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Differential Effects of Resveratrol and its Oligomers Isolated from Seeds of *Paeonia lactiflora* (Peony) on Proliferation of MCF-7 and ROS 17/2.8 Cells

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Abstract

A methanol extract from seeds of *Paeonia lactiflora* (Paeoniaceae, peony) was found to possess different antiproliferative activities against four different human cancer cell lines: Hela, MCF-7, HepG2 and HT-29. Furthermore, five different methanol (20, 40, 60, 80 and 100% MeOH) fractions obtained by fractionation of the methanol extract of the seeds on a Diaion HP-20 column exhibited differential antiproliferative effects against the above four cancer cell lines. Among five fractions, the 60% MeOH fraction showed relatively lower antiproliferative activity on MCF-7 estrogen-sensitive breast cancer cell than the other cancer cell lines. Systematic separation of 60% the MeOH fraction by silica gel and Sephadex LH-20 columns led to the isolation of four known stilbenes, *trans*-resveratrol (1), *trans*-(+)-ε-viniferin (2), gnetin H (3) and suffruticosol B (4). The four stilbenes (1~4) exerted differential biphasic effects on cell proliferation of MCF-7 cells in a similar manner as genistein, a soybean isoflavone used as a positive reference, in the concentration range from 1.0 to 200 μM. Three stilbenes (1~3) weakly stimulated the proliferation of MCF-7 cells at doses below 10 μM. However, strong antiproliferative effects on MCF-7 cell were exerted by extract 1 at a dose of 200 μM, and by 2 and 3 at doses above 25 μM. In contrast, 4 inhibited the proliferation of MCF-7 cell at a dose below 25 μM, but stimulated cell proliferation at concentrations of 50 and 100 μM. All four stilbenes (1~4) stimulated the proliferation of ROS 17/2.8 osteoblast-like cells in the range of 10^{-10}~10^{-1} μM. Compound 1 exhibited especially potent proliferative activity, although its activity was weaker than that of genistein. Additionally, three resveratrol oligomers (2~4) also exhibited concentration-dependently moderate proliferative activity, but less than that of 1. These results suggest that resveratrol, and its dimer and trimers from the seeds of *Paeonia lactiflora* may act as a phytoestrogen, but in a somewhat different manner from that of genistein.

Key words: phytoestrogen, *Paeonia lactiflora*, Paeoniaceae, stilbenes, *trans*-resveratrol, *trans*-(+)-ε-viniferin, gnetin H, suffruticosol B, MCF-7, ROS 17/2.8, MTT assay

INTRODUCTION

Recently, much attention has been focused on the development of naturally occurring phytoestrogens (PEs) whose structures and molecular weights are similar to steroid estrogens and that possess estrogenic and/or antiestrogenic effects either inherently or after conversion by intestinal flora (1,2). Isoflavones in soybeans and lignans in flaxseeds are well-known as phytoestrogens that play important physiological roles in prevention of several cancers, coronary heart disease and osteoporosis (3,4). In addition, phytoestrogens have been found to have a variety of biological effects, such as antioxidative and antihemolytic properties (5). For these reasons, extensive search for novel phytoestrogens from natural sources has been undertaken.

At present, synthetic estrogen (17β-estradiol, ethinylestradiol) replacement theraphy is widely used for the prevention and treatments of hypertension and osteoporosis in postmenopausal women caused by estrogen deficiency. However, some women are reluctant to take synthetic estrogen replacements because of potentially increased cancer risks (6,7). Ingestion of dietary phytoestrogens is a possible safe alternative to synthetic extrogens for controlling estrogen deficiency diseases. Therefore, there is a need for the development of safe and efficacious new phytoestrogens.

Resveratrol (*trans*-3,4′,5-trihydroxystilbene), a naturally occurring phytoalexin found in many plant species including grapes (8,9), has been reported to have a variety of biological and pharmacological activities (10,11). In particular, resveratrol is receiving renewed interest as

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a phytoestrogen (12) that potentially plays important roles in the prevention of several physical diseases which are common in postmenopausal women, such as osteoporosis and hypertension (13-15). However, specific estrogenic effects of resveratrol are still controversial due to inconsistent results from the *in vitro* and *in vivo* assay systems used (16). Meanwhile, many oligostilbenes from at least seven plant species have been identified as having possible phytoestrogenic activity with potential for drug development due to their biological effects (17,18). However, information on estrogenic activity of resveratrol oligomers is still very limited.

To date, several *in vitro* assays for the evaluation of estrogenic activity of natural products have been developed (19). Among them, the cell-proliferation assay (E-screen) is one of the most widely used *in vitro* assays for determining the estrogenic potency of natural compounds (19). In this system, the proliferation of estrogen-dependent human breast cancer cell lines like MCF-7 or T47-D, which are known to have specific estrogen-receptor (ER), is measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) microtiter assay (20,21). Additionally, an *in vitro* assay using osteoblastic MC3T3-E1 and ROS 17/2.8 cells can be used for screening for phytoestrogens capable of bone formation (13,22).

Recently, we have been screening Oriental crude drugs for estrogenic activity using the proliferation of MCF-7 (an estrogen sensitive cell line) cell by MTT assay. Among them, the seeds of *Paeonia lactiflora* (Paeoniaceae, peony) were shown to possess multiple cytotoxic activites against several cancer cell lines (23), and particularly to exhibit concentration-dependent proliferative effects on MCF-7 cells. This prompted us to investigate the active principles responsible for the different proliferative activites of the seed extracts.

In the present study, we isolated and identified resveratrol and its oligomers from seeds of *Paeonia lactiflora* and demonstrated multiple antiproliferative activities against several human cancer cell lines, such as Hela, MCF-7, HepG2 and HT-29, and further determined different their proliferative effects on MCF-7 and ROS 17/2.8 cell lines.

MATERIALS AND METHODS

Materials

The seeds of *Paeonia lactiflora* Pallas were directly harvested in mid August in the herb garden of the Medicinal Plant Experiment Station of Uisong, Gyeongbuk, Korea. A voucher specimen has been retained in the Department of Food Science and Nutrition, Catholic University of Daegu, Korea.

Chemicals

Culture supplies such as flasks and 96-well plates (PrimariaTM, flat-bottom) were obtained from Falcon (Franklin, NJ, USA). Dulbecco's modified Eagle medium (DMEM), minimum essential medium (MEM), RPMI 1640 medium, fetal bovine serum (FBS), fetal calf serum (FCS), penicillin-streptomycin, trypsin-EDTA, phosphate buffered saline (PBS) and other tissue culture reagents were purchased from Gibco BRL, Life Technologies (Gland Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), genistein, and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All reagents and solvents used were sterilized by passing through sterile membrane filters (Nylon, 0.2 μm pore size, Nalgene TM). All containers were thoroughly cleaned, rinsed with triple-distilled water, and sterilized by autoclaving at 121°C for 15 min. Ion exchange resin (Diaion HP-20, Mitsubishi, Tokyo, Japan), silica gel (70-230 mesh, Merck, Damstadt, Germany) and Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) were used for column chromatography. All fractions were screened on precoated silica gel thin-layer chromatography (TLC) plates (200 μm thickness, silica 60F254 gel-coated glass, Merck, Damstadt, Germany) with compounds revealed under UV light. All other reagents used for this study were of analytical and HPLC grades.

Extraction and isolation

Ground seeds (300 g) of *Paeonia lactiflora* were extracted continuously with MeOH at room temperature, then filtered and evaporated under reduced pressure. The methanol extract (37.5 g) was further solubilized in 80% aq. MeOH (500 mL) and then defatted twice with *n*-hexane (1 L). The concentrated 80% methanol extract (25.5 g) was suspended in 10% aqueous MeOH and loaded onto a Diaion HP-20 column (5.5 × 50 cm). The column was eluted successively with four liters each of 20, 40, 60, 80 and 100% MeOH. Each eluent was then concentrated to yield 20% MeOH fr. (4.85 g), 40% MeOH fr. (2.82 g), 60% MeOH fr. (3.89 g), 80% MeOH fr. (3.26 g) and a 100% MeOH fr. (0.33 g). Among them, the 60% MeOH fr. (3.89 g) was chromatographed on silica gel column (5.5 × 50 cm) with $CHCl_3$-MeOH (4:1, v/v) as the eluent, to afford five fractions; Fr. 1 (0.062 g), Fr. 2 (0.19 g), Fr. 3 (0.41 g), Fr. 4 (1.29 g) and Fr. 5 (0.11 g). The Fr. 2 was further chromatographed on a Sephadex LH-20 column (2.5 × 80 cm) with MeOH to separate compound **1** (51 mg). The Fr. 3. was also subjected to the same purification procedure on Sephadex LH-20 column which afforded pure compound **2** (63

mg) and compound **3** (146 mg). Finally, the Fr. 4 and Fr. 5 were combined and further chromatographed on Sephadex LH-20 column with 90% MeOH as an eluent to give pure compound **4** (850 mg). The schematic procedure for extraction and isolation of compounds **1**~**4** from the methanolic extract of the seeds is shown in Fig. 1.

Analytical HPLC

Each methanol fraction (10 mg) obtained previously was redissolved in 10 mL of 80% aq. MeOH and then passed through 0.45 μm membrane filter (Gelman Sci., USA). Samples were appropriately diluted with 80% aq. MeOH and then injected into a Gilson 506B HPLC System coupled with a Waters 996 photodiode array UV-visible detector and using Gilson Unipoint ^{TM} 3.0 software, and Gilson 231 XL autosampler with a 10 μL loop. HPLC analysis was carried out using a YMC-Pack Pro C_{18} column (46 mm id × 250 mm, YMC Inc., USA) with a Guard-Pak C_{18} precolumn insert. The separation was conducted using a linear gradient of two solvent systems; solvent A, pH 3.0 with H_3PO_4 in 20% aq. MeOH; solvent B, 80% aq. MeOH, at a flow rate of 1.0 mL/min. The elution profile was as follows: 0~2 min, 100% A, 0% B; 5~10 min, 80% A, 20% B; 15~20 min, 60% A, 40% B; 25~30 min, 40% A, 60% B; 35~40 min, 0% A, 100% B. The column was returned to initial conditions for 10 min before the next injection. The eluent was simultaneously monitored for absorbance at 280 and 320 nm, respectively, and the UV absorption spectra

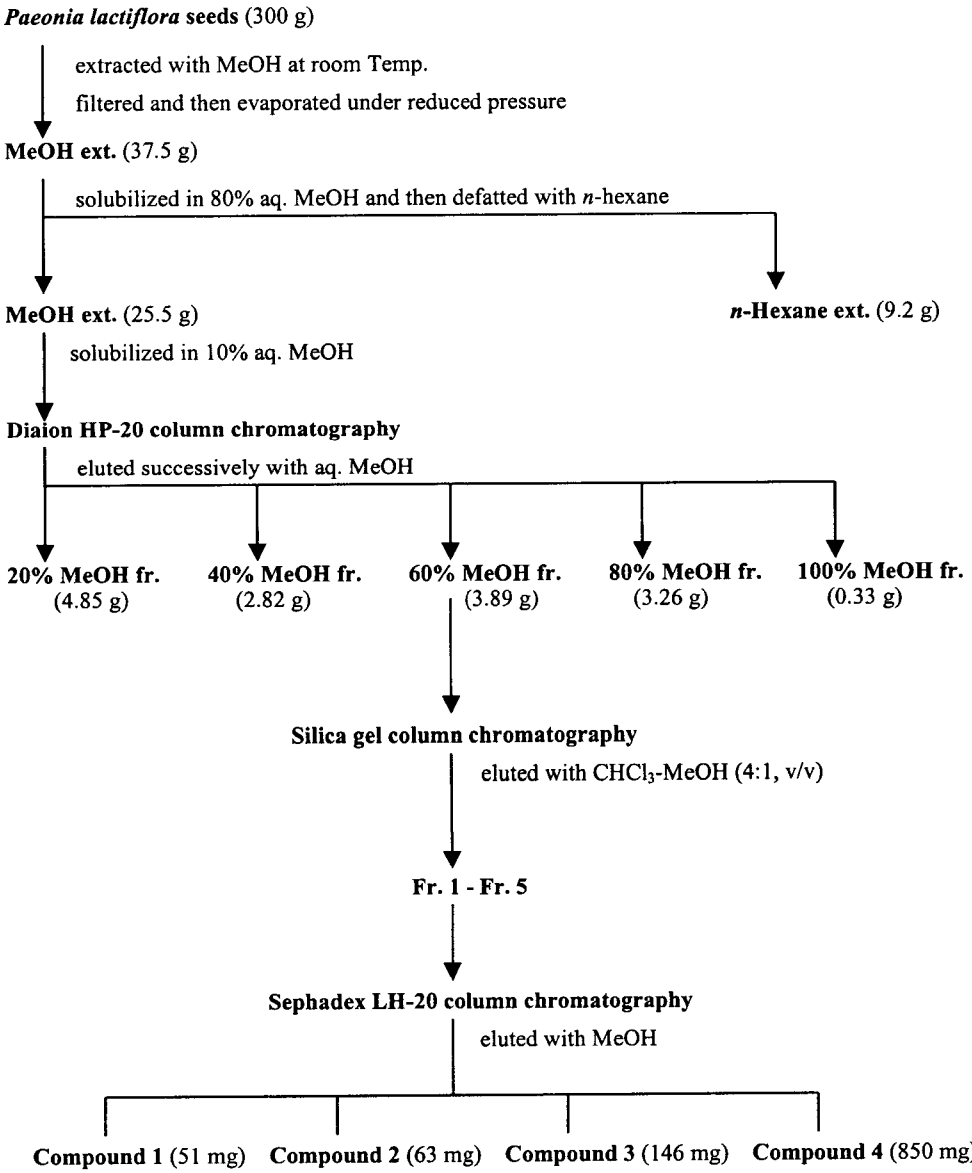

Fig. 1. Schematic of the procedure for the extraction and isolation of resveratrol and its oligomers from seeds of *Paeonia lactiflora*.

were recorded for all peaks.

Cell culture

The four human cancer cell lines, HepG2 (hepatocellular carcinoma cell), MCF-7 (breast adenocarcinoma cell), Hela (cervices adenocarcinoma cell), and HT-29 (colon adenocalcinoma cell), as well as ROS 17/2.8 (rat osteosarcoma cell) used in this study were obtained from Korea Cell Line Bank (Seoul, Korea).

HepG2, and Hela, MCF-7 and ROS 17/2.8, as well as HT-29 were cultured in MEM supplemented with 10% FBS, phenol red-free DMEM with 10% FCS, and RPMI 1640 with 10% FCS, respectively, containing 1% antibiotic-antimycotic mixture (100 Unit/mL penicillin and 50 g/mL streptomycin) in all cultures. All cells were maintained at 37°C, 5% CO_2 in a humidified atmosphere incubator. The media were changed twice or three times every week.

Cell proliferation assay

Effects of samples against proliferation of four different cancer cells were determined according to a slightly modified MTT colorimetric assay (24). Cultured cancer cells were washed with PBS and then dissociated with 0.05% trypsin-0.02% EDTA. The cells were further centrifuged and then seeded into 96-well culture plates (1×10^4 cells/mL). After 24 hr preincubation, the seeding media were replaced by new media containing various concentrations of each sample (200 μL). Genistein, a soy isoflavone, was used as positive control. After 48 hr incubation, MTT solution (20 μL of 5 mg MTT/mL PBS) was added and then further incubated for 4 hr. The supernatant was aspirated and 150 μL of DMSO was added to dissolve the formazan dye. The optical density was measured on an Elisa microplate reader (Model 550, Bio-Rad, USA) at 540 nm.

Statistical analysis

Data are expressed as means ± SE of six replicates. Significant differences between means were determined using Student's *t*-test at $p < 0.01$.

RESULTS AND DISCUSSION

Antiproliferative effects of methanol extracts on four cancer cell lines

Effects of methanol extracts from seeds of *Paeonia lactiflora* on the growth of four different human cancer cell lines, Hela, MCF-7, HepG2 and HT-29, were examined using the MTT assay and are shown in Table 1. The methanol extract inhibited the growth of all cancer cells lines examined, although the sensitivity was different in different cell lines. Then, the methanolic extract was further fractionated into five methanol fractions of 20, 40, 60, 80 and 100% MeOH on Diaion HP-20, and antiproliferative activity of each methanol fraction was tested. The 100% MeOH fr. exhibited the most potent cytotoxic effects with less than 5% cell viability of all cancer cell lines at a final concentration of 80 μg/mL, followed generally by 80% MeOH fr. > 60% MeOH fr. > 40% MeOH fr., in descending order. However, the antiproliferative effect of each MeOH fraction was different in different cancer cell lines. In contrast, the 20% MeOH fr. slightly increased cancer cell proliferation. It is very interesting to note that the 60% MeOH fr. did not exert greater antiproliferative effects on MCF-7 human breast cancer cell than on other cancer cell lines.

When proliferative effects of different concentrations of each MeOH fraction on MCF-7 cells were tested, the 80% and 100% MeOH fractions had potent concentration-dependent antiproliferative effects on MCF-7 cells as shown in Table 2. However, 20%, 40% and 60% MeOH fractions exerted very weak antiproliferative effects, with the weakest antiproliferative effect attained with the 60% MeOH fraction. Reasons for the different responses to different MeOH fractions are not clear, but one may speculate that the 60% MeOH fraction may contain several compounds with proliferative and antiproliferative effects on MCF-7 cells. So we further isolated major components in five MeOH fractions including the 60% MeOH fraction through HPLC, and investigated their UV absorption spectra.

Table 1. Antiproliferative activities of methanol extract from *Paeonia lactiflora* seeds and its five sequential methanol fractions against four different human cancer cell lines

Cell line	Cell viability (% of control)					
	MeOH crude extract	20% MeOH fr.	40% MeOH fr.	60% MeOH fr.	80% MeOH fr.	100% MeOH fr.
Hela	25.8	111.2	76.8	34.3	0.7	3.1
MCF-7	30.9	93.4	76.9	88.2	10.5	2.9
HepG2	71.9	116.7	88.4	47.3	32.4	3.6
HT-29	52.5	108.8	63.6	54.2	40.8	3.2

Each test sample was added into the culture system at a final concentration of 80 μg/mL. Four cancer cells were plated in 96-well plates (1×10^4 cells/well) and incubated for 48 h in the presence of various samples before determination of cell viability by the MTT assay. All experiments were performed in triplicate. Standard deviations have been omitted for simplicity.

Table 2. Concentration-dependent antiproliferative effects of five sequential methanol fractions obtained from methanol seed extracts of *Paeonia lactiflora* on MCF-7 human breast cancer cell growth

Cell line	Conc. (μg/mL)	Cell viability (% of control)				
		20% MeOH fr.	40% MeOH fr.	60% MeOH fr.	80% MeOH fr.	100% MeOH fr.
MCF-7	20	109.2	71.8	95.6	63.4	54.1
	40	98.4	65.8	99.2	44.5	8.5
	60	93.3	75.1	96.1	21.5	3.6
	80	93.4	76.9	88.2	10.5	2.9
	100	102.1	86.6	64.6	6.8	2.7

All experiments were performed in triplicate. Standard deviations have been omitted for simplicity. The conditions for determining cell viability were the same as described in Table 1.

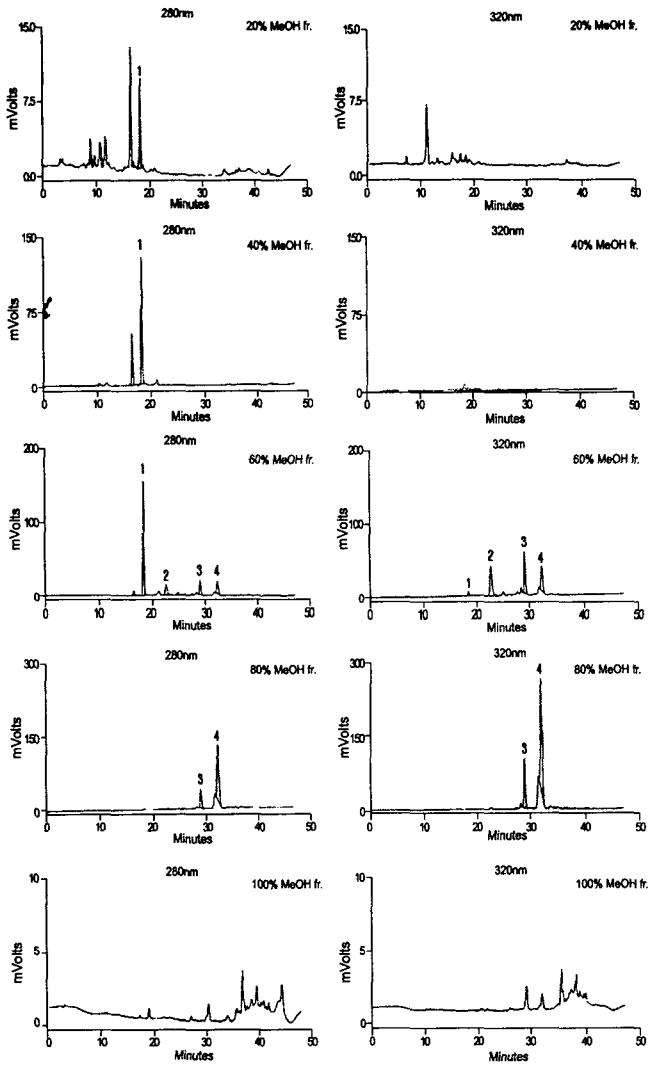

Fig. 2. HPLC chromatograms of five sequential methanol fractions obtained from the methanol extracts of seeds of *Paeonia lactiflora* using a Diaion HP-20 column. Peak 1, suffruticosol B; peak 2, *trans*-resveratrol; peak 3, *trans*-(+)-ε-viniferin; peak 4, gnetin H. HPLC conditions are as described in Materials and Methods.

HPLC analysis

To investigate major constituents in the five different MeOH fractions, HPLC analysis was carried out. As a result, several peaks were isolated from each of the five MeOH fractions (Fig. 2). In particular, the 60% MeOH fraction contained four different pure peaks **1**~**4**, which were also detected in the 40% and 80% MeOH fractions but not in the 100% MeOH fraction, although ratio of each component was different in five MeOH fractions. Furthermore, UV absorption spectra of the four peaks isolated from 60% MeOH fraction were determined (Fig. 3). Among four peaks isolated, peak **1** (t_R, 17 min) had UV absorption maxima (λ max) at 282 nm, indicating the presence of phenolic chromophore, which is characteristic for oligostilbenes (25). In contrast, peak **2** (t_R, 24 min) possessed UV maxima at 308 and 320 nm, which is attributable to a *trans*-resveratrol (8), peak **3** (t_R, 28 min) and peak **4** (t_R, 33 min) both had UV

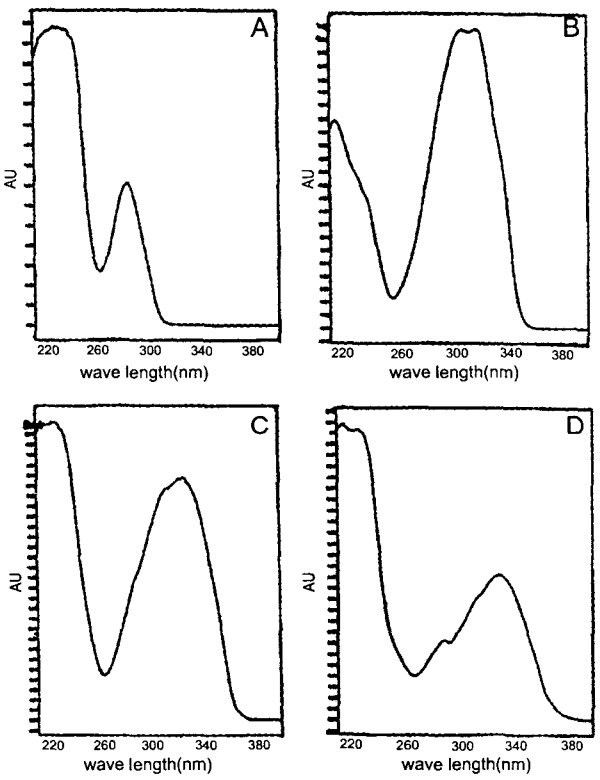

Fig. 3. UV absorption spectra of the four compounds isolated from 60% aqueous MeOH fraction of *Paeonia lactiflora* seeds. A, suffruticosol B; B, *trans*-resveratrol; C, *trans*-(+)-ε-viniferin; D, gnetin H.

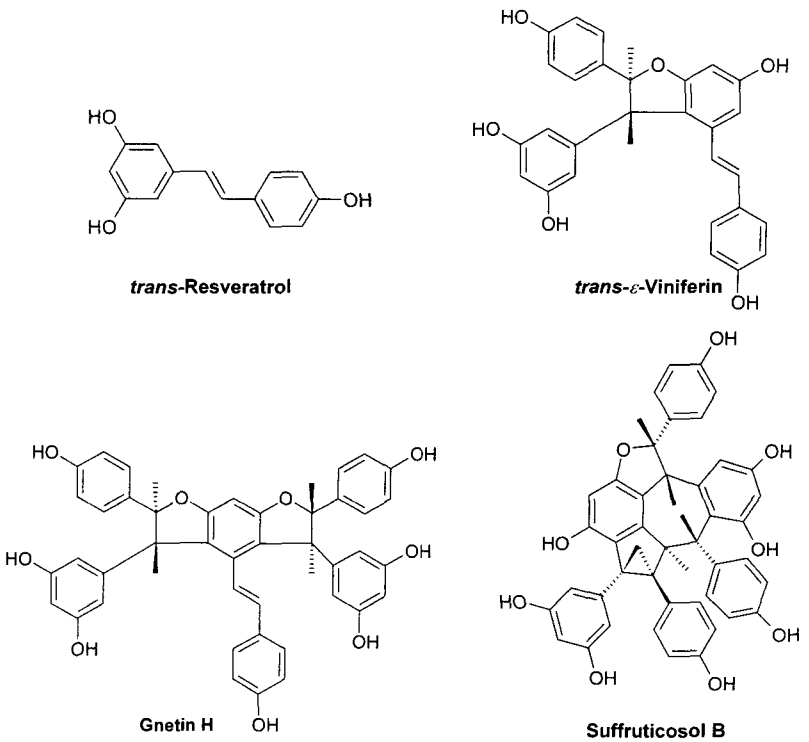

Fig. 4. Chemical structures of four compounds 1-4 isolated from seeds of *Paeonia lactiflora*.

maxima at 324 nm, which is indicative of resveratrol oligomers with *trans*-stilbene moiety (25).

Identification of four compounds 1~4

The 60% MeOH fraction was further subjected to repeated chromatographic separation on silica gel and Sephadex LH-20 to yield four pure four compounds **1~4**, as shown in Fig. 1. Four compounds were well coincided with peaks **1~4**, which were isolated from 60% MeOH fraction by HPLC (Fig. 2); compound **1** was corresponded with peak **2**, compound **2** with peak **3**, compound **3** with peak **4**, and compound **4** with peak **1**. Four compounds **1~4** were already identified as *trans*-resveratrol, *trans*-(+)- ε -viniferin, gnetin H and suffruticosol B by an extensive analysis of 1D- and 2D-NMR spectroscopy (Fig. 4) (23). These results suggest that resveratrol and its oligomers may be the main contributors to the different antiproliferative activities of the five different MeOH fractions, including the 60% MeOH fraction, against MCF-7 cells.

Effect of *trans*-resveratrol and its oligomers on the proliferation of MCF-7 cells

Concentration-dependent effects of *trans*-resveratrol and its oligomers on the proliferation of MCF-7 estrogen-sensitive breast cancer cells are shown in Fig. 5. Different doses of resveratrol and its dimer and trimers exerted differential biphasic effects on cell proliferation, much like the well-characterized phytoestrogen, genistein, a soy isoflavone which was used as a positive reference at 1~200 μM. *trans*-Resveratrol, resveratrol dimer, *trans*-(+)- ε -viniferin and resveratrol trimer, gnetin H stimulated the proliferation of MCF-7 cells at doses below 10 μM. However, strong cytotoxic effects on MCF-7 cells were exerted by *trans*-resveratrol at 200 μM, and by *trans*-(+)- ε -viniferin and gnetin H with doses above 25 μM. Among them, *trans*-(+)- ε -viniferin was the most cytotoxic, the concentration needed to kill 50% of MCF-7 cells in 24 hr being <25 μM. In contrast, suffruticosol B, a resveratrol trimer, inhibited the proliferation of MCF-7 cells with doses below 25 μM, but stimulated the cell proliferation at concentrations of 50 and 100 μM. These results suggest that resveratrol and its oligomers in the seeds of *Paeonia lactiflora* exhibited concentration-dependent differential estrogenic activity against MCF-7 cells, and *trans*-resveratrol and suffruticosol B could be largely responsible for the low antiproliferative activity of the 60% MeOH fraction against MCF-7 cells as shown in Table 2. Meanwhile, *trans*-(+)- ε -viniferin and gnetin H, having *trans*-resveratrol nuclei connected with dihydrobenzofuran ring, had markedly high antiproliferative activity against MCF-7 cells at a concentration of above 50 μM, whereas suffruticosol B stimulated significant proliferation of MCF-7 cell at 50 ~100 μM, which indicates that the special position of the resveratrol moiety may play an important role in different proliferative actions of oligostilbenes against

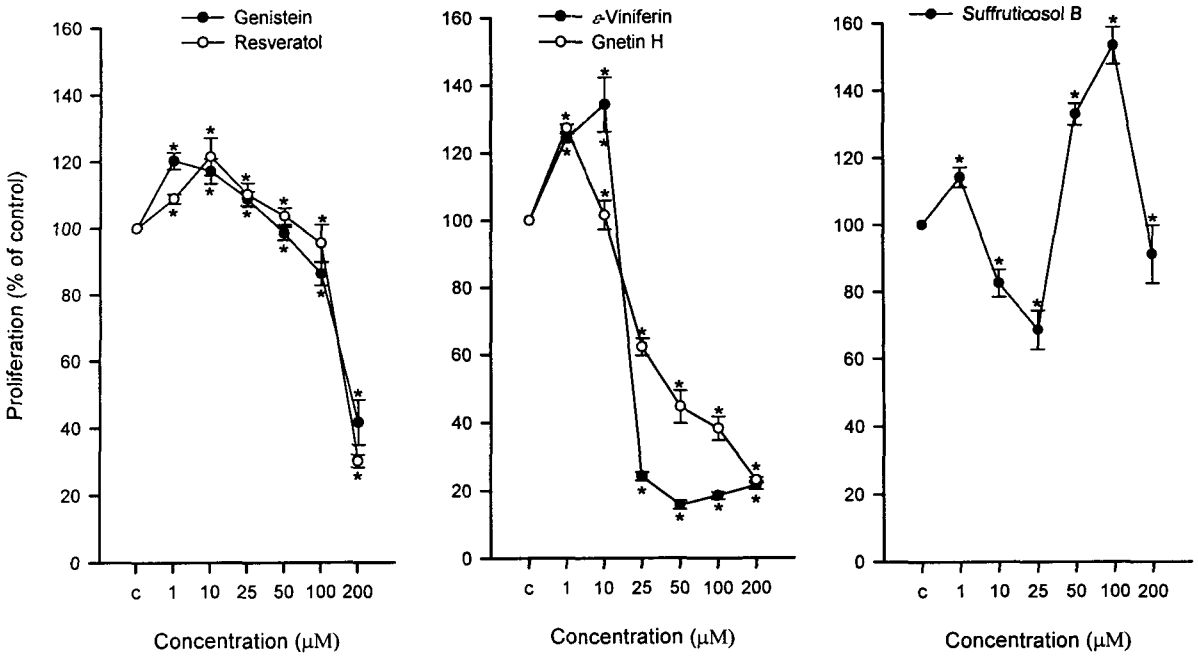

Fig. 5. Effects of four stilbenes isolated from seeds of *Paeonia lactiflora* on the viability of MCF-7 human breast cancer cells. Genistein, a soybean isoflavone, was used as a positive control. MCF-7 cells were plated in 96-well plates (1×10^4 cells/well) and incubated for 48 hr in the presence of various samples before determination of cell viability by the MTT assay. Values represent the mean ± standard error of six independent determinations. Significantly different from the control, $^*p < 0.01$.

MCF-7 cell.

Effect of *trans*-resveratrol and its oligomers on the proliferation of ROS 17/2.8 cells

Concentration-dependent effects of *trans*-resveratrol and its oligomers on the proliferation of ROS 17/2.8 osteoblast-like cells are shown in Fig. 6. Four stilbenes stimulated the proliferation of ROS 17/2.8 cells in a dose-dependent manner. In particular, *trans*-resveratrol significantly stimulated cell proliferation in the range of $10^{-10} \sim 10^{-4}$ μM, although its activity was lower than that of genistein. In addition, resveratrol dimer, *trans*-(+)-ε-viniferin and trimers, gnetin H and suffruticosol B exhibited moderate proliferative effects, but less than that of *trans*-resveratrol. Thus, resveratrol and its oligomers are assumed to have considerable effects on proliferation of ROS osteoblastic cells, although their proliferative activities were different from the other stilbene derivatives. This is the first report on the proliferative effects of oligostilbenes against osteoblastic cells, although resveratrol has already been reported to stimulate the proliferation and differentiation of osteoblastic cells (13).

trans-Resveratrol is a weak ligand for the estrogen receptor and is defined as a phytoestrogen (12). Many studies have been carried out on estrogenic effects of resveratrol in various cancer cell lines (11,12). However, the estrogen or antiestrogenic effects of *trans*-(+)-ε-viniferin and gnetin H against MCF-7 and ROS cells are reported for the first time in this paper. Resveratrol has been reported to stimulate the growth of estrogen-dependent breast cancer cells (12), and to inhibit both estrogen receptor-positive and -negative human breast cancer cell lines (26). The reasons for the discrepant results might be due to the concentrations of resveratrol examined in different studies. Lu and Serrero (27) showed that resveratrol is a growth stimulator of MCF-7 cells at low concentrations, but is a growth inhibitor at higher concentrations, something we also observed in this study. In addition, resveratrol trimers, suffruticosol A and B from *Paeonia suffruticosa* Andrews have been reported to act as antagonists for ecdysteroid, a steroid hormone of insects (28). Further studies are required to elucidate the underlying mechanisms of estrogenic and antiestrogenic activities of resveratrol and its dimer and trimers, and to develop these compounds as useful therapeutic agents for estrogen-dependent diseases such as cancers, osteoporosis and hypertension in postmenopausal women.

In conclusion, the methanol extract from seeds of *Paeonia lactiflora* showed differential antiproliferative effect against four human cancer cell lines. Bioassay-guided separation of the seed extract resulted in the isolation of resveratrol and its oligomers, *trans*-(+)-ε-viniferin, gnetin H and suffruticosol B. The structures of resveratrol and its oligostilbenes have been determined on the basis of spectroscopic evidence. Among them, *trans*-resveratrol stimulated proliferation of MCF-7 at low concentrations, similar to that of genistein, but resveratrol and its oligomers, *trans*-(+)-ε-viniferin, gne-

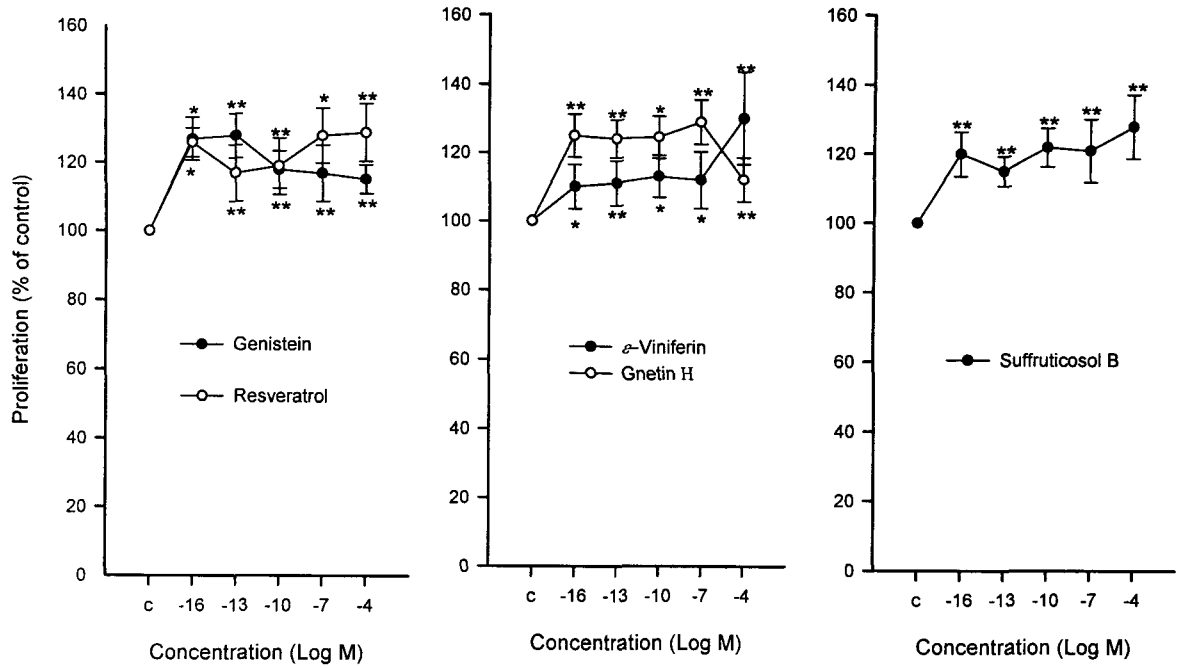

Fig. 6. Effects of four stilbenes isolated from seeds of *Paeonia lactiflora* on the viability of ROS 17/2.8 osteoblastic cells. Genistein was used as a positive control. Cells were treated with various concentrations of samples for 24 hr before determination of cell viability by the MTT assay. Values represent the mean±standard error of six independent determinations. Significantly different from the control, $^{*}p<0.05$, $^{**}p<0.01$.

tin H and suffruticosol B exhibited concentration-dependently biphasic effects on proliferation of MCF-7 human breast cancer cell. Meanwhile, resveratrol and its oligomers dose-dependently stimulated the proliferation of ROS 17/2.8 osteoblastic cells. Further study is needed to investigate the differential estrogenic effects of resveratrol and its oligomers using other *in vitro* assay systems.

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