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ORIGINAL ARTICLE

Sorbus rufopilosa Extract Exhibits Antioxidant and Anticancer Activities by Inducing Cell Cycle Arrest and Apoptosis in Human Colon Adenocarcinoma HT29 Cells

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Background: *Sorbus rufopilosa*, a tsema rowan, is a species of the small ornamental trees in the genus Sorbus and the family Rosaceae found in East Asia. The bioactivities of *S. rufopilosa* have not yet been fully determined. The objective of this study is to evaluate the antioxidant and anticancer effects of ethanol extract of *S. rufopilosa* (EESR) and to determine the molecular mechanism of its anticancer activity in human colon carcinoma HT29 cells.
Methods: To examine the antioxidant activity of EESR, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity assay was performed. Inhibitory effect of EESR on cancer cell growth and proliferation was determined by water-soluble tetrazolium salt assay. To investigate the mechanism of EESR-mediated cytotoxicity, HT29 cells were treated with various concentrations of EESR and the induction of cell cycle arrest and apoptosis was analyzed by flow cytometry, 4,6-diamidino-2-phenylindole staining, and Western blot analysis.
Results: EESR showed significant antioxidant activity and inhibitory effect on HT29 cell growth in a dose-dependent manner. EESR induced cell cycle arrest at G2/M phase in a dose-dependent manner by modulating cyclin B, cyclin-dependent kinase 1 (CDK1), and CDK inhibitor p21 expression. EESR-induced apoptosis was associated with the upregulation of p53, a death receptor Fas, and a pro-apoptotic protein Bax and the activation of caspase 3, 8, and 9, resulting in the degradation of PARP.
Conclusions: EESR possessing antioxidant activity efficiently inhibits proliferation of HT29 cells by inducing both cell cycle arrest and apoptosis. EESR may be a possible candidate for the anticancer drug development.
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Key Words: Anticancer, Antioxidant, Apoptosis, Cell cycle arrest, *Sorbus rufopilosa*

INTRODUCTION

Colorectal cancer is the second most prevalent malignancy and the fourth leading cause of cancer death globally, with 694,000 mortalities in 2012.[1] Colorectal cancer is the third most common cancer in men and the second most common in women, representing almost 10% of the global cancer incidence.[1] Due to its difficulty in early diagnosis, strong invasion, metastasis, and poor prognosis, colorectal cancer is highly lethal and aggressively malignant.[1,2] As the outcome of surgical resection, the main treatment for colorectal cancers, is not always satisfactory, chemopreventive and chemotherapeutic approaches of novel natural compounds and extracts have received increasing attention in recent years.[3-5]

The abnormal cell growth is one of the critical characteristics of cancer for invasion or spreading out to other parts of the body. Cancer cells can grow unusually via limitless number of cell division and can avoid apoptosis.[6,7] Therefore, the inhibition of cancer cell proliferation by inducing cell cycle arrest and apoptosis is being an important preventive strategy against cancer.[8,9] Cell cycle program is regulated by cyclin/cyclin-dependent kinase (CDK) complexes.[10,11] The major molecules responsible for G2/M

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transition are cyclin A/CDK1 and cyclin B/CDK1.[12] The activation of cyclin/CDK complex accelerates cell cycle progression, which are regulated by phosphorylation. Whereas damage signals of cells activate p53 via CHK2 to repair damaged DNA, leading to cell cycle arrest.[13] The expression of CDK inhibitor p21 is upregulated by activated p53, resulting in the suppression of G2/M transition by the inactivation of cyclin/CDK complex.[14,15]

Apoptosis, a type of programmed cell death, is characterized by cell membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation.[16,17] There are two basic apoptotic signaling pathways: the extrinsic and the intrinsic pathways.[18,19] The extrinsic pathway can be mediated by one of several death receptors (TNF receptor superfamily) when bound by the appropriate ligand (e.g., Fas ligand, TNF-related apoptosis inducing ligand, and TNF-α).[20] The interaction between death receptors and their ligands leads to the assembly of the death-inducing signaling complex, followed by the activation of an initiator caspase, caspase-8.[21] The intrinsic apoptotic pathway is activated by various intracellular stimuli, including DNA damage, growth factor deprivation, and oxidative stress. It has been reported that the modulation of pro-apoptotic Bcl-2 family proteins can cause the mitochondrial membrane permeabilization, leading to the release of cytochrome *c* and the activation of an initiator caspase, caspase-9.[22,23] Processed initiator caspases, caspase-8, and caspase-9, from extrinsic and intrinsic pathways, respectively, can activate an effector caspase, caspase-3, and then active caspase-3 degrade proteolytically intracellular proteins such as PARP to carry out the apoptosis.[24]

Sorbus rufopilosa, a tsema rowan, is a species of the small ornamental trees in the genus Sorbus and the family Rosaceae.[25] The fruit of European rowan (*S. aucuparia*) can be made into jams and other preserves and also can be a substitute for coffee beans. It has may uses in alcoholic beverages to produce country wine and to flavor liqueurs and cordials. It has been reported that phenolic extracts from *S. aucuparia* berry has antioxidant activity.[26] However, the bioactivity of *S. rufopilosa* remains still unclear. In this study, we investigated the antioxidant and anticancer activities of *S. rufopilosa* and the molecular mechanism of its anticancer effect on human colon carcinoma HT29 cells.

MATERIALS AND METHODS

1. Preparation of *Sorbus rufopilosa* extract solution

The ethanol extract of *S. rufopilosa* (EESR) was obtained from International Biological Material Research Center, Korea (FBM123-023). Plant material of *S. rufopilosa* was extracted with 95% ethanol at 45°C using a sonicator, evaporated and freeze-dried. EESR was dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C prior to use.

2. Cell culture

Human colon adenocarcinoma HT29 cells, human hepatocellular carcinoma HepG2 cells, and human lung adenocarcinoma A549 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin, and streptomycin at 37°C and 5% CO_2.

3. 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity assay

Antioxidant activity of EESR was determined using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Various concentrations (2.56-64 μg/mL) of EESR and standard compound (ascorbic acid) were added to DPPH solution. Absorbance was measured at 520 nm using a microplate reader (Beckman Coulter, Fullerton, CA, USA), after reacting for 30 minutes at room temperature. The assay was replicated three times and results are expressed as mean ± SD. The percentage of inhibition was assessed using the following formula:

DPPH radical scavenging activity (%) = $\{1 - (A - B)/C\} \times 100$

A: sample absorbance 520 nm

B: color control absorbance 520 nm

C: control absorbance 520 nm

The concentration of sample inhibiting 50% of free radical DPPH (IC_{50}) was determined.

4. Cell viability assay and morphological study

The cell viability was measured using the EZ-Cytox cell viability assay kit (Daeillab, Seoul, Korea). Cells were plated at a density of 2-5 × 10^4 cells/mL in 24-well plate and treated with media containing DMSO as a control or various concentrations of EESR for 48 hours. EZ-Cytox assay reagent (10 μL) was added to each cell culture well, and the mixture was incubated for 30 minutes at 37°C. The absorbance was measured at 450 nm using a microplate reader. For morphological study, HT29 cells were treated with EESR for 48 hours and directly photographed with an inverted microscope using Axio Vision program.

5. Cell cycle analysis

The effect of EESR on the cell cycle in HT29 cells was examined using the Muse™ Cell Cycle kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Briefly,

cells (2×10^5 cells/well) were plated in 6-well plate and treated with 0.1% DMSO as a vehicle control or with various concentrations of EESR for 48 hours. The cells were then harvested, washed once with PBS, and fixed in cold 70% ethanol for 3 hours at −20°C. Fixed cells were centrifuged at 300 × *g* for 5 minutes and resuspended in PBS. After addition of an equal volume of Muse™ Cell Cycle reagent, cells were incubated for 30 minutes at room temperature in the dark. Finally, flow cytometry was conducted (Muse™ Cell Analyzer; Merck Millipore) and the Muse analysis software (ver 1.4) was used to determine the relative DNA content.

6. Western blot analysis

EESR-treated cells were lysed with lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(2-aminoethyl ether) tetraacetic acid, 1% Triton X-100, 1 μg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride) for 1 hour at 4°C and centrifuged for 30 minutes at 13,000 rpm. Total soluble proteins in the supernatant were collected and the concentration of protein was determined by Bradford method. For Western blot analysis, 30 to 50 μg/mL of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Blots were incubated at 4°C overnight with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and visualized by an enhanced chemiluminescence detection system (FluoChem®FC2; Alpha-Innotech, San Leandro, CA, USA) using Western blotting luminol reagent (Santa Cruz Biotechnology, Dallas, TX, USA). CDK1, cyclin A, cyclin B, cell division cycle 25C (Cdc25C), p-Cdc25C, Wee1, p53, Fas, Fas-associated protein with death domain (FADD), Bax, caspase-3, caspase-8, caspase-9, PARP, actin primary antibodies and peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology. Primary antibodies against CHK2, p-CHK2, p21, p-CDK1, and Bcl-2 were purchased from Cell Signaling Technology (Beverly, CA, USA).

7. Nuclear staining with 4,6-diamidino-2-phenylindole

Cells (5×10^4 cells/well) were plated in 8-well chamber slide and treated with 0.1% DMSO as a vehicle control or with various concentrations of EESR for 48 hours. EESR-treated cells were fixed with 4% formaldehyde for 20 minutes at room temperature. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 10 minutes at room temperature. After washing with PBS, cells were incubated with 1 μg/mL of 4,6-diamidino-2-phenylindole (DAPI) for 10 minutes and then washed three times in PBS. Apoptotic nuclei (condensed chromatin) were examined by fluorescence microscopy (Carl Zeiss, Jena, Germany).

8. Statistical analysis

Data are presented as the mean ± SD from at least three independent experiments. Statistical comparisons between groups were performed by SPSS program followed by Student *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

1. *Sorbus rufopilosa* extract shows antioxidant activity

Since it has been reported that oxidative stress is one of the key components in the induction and the maintenance of cancer,[27,28] we first examined the antioxidant effect of EESR using DPPH radical scavenging activity assay. As shown in Table 1, EESR inhibited DPPH activity in a dose-dependent manner showing 47.40%, 95.32% and 98.71% inhibition rate at 2.56 μg/mL, 12.80 μg/mL and 64.00 μg/mL, respectively. EESR showed the IC_{50} (concentration at 50% inhibition) value of 3.11 μg/mL, which were comparable to reference compound (ascorbic acid) with IC_{50} value of 1.49 μg/mL. These results indicate that EESR have significant antioxidant activity.

2. *Sorbus rufopilosa* extract inhibits HT29 cell growth

To investigate the anticancer activity of EESR, we examined the inhibitory effect of EESR on cancer cell growth using human colon adenocarcinoma HT29 cells, human hepatocellular carcinoma HepG2 cells, and human lung adenocarcinoma A549 cells. Cells were treated with various concentrations of EESR for 48 hours and cell viability was measured by water-soluble tetrazolium salt assay. As shown in Figure 1A, EESR inhibited the growth of HT29 cells in a dose-dependent manner, whereas EESR did not show any significant inhibitory effect on cell proliferation in HepG2 and A549 cells at the highest concentration (200 μg/mL).

Table 1. DPPH radical scavenging activity by EESR

EESR		Ascorbic acid	
Concentration (μg/mL)	Inhibition rate (%), mean ± SD	Concentration (μg/mL)	Inhibition rate (%), mean ± SD
2.56	47.40 ± 0.63	0.51	22.70 ± 0.94
12.80	95.32 ± 0.13	2.56	79.66 ± 0.79
64.00	98.71 ± 0.25	12.80	97.57 ± 0.21

IC_{50} (μg/mL): EESR, 3.11; ascorbic acid, 1.49. DPPH, 2,2-diphenyl-1-picrylhydrazyl; EESR, ethanol extract of *Sorbus rufopilosa*.

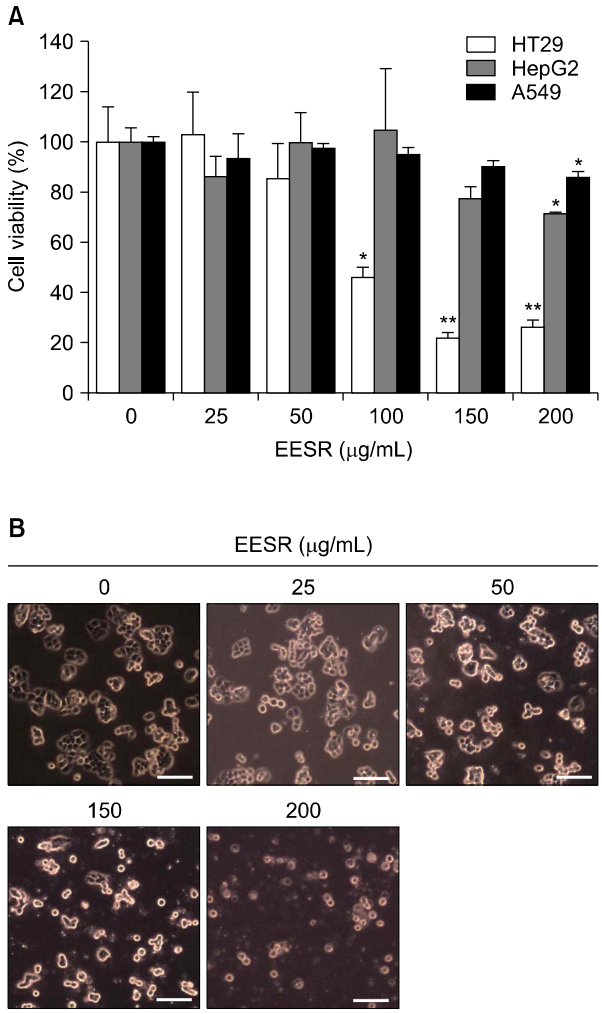

Figure 1. Effect of ethanol extract of *Sorbus rufopilosa* (EESR) on cancer cell growth and morphology of HT29 cells. (A) Human colon carcinoma HT29 cells, human lung carcinoma A549 cells, and human hepatocellular carcinoma HepG2 cells were treated with various concentrations of EESR for 48 hours. Cytotoxic effect of EESR was determined by water-soluble tetrazolium salt assay. Results are expressed as percentage of the vehicle treated control ± SD of three independent experiments. $*P < 0.05$, $**P < 0.005$ compared with dimethyl sulfoxide treated cells. (B) Morphological changes by EESR in HT29 cells. Cells were treated with indicated concentration of EESR for 48 hours, and then visualized by light microscopy. Scale bar, 100 μm.

In addition, the morphological changes of EESR-treated HT29 cells were observed using a phase contrast microscope. Increasing concentrations of EESR caused inducing the morphological change of HT29 cells and decreasing viable adherent HT29 cells (Fig. 1B). Therefore, we decided to examine the molecular mechanism of cytotoxicity of EESR in HT29 cells.

3. *Sorbus rufopilosa* extract induces HT29 cell accumulation at SubG1 and G2/M phase

To clarify the mechanisms involved in the cytotoxic effect of EESR in HT29 cells, cell cycle analysis was performed by flow cytometry. As shown in Figure 2, cell population at G2/M phase increased by EESR in a dose-dependent manner up to 100 μg/mL, accompanied by a decrease of cell population at G1 and S phases. Furthermore, EESR treatment induced significant accumulation of cells at SubG1 phase (apoptotic cell population) in a concentration-dependent manner, raising the possibility that EESR may induce apoptosis in HT29 cells. Figure 2 also shows the distribution of cells in various stages of the cell cycle. These results suggest that EESR may induce G2/M arrest and apoptosis in HT29 cells.

4. *Sorbus rufopilosa* extract regulates the expression and the activation of cell cycle checkpoint proteins

As we found that the cell population in G2/M phase increased in a dose-dependent manner by EESR, the expression of G2/M transition-related proteins was examined using Western blot analysis. As shown in Figure 3A, the expression of p53 and p21 increased, whereas the expression of cyclin B decreased significantly in EESR-treated HT29 cells. However, the expression of cyclin A did not show any big change after EESR treatment. In addition, EESR activated CHK2 and inactivated Cdc25C via phosphorylation (Fig. 3B). Figure 3B also showed that the expression of Wee1 kinase increased by EESR treatment. These results indicate that EESR modulates the checkpoint proteins and blocked the G2/M transition.

5. *Sorbus rufopilosa* extract induces apoptosis in HT29 cells

Since HT29 cell accumulation at SubG1 phase was detected by flow cytometry, we next observed the formation of apoptotic bodies, one of the hallmarks of apoptosis, in EESR-treated HT29 cells by DAPI staining. As shown in Figure 4A, the apoptotic bodies increased, whereas the number of cell decreased in a dose-dependent manner by EESR treatment. To investigate the molecular mechanisms involved in the apoptosis induction of EESR in HT29 cells, the expression of apoptosis-related molecules in the protein levels were determined by Western blot analysis. As shown in Figure 4B, exposure to EESR led to a significant increase of p53, a death receptor Fas, FADD, and Bax expression in HT29 cells. However, there was no big difference in Bcl-2 expression between vehicle control-treated and EESR-treated cells (Fig. 4B). As shown in Figure 4C, EESR activated caspases,

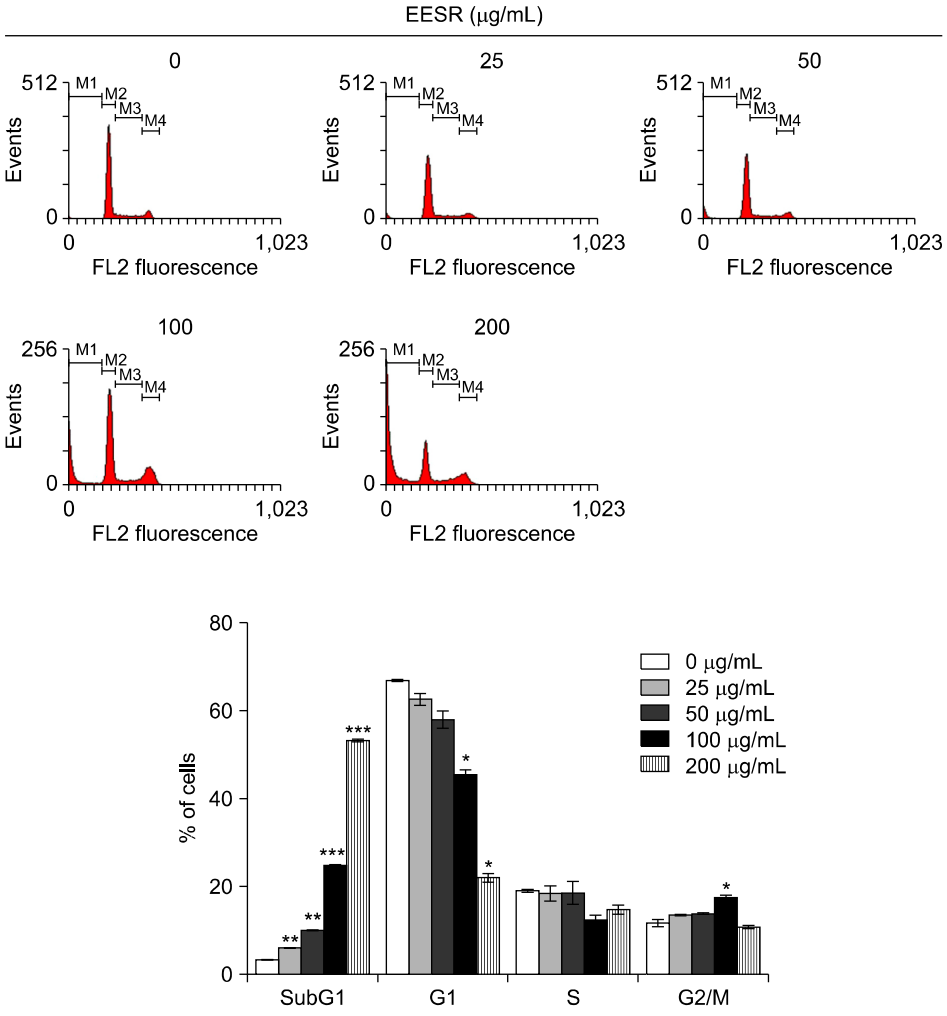

Figure 2. Cell accumulation at SubG1 and G2/M phase by ethanol extract of *Sorbus rufopilosa* (EESR) treatment. HT29 cells were cultured and treated with EESR for 48 hours. Cells were harvested, fixed and then incubated with Muse™ Cell Cycle reagent for 30 minutes and analyzed by flow cytometry. DNA-fluorescence histogram and the percentage of gated cells are shown. M1, SubG1 phase; M2, G1 phase; M3, S phase; M4, G2/M phase. $*P < 0.05$, $**P < 0.01$, and $***P < 0.005$ compared with cell population of dimethyl sulfoxide treated control.

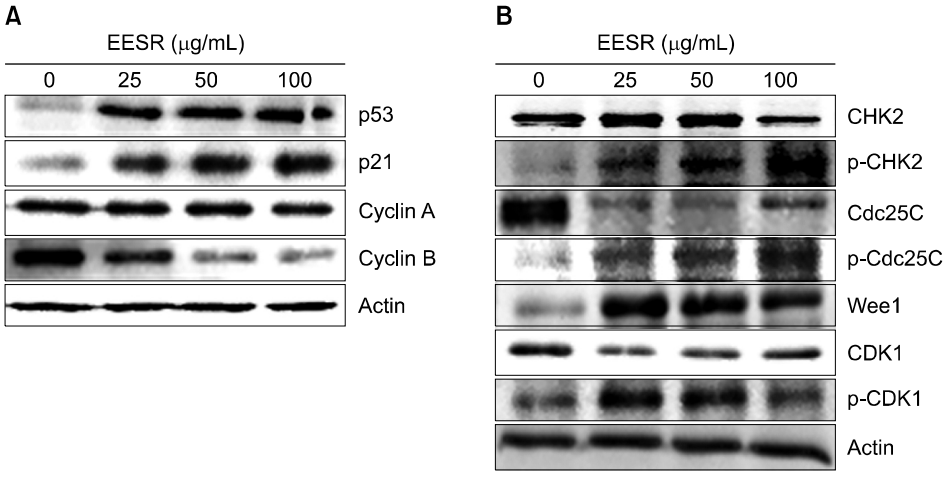

Figure 3. Modulation of G2/M checkpoint-related protein expression in HT29 cells by ethanol extract of *Sorbus rufopilosa* (EESR). HT29 cells were treated with various concentrations of EESR for 48 hours. The cells were lysed and cellular proteins were then separated by SDS-PAGE, followed by Western blot analysis using antibodies against G2/M checkpoint-related proteins (A: p53, p21, cyclin A, and cyclin B; B: CHK2, p-CHK2, Cdc25C, p-Cdc25C, Wee1, cyclin-dependent kinase 1 [CDK1], and p-CDK1). Actin was used as an internal control.

including caspase-8, caspase-9, and caspase-3 in a dose-dependent manner, leading to the cleavage of PARP, one of the target proteins of caspase-3 in apoptosis.

DISCUSSION

In the present study, the antioxidant and anticancer activities of *S. rufopilosa* extract and molecular mechanism of anticancer

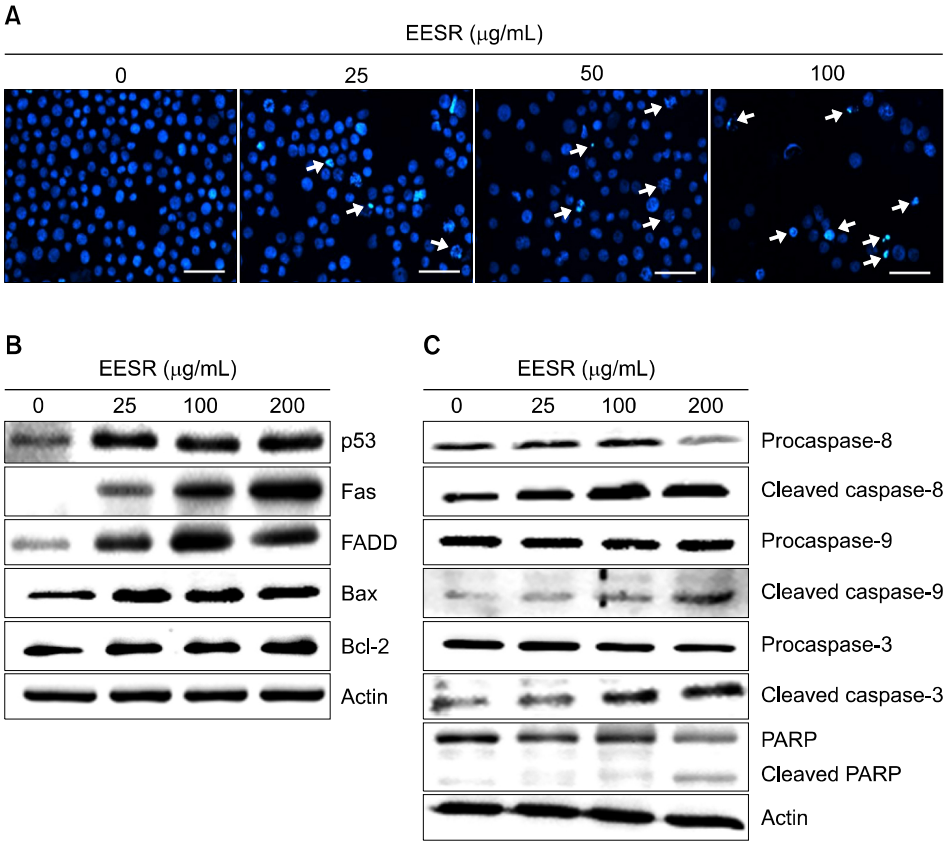

Figure 4. Apoptosis induction and apoptosis-related protein modulation by ethanol extract of *Sorbus rufopilosa* (EESR) in HT29 cells. (A) 4,6-diamidino-2-phenylindole (DAPI) staining. EESR-treated cells were fixed, permeabilized, and stained with DAPI for 20 minutes. Stained cells were observed by fluorescence microscopic analysis and imaged using Axio Vision program. Arrows indicate the apoptotic bodies. Scale bar, 50 μm. (B, C) HT29 cells were treated with various concentrations of EESR for 48 hours. The cells were lysed and cellular proteins were then separated by SDS-PAGE, followed by Western blot analysis using antibodies against apoptosis-related proteins (B: p53, Fas, Fas-associated protein with death domain [FADD], Bax, and Bcl-2; C: caspase-8, caspase-9, caspase-3, and PARP). Actin was used as an internal control.

activity were examined. Oxidative stress forced by reactive oxygen species such as superoxide radical (O_2^-), hydroxyl radical (· OH), and hydrogen peroxide (H_2O_2) can cause toxic effects that damage various components of the cell, including DNA damage, lipid peroxidation, and protein damage.[27,28] As oxidative stress is thought to be involved in human diseases, such as cancer, cardiovascular disease, arthritis, and neurodegenerative disease, it is important to identify anticancer active plant extracts possessing antioxidant potential.[29,30] Our data showed that EESR has significant antioxidant activity with IC_{50} value of 3.11 μg/mL, although the capability of scavenging DPPH radical of EESR was slightly lower than those of ascorbic acid.

G2/M transition is regulated by cyclin A/CDK1 and cyclin B/CDK1 complexes. In detail, cyclin A can associate with CDK1 in the late S phase and the cell will progress through the S phase to G2 phase.[31] In the late G2 phase, cyclin A is replaced by cyclin B, leading to the formation of cyclin B/CDK1 complex, and cyclin B/CDK1 complex triggers the progression of the cells into and out of M phase.[32] In this study, we found that EESR downregulated cyclin B expression, whereas expression of cyclin A did not show any big difference by EESR, suggesting that EESR may induce cell cycle arrest at the transition from G2 to M phase.

The negative regulation of the cyclin B/CDK1 complex occurs via p53-dependent and p53-independent pathways, resulting in cell cycle arrest.[14] p53, a multifunctional tumor suppressor, is known to regulate various cellular events, such as cell cycle arrest, transcription, differentiation, and apoptosis.[33] In the case of p53-dependent pathway, DNA damage signals trigger p53 upregulation, and then p53 induces the expression of p21, a CDK inhibitor, which can suppress G2/M transition by the inactivation of cyclin/CDK complex.[34] In the p53-independent pathway, CHK1 and CHK2 can inhibit the function of Cdc25C phosphatase through phosphorylation, leading to increase of p-CDK1, an inactive form of CDK1.[35,36] CDK1 is also negatively regulated by Wee1 kinase, which catalyzes the inhibitory phosphorylation of CDK1, resulting in G2/M arrest.[37] Our data from Western blot analysis revealed that EESR induced the increase of p53 and p21 protein expression, and the inactivation of CDK1 via phosphorylation by inactivation of Cdc25C and upregulation of Wee1. These results suggest that EESR may induce G2/M arrest by both p53-dependent and p53-independent pathways.

It has been reported that p53 can activate the extrinsic pathway of apoptosis through the induction of Fas gene expression and the enhancement of cell surface levels of Fas.[38,39]

In addition, p53 is known to upregulate the expression of Bax, a pro-apoptotic Bcl-2 family, which governs the release of cytochrome *c* to induce the intrinsic pathway of apoptosis.[40] In this study, we found that EESR upregulated p53, Fas, FADD and Bax expression, raising the possibility that EESR may induce p53-associated apoptosis through both extrinsic and intrinsic pathways. Moreover, our data showed that EESR activated caspase-8 and caspase-9, leading to the activation of caspase-3. Since it has been demonstrated that caspase cascade is initiated by caspase-8 and caspase-9 activation through extrinsic and intrinsic pathways, respectively, these results indicate that EESR may induce apoptosis through both extrinsic and intrinsic pathways in HT29 cells.

In conclusion, we found that EESR showed significant antioxidant activity and efficiently induced G2/M arrest and apoptosis in HT29 cells, followed by the suppression of HT29 cell proliferation. EESR-mediated cell cycle arrest at G2/M phase was associated with both p53-dependent and p53-independent pathways. In addition, EESR induced HT29 cell apoptosis associated with p53 upregulation through both extrinsic and intrinsic pathways. Our findings firstly show the antioxidant and anticancer activities of EESR and the mode of action, suggesting that EESR may be a possible nutraceutical candidate for chemoprevention and chemotherapy of cancer. Further studies will be needed to identify the bioactive compound(s) of EESR for its anticancer activity.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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