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Using the pER8:GUS Reporter System to Screen for Phytoestrogens from *Caesalpinia sappan*

Wan-Chun Lai,[†] Hui-Chun Wang,[†] Guan-Yu Chen,[‡] Juan-Cheng Yang,[†] Michal Korinek,[†] Chia-Jung Hsieh,[§] Hiroshi Nozaki,[⊥] Ken-Ichiro Hayashi,[⊥] Chih-Chung Wu,[†] Yang-Chang Wu,^{‡,||} and Fang-Rong Chang^{*,†,▽}

[†]Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan, Republic of China

[‡]Natural Medicinal Products Research Center, China Medical University Hospital, Taichung 40402, Taiwan, Republic of China

[§]Department of Traditional Chinese Medicine, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan, Republic of China

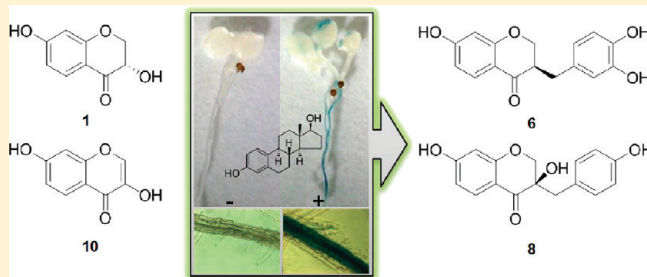
[⊥]Department of Biochemistry, Faculty of Science, Okayama University of Science, 1-1 Ridai-cho, Okayama 700-0005, Japan

^{||}Graduate Institute of Integrated Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan, Republic of China

[▽]Cancer Center, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan, Republic of China

Supporting Information

ABSTRACT: *Arabidopsis thaliana* pER8:GUS, a low-cost, highly efficient, and convenient transgenic plant system, was used to assay the estrogen-like activity of 30 traditional Chinese medicines. The MeOH extract of *Caesalpinia sappan* exhibited significant bioactivity in this assay, and subsequent bioactivity-guided fractionation of the extract led to the isolation of one new compound, (S)-3,7-dihydroxychroman-4-one (**1**), and 10 known compounds. Both the plant pER8:GUS and in vitro estrogen response element reporter assays were used to evaluate the estrogenic activity of the isolated compounds, and these two systems produced comparable results. Compounds **6**, **8**, and **11** showed significant estrogenic activity comparable to genistein. These active compounds were determined to be nontoxic new sources of phytoestrogens. In addition, compounds **2** and **3** inhibited ERE transcription induced by 17 β -estradiol. A docking model revealed that compounds **6**, **8**, and **11** showed high affinity to the estrogen receptor. The pER8:GUS reporter system was demonstrated to be a useful and effective technique in phytoestrogen discovery.



Phytoestrogens, such as isoflavones, coumestans, and lignans, are estrogen-like substances found in a wide variety of plants consumed by humans. Because they have fewer side effects and are associated with a lower risk of breast tumors compared to synthetic estrogens, phytoestrogens have been used to relieve postmenopausal ailments.¹ In addition, they have been reported to prevent breast cancer, osteoporosis,² cardiovascular diseases,³ and neurodegenerative disorders.⁴ However, recent studies suggested that phytoestrogens, such as resveratrol, genistein, and quercetin, can exhibit a complicated biphasic effect on the development of carcinogenesis or act as selective estrogen receptor modulators (SERMs).^{5,6}

In 2005, a cross-kingdom bioassay system was applied to discover phytoestrogens using a transgenic *Arabidopsis* plant, pER8:GFP.⁷ The pER8:GFP contains the XVE (LexA-VP16-ER) vector as an activator unit and the GFP (green fluorescent protein) gene as a reporter. The XVE system is an estrogen-receptor-based chemical-inducible system, which was developed by Zuo et al. in 2000.⁸ It comprises a DNA binding domain of LexA (X), an acidic transactivating domain of VP16 (V), and a

regulation region of the estrogen receptor (E; ER).⁸ In recent years, cross-kingdom bioassay systems were successfully established to detect estrogenic natural products and endocrine-disrupting chemicals.⁹ The technological evolution resulted in the development of highly sensitive chemically inducible systems.⁹ In 2006, Brand et al. developed another transgenic plant system, pER8:GUS, which was used in this study to examine the estrogen-like activity of natural products.¹⁰ The pER8:GUS system was developed using the vector pLB12, which contains both an activator unit (XVE) and a GUS (β -glucuronidase) reporter within a responder unit.¹⁰ To date, no study has been performed comparing the ER sensitivity of pER8:GUS with pER8:GFP.

In this study, we tested the efficacy of the new tool, pER8:GUS, to detect phytoestrogens. In addition, 30 extracts recommended by gynecological TCM (traditional Chinese medicine) physicians were screened for their estrogenic activity using this system. The MeOH extract of *Caesalpinia sappan* L. (Su Mu in

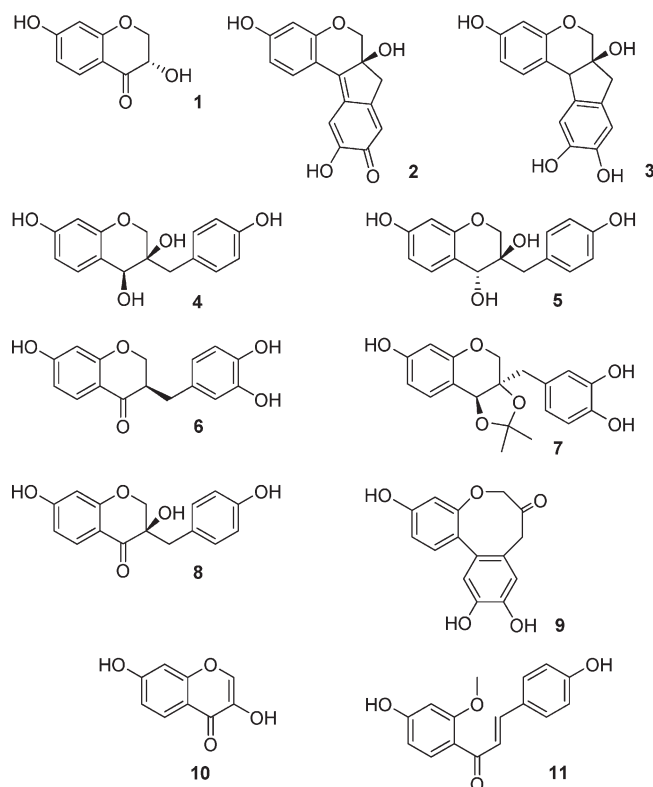
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Chinese) showed significant estrogenic activity with a minimum active concentration (MAC) of 1.56 $\mu\text{g/mL}$.

C. sappan is found in India, Peru, Malaya, etc.¹¹ The dried heartwood of *C. sappan* is an important oriental medicine used for the treatment of gynecologic diseases. Historically, it has been used to treat dysmenorrhea, amenorrhea, menorrhagia, traumatic swelling and pain, and blood stasis after delivery.¹² Recent investigations showed various pharmacological activities, including vasorelaxant,¹³ antihypercholesteremic,¹⁴ anti-inflammatory,¹⁴ antimicrobial,¹⁵ and anticomplementary effects.¹⁶ However, no study has been reported on its estrogen-like activity.

Bioassay-directed chromatographic fractionation of *C. sappan* led to the discovery of one new chromanone derivative, (S)-3,7-dihydroxychroman-4-one (**1**), and 10 known compounds, i.e., seven homoisoflavonoid derivatives, brazilein (**2**),¹⁷ brazilin (**3**),¹⁷ 3'-deoxysappanol (**4**),¹⁸ 3'-deoxyepisappanol (**5**),¹⁸ 3-deoxysappanone B (**6**),¹⁸ an isopropylidene derivative of sappanol (**7**),¹⁹ and 3'-deoxysappanone B (**8**);¹⁸ one biphenyl derivative, protosappanin A (**9**);²⁰ one chromenone derivative, 3,7-dihydroxychromen-4-one (**10**); and one chalcone derivative, 3-deoxysappanchalcone (**11**).²¹ The estrogenic and antiestrogenic activities of these compounds were investigated using a transgenic plant assay system and an in vitro reporter assay. In addition, docking analysis was utilized to deduce ER binding affinities of the active compounds.



RESULTS AND DISCUSSION

Dose-Dependent Activity of 17 β -Estradiol in the Transgenic Plant System pER8:GUS. Using the transgenic plant system pER8:GUS, the minimum active concentration of 17 β -estradiol (E2) was found to be 0.62–1.25 nM (Figure 1). The quantification of GUS gene expression was successfully determined by a fluorometric assay. Results showed that the gene expression of pER8:GUS exhibited dose-dependent activity at approximately

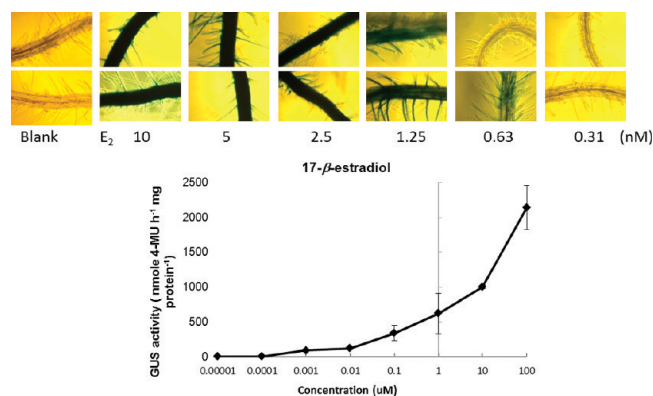


Figure 1. Minimum active concentration of E2 on the pER8:GUS system by the histochemical assay. Results of the fluorometric assay showed that E2 (0.01–100 μM) induced GUS activity of pER8:GUS in a dose-dependent manner. Each experiment represents the GUS activity and is expressed as mean \pm SEM ($n = 3$).

0.01–100 μM E2, suggesting that the use of the pER8:GUS reporter gene assay enhanced the sensitivity from the micromolar to the nanomolar level compared to pER8:GFP. Consequently, this system is stable and sensitive for testing estrogen-like effects.

The transgenic plant pER8:GUS, with the GUS gene as a gene fusion marker for the analysis of gene expression, expresses high estrogenic sensitivity and can be used to quantify the bioactivity of phytoestrogens. Moreover, it is a visible system, and primary results can be readily observed without using a microscope.

Estrogenic Activity of Extracts from TCM. On the basis of clinical and practical experiences of using TCM, 30 Chinese traditional herbs were selected for the assessment of estrogenic activity (Table 1). Plant materials of selected TCM were extracted with 95% aqueous MeOH or 95% aqueous EtOH. Extracts were evaporated to dryness under reduced pressure to obtain the MeOH or EtOH extracts. In order to discover potential phytoestrogens, each dry extract was redissolved in DMSO and screened by the pER8:GUS assay system. Estrogenic activity of extracts was detected via a simple histochemical assay for GUS activity, and the MAC of each estrogen-active extract was measured.²² Soybean, a typical dietary phytoestrogen, was extracted with 95% aqueous EtOH and selected as a positive control (MAC 0.25 $\mu\text{g/mL}$).

The results showed that the extracts of *Cuscuta chinensis* Lam., *Caesalpinia sappan* L., *Acorus gramineus* Soland, *Cyperus rotundus* L., and *Artemisia capillaris* Thunb. exhibited moderate to good estrogenic activity compared to soybean extract (Table 1). *Dianthus superbus* L. exhibited only weak estrogenic activity at concentrations greater than 100 $\mu\text{g/mL}$. Excluding *C. chinensis*²³ and *C. rotundus*,²⁴ the estrogenic activity of these extracts has not been reported. The data indicated that the transgenic plant system is useful in screening estrogenic activity and avoiding cytotoxicity, which is a major limitation of other screening models. The results may provide some answers to why these TCM herbs are used in the field of gynecology. Among these six active extracts, *C. sappan* was the most potent, with an MAC of 1.56 $\mu\text{g/mL}$. In addition to its significant estrogenic activity, the MeOH extract of *C. sappan* exhibited cytotoxicity ($\text{IC}_{50} < 20 \mu\text{g/mL}$) against liver, breast, and lung cancer cell lines. Therefore, the MeOH extract from *C. sappan* was selected for further investigation directed by bioactivity-guided fractionation.

Table 1. Estrogenic Activity of MeOH or EtOH Extracts from TCM

no.	plant name	family	part	extr ^a	MAC ($\mu\text{g/mL}$)
SB	<i>Glycine max</i> (L.) Merr.	Leguminosae	seed	E	0.25
S 1	<i>Achyranthes bidentata</i> Blume	Amaranthaceae	root	M	^b
S 2	<i>Asparagus cochinchinensis</i> (Lour.) Merr.	Liliaceae	root tuber	M	^b
S 3	<i>Angelica dahurica</i> Benth. et Hook.	Umbelliferae	root	M	^b
S 4	<i>Aster tataricus</i> L.F.	Compositae	rhizome	M	^b
S 5	<i>Cynanchum atratum</i> Bunge.	Asclepiadaceae	rhizome	M	^b
S 6	<i>Cuscuta chinensis</i> Lam.	Convolvulaceae	seed	M	50
S 7	<i>Cimicifuga fetida</i> Linn.	Ranunculaceae	rhizome	M	^b
S 8	<i>Cnidium monnieri</i> Cusson	Umbelliferae	fruit	M	^b
S 9	<i>Cervus nippon</i> Temminck	Cervidae	antler	M	^b
S 10	<i>Caesalpinia sappan</i> L.	Leguminosae	heartwood	M	1.56
S 11	<i>Eucommia ulmoides</i> Oliver	Eucommiaceae	cortex	M	^b
S 12	<i>Evodia rutaecarpa</i> (Juss.) Benth.	Rutaceae	fruit	M	^b
S 13	<i>Ficus formosana</i> Maxim.	Moraceae	root	M	^b
S 14	<i>Lycium chinense</i> Miller	Solanaceae	cortex of root	M	^b
S 15	<i>Ligustrum lucidum</i> Ait.	Oleaceae	fruit	M	^b
S 16	<i>Acorus gramineus</i> Soland	Araceae	rhizome	M	12.5
S 17	<i>Paeonia suffruticosa</i> Andr.	Ranunculaceae	cortex of root	M	^b
S 18	<i>Rubus chingii</i> Hu	Rosaceae	fruit	M	^b
S 19	<i>Rehmannia glutinosa</i> (Gaertn.) Libosch.	Scrophulariaceae	rhizome	M	^b
S 20	<i>Zizyphus jujuba</i> Mill. var. <i>inermis</i> (Bge.) Rehd	Rhamnaceae	fruit	M	^b
S 21	<i>Poria cocos</i> (Schw.) Wolf	Polyporaceae	sclerotium	M	^b
S 22	<i>Dianthus superbus</i> L.	Caryophyllaceae	plant	M	100
S 23	<i>Taraxacum mongolicum</i> Hand.	Compositae	plant	E	^b
S 24	<i>Paeonia suffruticosa</i> Andr.	Ranunculaceae	cortex of root	E	^b
S 25	<i>Cyperus rotundus</i> L.	Cyperaceae	rhizome	M	6.25
S 26	<i>Artemisia capillaris</i> Thunb.	Compositae	plant	M	12.5
S 27	<i>Prunus persica</i> (L.) Batsch	Rosaceae	kernel	M	^b
S 28	<i>Patrinia scabiosaeifolia</i> Fisch.	Valerianaceae	plant	E	^b
S 29	<i>Bupleurum scorzoniferifolium</i> Wild.	Umbelliferae	root	E	^b
S 30	<i>Prunella vulgaris</i> L.	Lamiaceae	plant	E	^b

^a E: 95% aqueous EtOH, M: 95% aqueous MeOH. ^b MAC >200 $\mu\text{g/mL}$.

Structure Elucidation of the Isolated Compounds from *C. sappan*. The dried heartwood of *C. sappan* was extracted with MeOH. The MeOH extract was partitioned between EtOAc and H₂O to obtain the estrogen-active EtOAc layer. The EtOAc layer was subjected to silica gel CC and eluted with EtOAc–MeOH to give four fractions, two of which showed both estrogenic and cytotoxic activities. Chromatographic fractionation of the active fractions afforded one new chromanone derivative, (S)-3,7-dihydroxychroman-4-one (**1**), and 10 known compounds.

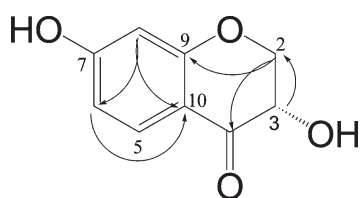
Compound **1** was obtained as a light yellow, amorphous solid. Its HREIMS showed a molecular ion at m/z 180.0424 [M]⁺, which in combination with ¹H and ¹³C NMR data indicated a molecular formula of C₉H₈O₄ (calcd 180.0423). The IR spectrum showed absorptions for hydroxy (3439 cm⁻¹), keto carbonyl (1645 cm⁻¹), aromatic (1539 and 1455 cm⁻¹), and ether C–O–C (1016 cm⁻¹, asymmetric stretch) functionalities. UV absorptions at 233, 274, and 312 nm indicated the presence of an aromatic system. The ¹H and ¹³C NMR data of compound **1** were similar to those of compound **10**, suggesting the presence of a chromenone skeleton (Table 2). Nine carbon resonances, including one methylene (δ_C 72.2), four methine (δ_C 69.9, 103.6, 112.1, and 130.1), and four quaternary carbons (δ_C 113.5, 165.5, 167.0,

and 193.8) were observed via ¹³C NMR and DEPT spectra. One quaternary carbon resonating at δ_C 193.8 was identified as a keto carbonyl group. Furthermore, in the ¹H NMR spectrum, ABX spin systems were recognized at δ_H 6.31 (1H, d, J = 2.0), δ_H 6.50 (1H, dd, J = 8.8, 2.0 Hz), and δ_H 7.70 (1H, d, J = 8.8 Hz). These data supported the presence of a chromanone skeleton (Table 2). Based on the ¹³C NMR and HSQC correlations, a downfield-shifted carbon at δ_C 167.0 indicated that a phenolic hydroxy group was substituted on the chromanone moiety. The HMBC correlations between H-2 and C-4/C-9 and between H-8 and C-6/C-10 confirmed the substitution of the phenolic hydroxy group at C-7 and a second hydroxy group at C-3. The specific rotation [α]_D²⁵ –98.8 (c 0.2, CHCl₃) of **1** was similar to those of (S)-3-hydroxy-2,3-dihydro-4H-chromen-4-one [–57 (c 2.0, CHCl₃)] and (S)-4-oxo-3,4-dihydro-2-chromen-3-yl acetate [–63 (c 0.5, CHCl₃)].²⁵ Therefore, the absolute configuration at C-3 was determined as S, and the new compound was identified as (S)-3,7-dihydroxychroman-4-one (**1**).

Compound **10** was isolated for the first time as a naturally occurring compound and was identified as 3,7-dihydroxychromen-4-one. Clear ¹H and ¹³C NMR data of **10** were never reported and are given here in Table 2.

Table 2. ^1H and ^{13}C NMR Data of **1** and **10** (400 and 100 MHz in CD_3OD)

position	1			10	
	δ_{C} , mult.	δ_{H} (J in Hz)	HMBC	δ_{C} , mult.	δ_{H} (J in Hz)
2	72.2, CH_2	4.13, dd, (10.6, 10.6) 4.48, dd, (10.6, 5.6)	3, 4, 9 3, 4, 9	141.0, CH	7.96, s
3	69.9, CH	4.41, dd, (10.6, 5.6)	4, 9	143.0, C	
4	193.8, C			174.8, C	
5	130.1, CH	7.70, d, (8.8)	4, 7, 9	127.8, CH	8.01, d, (8.8)
6	112.1, CH	6.50, dd, (8.8, 2.0)	8, 10	116.1, CH	6.90, dd, (8.8, 2.4)
7	167.0, C			164.2, C	
8	103.6, CH	6.31, d, (2.0)	6, 7, 9, 10	103.1, CH	6.80, d, (2.4)
9	165.5, C			159.6, C	
10	113.5, C			117.0, C	

Figure 2. HMBC correlations of (S)-3,7-dihydroxychroman-4-one (**1**).

Compounds **4** and **5** showed the same R_f values on normal phase TLC, but were separable by reversed-phase HPLC with retention times (t_R) of 13.04 and 12.56, respectively. Compounds **4** and **5** displayed similar ^1H and ^{13}C NMR data, suggesting that they are stereoisomers, namely, 3,4-*cis*- and -*trans*-di-*O*-substituted homoisoflavans, respectively. Accordingly, in their ^1H NMR spectra (400 MHz, methanol- d_4), the nonequivalent methylene H-9 protons of **4** appeared at δ_{H} 2.63 (1H, d, $J = 14.0$) and 2.67 (1H, d, $J = 14.0$), while those of **5** appeared at δ_{H} 2.71 (1H, d, $J = 14.0$) and 2.89 (1H, d, $J = 14.0$). Compound **4** showed a positive Cotton effect and **5** showed a negative Cotton effect at 275 nm in the CD spectra. On the basis of comparison with literature data, these two compounds were identified as 3'-deoxysappanol (**4**) and 3'-deoxyepisappanol (**5**).²¹

Estrogenic Activity of the Isolated Compounds Tested by the pER8:GUS Assay System. The isolated compounds from *C. sappan* were evaluated for estrogenic activity using the pER8:GUS assay system with E2 as a positive control. First, a histochemical assay was applied to afford the estrogen MAC of the tested compounds (Figure 3). A transgenic plant was incubated in the presence or absence of tested compounds and then soaked in a GUS assay solution. Compounds **6** and **8–11** showed estrogenic activity (MAC <20 μM) in the pER8:GUS reporter system (Figure 3B). While compounds **1–5** and **7** exhibited no GUS expression at 150–280 μM (Figure 3C), these compounds exhibited weak estrogenic activity at concentrations greater than 500 μM (data not shown).

Estrogenic activity was further evaluated by a quantitative fluorometric assay (Figure 4). Compounds **6** and **8–11** expressed estrogenic activity in a dose-dependent manner, which confirmed the results from the histochemical assay. Compounds **6**, **8**, **9**, and **11** exhibited comparable estrogenic activity to that of genistein.²⁶ Compound **8**, a homoisoflavanone, expressed the highest activity. Therefore, these compounds were determined to be new phytoestrogenic candidates. This study is the first to show

the estrogenic activity of the new biphenyl protosappanin A (**9**), one of the major components of *C. sappan* extract, in this transgenic plant assay.

Cytotoxic Effects of the Isolated Compounds. The MeOH extract of *C. sappan* showed cytotoxicity as previously reported.²⁷ The isolated compounds were evaluated in an MTT assay using MCF-7, MDA-MB-231, A549, Ca9-22, Hep3B, and HepG2 cancer cells with doxorubicin (Doxo) as a positive control (Table 3). Compounds **2** and **3** exhibited moderate cytotoxicity against all tested cancer cell lines, with the greatest potency toward MDA-MB-231 breast cancer cells (IC_{50} 8.31 and 10.80 μM , respectively). According to the analytical results, compounds **2** and **3** are the major components of *C. sappan*.¹⁷ Therefore, it may be suggested that **2** and **3** are responsible for the cytotoxic effects of the heartwood of *C. sappan*. Compound **11** also exhibited moderate cytotoxic effects against all tested cancer cell lines and was more potent toward HepG2 cancer cells (IC_{50} 7.37 μM). Compound **1** showed only weak cytotoxicity against Hep3B, Ca9-22, and A549 cancer cells (IC_{50} 47.22–80.67 μM). Compounds **4–8** showed no cytotoxicity against all tested cell lines.

Estrogen Response Element Reporter Assay in MCF-7 Cells. In order to confirm the estrogen-like activity demonstrated with the pER8:GUS assay system, the compounds were tested in another reporter assay using ER-positive MCF-7 cells. In this assay, the effects of the compounds on the estrogen responsive element (ERE) were examined by the detection of secreted alkaline phosphatase (SEAP) reporter protein. Results showed that the ERE was active when the cells were treated with compounds **6**, **8**, and **11** at a concentration of 20 μM (Figure 5). These effects were comparable to those of E2 at a concentration of 0.01 nM. Compounds **4** and **5** (a mixture), **7**, and **10** showed weak SEAP activity (Figure 5). Thus, the homoisoflavanones **6** and **8** showed stronger estrogenic activity than the mixture of homoisoflavans **4** and **5**. Compounds **1–3** and **9** did not elicit any estrogenic activity at a treatment dose of 20 μM (Figure 5). The results of the in vitro reporter assay in human cells confirmed the estrogenic activity of compounds **4–8**, **10**, and **11** identified by the transgenic plant assay system. Unexpectedly, compound **9**, which was active in the transgenic plant assay, did not show estrogenic activity in the in vitro human cell assay.

The SEAP activity of compounds **6** and **8** was also compared to that of genistein. All three compounds exhibited potent estrogenic activity in a dose-dependent manner (Figure 6).

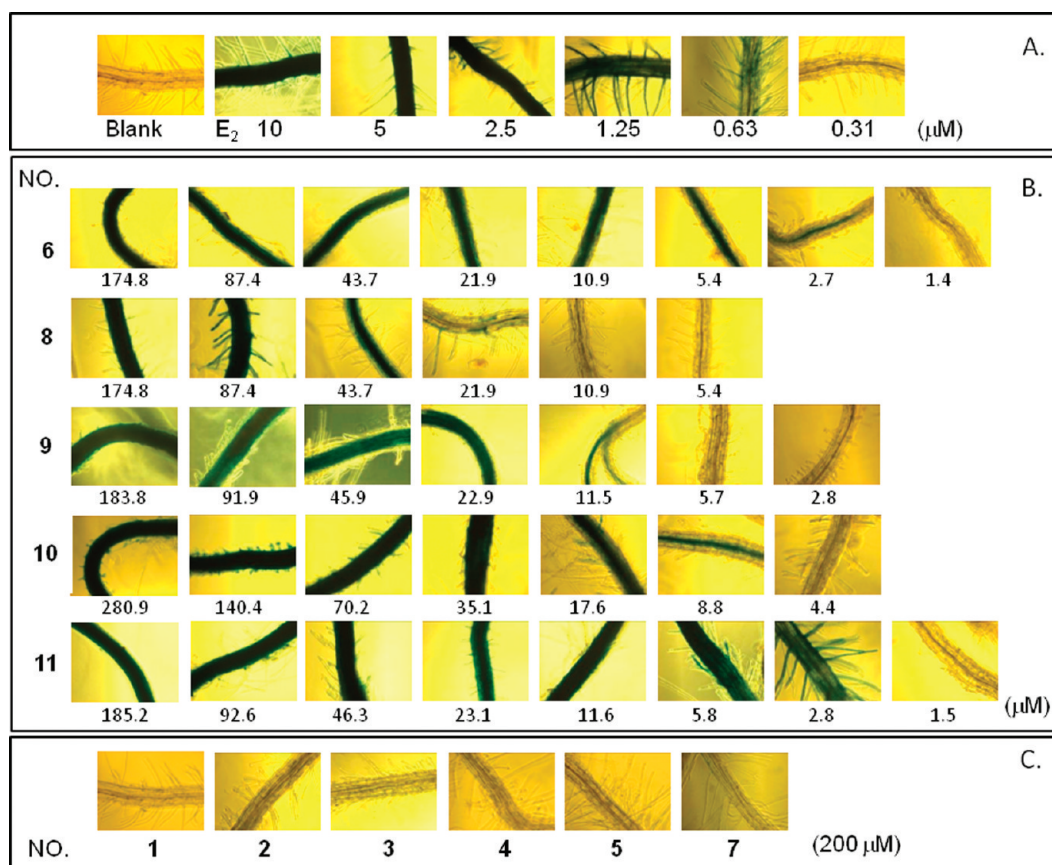


Figure 3. Estrogenic MAC of the isolated compounds in the histochemical assay. The MACs of active compounds 6, 8, 9, 10, and 11 were 2.7, 10.9, 11.5, 8.8, and 2.8 μM , respectively. Each histochemical assay was reproduced in three independent experiments.

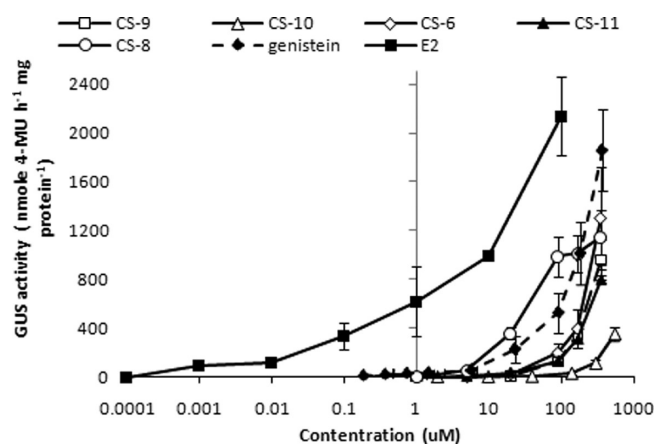


Figure 4. Quantification of estrogenic activity of the pure components from *C. sappan*. Each experiment represents the GUS activity and is expressed as mean \pm SEM ($n = 3$).

SERMs (selective estrogen receptor modulators), which act selectively on estrogen receptors, possess both estrogenic and antiestrogenic activities. The fact that genistein, like some of the SERMs, partially antagonizes the transcriptional activity of the ER,²⁸ motivated us to test the modulatory activities of the isolated compounds on ERE-mediated transcription. The positive controls tamoxifen and raloxifene significantly inhibited E2-induced reporter activity, while compounds 2 and 3 as well

as genistein exhibited only partial antiestrogenic activity in MCF-7 cells treated with 0.01 nM E2 (Figure 7). However, the cytotoxicity of 2 and 3 may have influenced their antiestrogenic effects on the MCF-7 cell line.

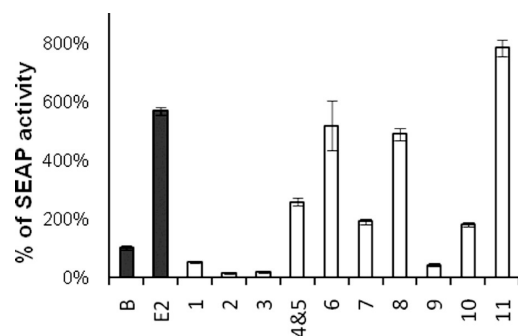
Molecular Docking. To further understand the interaction between the active compounds and the ER, we used the docking package LigandFit within the software Discovery Studio 2.5. According to the literature,^{29,30} when the rmsd (root-mean-square deviation) value is less than or equal to 2 in the conformation experiment, it corresponds to the best-docked conformation and is suggested as the best scoring model. The validation of the function achieved in LigandFit/Discovery Studio 2.5 was verified by the docking of the native ligand (E2) into its binding site to achieve the best prediction and highest accuracy. Figure 8A shows that the best-docked conformation for E2 (yellow) was exactly superimposed on the cocrystal structure of ligand (E2, pink) in the ER. The rmsd value of docked E2 was 0.3 Å.

The results showed that compounds 6, 8, and 11 docked well into the ligand binding site of ER. Compound 6 formed two hydrogen bonds to the Glu 353 residue, with calculated distances of 1.13 and 2.46 Å, and an additional hydrogen bond to the His 524 residue with a bond length of 1.72 Å (Figure 8B). Compound 8 formed three hydrogen bonds with Arg 394 (1.79 Å), Glu 353 (2.24 Å), and Thr 347 (1.93 Å) residues (Figure 8C). Compound 11 formed two hydrogen bonds with the Glu 353 residue (1.92 and 2.49 Å) (Figure 8D). Compounds 6, 8, and 11 formed hydrogen bonds with the same Glu 353 residue in the native E2 binding site of the docking model, which suggested that

Table 3. Cytotoxicity of the Isolated Compounds from *C. sappan*

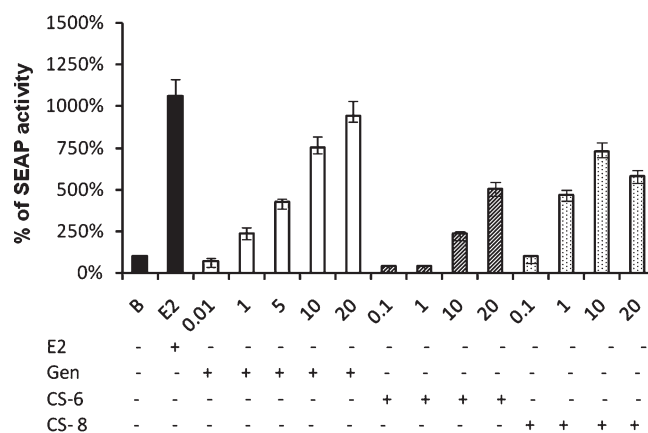
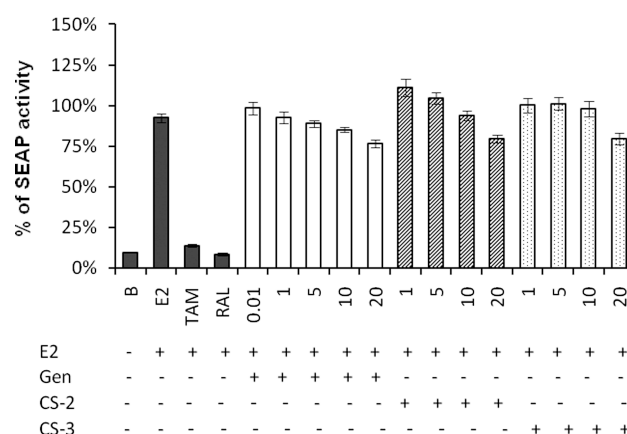
compound	IC ₅₀ (μM) ^a /cell line					
	MCF-7	MDA-MB-231	A549	Ca9-22	Hep3B	HepG2
1	<i>b</i>	<i>b</i>	80.67	62.17	47.22	<i>b</i>
2	13.94	8.31	34.08	30.39	12.04	11.09
3	21.99	10.80	54.41	34.20	15.87	12.03
4	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>
5	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>
6	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>
7	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>
8	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>
9	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>
10	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>
11	51.41	44.44	32.60	57.56	17.89	7.37
Doxo	0.77	1.93	0.59	0.26	1.03	0.63

^aData are expressed as mean ($n = 2$). ^bInactive. Doxo: doxorubicin, a positive control.

**Figure 5.** Estrogenic activity in MCF-7 cells of the pure components from *C. sappan*. The SEAP activity induced by E2 (0.01 nM) was selected as the positive control. Compounds (20 μM) were tested individually, and the SEAP activity was compared to B (blank), which was set to 100% SEAP activity. Each column represents a percentage of SEAP activity and is expressed as mean ± SEM ($n = 4$).

these compounds should possess good binding affinities to the ER. The docking results taken together with the results of the in vitro reporter gene assay suggest that the active compounds induced ERE transcription in an ER-dependent manner.

In conclusion, this is the first study using the pER8:GUS system for the bioactivity-guided fractionation of estrogen-like natural products. The applicability of this transgenic plant assay system in the discovery of phytoestrogens was confirmed using an in vitro MCF-7 reporter assay. The transgenic plant system pER8:GUS exhibited higher sensitivity toward E2 compared to the reported technique using pER8:GFP (Figure 1).⁷ As it uses a histochemical method and visual observation, pER8:GUS should be a low-cost and convenient screening system. While cytotoxicity is a limiting factor of in vitro cell-based models, the transgenic plant system expressed tolerance toward higher doses of cytotoxic compounds, and this new screening approach can be widely used in phytoestrogen discovery. Cytotoxic compounds 2 and 3 are potential estrogen partial agonists (active at a higher concentration in the plant model)/antagonists (active at a lower concentration in the MCF-7 cell model) and should be further

**Figure 6.** Estrogenic activity in MCF-7 cells of compounds 6 (CS-6) and 8 (CS-8). The SEAP activities induced by E2 (0.01 nM) and genistein (Gen, 0.01–20 μM) were selected as positive controls. Compounds 6 and 8 (0.1–20 μM) were tested alone, and the bioactivity was compared to B (blank), which was set to 100% SEAP activity. Each column represents a percentage of SEAP activity and is expressed as mean ± SEM ($n = 4$).**Figure 7.** Antiestrogenic activity in MCF-7 cells of compounds 2 (CS-2) and 3 (CS-3). The SEAP activity induced by E2 (0.01 nM) was set to 100%. Tamoxifen (TAM, 20 nM) and raloxifene (RAL, 2 nM) [induced with E2 (0.01 nM)] were selected as positive controls. Compounds 2, 3, and genistein (1–20 μM) were tested individually in the presence of E2 (0.01 nM). Each column represents a percentage of SEAP activity and is expressed as mean ± SEM ($n = 4$).

investigated for their pharmacological activity through in vivo studies. The major limitation of this transgenic plant assay may be its relative lower sensitivity (MAC of E2 at 0.63–1.25 nM) when compared to an in vitro reporter assay in MCF-7 human cancer cells (MAC of E2 at 0.01 nM). Estrogen-dependent activities of the isolated compounds from *C. sappan* supported the folk medicinal use of *C. sappan* in treating gynecological diseases. The pER8:GUS reporter assay system was demonstrated as a useful and effective technique in phytoestrogen discovery.

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were obtained using Jasco UV-530 ultraviolet spectrophotometers. IR spectra were obtained on a Mattson Genesis II infrared spectrophotometer.

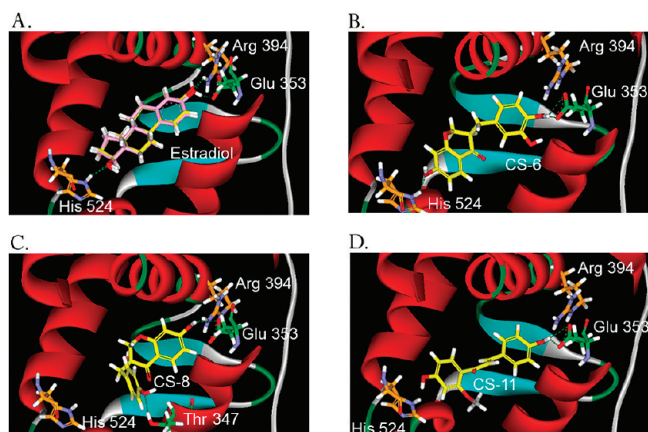


Figure 8. Simulations of compounds **6**, **8**, and **11** bound into the binding site of the estrogen receptor (PDB code: 1A52). (A) Superimposition of the best-docked E2 (yellow) compared to the naturally bound E2 (pink) in the ER. The rmsd value of docked ligand was 0.3 Å. The green dashed lines are hydrogen bonds. E2 in the active binding site formed three hydrogen bonds with the residues Arg 394 (2.31 Å), Glu 353 (2.02 Å), and His 524 (2.02 Å). (B) Compound **6** (CS-6) (yellow) docked into the binding site of the ER formed three hydrogen bonds with the residues Glu353 (2.46 and 1.13 Å) and His 524 (1.72 Å). (C) Compound **8** (CS-8) (yellow) docked into the binding site of ER formed three hydrogen bonds with the residues Arg 394 (1.79 Å), Glu353 (2.24 Å), and Thr 347 (1.93 Å). (D) Compound **11** (CS-11) (yellow) docked into the binding site of ER formed two hydrogen bonds with the residue Glu353 (2.49 and 1.92 Å).

Optical rotations were measured with Jasco DIP 370 and P-1020 digital polarimeters. The CD spectra were obtained using a Jasco J-810 spectrophotometer. The instrumentation for HPLC was composed of dual Shimadzu LC-10AT pumps and a Shimadzu SPD-10A UV-vis detector, as well as a Waters Atlantis T3 RP 150 × 4.6 mm, 5 μm preparative column, Thermo ODS Hypersil 250 × 4.6 mm, 5 μm preparative column, or Thermo ODS Hypersil 21.2 × 250 mm, 5 μm preparative column. NMR (400 and 600 MHz) spectra were obtained on a Varian Unity 400 MHz FT-NMR and a Varian Unity 600 MHz FT-NMR. ESIMS data were collected on a VG Biotech Quattro 5022 mass spectrometer. High-resolution EIMS data were obtained on a JEOL JMS-700 mass spectrometer (EI/CI/FAB).

Plant Material. The dried heartwood of *C. sappan* (*Caesalpinia* 001) was collected from Taichung City, Taiwan, in October 2006. The material was identified by Dr. Ming-Hong Yen and deposited in the Graduate Institute of Natural Products, Kaohsiung, Taiwan.

Extraction and Isolation. Each TCM (36.0 g) was extracted with 95% aqueous MeOH (200 mL) or 95% aqueous EtOH (200 mL) at room temperature three times. The solutions were separately concentrated under reduced pressure to yield an MeOH or EtOH extract. All 30 extracts were dissolved in DMSO to obtain 10 mg/mL stock solutions. Each TCM extract (200 μg/mL) was screened for estrogenic activity using the pER8:GUS reporter system.

The dried heartwood of *C. sappan* (2.6 kg) was extracted with MeOH (8 L × 7) at room temperature to yield an MeOH extract (274.4 g). The MeOH extract was partitioned with EtOAc and H₂O to yield EtOAc (203.0 g), insoluble (12.7 g), and aqueous (58.7 g) layers. The layers were assayed for estrogen-like activity using the transgenic plant pER8:GUS reporter system, and only the EtOAc layer exhibited estrogen-like activity. The initial fractionation of the insoluble layer was conducted via recrystallization to yield brazilein (**2**, 10.2 g).

The EtOAc layer was further separated into three fractions by silica gel CC eluting with EtOAc–MeOH (1:0 → 0:1). Fractions CSE1 and

CSE2 showed significant estrogenic activity (MAC < 50 μg/mL). Fraction CSE1 was separated on silica gel with CHCl₃–MeOH (35:1) to yield 14 subfractions, CSE1-1 to CSE1-14. Among these subfractions, CSE1-5 to CSE1-10 showed estrogenic activity and were subjected to further chromatography.

Fraction CSE1-5 (1.7 g) was separated into 10 fractions by silica gel CC eluting with CHCl₃–EtOAc (15:1). Subfraction 1-5-7 (560.5 mg) was purified over reversed-phase SPE (solid phase extraction) eluting with MeOH–H₂O (3:2) to provide (S)-3,7-dihydroxychroman-4-one (**1**, 12.0 mg). Subfraction 1-5-8 (646.6 mg) was separated by silica gel CC eluting with CHCl₃–MeOH (30:1), and subfraction 1-5-8-9 (106.5 mg) was subjected to RP-HPLC chromatography (Thermo ODS preparative: 21.2 × 250 mm, 5 μm, MeOH–H₂O, 7:3, flow rate 3 mL/min; UV detector setting at 254 nm), which produced a mixture of **4** and **5** (44.0 mg). The mixture was purified over reversed-phase recycle HPLC (Thermo ODS preparative: 250 × 21.2 mm, 5 μm, MeOH–H₂O, 65:35, flow rate 3 mL/min; IR detector, recycle for three times) to provide 3'-deoxysappanol (**4**, 16.0 mg) and 3'-deoxyepisappanol (**5**, 5.0 mg).

Fraction CSE1-6 (2.4 g) was chromatographed on silica gel and eluted with CHCl₃–MeOH (20:1) to give nine subfractions. Subfraction 1-6-4 (191.4 mg) was subjected to silica gel CC, and subfraction 1-6-4-3 (80.3 mg) was purified over RP-HPLC (Thermo ODS preparative: 250 × 21.2 mm, 5 μm, MeOH–H₂O, 3:2, flow rate 3 mL/min; UV detector setting at 254 nm) to yield 3-deoxysappanchalcone (**11**, 18.3 mg). Subfractions 1-6-5 and 1-6-6 (343.8 mg) were combined and separated on silica gel CC to yield seven subfractions. Subfraction 1-6-6-2 (22.4 mg) was purified over RP-HPLC (Thermo ODS preparative: 250 × 21.2 mm, 5 μm, MeOH–H₂O, 65:35, flow rate 3 mL/min; UV detector setting at 254 nm) to provide an isopropylidene derivative of sappanol (**7**, 4.1 mg) and 3-deoxysappanone B (**8**, 5.2 mg). Subfraction 1-6-6-3 (148.8 mg) was purified by RP-HPLC (Thermo ODS preparative: 250 × 21.2 mm, 5 μm, MeOH–H₂O, 65:35, flow rate 3 mL/min; UV detector setting at 254 nm) to provide 3-deoxysappanone B (**8**, 16.0 mg).

Fraction CSE1-7 (11.9 g) was recrystallized with CHCl₃–MeOH to yield protosappanin A (**9**, 394.1 mg). Fraction CSE1-10 (33.2 g) was separated on silica gel eluted with CHCl₃–MeOH (20:1 → 6:1) to yield four subfractions, and then subfraction 1-10-1 (188.0 mg) was purified on a silica gel column using CHCl₃–EtOAc (9:1) to yield 3,7-dihydroxychromen-4-one (**10**, 20.0 mg).

Fraction CSE2 (35.9 g) was chromatographed on silica gel with CHCl₃–MeOH (8:1) to yield 10 subfractions. Subfractions from CSE2-1 to CSE2-5 exhibited estrogenic activity. Subfraction 2-5 (6.6 g) was subjected to silica gel CC eluting with CHCl₃–EtOAc (2:1) to yield brazilein (**3**, 850.5 mg). The known compounds were identified by comparing their physical and spectroscopic data with reported data.

(S)-3,7-Dihydroxychroman-4-one (**1**): light yellow, amorphous solid; [α]_D²⁵ –98.8 (c 0.2, CHCl₃); UV (MeOH) λ_{\max} (log ϵ) 312 (2.57), 274 (4.32), 233 (3.45) nm; IR (neat) γ_{\max} 3439, 1645, 1557, 1455, 1016 cm^{–1}; ¹H and ¹³C NMR data, see Table 2; HREIMS *m/z* 180.0424 [M]⁺ (calcd for C₉H₈O₄, 180.0423).

Transgenic Plant Material and Estrogen-Like Reporter Assay. The *Arabidopsis* pER8:GUS line, with an estrogen receptor-based transactivator XVE (pER8) system, was originally developed by Brand et al.¹⁰ pER8:GUS seeds were grown in the dark for 24–36 h at 4 °C on medium (1/2MS, 1% sucrose, 0.8% phytoagar) for vernalization and then germinated under white light for 72 h at 24 °C. The plants were transferred to a 24-well microtiter plate in the presence or absence of test samples and incubated at 24 °C for 48 h. Plants cultured with 0.31–10 nM 17 β -estradiol were taken as a positive control.

Histochemical Assay. After incubation in the presence or absence of test samples, transgenic plants were soaked in 0.2 mL per well of the GUS assay solution [50 mM Na₂PO₄ buffer (pH 7.0), 10 mM EDTA (pH 8.0), 2 mM X-Gluc, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, and

0.1% Triton X-100] in a 24-well plate and incubated for 3 h or overnight at 37 °C. The sections were rinsed with 70% aqueous EtOH for 1 h to remove the chlorophyll. Using a ZEISS Axiovert 200 inverse microscope, samples were examined for GUS staining and photographed with a digital camera.

Fluorometric Assay. For the quantitative assay, GUS activity was determined by the fluorometric assay as described by Jefferson et al.²² At the end of the bioassay, plants were frozen in liquid N₂ and homogenized. Proteins were extracted in extraction buffer [50 mM Na₃PO₄ buffer (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 10 mM β -mercaptoethanol] and centrifuged at 10000g for 15 min at 4 °C to remove insoluble cell debris. The total protein concentrations of the plant extracts were determined by the dye-binding method proposed by Bradford³¹ with a kit supplied by Bio-Rad Laboratories. The GUS activity was determined with the fluorescence substrate 4-methylumbelliferyl- β -D-glucuronide (4-MUG, 1 mM) in extraction buffer at 37 °C, and the reaction was terminated by the addition of a stop buffer (0.2 M Na₂CO₃). Fluorescence was measured at 365 nm (excitation) and 455 nm (emission) using a microtiter reader. The fluorometer was calibrated with freshly prepared 4-methylumbelliferone (4-MU) standards of 0.05 to 1 μ M in the extraction buffer.

Cytotoxicity Assay. Six human cancer cell lines were used in the MTT assay as described by Wu et al.³² Cell viability was measured by the MTT colorimetric method. Human liver (Hep3B and Hep G2), lung (A549), oral (Ca 9-22), and breast (MCF-7 and MDA-MB-231) cancer cell lines were obtained from the American Type Culture Collection. All cell lines were cultured in DMEM/F-12 media containing 10% (v/v) FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.³²

Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000–10 000 cells per well in the absence or presence of tested samples, which were diluted with DMSO. Doxorubicin was used as a positive control. After three days of cultivation, attached cells were incubated with MTT (0.5 μ g/mL) for 1 h. The absorbance at 550 nm was measured using a microplate reader. These results represented the average from at least two independent experiments.

Reporter Gene Assay. Human breast adenocarcinoma cells MCF-7 obtained from Bioresource Collection and Research Center were cultured in phenol-red free minimum essential medium Eagle (MEM) supplemented with dextran-charcoal-treated serum, 2 mM L-glutamine, 10% fetal bovine serum (Gibco), penicillin, and streptomycin. The cells were incubated in an atmosphere of 5% CO₂ at 37 °C. The transfections were made using a liposome-based method (Lipofectamine 2000, Invitrogen) according to the manufacturer's protocol in 96-well plates. Briefly, 0.2 μ g of pERE-TA-SEAP plasmid (Clontech) was transfected into 2×10^4 cells in 100 μ L of growth medium per well and incubated for 6 h. Cells were washed and treated with samples of interest in growth medium for 48 h. Aliquots of culture media were analyzed for secreted alkaline phosphatase activity using the Phospha-Light reporter chemiluminescence assay kit (Applied Biosystems). The MTT colorimetric assay was performed on the cells for assessing their corresponding cytotoxicity.^{33,34} Finally, the estrogenic and antiestrogenic data were determined by the formula, final SEAP% = SEAP%/cell viability%, to avoid false data result from cytotoxicity. Tests were done in triplicate or quadruplicate.

Molecular Docking Model. The docking package LigandFit within the software Discovery Studio 2.5 (Accelrys, San Diego, CA) was used to predict the binding mode of the compounds in the estrogen receptor.³⁵ The crystal structures of the estrogen receptor complex with 17 β -estradiol (PDB code: 1A52) were retrieved from the RCSB Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>).³⁶ First, water molecules and gold were removed from the receptor. The hydrogen atom and the charge were added to the receptor and optimized by the Charm force field. A binding pocket of the native bonded 17 β -estradiol

in the receptor was selected as the binding site for a docking cavity. The Dreiding force field was used to search the conformations when compounds were docked in the ER- α . The rmsd was measured as the distance between the docked compounds and the native binding ligand. The energy minimizations of structures were performed by a MMFF94 force field using the software package ChemBio3D Ultra 11.0 (CambridgeSoft Co.).

■ ASSOCIATED CONTENT

S Supporting Information. 1D and 2D NMR spectra of compounds **1** and **10** are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +886-7-3121101-2162. Fax: +886-7-3114773. E-mail: aaronfrc@kmu.edu.tw.

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■ REFERENCES

- (1) Nelson, H. D.; Humphrey, L. L.; Nygren, P.; Teutsch, S. M.; Allan, J. D. *JAMA, J. Am. Med. Assoc.* **2002**, 288, 872–881.
- (2) Tsuang, Y. H.; Chen, L. T.; Chiang, C. J.; Wu, L. C.; Chiang, Y. F.; Chen, P. Y.; Sun, J. S.; Wang, C. C. *J. Orthop. Surg. Res.* **2008**, 3, 1–9.
- (3) Siow, R. C.; Li, F. Y.; Rowlands, D. J.; de Winter, P.; Mann, G. E. *Free Radical Biol. Med.* **2007**, 42, 909–925.
- (4) Brann, D. W.; Dhandapani, K.; Wakade, C.; Mahesh, V. B.; Khan, M. M. *Steroids* **2007**, 72, 381–405.
- (5) Moutsatsou, P. *Hormones (Athens)* **2007**, 6, 173–193.
- (6) Bhat, K. P.; Lantvit, D.; Christov, K.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Cancer Res.* **2001**, 61, 7456–7463.
- (7) Chang, F. R.; Hayashi, K.; Chua, N. H.; Kamio, S.; Huang, Z. Y.; Nozaki, H.; Wu, Y. C. *J. Nat. Prod.* **2005**, 68, 971–973.
- (8) Zuo, J.; Niu, Q. W.; Chua, N. H. *Plant J.* **2000**, 24, 265–273.
- (9) Takahashi, Y.; Tojo, T.; Nagahora, S.; Yamazaki, K. *J. Agric. Food Chem.* **2007**, 55, 2923–2929.
- (10) Brand, L.; Horler, M.; Nuesch, E.; Vassalli, S.; Barrell, P.; Yang, W.; Jefferson, R. A.; Grossniklaus, U.; Curtis, M. D. *Plant Physiol.* **2006**, 141, 1194–1204.
- (11) Badami, S.; Moorkoth, S.; Rai, S. R.; Kannan, E.; Bhojraj, S. *Biol. Pharm. Bull.* **2003**, 26, 1534–1537.
- (12) *Encyclopedia of Chinese Materia Medica*; Shanghai Science and Technology Press: Shanghai, 1977; p 1083.
- (13) Hu, C. M.; Kang, J. J.; Lee, C. C.; Li, C. H.; Liao, J. W.; Cheng, Y. W. *Eur. J. Pharmacol.* **2003**, 468, 37–45.

- (14) Hikino, H.; Taguchi, T.; Fujimura, H.; Hiramatsu, Y. *Planta Med.* **1977**, *31*, 214–220.
- (15) Kim, K. J.; Yu, H. H.; Jeong, S. I.; Cha, J. D.; Kim, S. M.; You, Y. O. *J. Ethnopharmacol.* **2004**, *91*, 81–87.
- (16) Oh, S. R.; Kim, D. S.; Lee, I. S.; Jung, K. Y.; Lee, J. J.; Lee, H. K. *Planta Med.* **1998**, *64*, 456–458.
- (17) Kim, D. S.; Baek, N. I.; Oh, S. R.; Jung, K. Y.; Lee, I. S.; Lee, H. K. *Phytochemistry* **1997**, *46*, 177–178.
- (18) Namikoshi, M.; Saitoh, T. *Chem. Pharm. Bull.* **1987**, *35*, 3597–3602.
- (19) Namikoshi, M.; Nakata, H.; Yamada, H.; Nagai, M.; Saitoh, T. *Chem. Pharm. Bull.* **1987**, *35*, 2761–2773.
- (20) Nagai, M.; Nagumo, S.; Lee, S. M.; Eguchi, I.; Kawai, K. I. *Chem. Pharm. Bull.* **1986**, *34*, 1–6.
- (21) Namikoshi, M.; Nakata, H.; Nuno, M.; Ozawa, T.; Saitoh, T. *Chem. Pharm. Bull.* **1987**, *35*, 3568–3575.
- (22) Jefferson, R. A.; Kavanagh, T. A.; Bevan, M. W. *Embo. J.* **1987**, *6*, 3901–3907.
- (23) Umehara, K.; Nemoto, K.; Ohkubo, T.; Miyase, T.; Degewa, M.; Noguchi, H. *Planta Med.* **2004**, *70*, 299–304.
- (24) Indira, M.; Sirsi, M.; Senich, R.; Dev, S. J. *Sci. Ind. Res. India* **1956**, *15*, 202–204.
- (25) Demir, A. S.; Aybey, A.; Sesenoglu, Ö.; Polat, F. *Tetrahedron: Asymmetry* **2003**, *14*, 1489–1493.
- (26) Song, T. T.; Hendrich, S.; Murphy, P. A. *J. Agric. Food Chem.* **1999**, *47*, 1607–1610.
- (27) Kim, E. C.; Hwang, Y. S.; Lee, H. J.; Lee, S. K.; Park, M. H.; Jeon, B. H.; Jeon, C. D.; Yu, H. H.; You, Y. O. *Am. J. Chinese Med.* **2005**, *33*, 405–414.
- (28) Duffy, C.; Perez, K.; Partridge, A. *CA Cancer J. Clin.* **2007**, *57*, 260–277.
- (29) Shrestha, A. R.; Shindo, T.; Ashida, N.; Nagamatsu, T. *Bioorg. Med. Chem.* **2008**, *16*, 8685–8696.
- (30) Wang, R.; Lu, Y.; Wang, S. J. *Med. Chem.* **2003**, *46*, 2287–2303.
- (31) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
- (32) Wu, C. C.; Chan, M. L.; Chen, W. Y.; Tsai, C. Y.; Chang, F. R.; Wu, Y. C. *Mol. Cancer Ther.* **2005**, *4*, 1277–1285.
- (33) Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 589–601.
- (34) Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 4827–4833.
- (35) Venkatachalam, C. M.; Jiang, X.; Oldfield, T.; Waldman, M. *J. Mol. Graph. Model* **2003**, *21*, 289–307.
- (36) Tanenbaum, D. M.; Wang, Y.; Williams, S. P.; Sigler, P. B. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 5998–6003.