent mechanisms [26-29]. Previous studies indicated that G<sub>1</sub> arrest could also be achieved by p53, which is phosphorylated on Ser15 by ATM/ATR and on Ser20 by Chk2, through induction of p21<sup>WAF-1/Cip1</sup> 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<sub>1</sub>/D<sub>3</sub>, cdk4/6, and upregulation of p21<sup>WAF-1/Cip1</sup> 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 (<sup>1</sup>H) and 75 MHz (<sup>13</sup>C). CDCl<sub>3</sub> 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,  $y = 25.249x + 156.91$ , 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<sub>2</sub>O to give EtOAc-soluble (18.4 g) and H<sub>2</sub>O-soluble fractions. The EtOAc-soluble fraction, which displayed potent cytotoxicity (IC<sub>50</sub> = 30.3  $\mu\text{g/mL}$ ), 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<sub>50</sub> = 14.6  $\mu\text{g/mL}$ ) and was further separated by HPLC using a normal-phase column (250 × 10 mm, 5  $\mu\text{m}$ , 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<sub>2</sub>. All cells (1 × 10<sup>3</sup> 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 ( $2 \times 10^5$  cells/dish), were treated with 0, 0.5, 1, 2, and 4  $\mu$ M Taiwanin 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  $-20^\circ\text{C}$  overnight. The cell pellets were collected by centrifugation and rinsed with ice-cold PBS. Cells were stained with 1 mL of 50  $\mu\text{g/mL}$  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 ( $1 \times 10^6$  cells per dish) were treated with Taiwanin E at dosages of 0, 0.5, 1, 2, and 4  $\mu\text{M}$  for 48 h. 40  $\mu\text{g}$  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<sup>Kip1</sup>, cdk2, cdk4, phosphor (Ser807/811)-pRb, and Chk2; mouse polyclonal antibodies to cdk6, cdk4, cyclin D<sub>1</sub>, cyclin D<sub>3</sub>, cyclin E, p21<sup>Waf1/Cip1</sup>, phosphor (Ser15)-p53, and  $\beta$ -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  $\beta$ -actin, for which a working dilution of 1:10000 was employed.

**Statistical analysis:** Data are expressed as means  $\pm$  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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<b>New Mechanism of Magnolol and Honokiol from <i>Magnolia officinalis</i> against <i>Staphylococcus aureus</i></b> Tao Liu, Yalin Pan and Renfu Lai	1307
<b>Molecular Cloning and Characterization of Tyrosine Aminotransferase and Hydroxyphenylpyruvate Reductase, and Rosmarinic Acid Accumulation in <i>Scutellaria baicalensis</i></b> Yeon Bok Kim, Md Romij Uddin, YeJi Kim, Chun Geon Park and Sang Un Park	1311
<b>Phenols and Antioxidant Activity <i>in Vitro</i> and <i>in Vivo</i> of Aqueous Extracts Obtained by Ultrasound-Assisted Extraction from Artichoke By-Products</b> Rossana Punzi, Annalisa Paradiso, Cristina Fasciano, Antonio Trani, Michele Faccia, Maria Concetta de Pinto and Giuseppe Gambacorta	1315
<b>Bioactive Metabolites from <i>Cnidocolus souzae</i> and <i>Acmella pilosa</i></b> Hiatzy E. Zapata-Estrella, Azeret D. M. Sánchez-Pardenilla, Karlina García-Sosa, Fabiola Escalante-Erosa, Fátima de Campos-Buzzi, Nara Lins Meira-Quintão, Valdir Cechinel-Filho and Luis M. Peña-Rodríguez	1319
<b>Dipterostilbenosides A and B, Oligostilbene Glycosides from <i>Dipterocarpus tuberculatus</i></b> Serm Surapinit, Jonkolnee Jong-aramruang, Pongpun Siripong, Suttira Khumkratok and Santi Tip-pyang	1323
<b>Isolation of <math>\beta</math>-Indomycinone Guided by Cytotoxicity Tests from <i>Streptomyces</i> sp. IFM11607 and Revision of its Double Bond Geometry</b> Kentaro Tsukahara, Kazufumi Toume, Hanako Ito, Naoki Ishikawa and Masami Ishibashi	1327
<b>Daurichromenic Acid-producing Oxidocyclase in the Young Leaves of <i>Rhododendron dauricum</i></b> Futoshi Taura, Miu Iijima, Jung-Bum Lee, Toshihiro Hashimoto, Yoshinori Asakawa and Fumiya Kurosaki	1329
<b>Synthesis and Characterization of 4-Aryl-4H-chromenes from H-Cardanol</b> Hulluru Surya Prakash Rao and Mani Kamalraj	1333
<b>Galactans of <i>Gracilaria pudumadamensis</i> (Gracilariales, Rhodophyta) of Indian Waters</b> Stalin Kondaveeti, Sanjay Kumar, Meenakshi S. Ganesan and Arup K. Siddhanta	1341
<b>The Effect of <i>Ginkgo biloba</i> and <i>Camellia sinensis</i> Extracts on Psychological State and Glycemic Control in Patients with Type 2 Diabetes Mellitus</b> Lina Lasaitė, Asta Spadiene, Nijole Savickienė, Andrejs Skesters and Alise Silova	1345
<b>Comparative Anti-inflammatory Effects of Anti-arthritis Herbal Medicines and Ibuprofen</b> Joshua J. Kang, Mohammed A. Samad, Kye S. Kim and Soochan Bae	1351
<b>Quantitative Analysis Coupled with Toxic Evaluation to Investigate the Influence of Sulfur-Fumigation on the Quality of <i>Chrysanthemum morifolium</i></b> Ke Ding, Gang Cao, Zhiwei Xu and Xiaocheng Chen	1357
<b>Chemical Composition of the Leaf Oil of <i>Actephila excelsa</i> from Vietnam</b> Do N. Dai, Tran D. Thang, Dau B. Thin and Isiaka A. Ogunwande	1359
<b>Composition and Chemical Variability of Corsican <i>Pinus halepensis</i> Cone Oil</b> Anne-Marie Nam, Joseph Casanova, Félix Tomi and Ange Bighelli	1361
<b>Hawaiian Sandalwood: Oil Composition of <i>Santalum paniculatum</i> and Comparison with Other Sandal Species</b> Norbert A. Braun, Sherina Sim, Birgit Kohlenberg and Brian M. Lawrence	1365
<b>Aroma Compounds of Mountain Tea (<i>Sideritis scardica</i> and <i>S. raeseri</i>) from Western Balkan</b> Bujar Qazimi, Gjoshe Stefkov, Marija Karapandzova, Ivana Cvetkovikj and Svetlana Kulevanova	1369
<b>Chemical Composition of the Essential Oil of the Local Endemics <i>Centaurea davidovii</i> and <i>C. parilica</i> (Asteraceae, sect. <i>Leptanthus</i>) from Bulgaria</b> Antonella Maggio, Luana Riccobono, Svetlana Bancheva, Maurizio Bruno and Felice Senatore	1373
<b>Compositional Analysis and <i>in vitro</i> Protective Activity against Oxidative Stress of Essential Oils from Egyptian Plants Used in Traditional Medicine</b> Tarek F. Eissa, Elena González-Burgos, M. Emilia Carretero and M. Pilar Gómez-Serranillos	1377
<b>Chemical Composition and Antifungal Activity of the Essential Oils of <i>Schinus weinmannifolius</i> Collected in the Spring and Winter</b> Camila Hernandez, Silvia H. Taleb-Contini, Ana Carolina D. Bartolomeu, Bianca W. Bertoni, Suzelei C. França and Ana Maria S. Pereira	1383
<b>The Essential Oil Profiles and Antibacterial Activity of Six Wild <i>Cinnamomum</i> species</b> Charles Santharaju Vairappan, Thilaghavani Nagappan and Julius Kulip	1387

### Accounts/Reviews

<b>Dissecting Traditional Chinese Medicines by Omics and Bioinformatics</b> Yuan Quan, Zhong-Yi Wang, Min Xiong, Zheng-Tao Xiao and Hong-Yu Zhang	1391
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# Natural Product Communications

## 2014

Volume 9, Number 9

### Contents

<u>Original Paper</u>	<u>Page</u>
<b>The Leaf, Wood and Bark Oils of three Species of <i>Myodocarpus</i> (Myodocarpaceae) Endemic to New Caledonia</b> Nicolas Lebouvier, Douglas Lawes, Edouard Hnawia, Michael Page, Joseph Brophy and Mohammed Nour	1223
<b>Iridoids and a Norsesquiterpenoid from the Leaves of <i>Villaria odorata</i></b> Mario A. Tan, Raychel Ann U. Villacorta, Grecebio Jonathan D. Alejandro and Hiromitsu Takayama	1229
<b>Induction, Cloning and Functional Expression of a Sesquiterpene Biosynthetic Enzyme, <math>\delta</math>-Guaiene Synthase, of <i>Aquilaria microcarpa</i> Cell Cultures</b> Jung-Bum Lee, Syun Hirohashi, Yoshimi Yamamura, Futoshi Taura and Fumiya Kurosaki	1231
<b>Zerumbone Induces G2/M Cell Cycle Arrest and Apoptosis via Mitochondrial Pathway in Jurkat cell Line</b> Heshu Sulaiman Rahman, Abdullah Rasedee, Max Stanley Chartrand, Hemn Hassan Othman, Swee Keong Yeap and Farideh Namvar	1237
<b>Diterpenoids from <i>Fagonia mollis</i></b> Amal Sallam, Alfarius Eko Nugroho, Yusuke Hirasawa, Wong Chin-Piow, Toshio Kaneda, Osamu Shirota, Sahar R. Gedara and Hiroshi Morita	1243
<b>Cytotoxicity of the Diterpene 14-O-Methyl-ryanodanol from <i>Erythroxylum passerinum</i> in an Astrocytic Cells Model</b> Noélio de Jesus Menezes-Filho, Cleide dos Santos Souza, Tereza Cristina Silva Costa, Victor Diógenes Amaral da Silva, Cátia Suse de Oliveira Ribeiro, Marizeth Liborio Barreiros, Jose Fernando Oliveira Costa, Jorge Mauricio David, Juceni P.L. David and Silvia Lima Costa	1245
<b>Absolute Configuration of Cembrane Diterpenoids from <i>Bursera multijuga</i></b> Juan D. Hernández-Hernández, Hugo A. García-Gutiérrez, Luisa U. Román-Marín, Yunuen I. Torres-Blanco, Carlos M. Cerda-García-Rojas and Pedro Joseph-Nathan	1249
<b>Trichostemoneate, a New Anticancer Triterpene from the Stem Bark of <i>Walsura trichostemon</i></b> Kiattipum Phontree, Jirapast Sichaem, Suttira Khumkratok, Pongpun Siripong and Santi Tip-pyang	1253
<b>A New Cycloartane Glucoside from <i>Rhizophora stylosa</i></b> Phan Thi Thanh Huong, Chau Ngoc Diep, Nguyen Van Thanh, Vu Anh Tu, Tran Hong Hanh, Nguyen The Cuong, Nguyen Phuong Thao, Nguyen Xuan Cuong, Do Thi Thao, Tran Huy Thai, Nguyen Hoai Nam, Ninh Khac Ban, Phan Van Kiem and Chau Van Minh	1255
<b>Kolgaosides A and B, Two New Triterpene Glycosides from the Arctic Deep Water Sea Cucumber <i>Kolga hyalina</i> (Elasipodiida: Elpidiidae)</b> Alexandra S. Silchenko, Anatoly I. Kalinovskiy, Sergey A. Avilov, Pelageya V. Andryashchenko, Sergey N. Fedorov, Pavel S. Dmitrenok, Ekaterina A. Yurchenko, Vladimir I. Kalinin, Antonina V. Rogacheva and Andrey V. Gebruk	1259
<b>Acaricidal Activity against <i>Panonychus citri</i> and Active Ingredient of the Mangrove Plant <i>Cerbera manghas</i></b> Yecheng Deng, Yongmei Liao, Jingjing Li, Linlin Yang, Hui Zhong, Qiuyan Zhou and Zhen Qing	1265
<b>Three New Steroid Biglycosides, Plancisides A, B, and C, from the Starfish <i>Acanthaster planci</i></b> Alla A. Kicha, Thi H. Dinh, Natalia V. Ivanchina, Timofey V. Malyarenko, Anatoly I. Kalinovskiy, Roman S. Popov, Svetlana P. Ermakova, Thi T. T. Tran and Lan P. Doan	1269
<b>Unusual 2(1H)-Pyrazinones Isolated from a Culture of a Brazilian Marine-Derived <i>Streptomyces</i> sp.</b> Sérgio S. Thomasi, Ana C. Granato, Luis H. Romano, Liene Dhooche, Eduardo S. P. do Nascimento, Alberto C. Badino, Maria F. G. F. da Silva, Antonio G. Ferreira and Tiago Venâncio	1275
<b>An HPLC Evaluation of Cytochalasin D Biosynthesis by <i>Xylaria arbuscula</i> Cultivated in Different Media</b> Luciana da S. Amaral, Edson Rodrigues-Filho, Carolina A. A. Santos, Lucas M. de Abreu and Ludwig H. Pfennig	1279
<b>A Simple Method for Isolation and Purification of DIBOA-Glc from <i>Tripsacum dactyloides</i></b> Cammy D. Willett, Robert N. Lerch, Keith W. Goyne, Nathan D. Leigh, Chung-Ho Lin and Craig A. Roberts	1283
<b>Antimicrobial Metabolites from Endophytic <i>Streptomyces</i> sp. YIM61470</b> Xueqiong Yang, Yun Liu, Shuquan Li, Fangfang Yang, Lixing Zhao, Li Peng and Zhongtao Ding	1287
<b>Genkwanin 4'-O-glucosyl-(1<math>\rightarrow</math>2)-rhamnoside from New Chemotype of <i>Asplenium normale</i> in Japan</b> Tao Fujiwara, Ayumi Uehara, Junichi Kitajima, Tsukasa Iwashina, Sadamu Matsumoto and Yasuyuki Watano	1289
<b>Potent SIRT1 Enzyme-stimulating and Anti-glycation Activities of Polymethoxyflavonoids from <i>Kaempferia parviflora</i></b> Asami Nakata, Yuka Koike, Hirofumi Matsui, Tsutomu Shimada, Masaki Aburada and Jinwei Yang	1291
<b>Protective Activity of C-Geranylflavonoid Analogs from <i>Paulownia tomentosa</i> against DNA Damage in 137Cs Irradiated AHH-1 Cells</b> Hyung-In Moon, Min Ho Jeong and Wol Soon Jo	1295
<b>Antibacterial Activities of Oxyprenylated Chalcones and Naphthoquinone against <i>Helicobacter pylori</i></b> Charles Bodet, Christophe Burucoa, Steeve Rouillon, Nicolas Bellin, Vito Alessandro Taddeo, Serena Fiorito, Salvatore Genovese and Francesco Epifano	1299
<b>Anti-proliferation Effect on Human Breast Cancer Cells via Inhibition of pRb Phosphorylation by Taiwanin E Isolated from <i>Eleutherococcus trifoliatus</i></b> Hui-Chun Wang, Yen-Hsueh Tseng, Hui-Rong Wu, Fang-Hua Chu, Yueh-Hsiung Kuo and Sheng-Yang Wang	1303

Continued inside backcover