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ORIGINAL ARTICLE

The Reactive Oxygen Species/AMP-Activated Protein Kinase Signaling Pathway's Role in the Apoptotic Induction of MCF-7 Human Breast Cancer Cells Caused by the Ethanol Extract of *Citrus Unshiu* Peel

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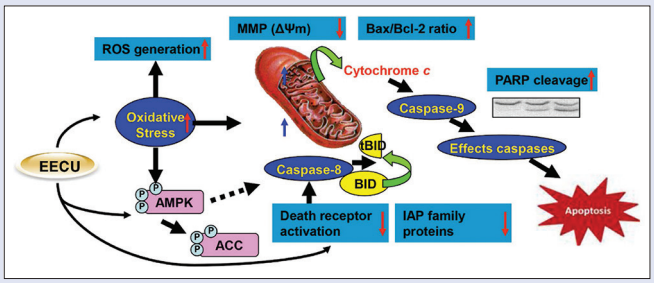
ABSTRACT

Objective: *Citrus unshiu* Markovich, which has been used for many different purposes in traditional medicine, has been reported to possess various pharmacological properties; however, its anticancer potentials are relatively unknown. This study aimed to investigate the effect of the ethanol extract of *C. unshiu* peel (EECU) on MCF-7 human breast cancer cells. **Materials and Methods:** Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis was detected using DAPI staining and flow cytometry. Mitochondrial membrane potential (MMP), reactive oxygen species (ROS) assay, caspase activity, and Western blotting analysis were used to confirm the basis of apoptosis. **Results:** Our results demonstrated that the inhibition of MCF-7 cell survival by EECU was associated with the induction of apoptosis. EECU-induced apoptosis resulted in a sequence of events, which began with the increased expression of death receptor-related proteins and a Bax/Bcl-2 expression ratio. This led to the collapse of MMP and the cytosolic release of cytochrome *c*, which was accompanied by and the activation of caspase-9 and caspase-8 and proteolytic degradation of poly (ADP-ribose) polymerase. EECU also induced apoptosis of MCF-7 cells by stimulating AMP-activated protein kinase (AMPK), through the generation of ROS. However, compound C, a pharmacological inhibitor of AMPK, significantly weakened EECU-induced apoptosis. Furthermore, the activation of AMKP, induction of apoptosis, and reduction of cell viability by EECU were effectively prevented when ROS production was blocked. **Conclusions:** These results demonstrate that EECU inhibits MCF-7 cell proliferation by activating the intrinsic and extrinsic apoptosis pathways through the ROS-dependent activation of the AMPK pathway.

Keywords: AMP-activated protein kinase, apoptosis, *Citrus unshiu Markovich,* reactive oxygen species

SUMMARY

- Ethanol extract of *Citrus unshiu* peel (EECU) suppressed cell viability by inducing apoptosis through the activation of both intrinsic and extrinsic pathways in MCF-7 human breast cancer cells
- EECU-induced apoptosis is associated with the mitochondrial dysfunction, release of cytochrome *c*, and increased reactive oxygen species production in MCF-7 cells
- AMP-activated protein kinase activation is associated with EECU-induced apoptosis, which was reactive oxygen species dependent in MCF-7 cells.



Abbreviations used: ACC: Acetyl-CoA carboxylase; AMPK: 5′-AMP-activated protein kinase; DCF-DA: 2′,7′-dichlorofluorescin diacetate; DMSO: Dimethylsulfoxide; DR: Death receptor; ECL: Enhanced chemiluminescence; EECU: Ethanol extract of *Citrus unshiu* peel; ELISA: Enzyme-linked immunosorbent assay; FADD: Fas-associated protein with death domain; FasL: Fas ligand; FBS: fetal bovine serum; FITC: Fluorescein isothiocyanate; HRP: Horseradish peroxidase; IETD: Ile-Glu-Thr-Asp; JC: 1: 5,5′,6,6′-tetrach loro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; LEHD: Leu-Glu-His-Asp; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC: N-acetyl-L-cysteine; PARP: poly (ADP-ribose) polymerase; PBS: Phosphate-buffered saline; PI: Propidium Iodide; pNA: p-nitroaniline; ROS: Reactive oxygen species; SD: Standard deviation; SDS: Sodium-dodecyl sulfate; tBid: Truncated Bid; TRAIL: TNF-related apoptosis-inducing ligand

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INTRODUCTION

Inhibiting cancer cell proliferation through the activation of the apoptotic pathway (which is the most typical cell death mechanism) is the most fundamental research area in the attempt to discover anticancer agents. Apoptosis is largely divided into death receptor (DR)-initiated extrinsic pathways and mitochondria-mediated intrinsic pathways.[1,2] The former pathways are induced by the activation of caspase-8 by the

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binding of death ligands to the DRs and the latter are induced by the activation of caspase-9 associated with mitochondrial damage associated with the altered expression of Bcl-2 family members.[3,4] Activation of the initiator caspases of both types of pathways induces the activity of downstream effector caspases, such as caspase-3 and caspase-7; this results in the degradation of various substrate proteins that are required for cell survival and complete apoptosis.[1,5]

Induction, such as apoptosis, is regulated in a complicated manner by the activation and inactivation of various intracellular signaling pathways that are involved in the cellular metabolism. One of the most important molecules for inducing apoptosis in cancer cells is 5′-AMP-activated protein kinase (AMPK), which is a serine/threonine kinase; this molecule plays an essential role in regulating cellular homeostasis and protecting cells under conditions of metabolic stress.[6,7] AMPK is activated in response to the phosphorylation in the Thr 172 residue of the α subunit among the three subunits (α, β, and γ), constituting AMPK.[8,9] Several previous studies have shown that an activated form of AMPK plays an important role in inducing apoptosis in cancer cells.[10,11] Although research on reactive oxygen species (ROS) production and the role of AMPK is not fully understood, the activation of AMPK has frequently been observed to be accompanied by an increase in ROS levels.[12,13] These observations suggest that the AMPK signaling pathway may be a potential therapeutic target for inducing apoptosis associated with mitochondrial dysfunction.[14,15]

Plants that have been used for a long time in traditional medicine are constantly reviewed when developing new drugs. This is because the plants may contain many pharmacologically active substances and come with relatively few side effects; they have the potential to be an alternative strategy for the prevention and treatment of various diseases–including cancer.[16,17] Among such plants, citrus and dried peels have long been used as traditional medicines for therapeutic purposes. They have been used to treat colds, indigestion, and bronchial pain, and the peels have been reported to have anti-inflammatory, anti-allergic, antidiabetic, and antiviral effects.[18-21] In one study, which used a tumor-bearing mouse model, *Citrus unshiu* Markovich peel extract was shown to inhibit tumor growth and was associated with decreased production of cytokines, such as interferon-γ and tumor necrosis factor-α.[22] According to a recent study by Kim *et al.*,[23] *C. unshiu* peel was reported to reduce systemic inflammation in tumor-bearing mice and to prevent the production of pro-cachectic cytokines in tumors; this was connected with the prevention of skeletal muscle atrophy and weight loss. Furthermore, polysaccharides or flavonoids found in the peels of *C. unshiu* have been reported to inhibit the metastasis of cancer cells.[24,25] However, the exact anticancer effects of *C. unshiu* peel on human cancer cells remain unknown. Therefore, we investigated the pro-apoptotic activity of the ethanol extract of *C. unshiu* peel (EECU) on MCF-7 human breast cancer cells as part of our search for traditional medicinal products with anticancer abilities. In addition, we observed the major signaling pathways stimulated during apoptosis by EECU treatment and examined whether ROS-mediated AMPK activation is involved in these signaling pathways.

MATERIALS AND METHODS

Preparation of extract of *Citrus unshiu* peel

For the preparation of EECU, the dried peels of *C. unshiu* were provided from Dongeui Korean Medical Center (Busan, Republic of Korea) and pulverized into fine powder. The powder (100 g) was mixed into 1 L of 70% ethanol by sonication for 24 h. After filtering, the filtrate was concentrated with a vacuum rotary evaporator (BUCHI, Switzerland). The residue was then freeze-dried in a freezing-dryer and stored at −80°C. The powder (EECU) was dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) to obtain a final concentration of 100 mg/ml (extract stock solution) and was stored at 4°C. The stock solution was diluted with a culture medium to obtain the desired concentrations prior to use.

Cell culture

MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in a RPMI 1640 medium (WelGENE Inc., Daegu, Republic of Korea) containing 10% fetal bovine serum, 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 mg/mL of streptomycin at 37°C in a humidified incubator with a mixture of 95% air and 5% CO_2.

Cell viability assay

Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MCF-7 cells were seeded in 6-well plates at a density of 2×10^4 cells per well. After incubation overnight, the cells were treated with different concentrations of EECU. At 24 h after EECU treatment, an MTT solution was added to each well at a concentration of 0.5 mg/mL, and the cells were incubated at 37°C in the dark, resulting in the formation of MTT formazan crystals. Following a 3-h incubation, the supernatants were carefully removed, and the formazan crystals were solubilized in DMSO. The plates were thoroughly shaken, and the absorbance of each well was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Dynatech Laboratories, Chantilly, VA, USA). The optical density of the formazan in the control (untreated) cells was used to represent 100% viability. To evaluate the morphological changes of the MCF-7 cells after EECU treatment, we took photographs of the cells using a phase-contrast microscope at ×200 (Carl Zeiss, Oberkochen, Germany).

Analysis of apoptosis by flow cytometry

To determine the magnitude of the apoptosis by EECU, the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA) was used. The cells were harvested, washed with cold phosphate-buffered saline (PBS), and resuspended in a supplied binding buffer at a final concentration of 1×10^6 cells/ml according to the manufacturer's instructions. The cells were stained with FITC-conjugated annexin V and propidium iodide (PI) for 20 min in the dark. The cells were then analyzed using a flow cytometer (Becton Dickinson, San Jose, CA, USA). The annexin$^-$/PI$^-$ cell population was considered to be normal, while the annexin V-FITC$^+$/PI$^-$ and annexin$^+$/PI$^+$ cell populations were considered to be indicators of early and late apoptotic cells, respectively.[26] At least 10,000 cells from each sample were analyzed.

Protein isolation and Western blot analysis

After treatment with EECU, the cells were harvested, rinsed twice with cold PBS, and incubated for 30 min in a lysis buffer.[27] In a parallel experiment, extracts of the cytoplasm and mitochondria were prepared using the Mitochondria/Cytosol Isolation Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The protein concentrations in the lysates were quantified using a Bio-Rad protein assay (Bio-Rad Lab., Hercules, CA, USA). For Western blotting analysis, equal amounts of protein samples were separated by sodium-dodecyl sulfate (SDS) gel electrophoresis and were then transferred onto polyvinylidene difluoride membranes (Schleicher and Schuell, Keene, NH, USA). The membranes were blocked with 5% skim milk in 20 mM of Tris (pH 7.4), 150 mM of NaCl, and 0.1% Tween 20 for 2 h at room temperature. The membranes were probed with primary antibodies,

which were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, and Cell Signaling Technology Inc., Danvers, MA, USA, at 4°C overnight. Then, they were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Westborough, MA, USA) for 2 h at room temperature. The protein bands were detected using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences) according to the manufacturer's instructions.

Measurement of caspase activity

The activities of the caspases (caspase-8 and-9) were detected using Colorimetric Assay Kits (R and D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The cells were briefly lysed in the supplied lysis buffer. The equal amounts of proteins were incubated with the supplied reaction buffer, which contained dithiothreitol and Ile-Glu-Thr-Asp (IETD)-p-nitroaniline (pNA) or Leu-Glu-His-Asp (LEHD)-pNA as substrates for caspase-8 and caspase-9, respectively. They were incubated at 37°C for 2 h in the dark. Using an ELISA reader, the reactions were measured by observing changes in absorbance at 405 nm.

Detection of mitochondrial membrane potential (Δψm)

Changes in the mitochondrial membrane potential (MMP) were assessed using the dual-emission potential-sensitive probe, 5,5′,6,6′-tetrachlor o-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1, Sigma-Aldrich Chemical Co.), which exhibits potential-dependent accumulation in mitochondria by a fluorescence emission shift from green to red. Therefore, mitochondrial depolarization is indicated by a decrease in the red-green fluorescence intensity ratio. Following treatment with EECU, the cells were collected, washed with PBS, and incubated with 10 μM of JC-1 for 30 min at 37°C in the dark. After the cells were washed with PBS to remove any unbound dye, the amount of JC-1 retained by 10,000 cells per sample was measured at 530 nm (green fluorescence) and 590 nm (red fluorescence) using a flow cytometer; it was then analyzed with CellQuest software (Becton Dickinson).

Measurement of intracellular reactive oxygen species

For the detection of intracellular ROS production, 2′,7′-dichlorofluorescin diacetate (DCF-DA, Molecular Probes, Leiden, Netherlands) dye was used. After collecting the cells treated with EECU for a certain period of time, the cells were harvested, rinsed with PBS, and then stained with 10 μM of DCF-DA for 20 min at 37°C in the dark. The cells were immediately washed, resuspended in PBS, and analyzed for fluorescence intensity using a flow cytometer. The values were expressed as a percentage of fluorescence intensity relative to blank control cells.[28] To confirm whether intracellular ROS levels play a role in the cytotoxicity of EECU, cells were pretreated with N-acetyl-L-cysteine (NAC) (Sigma-Aldrich Chemical Co.), which is a well-established antioxidant, for 1 h prior to treatment with EECU.

Data analysis

Experiments were carried out at least three times, and the results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using the SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc., Chicago, IL, USA). The difference between two groups was analyzed with the two-tailed Student's *t*-test, and the difference between three or more groups was analyzed with one-way ANOVA multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Ethanol extract of *Citrus unshiu* peel inhibits cell viability and induces apoptosis in MCF-7 cells

To investigate the antiproliferative effects of EECU on MCF-7 cells *in vitro*, the MTT assay and flow cytometry analyses were performed. As shown in Figure 1a, a high dose of EECU (200 μg/ml or more) for 24 h resulted in reduced cell viabilities, whereas a low dose of EECU (100 μg/ml) or the DMSO control treatment had no effect on cell viability. At the same time, various morphological changes were evident after treatment with EECU. In the presence of EECU, MCF-7 cells became smaller and rounded; density also became lower, resulting in a loss of adhesion [Figure 1b]. In addition, EECU markedly enhanced the percentage of annexin V^+/PI^- and V^+/PI^+ cells, which represent early and late apoptotic cells, respectively. This was compared to the untreated control group [Figure 1c] and indicated that EECU suppressed cell viability by inducing apoptosis in MCF-7 cells.

Ethanol extract of *citrus Unshiu* peel enhances the activation of caspase-8 and caspase-9 and the degradation of poly (ADP-ribose) polymerase in MCF-7 cells

To gain insight into the EECU-mediated apoptotic pathway, we examined whether caspase activation is involved in the EECU-induced apoptosis of MCF-7 cells. The immunoblotting results showed that the expression of pro-caspase-8 and-9 (as well as the representative initiator caspases of the extrinsic and intrinsic apoptosis pathway)[1,2] was apparently decreased with increasing EECU concentration, while their active forms were increased [Figure 2a]. A subsequent degradation of poly (ADP-ribose) polymerase (PARP), which is a representative substrate protein of activated effector caspases,[29] was also observed. Consistent with the results of the Western blot analysis, the *in vitro* activity of caspase-8 and caspase-9 was significantly enhanced as a result of EECU treatment [Figure 2b], indicating that both of the apoptosis pathways might be activated during the induction of apoptosis by EECU.

Ethanol extract of *Citrus Unshiu* peel modulates the expression of death receptor-related and Bcl-2 family proteins in MCF-7 cells

Next, we investigated the effect of EECU on the expression of DR-related proteins that are directly related to the activation of the extrinsic apoptotic pathway. The immunoblotting data indicated that the expression of Fas, Fas ligand (FasL), Fas-associated protein with death domain, DR4, and DR5 increased in a concentration-dependent manner with the treatment of EECU, but the TNF-related apoptosis-inducing ligand did not increase [Figure 3a]. Since mitochondrial dysfunction is crucial in the intrinsic apoptotic pathway, we also investigated the effect of EECU on the expression of Bcl-2 family proteins, which are the key factors controlling the function of mitochondria.[1,5] Among the Bcl-2 family proteins, anti-apoptotic Bcl-2 expression was reduced remarkably in EECU-treated cells, but the expression of pro-apoptotic Bax increased in response to EECU treatment [Figure 3b]. In addition, the total amount of Bid protein was decreased by EECU treatment, but truncated Bid (tBid) expression was progressively increased depending on the concentration of EECU treatment, which presumably resulted from truncation due to the activated caspase-8.

Ethanol extract of *Citrus unshiu* peel-induced apoptosis is associated with the mitochondrial dysfunction in MCF-7 cells

Since a loss of MMP (meaning the mitochondrial function impairment) is characteristic of the activation of the intrinsic apoptosis pathway along

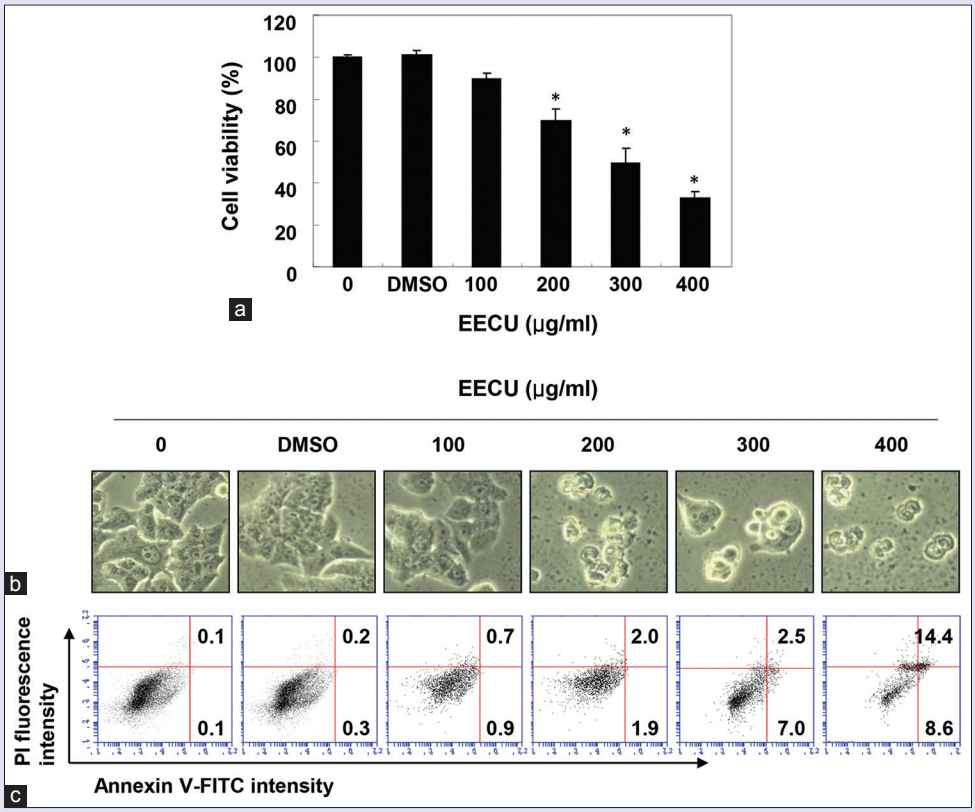

Figure 1: The effect of ethanol extract of *Citrus unshiu* peel on MCF-7 cell viability. MCF-7 cells were incubated with different concentrations of ethanol extract of *Citrus unshiu* peel for 24 h. (a) The cell viability was measured by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in the Materials and Methods section. The data are expressed as the percentage of cell viability, and they represent the mean ± standard deviation of three independent experiments (*$P < 0.05$ vs. untreated control). (b) The morphological changes of MCF-7 cells treated with ethanol extract of *Citrus unshiu* peel in various concentrations were observed under a phase-contrast microscope (×200). (c) The percentage of Annexin V-fluorescein isothiocyanate-positive cells cultured under the same conditions in the top (propidium iodide negative) and bottom (propidium iodide positive) of the right quadrant was indicated. Each point represents the means of two independent experiments

with the cytoplasmic release of cytochrome *c*,[4,5] we further investigated whether this phenomenon is involved in EECU-induced apoptosis in MCF-7 cells. We found that EECU markedly destroys the integrity of the mitochondria, and we measured this by the concentration-dependent loss of MMP [Figure 4a]. Subsequently, the release of cytochrome *c* from the mitochondria into the cytosol increased due to treatment with increased concentrations of EECU [Figure 4b].

AMP-activated protein kinase activation is associated with ethanol extract of *Citrus Unshiu* peel-induced apoptosis in MCF-7 cells

Numerous studies have shown that AMPK activation under stress conditions promotes cancer cell apoptosis and growth inhibition.[7,13] Therefore, we investigated whether EECU activates AMPK. AMPK activation is reflected by an increased phosphorylation of AMPKα and its downstream target kinase acetyl-CoA carboxylase (ACC).[8,9] Western blot results indicated that the phosphorylation of AMPK (Thr 172) and ACC (Ser 79) was increased depending on the concentration of the EECU treatment [Figure 5a], indicating that they were converted to the activated state. To address whether AMPK activation is an effective method of EECU-induced MCF-7 growth inhibition, the effects of EECU on cell viability after pretreatment of compound C (which is an inhibitor of AMPK) were investigated. As shown in Figure 5b, the suppression of cell viability was significantly abrogated in EECU-treated cells in the presence of compound C, indicating that the activation of AMPK is implicated in EECU-induced cytotoxicity in MCF-7 cells.

Ethanol extract of *Citrus unshiu* peel -induced activation of AMP-activated protein kinase is reactive oxygen species dependent in MCF-7 cells

It has been suggested that ROS, which are mainly produced by the mitochondria during the execution phase of apoptosis, may lead to the activation of AMPK.[12,13] Due to this suggestion, we further examined whether the activation of AMPK by EECU is dependent on ROS. Flow cytometry results demonstrated that the ROS levels increased rapidly within 30 min after EECU treatment and gradually decreased thereafter compared to the untreated cells [Figure 6a]. However, the EECU-induced increase in ROS contents was reduced by the addition of NAC, which is an ROS scavenger [Figure 6b]. NAC also almost completely reduced the EECU-mediated phosphorylation of AMPK and ACC, meaning that the AMPK signaling pathway was activated by EECU-induced ROS generation [Figure 6c]. Furthermore, the EECU-induced degradation of PARP and the reduction of cell viability were significantly lowered by the addition of NAC [Figure 6d]. These findings suggest that EECU increases ROS generation, which is required for AMPK activation and induces apoptosis in MCF-7 cells.

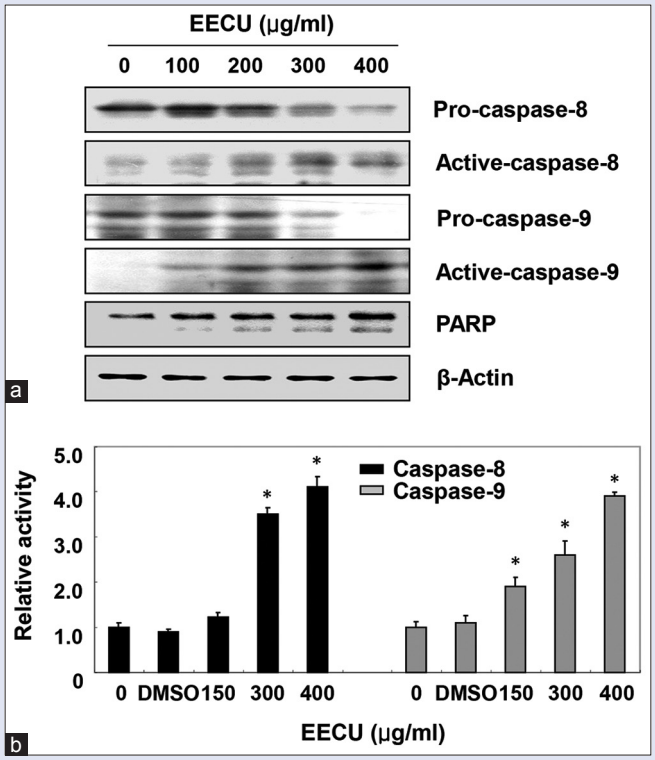

Figure 2: The activation of caspase-8 and caspase-9 by ethanol extract of *Citrus unshiu* peel in MCF-7 cells. MCF-7 cells were treated with the indicated concentrations of ethanol extract of *Citrus unshiu* peel for 24 h. (a) The cell lysates were prepared, and equal amounts of cellular proteins were separated on sodium-dodecyl sulfate-polyacrylamide gels and transferred to membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized using an enhanced chemiluminescence detection system. Actin was used as an internal control. Representative images of at least three independent experiments are shown. (b) The activities of caspases were evaluated using caspase colorimetric assay kits. An example of representative results according to each treatment concentration is presented. The data are expressed as the mean ± standard deviation of three independent experiments (*$P < 0.05$ vs. untreated control)

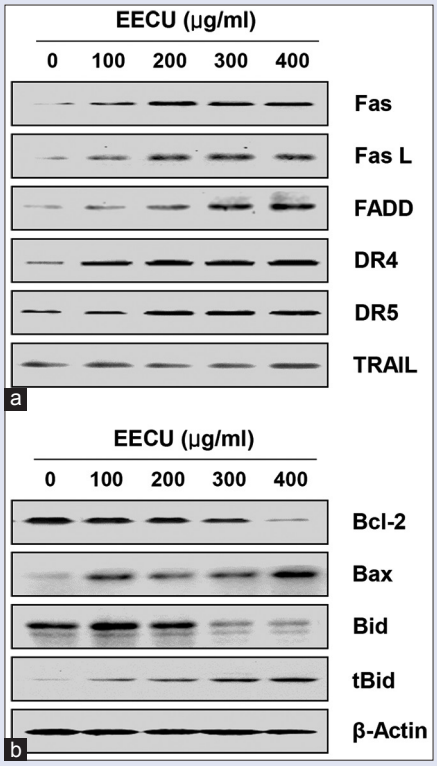

Figure 3: The effects of ethanol extract of *Citrus unshiu* peel on the levels of death receptor-related (a) and Bcl-2 family (b) proteins in MCF-7 cells. After 24 h of incubation with the indicated concentrations of ethanol extract of *Citrus unshiu* peel, the cells were lysed, and cellular proteins were separated with sodium-dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the membranes. The membranes were probed with the desired antibodies. Proteins were visualized using an enhanced chemiluminescence detection system. Equal protein loading was confirmed by an analysis of actin in the protein extracts. Representative images of at least three independent experiments are shown

DISCUSSION

In this study, we demonstrated that EECU induces apoptosis in MCF-7 human breast cancer cells by activating both extrinsic and intrinsic apoptotic pathways. Our data also showed that the intracellular accumulation of ROS resulted in the activation of AMPK, which then induced apoptosis; this indicated that ROS-dependent AMPK activation plays an important role in this process.

Among the various types of programed cell deaths associated with the inhibition of cancer cell proliferation, apoptosis is characterized by the activation of common caspases.[1,2] In the two representative pathways of apoptosis, the extrinsic pathway begins with the binding of the cell surface DRs of the death ligands to activate caspase-8, which follows the mobilization of the adapter molecule.[30,31] The intrinsic pathway is activated by the activation of caspase-9 through the loss of internal mitochondrial membrane integrity and the release of pro-apoptotic factors, such as cytochrome *c*, into the cytoplasm.[1,5] The activation of the effector caspases (-3 and -7) by activated initiator caspases (-8 and -9) in both pathways leads to the cleavage of the death substrates, such as PARP.[30,31] The results of this study showed that the caspase-8 activity was increased in cultured MCF-7 cells in media containing EECU, which was associated with an increased expression of DR-related genes and the degradation of PARP. In addition, the activity of caspase-9 was also enhanced by EECU treatment, suggesting that both extrinsic and intrinsic pathway activities might be involved in the induction of apoptosis by EECU in MCF-7 cells.

When the typical intrinsic pathway is activated, the loss of MMP and the cytosolic release of cytochrome *c* require the oligomerization of pro-apoptotic Bax to be inserted on the mitochondrial membrane.[5,30] Thus, increased expression of the Bax protein plays a crucial role in the activation of the intrinsic pathway, and Bcl-2 is a typical anti-apoptotic protein that suppresses this phenomenon.[4,5] Current data clearly show that Bax expression is markedly increased in EECU-treated cells and that Bcl-2 expression is reduced, which is followed by the loss of MMP associated with the increased release of cytochrome *c*. This evidence clearly suggests that the intrinsic pathway is apparently involved in inducing apoptosis of MCF-7 cells by EECU. Furthermore, the truncation of Bid, which is a pro-apoptotic BH3-interacting domain death agonist, was increased depending on the concentration of the EECU treatment. The truncated form of Bid (tBid) by activated caspase-8 acts as a linker molecule, connecting the DR and the mitochondria-dependent pathways.[31,32] Thus, this result suggests that the extrinsic pathway by EECU ultimately amplifies the intrinsic pathway through the caspase-8-mediated truncation of Bid.

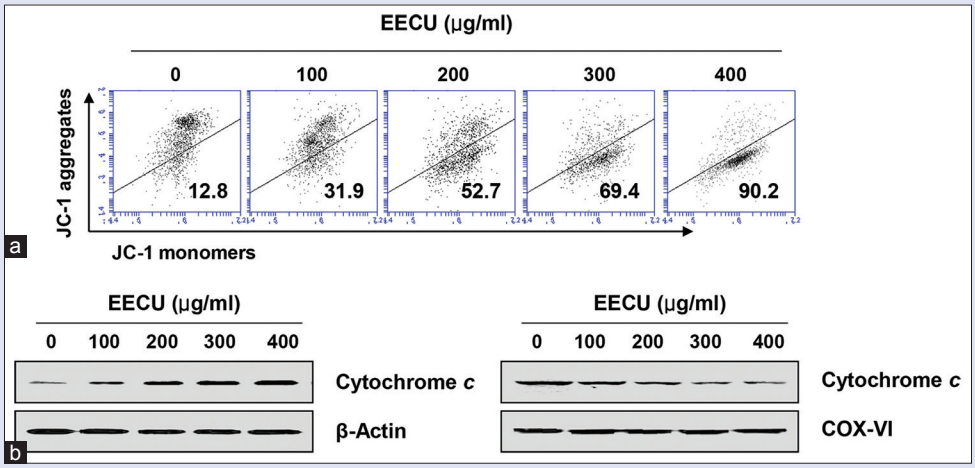

Figure 4: The loss of mitochondrial membrane potential and cytosolic release of cytochrome *c* by ethanol extract of *Citrus unshiu* peel in MCF-7 cells. (a) After 24 h of incubation with the indicated concentrations of ethanol extract of *Citrus unshiu* peel, the cells were stained with 1: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocy anine iodide-1 dye and were then analyzed on a flow cytometer. This was done in order to evaluate the changes in mitochondrial membrane potential. An example of representative results according to each treatment concentration is presented. (b) Cells cultured under the same conditions were lysed, and cytosolic and mitochondrial proteins were separated by sodium-dodecyl sulfate polyacrylamide gel electrophoresis and transferred to the membranes. The membranes were probed with an anti-cytochrome *c* antibody. Proteins were visualized using an enhanced chemiluminescence detection system. Equal protein loading was confirmed by the analysis of actin and cytochrome oxidase subunit VI (COX VI) in each protein extract. Representative images of at least three independent experiments are shown

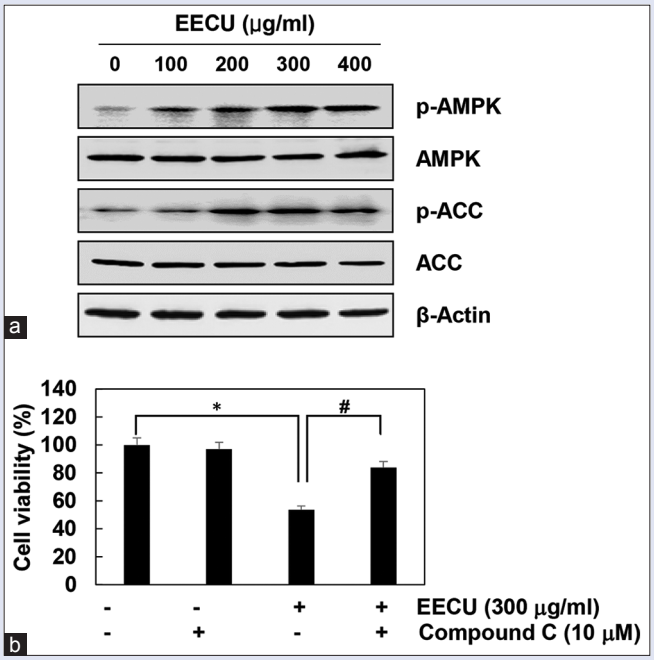

Figure 5: The activation of AMP-activated protein kinase by ethanol extract of *Citrus unshiu* peel in MCF-7 cells. MCF-7 cells were treated with different concentrations of ethanol extract of *Citrus unshiu* peel for 24 h (a), or they were pretreated with compound C, which is an AMP-activated protein kinase inhibitor, for 1 h and then treated with 300 μg/m ethanol extract of *Citrus unshiu* peel for 24 h (b). (a) Equal amounts of cell lysate were resolved with sodium-dodecyl sulfate-polyacrylamide gels, transferred to membranes, and probed with the indicated antibodies. The proteins were visualized using an enhanced chemiluminescence detection system. Actin was used as an internal control. Representative images of at least three independent experiments are shown. (b) The viability of cells was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The data are expressed as the mean ± standard deviation of three independent experiments (*$P < 0.05$ vs. untreated control; #$P < 0.05$ vs. ethanol extract of *Citrus unshiu* peel-treated cells)

Recent studies have shown that the activation of AMPK is directly related to apoptosis induced by various stimuli, including chemotherapeutic agents.[33-35] Anomalies in ATP production may be directly associated with the impaired mitochondrial function associated with increased ROS production, and ROS can act as a potent AMPK activator.[12,13] According to the results of this study, the induction of apoptosis by EECU was accompanied by a significant increase in the production of ROS as well as a loss of MMP. In addition, the levels of AMPK and ACC phosphorylation increased remarkably in EECU-treated MCF-7 cells, which mean that the AMPK signaling pathway was activated by the treatment of EECU. However, a blockade of AMPK activation by compound C, which is an inhibitor of AMPK activity, improved the EECU-induced inhibition of MCF-7 cell viability. Furthermore, the phosphorylation of AMPK and ACC by EECU was markedly suppressed under the condition of inhibition of ROS production using NAC; the degradation of PARP was attenuated and the inhibition of cell viability was significantly reversed. Although further studies on the inhibition of ATP production and related mechanisms of mitochondrial transport system disturbance are required, these data indicate that ROS-mediated AMPK activation may be involved in EECU-induced apoptosis in MCF-7 cells and that AMPK is likely to act as an upstream regulator in this process.

CONCLUSIONS

Our results demonstrated that EECU induces apoptosis in MCF-7 cells through the activation of both intrinsic and extrinsic pathways due to the enhancement of MMP loss, the cytosolic release of cytochrome *c*, and the generation of ROS with an increasing Bax/Bcl-2 ratio and Bid truncation. EECU also promoted AMPK activation, and the inhibition of AMPK activity blocked the EECU-induced reduction of cell viability. In addition, when ROS production was blocked, EECU-induced activation of AMPK and apoptosis were blocked, indicating that ROS is a potential upstream molecule of the cytotoxic effect induced by EECU in MCF-7 cells. Although the search for active ingredients in EECU and their specific anticancer activities should be continued, the results of this study indicate that EECU is an interesting natural compound with anticancer activity.

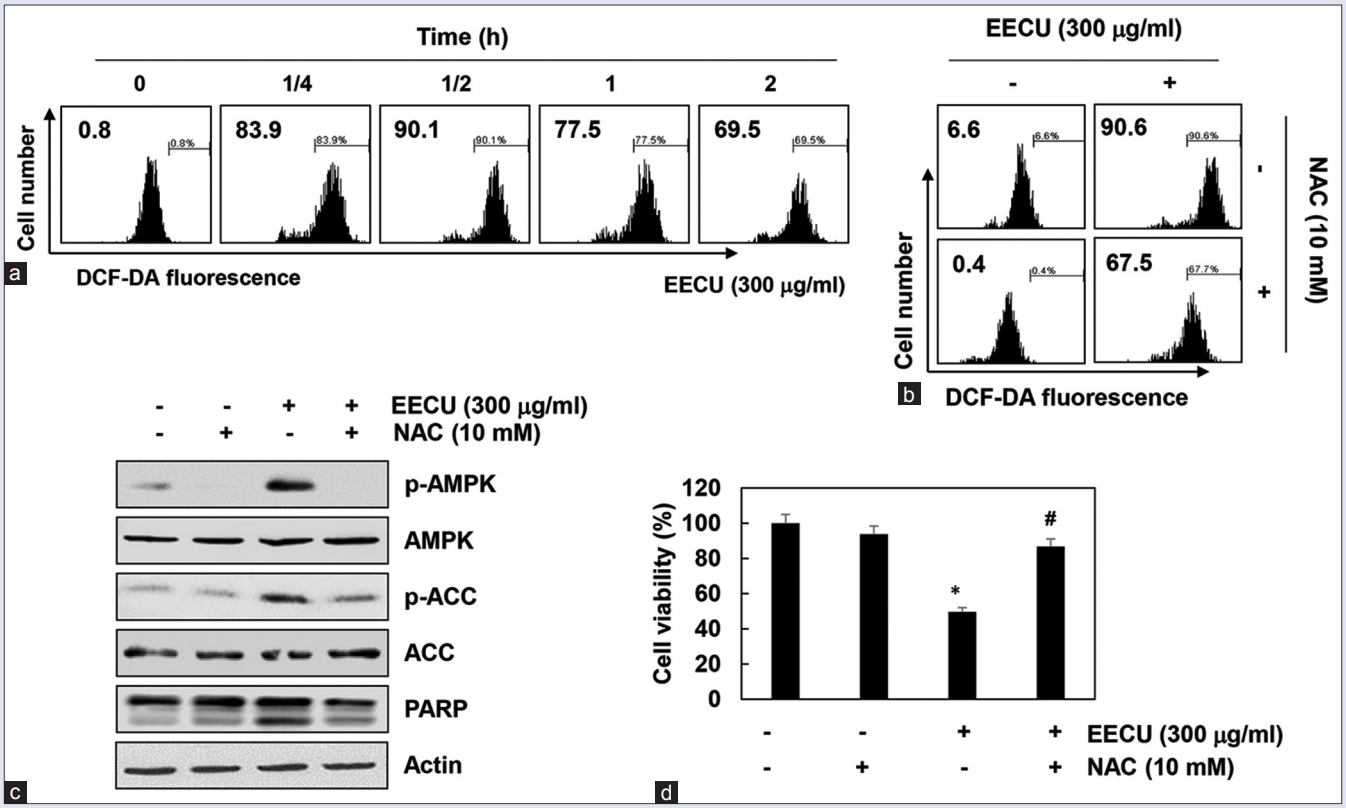

Figure 6: The reactive oxygen species-dependent activation of AMP-activated protein kinase by ethanol extract of *Citrus unshiu* peel in MCF-7 cells. (a and b) The cells were treated with 300 μg/m of ethanol extract of *Citrus unshiu* peel for the indicated times (a) or pretreated with N-acetyl-L-cysteine (10 mM) for 1 h before ethanol extract of *Citrus. unshiu* peel treatment for 30 min, which was followed by collection (b). The medium was discarded, and the cells were incubated at 37°C in the dark for 20 min with a new culture medium, which contained 10 μM of 2′,7′-dichlorofluorescin diacetate. Reactive oxygen species generation was measured by a flow cytometer. The data are the means of the two different experiments. (c and d) The cells were pretreated with 10 mM of N-acetyl-L-cysteine for 1 h before being treated with 300 μg/m of ethanol extract of *Citrus unshiu* peel. (c) After 24 h of incubation, the cellular proteins were separated by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized using an enhanced chemiluminescence detection system. Actin was used as an internal control. Representative images of at least three independent experiments are shown. (d) The viability of cells was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The data are expressed as the mean ± standard deviation of three independent experiments (*$P < 0.05$ vs. untreated control; #$P < 0.05$ vs. ethanol extract of *Citrus unshiu* peel-treated cells)

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Conflicts of interest

There are no conflicts of interest.

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