Dual effects of isoflavonoids from *Pueraria lobata* roots on estrogenic activity and anti-proliferation of MCF-7 human breast carcinoma cells

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ABSTRACT

Pueraria lobata root (PLR), well known as Kudzu root, has recently become commercially available in Western dietary supplements for menopausal symptoms. The scientific basis for its action has been attributed to the action of phytoestrogens. This study aimed to investigate the estrogen-like activity of isoflavonoids isolated from *P. lobata* root and their safety with respect to their effect on breast cancer cell proliferation. In an E-screen assay, crude MeOH extract of PLR significantly increased the proliferation of MCF-7 cells in a concentration-dependent manner. Among the four fractions obtained by solvent fractionation of MeOH extract, the *n*-BuOH fraction had significant estrogen-like activities at all concentrations tested. Phytochemical analysis of the *n*-BuOH fraction led to the isolation of 10 isoflavones (**1–10**), among which genistein (**10**) had significant estrogen-like activities at all concentrations tested. These activities were significantly enhanced by treatment with genistein and 17β-estradiol compared with 17β-estradiol alone, and this effect was mediated by decreased expression of estrogen receptor (ER)α and phospho-ERα in MCF-7 cells. In a cell cytotoxicity assay, genistein (**10**) exhibited significant cytotoxicity in both ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. This cytotoxicity was characterized by the induction of apoptotic cells stained with annexin V conjugated with Alexa Fluor 488 and involved activation of mitochondria-independent and -dependent apoptosis pathways in MCF-7 cells. Our results demonstrated that genistein (**10**) has estrogen-like effects dependent on ER pathway activation and anti-proliferative effects mediated by the apoptosis pathway rather than the ER pathway in MCF-7 breast cancer cells.

1. Introduction

Estrogen exerts a wide variety of effects on growth, development, differentiation, and reproduction through binding to a specific nuclear receptor protein, the estrogen receptor (ER). The ER exists in two forms, ERα and ERβ, that function as transcription factors to regulate the expression of target genes [1]. Hormone replacement therapy (HRT) is used to treat symptoms of menopause such as hot flashes and osteoporosis and to reduce the incidence of cardiovascular disease associated with menopause [2,3]. However, current HRT regimens appear to be associated with increased risks of developing breast and ovarian cancer in healthy women [4,5]. To overcome the shortcomings of HRT, phytoestrogens derived from plants have emerged as an alternative approach to alleviate the symptoms of menopause, although their safety needs to be evaluated further.

Pueraria lobata (Willd.) Ohwi (Leguminosae) is a creeping, climbing, and trailing perennial vine. *P. lobata* root (PLR), which is well known as Kudzu root, contains starch and has been used as a food ingredient in East Asia. In Vietnam, the starch is flavored with pomelo oil and consumed as a drink in the summer. In Japan, the starch named kuzuko is used in many dishes including *kuzumochi* and *kuzuyu*. The high nutritional value of PLR has been recognized for centuries in East Asia [6]. Extract of PLR was reported to prevent obesity and improve glucose metabolism [7]. The extract has also been reported to have anti-dipsotropic activity [8]. Furthermore, among the isolated compounds from PLR, daidzein, a major isoflavonoid, showed anti-inflammatory and antioxidant activities, and lupenone exhibited cytoxicity [9]. PLR has recently become commercially available in Western dietary
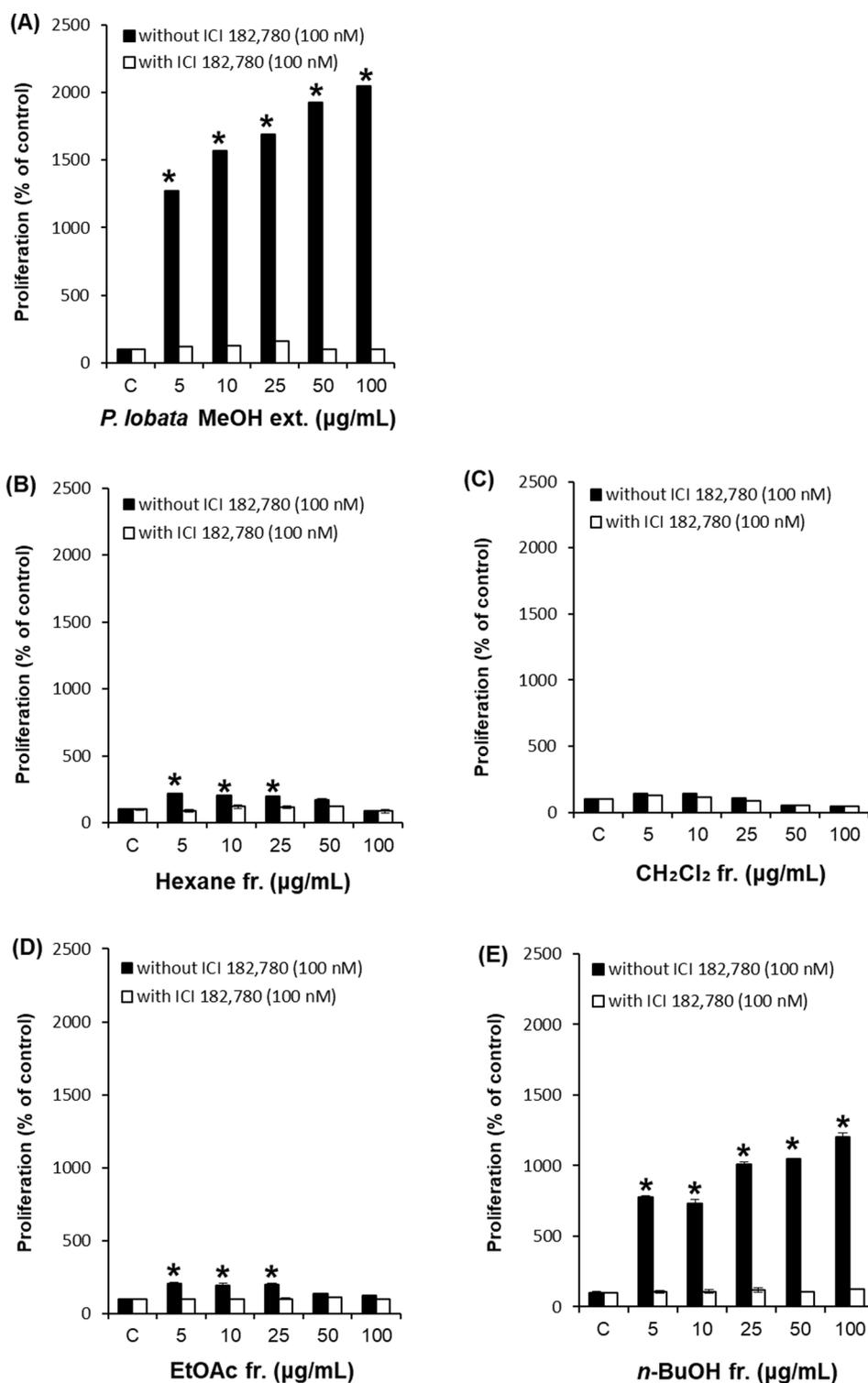

Fig. 1. Comparison of estrogenic activities of (A) *P. lobata* MeOH extract and its four fractions (B: Hexane, C: CH$_2$Cl$_2$, D: EtOAc, E: *n*-BuOH) in the absence or presence of ICI 182,780 as determined by MCF-7 cell proliferation measured by E-screen assay. The data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). *$p < 0.05$ compared to untreated control.

supplements for menopausal symptoms. Isoflavones in PLR, such as puerarin, have been reported to have estrogenic and anticancer activities [10,11]. Since HRT using hormones like estrogen increases the risks of breast and ovarian cancer, these isoflavones of PLR might be considered as promising natural alternatives to HRT for the treatment of postmenopausal symptoms with reduced risks of cancer.

This study aimed to investigate the estrogen-like activity of isoflavonoids isolated from *P. lobata* root and their safety regarding breast

cancer cell proliferation. To investigate active compounds from *P. lobata* root with dual effects of estrogen-like and anti-proliferative activities we performed phytochemical investigations combined with bioactivity-guided fractionation. This led to the successful isolation of 10 isoflavones (**1**–**10**), which were subjected to cell-based biological evaluation for dual activities. In this paper, we describe the isolation and structural elucidation of compounds **1**–**10**, together with their estrogenic and anti-proliferative effects.
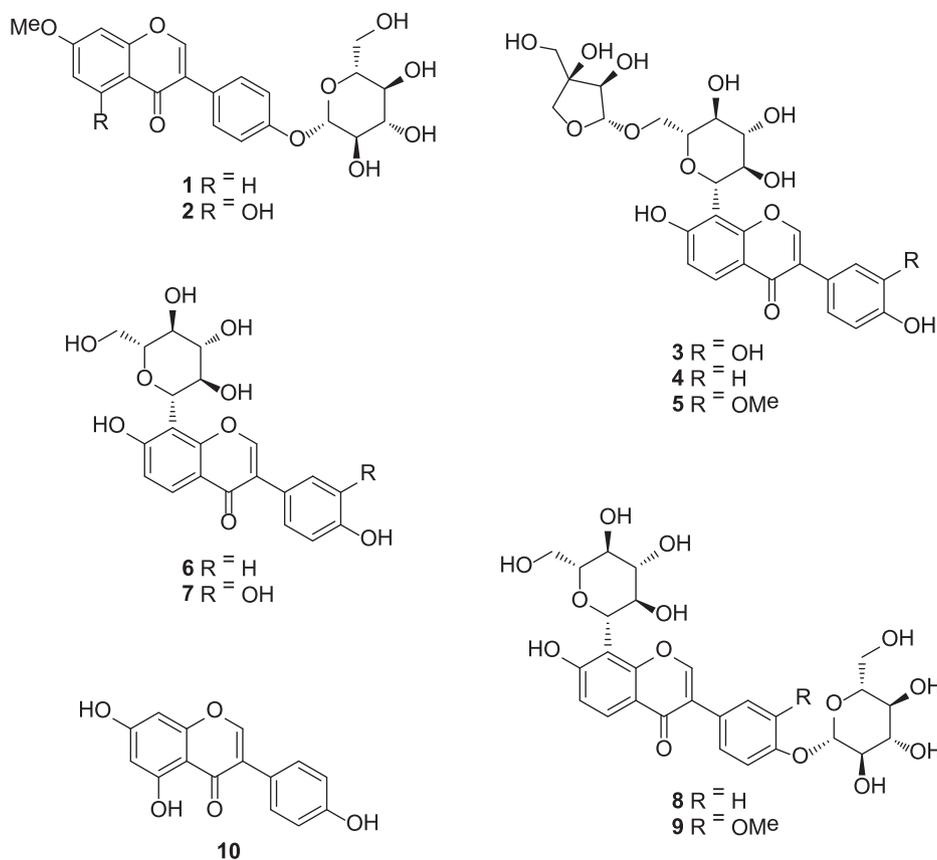
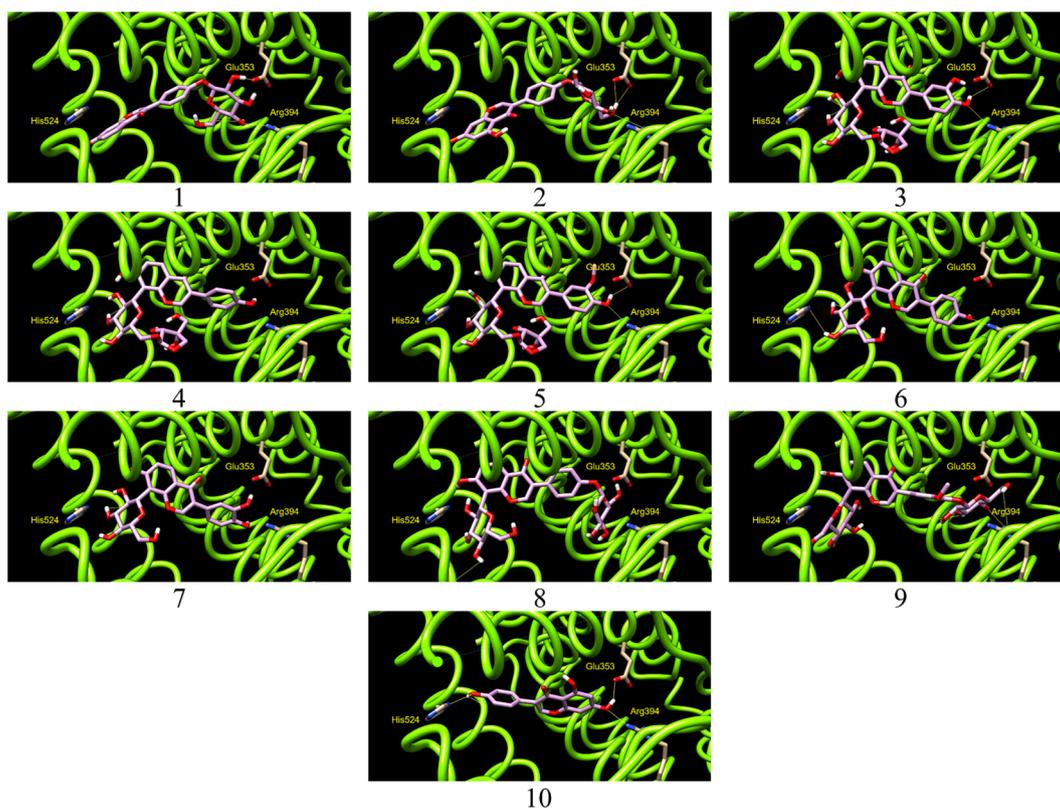**Fig. 2.** Chemical structures of compounds **1–10** identified from *P. lobata* root.

Fig. 3. Docking binding mode of compounds **1–10**. Key amino acid residues are labeled and putative hydrogen bonding is depicted as yellow lines. Molecular docking graphics were performed with the UCSF Chimera package.

Table 1
Calculated lowest binding affinity of compounds **1–10**.

Ligand	ERα binding affinity(kcal/mol)
1	−1.38
2	−1.19
3	−1.45
4	−2.66
5	+5.28
6	−7.13
7	−6.69
8	+79.71
9	+87.08
10[a]	−9.07

[a] Represents the co-crystallized ligand of the original X-ray crystal.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured using a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA). IR spectra were recorded with a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). UV spectra were acquired on an Agilent 8453 UV–visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). NMR spectra were measured on a Bruker Avance III HD 800 NMR spectrometer with a 5 mm TCI Cryoprobe (Bruker). Preparative and semi-preparative HPLC were performed on a Waters 1525 binary HPLC pump with a Waters 996 photodiode array detector using an analytical Agilent Eclipse XDB-C18 column (250 × 21.2 mm, 7 μm) (Waters Corporation, Milford, CT, USA) and a Phenomenex Luna Phenyl-hexyl 100 A column (250 × 10 mm, 10 μm), respectively. LC/MS analysis was performed with an Agilent 1200 Series HPLC system equipped with a diode array detector and a 6130 Series ESI mass spectrometer using an analytical Kinetex (2.1 × 100 mm, 5 μm) (Agilent Technologies). Column chromatography was performed with a silica gel 60 (Merck, 230–400 mesh) and an RP-C18 silica gel (Merck, 230–400 mesh). The packing material for open-column chromatography was Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan). Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was used for size exclusion chromatography. First-grade solvents (Samchun Pure Chemicals Co., Ltd., Pyeongtaek, Korea) were used for fractionation and isolation. Merck precoated silica gel F_{254} plates and reversed-phase (RP)-18 F_{254} plates were used for TLC. Spots were detected on TLC under UV light (dual wavelength 254/365 nm) or by heating after spraying with anisaldehyde-sulfuric acid.

2.2. Plant material

Roots of *P. lobata* collected from Geochang, Gyeongnam Province, South Korea in 2014 were purchased from Okchundang Co., Ltd.. The material was identified by one of the authors (K. H. Kim) and a voucher specimen (GK-14-063) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

2.3. Extraction and isolation

Dried root of *P. lobata* (500 g) was extracted with 80% MeOH at room temperature (20 h × 3). The extract was filtered and the filtrate was evaporated under reduced pressure using a rotary evaporator to obtain the MeOH extract (206.7 g). This extract was successively partitioned with hexane (4.5 g), CH_2Cl_2 (0.9 g), EtOAc (18.1 g), and *n*-BuOH (110 g), as well as water residue. Each fraction was subjected to an E-screen assay for estrogenic effect using MCF-7 cells. The *n*-BuOH fraction, which showed the highest bioactivity, was subjected to HP-20 column chromatography to yield water and MeOH layers, and the MeOH layer was fractionated using silica gel column chromatography

with the gradient solvent system of CH_2Cl_2/MeOH/H_2O (18:6:1 – 14:6:1 – 1:1:0) to yield four subfractions (B1 – B4). Fraction B3 (18.1 g) was subjected to C18 reversed-phase silica gel column chromatography using the gradient solvent system of MeOH (40% – 100%) to yield two subfractions (B31 and B32). Subfraction B31 (14.6 g) was fractionated using HP-20 column chromatography with the gradient solvent system of MeOH (0% – 20% – 40% – 60% – 80% – 100%) to yield six sub-fractions (B311 – B316). Silica gel chromatography was performed to fractionate subfraction B313 (4.3 g) with the gradient solvent system of CH_2Cl_2/MeOH/H_2O (5:1:0.15 – 1:1:0.2), yielding seven subfractions (B3131 – B3137). Subfraction B3134 (1.28 g) was subjected to C18 reversed-phase silica gel column chromatography using the gradient solvent system of MeOH (30% – 50%) to yield six subfractions (B31341 – 31346). Subfraction B31345 (183.3 mg) was loaded on a Sephadex LH-20 column to obtain five subfractions (B313451 – 313455), and subfraction B313454 (47.0 mg) was then purified by semi-preparative HPLC (2 mL/min, 10% acetonitrile) using a phenyl-hexyl column to yield compounds **5** (0.4 mg) and **7** (14.4 mg). Fraction B3135 (149.8 mg) was purified using preparative HPLC (5 mL/min, 14% acetonitrile) using a C18 reversed-phase column to yield compounds **6** (6.7 mg) and **10** (8.0 mg). Fraction B3136 (235.5 mg) was isolated using preparative HPLC (5 mL/min, 35% MeOH) with a C18 column to yield compounds **3** (2.3 mg), **4** (25.3 mg), **8** (5.3 mg), and **9** (1.5 mg). Preparative HPLC fractionation (5 mL/min, 55% – 100% MeOH) of fraction B316 (213.3 mg) using a C18 column yielded five subfractions (B3161 – B3165). Subfraction B3162 (45.0 mg) was purified by semi-preparative HPLC (2 mL/min, 10% acetonitrile) using a phenyl-hexyl column to yield five subfractions (B31621 – 31625), and solid-phase extraction of subfraction B31623 (10.1 mg) was then performed with a silica sep-pak cartridge using the gradient solvent system of CH_2Cl_2/MeOH (17:1 – 10:1) to yield compound **1** (8.7 mg). Fraction B3163 (49.6 mg) was purified by semi-preparative HPLC (2 mL/min, 10% acetonitrile) using a phenyl-hexyl column to yield compound **2** (3.2 mg).

2.4. Estrogen receptor/coactivator molecular docking

Molecular modeling was performed as previously reported [12]. The ligand 3D structure was constructed and minimized by MM2 using Chem 3D pro 12.0 software. The structure of ER-α co-crystallized with genistein was downloaded from the RCSB protein data bank (PDB ID: 1X7R). Chain A of ER-α was prepared for docking simulation using UCSF chimera 1.11 by removal of the other chain and all non-standard residues, including genistein. The protein and ligand files were prepared by the AutoDock Protocol [13]. Molecular docking analysis was performed using AutoDock 4.2.6 and AutoDock Tools 1.5.6 [14]. Analysis of putative hydrogen bonding and visual investigation were conducted with UCSF Chimera 1.11 [15].

2.5. Cell culture

ER-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI1640 medium (Cellgro, Manassas, VA, USA) supplemented with 10% FBS (Gibco BRL, Grand Island, NY, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin. Cultures were maintained at 37 °C in a humidified incubator gassed with 95% air and 5% CO_2.

2.6. Cell viability assay

The Ez-Cytox cell viability detection kit (Daeil Lab Service Co., Seoul, South Korea) was used for quantification of cell viability. MCF-7 and MDA-MB-231 cell lines were seeded in 96-well plates (1 × 10⁴ cells per well) for 24 h and then treated for the indicated concentrations of test samples in RPMI1640 medium supplemented with 10% FBS,
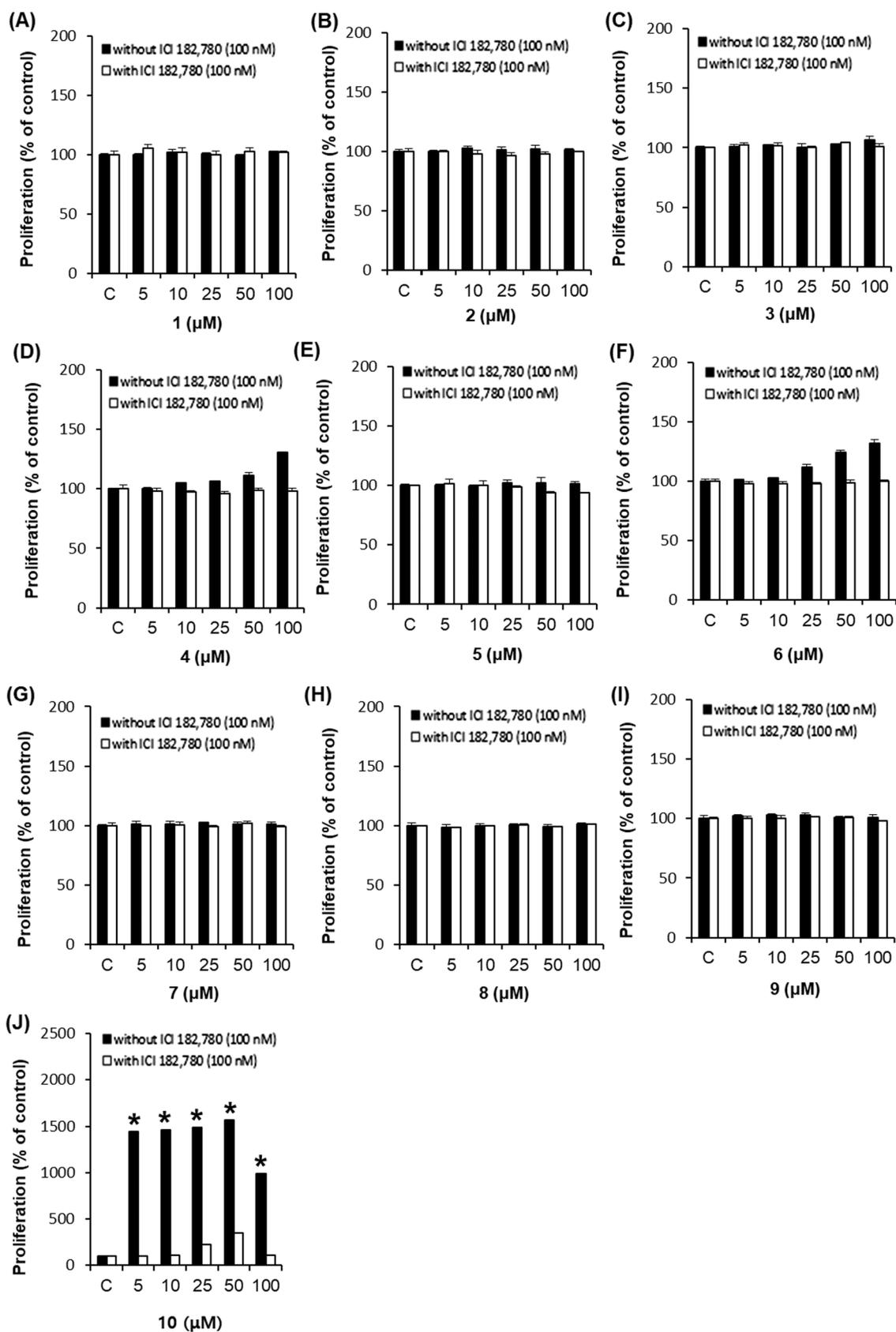
Fig. 4. Comparison of estrogenic activities of isolated compounds **1–10** (A–J) in the absence or presence of ICI 182,780 as determined by MCF-7 cell proliferation measured by E-screen assay. The data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). *$p < 0.05$ compared to untreated control.

(A)

(B)

Fig. 5. (A) Comparison of estrogenic activities of genistein (**10**) and 17β-estradiol in the absence or presence of ICI 182,780 on MCF-7 cell proliferation measured by E-screen assay. (B) Effects of genistein (**10**) and 17β-estradiol on the protein expression of phospho-ERα (66 kDa), ERα (66 kDa), and GAPDH (37 kDa) in MCF-7 cells. The data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). *$p < 0.05$ compared to untreated control.
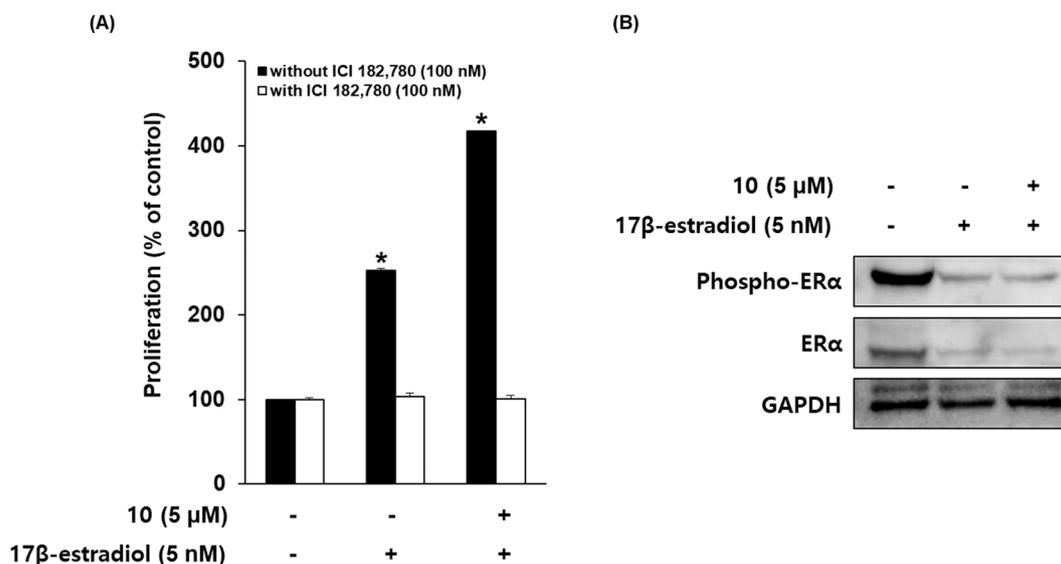
100 μg/mL streptomycin, and 100 U/mL penicillin for 24 h. The Ez-Cytox reagents were added to each well and the absorbance at 450 nm was measured using a microplate reader (PowerWave XS; Bio-Tek Instruments, Winooski, VT, USA).

2.7. E-screen assay

ER-positive MCF-7 human breast cancer cells were seeded in 24-well plates (2×10^4 cells per well) in RPMI1640 medium supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin for 24 h. Cells were treated with the indicated concentrations of test samples in phenol red–free RPMI medium (Gibco BRL) supplemented with 5% charcoal-dextran-stripped human serum (Innovative Research, Novi, MI, USA) for 144 h. When indicated, 100 nM ICI 182,780 compound (ER antagonist) was added with the test samples. The Ez-Cytox reagents were added to each well and the absorbance at 450 nm was measured using a microplate reader (PowerWave XS).

2.8. Western blotting analysis

MCF-7 human breast cancer cell lines were seeded in 6-well plates (4×10^5 cells per well) for 24 h and treated with the indicated concentrations of test samples in RPMI1640 medium supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin for 24 h. Cells were harvested and lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing 1 mM phenylmethylsulfonyl fluoride on ice, and the amount of protein in each lysate was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA), which is based on color formation with bicinchoninic acid (BCA). Bovine serum albumin (BSA) was used as a standard of common protein. 10 μL of BSA standards or protein in each lysate was added to each well of 96-well microplates. Consecutively, 200 μL of BCA protein reagent mix was added and incubated at 37 °C for 30 min. The absorbance at 562 nm was measured using a microplate reader (PowerWave XS). Equal amounts of protein (20 μg/lane) were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were incubated overnight with primary antibodies against phospho-ERα, ERα, Bax, Bcl-2, cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, and GAPDH (Cell Signaling Technology). Detection was performed using ECL Advance western blotting detection reagents (GE Healthcare, Little Chalfont, UK) and a

FUSION Solo Chemiluminescence System (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's instructions.

2.9. Image-based cytometric assay

An image-based cytometric assay was used to assess annexin V-positive stained apoptotic cells. MCF-7 cells were seeded in 6-well plates (4×10^5 cells per well) for 24 h and treated with the indicated concentrations of test samples in RPMI1640 medium supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin for 24 h. Cells were harvested and resuspended in binding buffer (Life Technologies, Carlsbad, CA, USA). Cells were stained with annexin V–Alexa Fluor 488 (Invitrogen, Temecula, CA, USA) for 30 min in the dark and positive cells were counted using a Tali image-based cytometer (Invitrogen).

2.10. Statistical analysis

All statistical analyses were performed using Statview software. Mean values for each group were compared by ANOVA. A p value < 0.05 was used as the criterion for a statistically significant difference.

3. Results and discussion

3.1. Estrogenic activity bioassay-guided fractionation

The roots of *P. lobata* were extracted with 80% MeOH and filtered. Evaporation of the filtrate yielded the MeOH extract. E-screen is a cell proliferation assay based on the ability of human breast cancer cells (MCF-7) to proliferate in the presence of estrogen active substances, which has been a reliable tool to easily and rapidly assess estrogenic activity of natural products [16]. In the E-screen assay, the crude MeOH extract of *P. lobata* roots (PLR) significantly increased the proliferation of MCF-7 cells in a concentration-dependent manner; specifically, 100 μg/mL of extract increased proliferation by 2044.78 ± 6.48% compared with untreated control that was set as 100%. Addition of ICI 182,780, an inhibitor of the estrogen receptor, to the extract completely blocked cell proliferation, confirming the estrogenic effects of the extract (Fig. 1A). The MeOH extract was then fractionated with hexane, CH_2Cl_2, EtOAc, and *n*-BuOH, yielding four solvent-partitioned fractions.
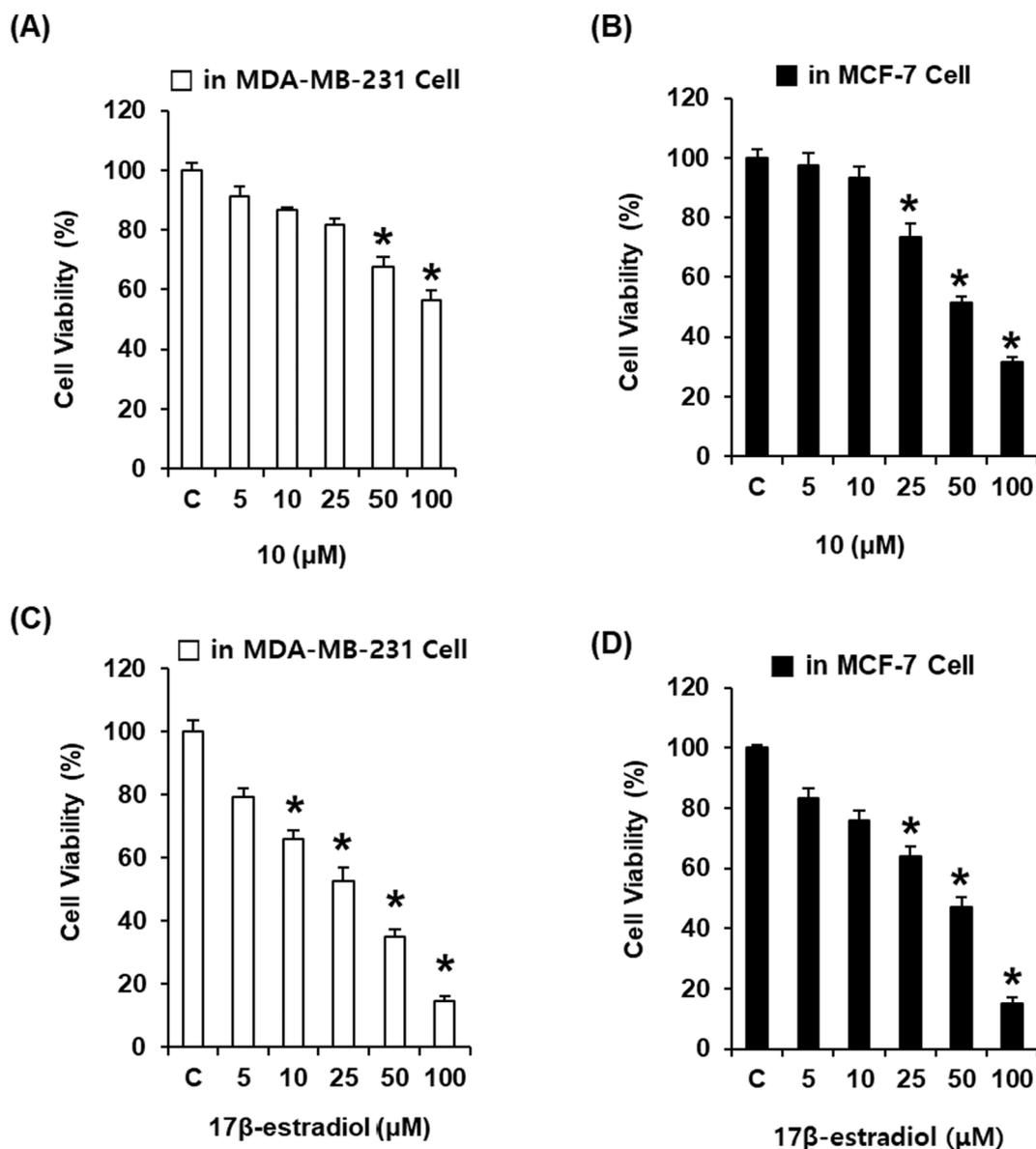
Fig. 6. Comparison of cytotoxic effect of genistein (**10**) (A and B) and 17β-estradiol (C and D) in human breast cancer cell lines MDA-MB-231 and MCF-7 by cell viability assay. The data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). *$p < 0.05$ compared to untreated control.

Each fraction was subjected to the E-screen assay, which indicated that the *n*-BuOH fraction had significant estrogen-like activities at all concentrations tested (Fig. 1). The greatest estrogenic effects were observed for 100 μg/mL *n*-BuOH fraction, which increased cell proliferation by 1199.70 ± 34.66% compared with untreated control that was set as 100% (Fig. 1E).

3.2. Isolation and structural characterization of compounds

Through repeated column chromatography over RP-C18 silica gel and HPLC purification, phytochemical analysis of the *n*-BuOH fraction led to the isolation of 10 isoflavones (**1**–**10**) that might be responsible for the observed bioactivity (Fig. 2). The isolated compounds were identified as isoononin (**1**) [17], prunetrin (**2**) [18], 3′-hydroxymirificin (**3**) [19], mirificin (**4**) [20], 3′-methoxymirificin (**5**) [21], puerarin (**6**) [21], pueraria glycoside (**7**) [20], puerarin-4′-*O*-ᴅ-glycoside (**8**) [22], 3′-methoxyl-4′-*O*-glucosylpuerarin (**9**) [23], and genistein (**10**) [20] by comparison of their NMR spectroscopic data with previously reported values and by LC/MS analysis.

3.3. Molecular docking study and estrogenic activity of compounds

The isolates (**1**–**10**) were analyzed in a molecular docking study. Binding between estrogen and the ligand-binding domain (LBD) of the estrogen receptor (ER) initiates a biological cascade that ultimately results in cell proliferation. The structure of the ERα ligand-binding domain with the ligand-binding pocket site is shown in Fig. 3. Genistein (**10**) formed the most similar hydrogen bonding interactions to 17β-estradiol, explaining the high affinity of genistein for ERα. However, the diglycosylated isoflavones (compounds **8** and **9**) exhibited low binding affinities (Table 1), possibly due to the hydroxy group substitutions in sugar moieties. The estrogenicity of the isolated isoflavones (**1**–**10**) was also tested in an E-screen assay. As expected, genistein (**10**) had significant estrogen-like activities at all concentrations tested (Fig. 4); 50 μM genistein increased proliferation of MCF-7 cells by 1566.59 ± 2.11% compared with untreated control that was set as 100% (Fig. 4J).

(A)
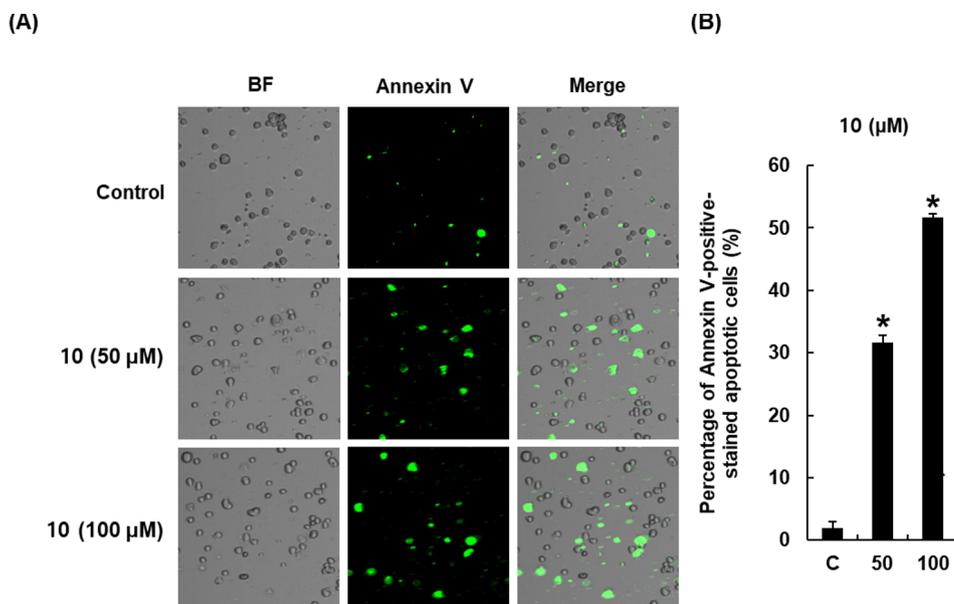

(B)

10 (µM)

Fig. 7. Effect of genistein (**10**) on MCF-7 cell apoptosis measured by image-based cytometric assay. (A) Representative images of apoptosis detection. (B) Percentage of Annexin V-positive stained apoptotic cells. The data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). *$p < 0.05$ compared to untreated control.
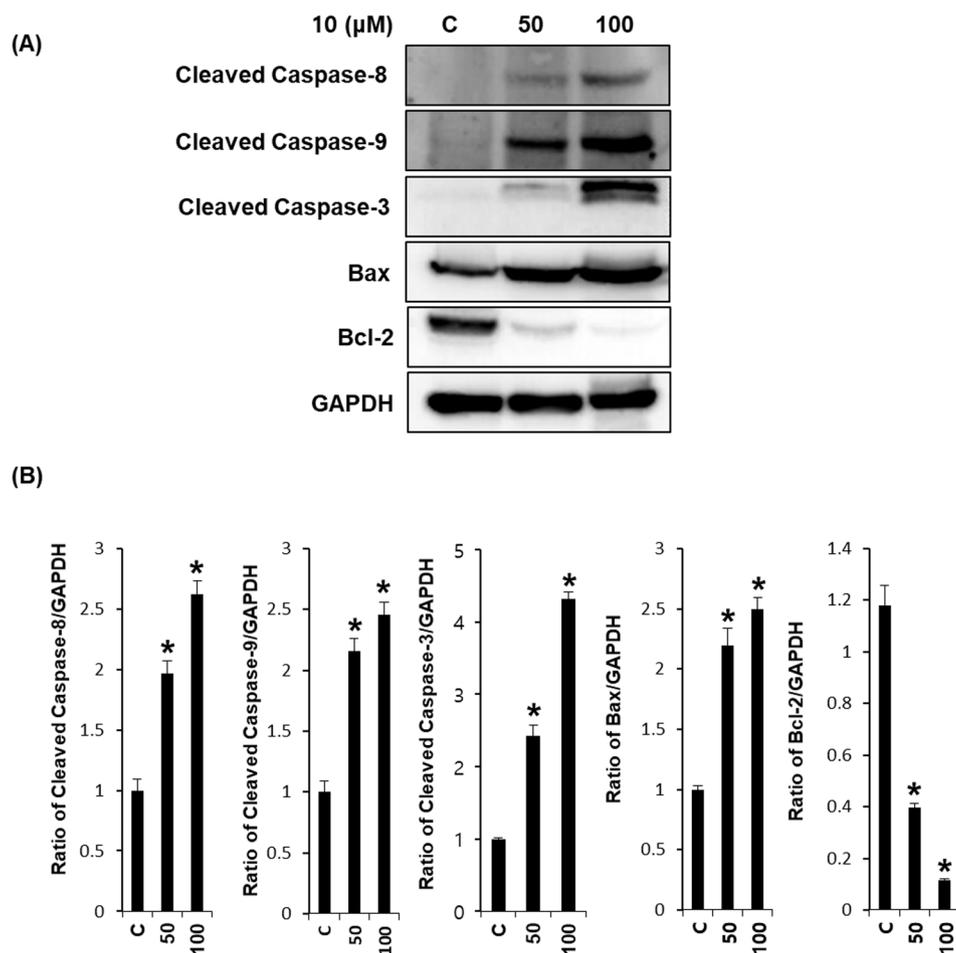
(A)

(B)

Fig. 8. Effects of genistein (**10**) on the protein expression of cleaved caspase-8, -9, -3, Bax, Bcl-2, and GAPDH in MCF-7 cells. (A) A representative Western blot demonstrating the levels of cleaved caspase-8 (18 kDa), -9 (37 kDa) and -3 (19 kDa), Bax (20 kDa), Bcl-2 (26 kDa), and GAPDH (37 kDa) in MCF-7 cells treated with compound **10** (50 and 100 µM) for 24 h. GAPDH was used as an internal control. (B) Bar graphs presenting densitometric quantification of western blot bands. The data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). *$p < 0.05$ compared to the untreated control.

3.4. Effects of genistein and 17β-estradiol on protein expression

Treatment of MCF7 cells with 5 μM genistein (**10**) and 5 nM 17β-estradiol enhanced cell proliferation significantly more effectively than treatment with 5 nM 17β-estradiol alone. The 5 nM 17β-estradiol increased proliferation of MCF-7 cells by 252.72 ± 2.06% compared with untreated control that was set as 100%, while 5 μM genistein (**10**) and 5 nM 17β-estradiol increased the proliferation by 417.55 ± 0.62% compared with untreated control (Fig. 5A). Western blot analysis was performed to investigate the effects of 5 μM genistein (**10**) and 5 nM 17β-estradiol on phosphorylation of ERα in MCF-7 cells. As shown in Fig. 5B, expression of ERα and phospho-ERα decreased after treatment with 5 μM genistein (**10**) and 5 nM 17β-estradiol or 5 nM 17β-estradiol alone. These results were consistent with previous studies in which exposure to estrogenic compounds and phytoestrogens with estrogenic activity suppressed gene and protein expression of ERα in ovaries of rats and mice [24–26]. Although estrogenic compounds and phytoestrogens induce proliferation of MCF-7 cells through an ER-dependent mechanism, their anti-proliferative effects are mediated through apoptosis via an ER-independent mechanism [27–29]. These experimental data demonstrated that estrogenic compounds and phytoestrogens have estrogen-like effects in MCF-7 cells through ER pathway activation and anti-proliferative effects independent of the ER pathway.

3.5. Cytotoxic effects of genistein in human breast cancer cells

Earlier studies have pointed out that phytoestrogens can have both positive and negative effects on the proliferation of breast cancer cells depending on amount of phytoestrogens, timing of exposure, or capacity of individual absorption and metabolism [30]. In addition, current HRT regimens appear to be associated with increased risks of developing breast cancer [4,5]. To evaluate the safety regarding the adverse effects associated with HRT treatment, the cytotoxic effects of genistein (**10**) was measured in ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells using a cell viability assay. Treatment with genistein (**10**) or 17β-estradiol at concentrations of 5–100 μM for 24 h showed significant cytotoxicity in both MCF-7 and MDA-MB-231 breast cancer cells (Fig. 6). After treatment with genistein (**10**) at 100 μM, MDA-MB-231 cell viability decreased to 56.63 ± 3.08% compared with untreated control (Fig. 6A) and MCF-7 cell viability decreased to 31.66 ± 1.62% (Fig. 6B, IC$_{50}$: 53.66 ± 1.80 μM). After treatment with 17β-estradiol at 100 μM, MDA-MB-231 cell viability decreased to 14.47 ± 1.66% (Fig. 6C, IC$_{50}$: 28.77 ± 3.45 μM) and MCF-7 cell viability decreased to 15.24 ± 1.73% (Fig. 6D, IC$_{50}$: 45.76 ± 3.17 μM).

3.6. Effects of genistein on apoptosis in MCF-7 cells

We further explored whether genistein (**10**) induced apoptosis in MCF-7 cells. Cells were exposed to genistein (**10**) at 50 and 100 μM and stained with annexin V conjugated with Alexa Fluor 488. As shown in Fig. 7, the percentage of Annexin V-positive cells indicating apoptotic cells significantly increased to 31.66 ± 1.15% and 51.66 ± 0.57% after treatment with 50 and 100 μM genistein (**10**), respectively. Western blot analysis was performed to further investigate the effects of genistein (**10**) on apoptotic cell death pathways in MCF-7 cells. As shown in Fig. 8, cleaved forms of caspase-8, -9 and -3 were detected after treatment with genistein (**10**) at 50 and 100 μM. Expression of Bcl-2 and Bax was also examined in MCF-7 cells after treatment with genistein (**10**) at 50 and 100 μM. Bcl-2 and Bax are proteins of the BCL-2 family; Bcl-2 is an inhibitor and Bax is a promoter of the apoptotic signaling pathway [31,32]. The expression of Bax increased, while that of Bcl-2 decreased, after treatment with genistein (**10**) at 50 and 100 μM. These proteins promote release of cytochrome *c* from mitochondria only at the lowest caspase-8 concentrations. Caspase-8 is a major initiator that plays an important role in the extrinsic apoptotic signaling pathway [33–35] whereas caspase-9 is a major initiator that

plays an important role in the intrinsic apoptotic signaling pathway [36]. Both pathways converge at caspase-3, an effector caspase that plays an important role in efficient execution of the apoptotic signaling pathway [37]. Our results suggest that genistein (**10**) induces both the extrinsic apoptotic signaling pathway and intrinsic apoptotic signaling pathway in MCF-7 cells.

4. Conclusions

In conclusion, we performed phytochemical investigation combined with bioactivity-guided fractionation of the MeOH extract of PLR and cell-based biological evaluations of the isolated isoflavones (**1–10**) to investigate their estrogen-like activity and effect on breast cancer cell proliferation in order to assess the efficacy and safety of PLR as an alternative to HRT. Genistein (**10**) exhibited estrogen-like effects dependent on ER pathway activation and anti-proliferative effects through mitochondria-independent and mitochondria-dependent apoptosis pathways rather than through the ER pathway in breast cancer cells. Our data suggest that genistein (**10**) may function as a phytoestrogen without the adverse effects of HRT treatment. Future studies will be conducted to confirm the beneficial effects of PLR on HRT using ovariectomized animal model.

Conflicts of interest

The authors declare no competing financial interest.

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