Cytotoxic Flavonoids as Agonists of Peroxisome Proliferator-Activated Receptor γ on Human Cervical and Prostate Cancer Cells

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We conducted *in silico* screening for human peroxisome proliferator-activated receptor gamma (hPPARγ) by performing an automated docking study with 450 flavonoids. Among the eight flavonoids as possible agonists of hPPARγ, only 3,6-dihydroxyflavone (**4**) increased the binding between PPARγ and steroid receptor coactivator-1 (SRC-1), approximately 5-fold, and showed one order higher binding affinity for PPARγ than a reference compound, indomethacin. The 6-hydroxy group of the A-ring of 3,6-dihydroxyflavone (**4**) participated in hydrogen-bonding interactions with the side chain of Tyr327, His449, and Tyr473. The B-ring formed a hydrophobic interaction with Leu330, Leu333, Val339, Ile341, and Met364. Therefore, 3,6-dihydroxyflavone is a potent agonist of hPPAR with cytotoxic effects on human cervical and prostate cancer cells.

Peroxisome proliferator-activated receptors (PPARs) are a subfamily of nuclear receptors (NRs). The NR family is one of the largest families of transcription factors. Members of this family are activated by small lipophilic molecules, including hormones and vitamins.[1,2] PPARs play critical roles in the regulation of cellular differentiation and development and are, therefore, therapeutic targets in metabolic disorders such as obesity, type 2 diabetes, atherosclerosis, and cancer.[3−6]

Three PPAR subtypes, i.e., PPARα, PPARγ, and PPARδ, have been identified in humans, and their structures and functions are well known. PPARs are activated by various naturally occurring lipids.[1] Activated PPARs form a heterodimeric complex with retinoid-X receptors (RXRs), and this complex recruits coactivators to regulate the transcription of genes involved in the control of lipid metabolism.[7,8] Human PPARγ (hPPARγ), the best-studied PPAR, regulates the proliferation, apoptosis, and differentiation of various human cancer cells, including lung, breast, colon, and prostate cancer cells.[9−11] Thus, it is a well-known target protein for anticancer therapy. Activation of PPARγ has been found to increase phosphatase and tensin homologue (PTEN) protein levels or decrease transforming growth factor β1 (TGFβ1) levels, resulting in apoptosis and inhibition of cellular growth or cellular differentiation of cancer cells.[12−14] This mechanism is represented in Figure 1.

PPARγ ligands such as thiazolidinediones (TZDs), polyunsaturated fatty acids, and nonsteroidal anti-inflammatory drugs (NSAIDs) have high binding affinities for the ligand-binding domain (LBD) of PPARγ and induce a conformational change within PPARγ.[15,16] Ligand-bound PPARγ recruits coactivators such as steroid receptor coactivator-1 (SRC-1) and p300 after forming a heterodimer with retinoid X receptor α (RXRα). The NR complex settles on the peroxisome proliferator response element (PPRE) of target genes and effectively stimulates the transcription of genes associated with glucose and lipid metabolism.[17] This intracellular activation mechanism of PPARγ was used to develop a simple method to screen PPARγ ligands. In brief, *Escherichia coli* lysates containing recombinant PPARγ protein and PPARγ ligand or ligand candidates were added to 96-well plates precoated with a coactivator protein. The complex, comprising PPARγ and the coactivator, was identified using an anti-PPARγ antibody.[18]

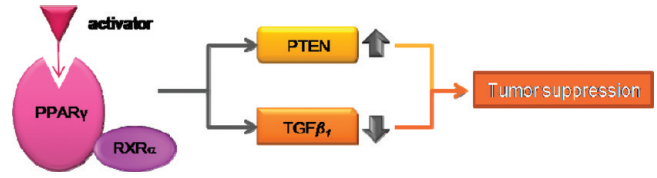

Figure 1. Schematic diagram of the mechanism of tumor suppression by PPARγ.

Glitazones (also called TZDs) are the representative family of ligands of PPARγ, and PPARγ is a known glitazone receptor. Glitazones have therapeutic effects against type 2 diabetes and cancer, and they are marketed as drugs by pharmaceutical companies. However, they have many side effects.[15] Therefore, it is necessary to develop more potent, safe drugs that activate PPARγ. Flavonoids are ligands of PPARγ and good candidates for this purpose.

Flavonoids exhibit a wide range of activities, including antioxidant, antiviral, antibacterial, and anticancer activities.[19,20] Since many flavonoids have low toxicity in mammals, some of them are commonly used in medicines. Flavonoids have been shown to inhibit the growth of tumors in various cancer cell types.[21,22] Flavonoids are agonists of NR family members, and the crystallographic complex structure of the human estrogen receptor (ER), an NR, with the isoflavonoid genistein has been solved.[23,24] Thus, it is likely that flavonoids may also function as PPARγ activators, promoting anticancer effects.

Since the clinical value of PPARγ activators as anticancer agents is well established, we performed a virtual screening for PPARγ using flavonoids and selected candidate agonists of PPARγ. Several *in vitro* assays were performed, including an investigation of the cytotoxicity of candidates on various human cancer cells.

Results and Discussion

To determine the active site of PPARγ, we referred to the two published crystal structures of PPARγ complexed with its agonists, rosiglitazone and GW409544 (a modified derivative of farglitazar).[25,26] 2D structures of these two agonists and the structure of the ligand-binding domain of PPARγ are represented in Figure 2. In the crystal structures, the headgroup of each agonist formed two or three hydrogen bonds with His323 in helix 5, Tyr327 in helix 3, and His449 in helix 10 of PPARγ.[25,26] Since most PPAR agonists show similar hydrogen-bonding patterns, which are key interactions

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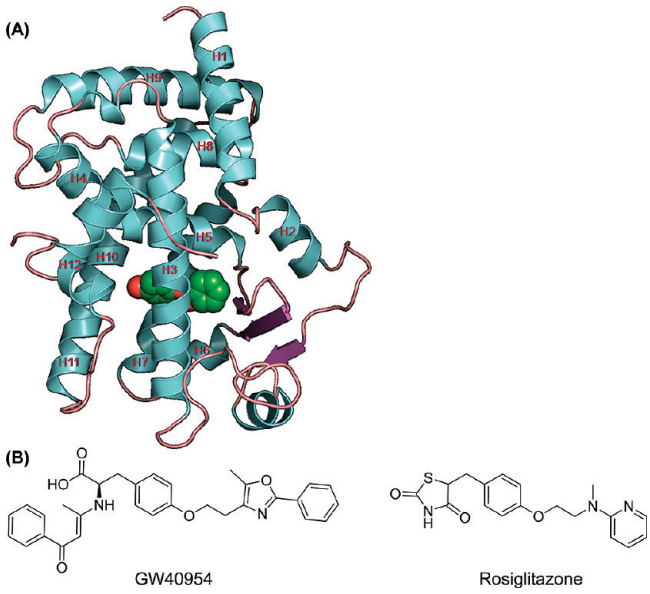

Figure 2. The structure of PPARγ and its known agonists. (A) Ligand-binding domain (LBD) of PPARγ. (B) Known PPARγ agonists, GW409544 and rosiglitazone racemate. Helix 1 is denoted as H1, and all 12 helices are denoted similarly.

for the stimulation of PPARγ activity, we determined the active site to include these interactions.

Of the 450 flavonoids we screened, 44 were found to fit within the active site of PPARγ. We selected eight flavonoids (four flavones, two flavonols, and two isoflavones) as candidate agonists of PPARγ on the basis of their high LigScores.[27] The LigScores of the selected candidates ranged between 4.8 and 5.3. The 2D structures of the candidates are represented in Figure 3. In the next step, we determined the agonist activity of the chosen candidates, using a simple screening method.

To discover new PPARγ ligands, eight candidates were screened using a PPARγ ligand screening system. This assay exploits the mechanism of PPARγ activation by its known ligands by measuring the ligand-stimulated interaction of PPARγ with SRC-1 as readout. Of the eight candidates screened, only compound **4**, 3,6-dihydroxyflavone, significantly increased the binding between PPARγ and SRC-1 in a concentration-dependent manner (Figure 4). Binding of PPARγ to SRC-1 was stimulated approximately 7-fold over the baseline in the presence of 320 μM compound **4** compared to only 2-fold over the baseline in the presence of indomethacin at the same concentration.

Although the NSAID indomethacin is not as selective a PPARγ ligand as TZDs, it is nonetheless recognized to activate PPARγ.[16] We used indomethacin as a positive control in this assay because of the high cost of TZDs. These data demonstrate that **4** can act as a PPARγ agonist for activating PPARγ *in vitro*.

We performed fluorescence-quenching experiments in order to determine the binding constant (K) of the eight candidate flavonoids and the reference compound indomethacin. Since PPARγ has one tyrosine (Tyr327) on the H3 helix of the active site, a decrease in protein fluorescence should be evident after ligand binding to the active site. Of the eight candidates, only **4** exhibited a binding affinity greater than that of indomethacin. The binding affinity of **4** ($K = 2.36 \times 10^5$ M^{-1}) was found to be an order of magnitude greater than that of indomethacin ($K = 3.39 \times 10^4$ M^{-1}). The remaining seven flavonoids had lower K values than indomethacin. This result agrees with the result of the biological screening described in section 3.2, suggesting that **4** is a potent agonist of PPARγ. The fluorescence spectra of indomethacin and **4** are shown in Figure 5.

In the docking model of **4** and PPARγ, the 6-hydroxy group of the A-ring of **4** participated in hydrogen-bonding interactions with the side chain of Tyr327 and His449. As mentioned in section 3.1, these hydrogen bonds are conserved in most agonists of PPARγ and are important for PPARγ activity. Additionally, this hydroxy group formed hydrogen bonds with the side chain of Tyr473. The C-ring of **4** participated in a hydrophobic interaction with Leu330, Leu333, Val339, Ile341, and Met364.[25,26] These residues are part of a hydrophobic pocket involving H-2, H-3, H-5, and H-6 of PPARγ. Thus, three hydrogen bonds and one hydrophobic interaction contributed to the high-affinity binding of **4** to PPARγ. The docking model and interactions are depicted in Figure 6.

To establish the cytotoxic activity of **4**, we performed an MTT assay to examine the effects of **4** on the proliferation of various human cancer cell lines. Five human cancer cell lines were selected; the criterion for selection was that PPARγ had anticancer effects on the cell line. The cytotoxic activities of **4** and indomethacin are shown in Table 1. Indomethacin showed cytotoxic activity only against HeLa cells (IC_{50}, 50 μM) and had no effect on the four other cancer cell lines. In contrast, **4** exhibited potent effects against

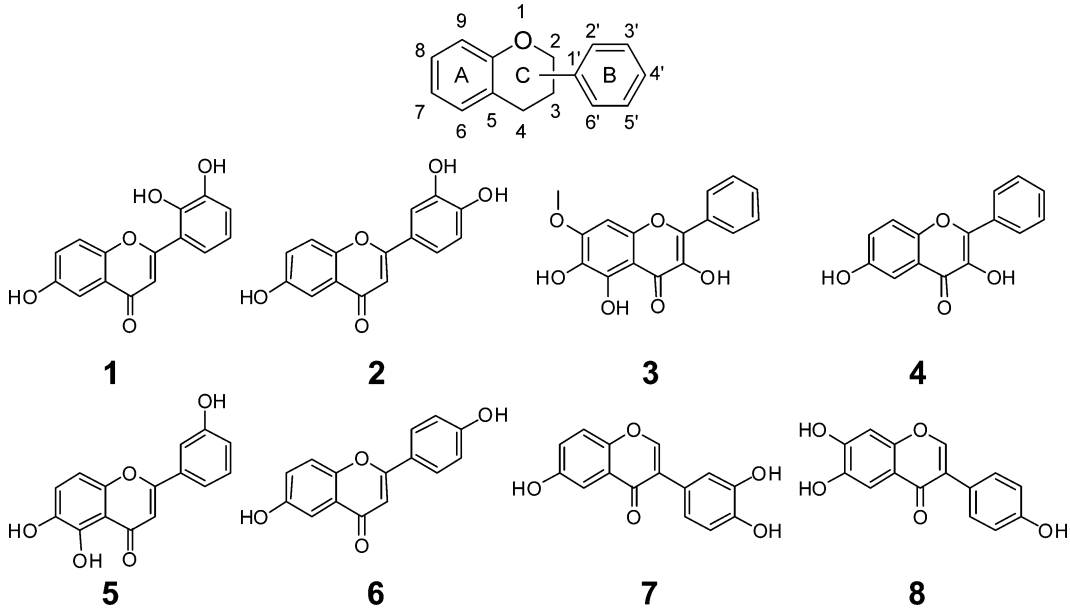

Figure 3. 2D structures of potential PPARγ ligands identified by the docking study.

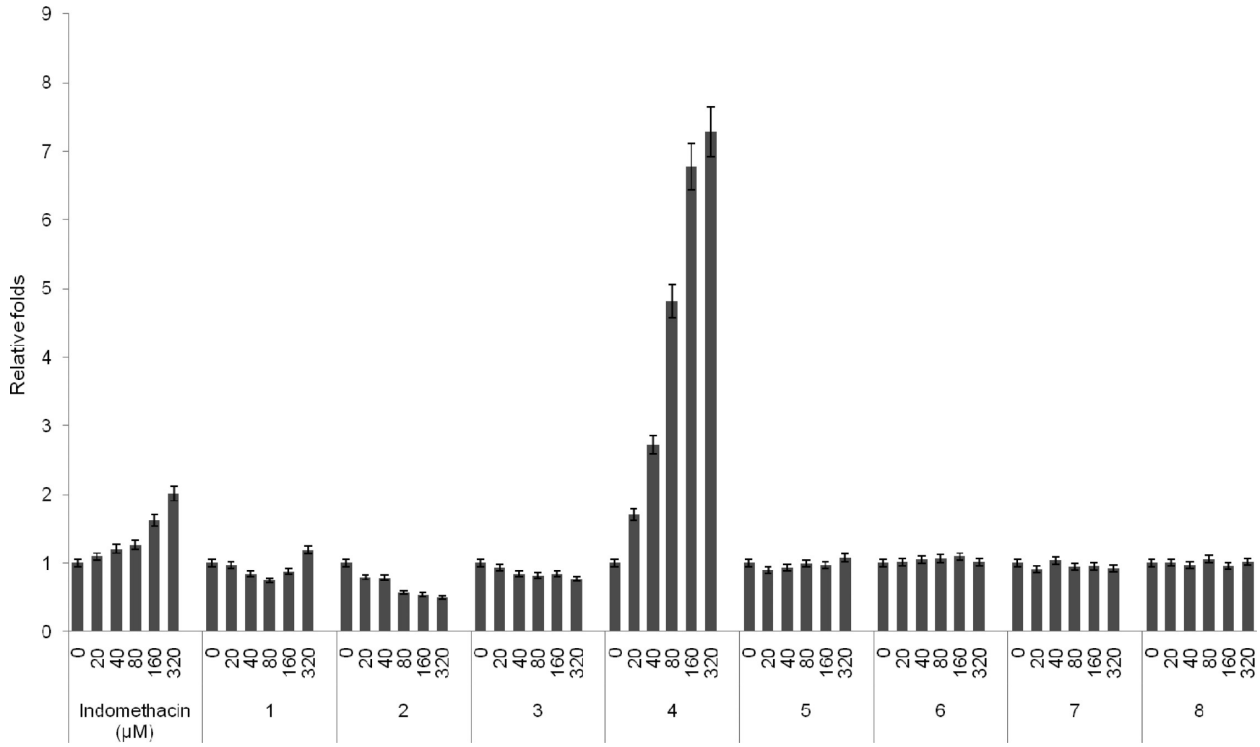

Figure 4. ELISA-based PPARγ activation assay.

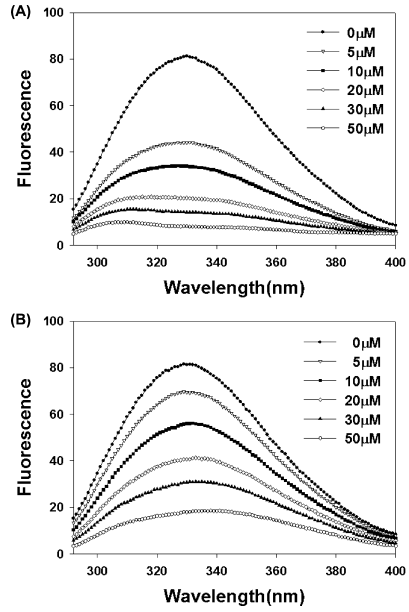

Figure 5. Fluorescence spectra of PPARγ with 0−100 μM (A) indomethacin and (B) **4**.

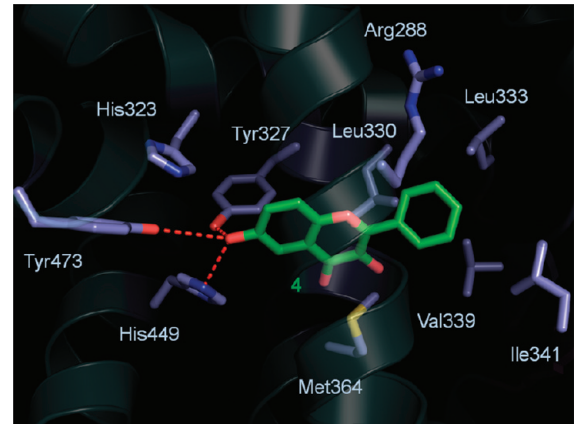

Figure 6. Binding model and key interactions between **4** and PPARγ.

Table 1. Binding Constant (*K*) and Cytotoxic Activity of Compound **4**

		cytotoxic activity (IC_{50}, μM)				
	K (M^{-1})	HeLa	MCF-7	MDA-MB-231	PC3	A549
indomethacin	3.39×10^5	50	>100	>100	>100	>100
4	2.36×10^5	12.5	>100	>100	50	100

both HeLa and PC3 cells, with IC_{50} values of 12.5 and 50 μM, respectively. We also verified that **4** altered the morphology of HeLa cells (Figure 7).

On the basis of results of the PPARγ activation assay and the MTT proliferation assay, we propose that the flavonoid **4** is a novel agonist of PPARγ, with cytotoxic activity against human cervical and prostate cancer cells.

Experimental Section

Chemicals. Flavonoids were purchased from Indofine Chemical Company, Inc., and DMSO-d_6 and D_2O were purchased from Cambridge Isotope Laboratories (Andover, MA).

***In Silico* Screening for PPARγ with Flavonoids.** On the basis of the crystal structure of estrogen receptor β ($ER_β$) complexed with the flavonoid genistein (1X7J.pdb and 1X7R.pdb),[23] we concluded that flavonoids potentially bind to the activator binding site of hPPARγ. We performed *in silico* screening for hPPARγ (chain B of 2PRG.pdb and chain D of 1K74.pdb) with flavonoids by conducting an automated docking study.[25,26] All 450 flavonoids, which were obtained from Indofine Chemical Company (Belle Mead, NJ), were potential agonists of PPARγ. The computational analysis was performed on a Linux platform, using DS modeling/CDOCKER (Accelrys Inc., San Diego, CA) with the CHARMm force field.[28−30]

Expression and Purification of PPARγ. A hexahistidine-tagged hPPARγ expression vector, pET-28a-hPPARγ-His, was constructed by cloning the appropriate nucleotide sequence into the *Bam*HI/*Xho*I restriction sites of the plasmid. The expression vector was transformed

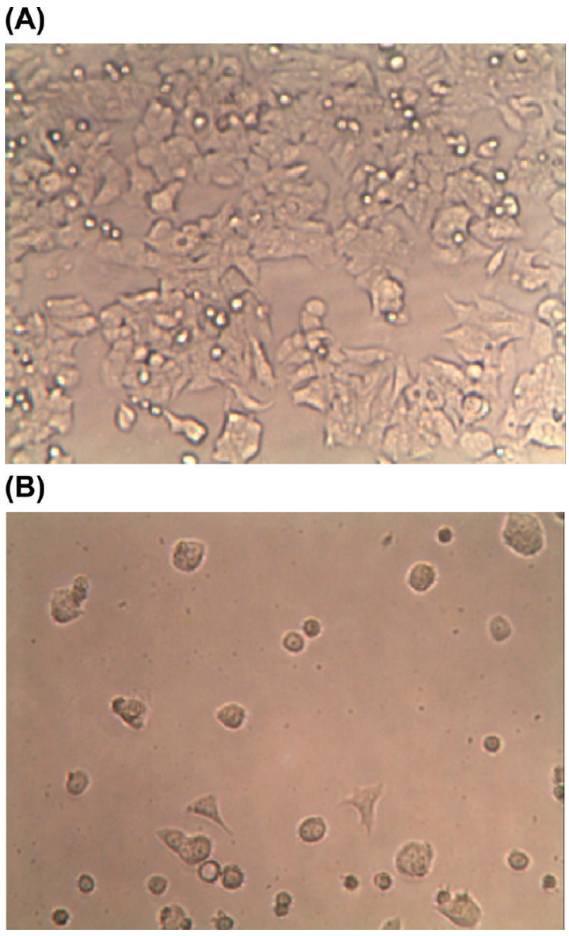

Figure 7. Cell morphology of human cervical breast cancer cells (HeLa) captured by phase contrast microscopy (A) in the absence of **4** and (B) in the presence of 12.5 μM **4** for 24 h.

into the *Escherichia coli* strain BL21. To acquire the recombinant protein, transformed bacteria cultured in Luria−Bertani (LB) medium were treated with 1 mM IPTG at OD_{600} 0.4−0.6 and induced overnight at 20 °C. After harvesting, the cells were resuspended in a buffer of 20 mM HEPES (pH 7.4), 250 mM NaCl, and 50 mM imidazole. The cell lysate was centrifuged at 15 000 rpm for 30 min, and the supernatant was loaded onto a HiTrap chelating column (GE Healthcare) that had been pre-equilibrated with buffer A [20 mM HEPES (pH 7.4) and 10% glycerol]. The column was washed with buffer A, and then, the bound material was eluted along a linear gradient of 0−500 mM imidazole. The hPPARγ-containing fractions were loaded onto a Superdex 75 column (GE Healthcare) pre-equilibrated with buffer B [10 mM Tris (pH 8.0), 0.5 mM EDTA, 50 mM NaCl, and 10 mM β-mercaptoethanol].

Simple Method to Screen PPARγ Ligands. We optimized and developed a simple method to screen PPARγ ligands, as described in previous reports.[18,31] In brief, each candidate ligand was diluted into the bacterial cell lysate containing histidine-tagged human PPARγ, and the mixture was added to 96-well plates precoated with SRC-1 recombinant protein. After incubation for 1 h, the wells were washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBST) before incubation with monoclonal anti-PPARγ antibody (Pγ48.34A) in 5% skim milk for 1 h. After three washes with PBST, horseradish peroxidase (HRP)-conjugated anti-mouse IgG in 5% skim milk was added to the wells, and the plates were incubated for another 1 h. After the plates were washed, SureBlue TMB microwell peroxidase substrate (KPL, Inc., Gaithersburg, MD) was added. When the optical density (OD) of the positive control reached unity, the enzyme reaction was stopped by adding 2.5 N H_2SO_4. Enzyme activity was detected at 450 nm, using an ELISA reader (Apollo LB 9110, Berthold Technologies GmbH, Germany).

Indomethacin, purchased from Sigma (St. Louis, MO), was used as the positive control, and Pγ48.34A was prepared as described previously.[18] Secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All the other reagents used in this study were of analytical grade and obtained commercially.

Determination of Binding Constant with Fluorescence-Quenching Experiments. Experiments were performed at 25 °C on an RF-5301PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). First, hPPARγ (5 μM) was dissolved in buffer B. Each flavonoid was titrated to give a protein:ligand molar ratio of 1:10. Next, the sample was measured in a 2 mL thermostated cuvette, with excitation and emission path lengths of 10 mm. Samples were excited at 280 nm, and emission spectra were recorded for light-scattering effects from 260 to 600 nm. Finally, we estimated K, using the Stern−Volmer equation.[32]

Cytotoxic Activity Assay. Human cervical cancer HeLa cells (KCLB10002), human breast cancer MCF-7 (KCLB30026) and MDA-MB-231 cells (KCLB30026), human lung cancer A549 cells (KCLB10185), and human prostate cancer PC3 cells (KCLB21435) were obtained from a Korean cell line bank (KCLB, Seoul, Korea). HeLa, MDA-MB-231, and PC3 cells were cultured at 37 °C in a 5% CO_2 atmosphere, using Dulbecco's modified Eagle's medium (DMEM, Welgene Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). MCF-7 and A549 cells were cultured under the same conditions but with RPMI-1640 medium (Welgene Inc.) containing 10% FBS and 1% antibiotics. The cells were maintained in suspension or as monolayer cultures and subsequently subcultured.

The cytotoxic activity of the flavonoids was evaluated using an MTT assay. The cells were seeded by adding 100 μL of cell suspension to each well to give a final seeding density of 2×10^4 cells/well before incubation at 37 °C in a 5% CO_2 atmosphere for 24 h. Various concentrations of the flavonoids were then added to the wells, followed by an additional 24 h incubation before the addition of 20 μL of MTT solution to each well and substrate development for 4 h. The amount of resulting formazan was determined by measuring the absorbance at 570 nm, using a microplate reader.[33−35]

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