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Short Communication

Orostachys japonicus induce caspase-dependent apoptosis in HeLa human cervical cancer cells

Seon-Hee Kim [1§] **and Dong Seok Lee** [1,2§]

[1]Department of Biomedical Laboratory Science, Inje University, Gimhae 50834, Korea
[2]Institute of Digital Anti-aging Healthcare, Graduate School, Inje University, Gimhae 50834, Korea

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§Corresponding Authors:
Seon-Hee Kim
Department of Biomedical Laboratory Science, Inje University, 197 Inje-ro, Gimhae 50834, Korea.
Tel. +82-55-320-3503
Fax. +82-55-321-4038
Email. seonhee@inje.ac.kr

Dong Seok Lee
Institute of Digital Anti-aging Healthcare, Graduate School, Inje University, 197 Inje-ro, Gimhae 50834, Korea.
Tel. +82-55-320-3262
Fax. +82-55-321-4038
Email. mbdslee@inje.ac.kr

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ORCID iDs
Seon-Hee Kim
https://orcid.org/0000-0001-9349-4318
Dong Seok Lee
https://orcid.org/0000-0001-6348-8937

ABSTRACT

BACKGROUND/OBJECTIVES: *Orostachys japonicus* A. Berger (*O. japonicus*) is a perennial herb belonging to the Crassulaceae family that has been traditionally used to treat inflammation, fever, and poisoning. Although studies on the anticancer activity of *O. japonicus* have been conducted, its effect on virus-induced cancers has yet to be elucidated.
MATERIALS/METHODS: In the present study, we investigated the effects and mechanisms of action of the ethyl acetate fraction of *O. japonicus* extract (E-OJ) on the viability and apoptosis of HeLa cervical cancer cells.
RESULTS: The effect of E-OJ on HeLa cells was compared to that of kaempferol, quercetin, and gallic acid, which are components of *O. japonicus*. Treatment with E-OJ induced a concentration-dependent decrease in cell viability, as confirmed by MTS assay. Pretreatment with a broad-spectrum caspase inhibitor resulted in the recovery of cell viability. Western blot analysis was conducted to determine whether the induction of apoptosis was caspase-dependent. E-OJ induced apoptosis by increasing Bax/Bcl-2 ratio. Furthermore, it modulated the levels of cleaved caspase-3, -8, and -9, indicative of an impact on both the intrinsic and extrinsic pathways of apoptosis. Pretreatment with caspase inhibitors reduced caspase activity.
CONCLUSION: These results suggest that the anticancer activity of *O. japonicus* is mediated by caspases, resulting in a decrease in the viability of HeLa cells.

Keywords: Extract; apoptosis; caspase; Bcl-2; Bax; HeLa cells

INTRODUCTION

Orostachys japonicus, a perennial herb belonging to the Crassulaceae family, has traditionally been used as a natural remedy for diverse ailments, including arthritis, hepatitis, fever, eczema, poisoning, and bleeding. Numerous studies have highlighted its therapeutic properties, revealing associations with antipyretic, hemostatic, and detoxifying actions, as well as anticancer and anti-inflammatory effects [1-13]. Previous investigations have identified *O. japonicus* as containing compounds such as friedelin, epifriedelanol, glutinone, glutinol, triterpenoids, β-sitosterol, campesterol, fatty acid ester, kaempferol, quercetin,

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Conflict of Interest

The authors declare no potential conflicts of interests.

Author Contributions

Conceptualization: Kim SH; Formal analysis: Kim SH; Investigation: Kim SH, Lee DS; Methodology: Kim SH; Supervision: Lee DS; Writing - original draft: Kim SH; Writing - review & editing: Kim SH, Lee DS.

flavonoids, and aromatic acids [14-16]. In addition, its safety was confirmed by its lack of effect on the survival and proliferation of normal macrophages, with no clinical signs of toxicity observed after administration in mice [14,17]. Notably, the sequential extraction of dried *O. japonicus* powder using organic solvents, including n-hexane, dichloromethane, ethyl acetate, n-butanol, and water, revealed that the ethyl acetate-soluble fraction exhibited anticancer effects in various cancer cell lines [1,8,16]. Previous research has demonstrated its significant anticancer activity against colon cancer (HT-29), breast cancer (MDA-MB-231), and gastric cancer (AGS). However, further investigations are warranted to explore its potential effects on virus-induced cancers, such as those caused by hepatitis B virus, Epstein-Barr virus, and human papillomavirus.

Cervical cancer, characterized by the uncontrolled proliferation of abnormal cells in the cervix, is the fourth most common cancer among women. Treatment modalities for cervical cancer include radiation therapy, chemotherapy, surgical intervention, and targeted therapy [18,19]. Despite a dramatic reduction in cancer incidence in recent years owing to the introduction of cervical cancer vaccines targeting human papillomavirus (HPV), the disease remains a significant threat to women in low- and middle-income countries, where limited resources often result in the progression of the disease to advanced and untreated stages [20,21]. Notably, conventional chemotherapeutic agents, while effective in destroying cancer cells, concurrently suppress essential components of the immune system, such as lymphocytes and bone marrow cells, leading to a compromised immune system. Therefore, the imperative to explore safe and effective anticancer agents derived from traditional herbal sources is underscored.

This study investigated the cytotoxic effects of *O. japonicus*-derived substances on cervical cancer cells. We compared the apoptotic effects of the ethyl acetate fraction of *O. japonicus* extract (E-OJ) and its known constituents kaempferol, quercetin, and gallic acid. Additionally, we examined changes in the expression of molecules associated with apoptosis. In this study, we sought to elucidate the therapeutic efficacy of *O. japonicus* extract against virus-induced cancers and the characteristics of the associated apoptosis. The results are expected to provide insights useful for the future development of anticancer agents that utilize these compounds.

MATERIALS AND METHODS

Plant extracts

O. japonicus was sourced from Geobugiwasong Ltd. (Miryang, Korea), naturally air-dried, and finely ground into powder. The ethyl acetate (EtOAc) fraction from *O. japonicus* was fractioned using a solvent, as described by our team [1,6,8-10]. A total of 200 g of *O. japonicus* powder was mixed with 1 liter of 95% ethyl alcohol (EtOH), followed by three rounds of reflux boiling using a reflux condenser (SciLab®, Seoul, Korea). Crude *O. japonicus* extract was concentrated using a rotary evaporator (IKA-Werke GmbH, Co. KG, Staufen, Germany). Subsequently, the concentrated extract was fractionated using the following organic solvents in sequential order: *n*-hexane (hexane), dichloromethane (DCM), ethyl acetate (EtOAc), *n*-butanol (BuOH), and water (H_2O). Among the obtained fractions, the ethyl acetate fraction of *O. japonicus* (E-OJ) was dried using rotary evaporation at 40°C until the solvent was completely evaporated, and then stored at –20°C. The E-OJ used in the experiments was dissolved in dimethyl sulfoxide (DMSO) as a solvent before treatment.

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Cell line and reagents

HeLa cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Penicillin, streptomycin, DMEM, and FBS were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Monoclonal antibodies targeting proteins, such as pro-caspase-3 (Cat. No. 9662), -8 (Cat. No. 9746), -9 (Cat. No. 9502), cleaved caspase-3 (Cat. No. 9661), -8 (Cat. No. 9496), -9 (Cat. No. 9505), Bcl-2 (Cat. No. 15071), Bax (Cat. No. 2772), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat. No. 2118) were acquired from Cell Signaling Technology (Beverly, MA, USA). Peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Z-VAD-FMK (Cat. No. 5503707), Z-DEVD-FMK (Cat. No. 550378), Z-IETD-FMK (Cat. No. 550380), and Z-LEHD-FMK (Cat. No. 550381) were purchased from BD Pharmingen™ (Franklin Lakes, NJ, USA), and kaempferol, quercetin, and gallic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

HeLa cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and maintained at 37°C under 5% CO_2 conditions. The cell culture medium was replaced every two days, and subculturing was performed upon reaching 80% confluence.

Cell viability assay

Cell viability was assessed using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, HeLa cells were cultured in a 96-well plate in serum-free medium at 37°C for 6 h before treatment with various concentrations of E-OJ (0, 2.5, 5, 7.5, and 10 μg/mL) for 12 or 24 h. Subsequently, MTS reagent was added, and the cell culture plate was incubated at 37°C for 2 h. The absorbance was measured at 490 nm using a fluorescence multi-detection reader (BioTek, Winooski, VT, USA). All experiments were performed in triplicate.

For the experiments used to confirm caspase dependence, the cells were pre-incubated with 20 μM Z-VAD-FMK for 30 min before treatment with E-OJ and respective compounds. Following treatment, the cell survival rates were measured using the MTS assay, as described above.

Western blotting analysis

HeLa cells were treated with E-OJ, kaempferol, quercetin, or gallic acid for 12 h. In experiments validating caspase dependence, cells were pre-treated with 20 μM each of Z-VAD-FMK, Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK for 30 min before treatment with E-OJ or respective compounds.

Subsequently, the cells were washed with phosphate-buffered saline (PBS), pelleted, and dissolved in cold lysis buffer for 1 h. The extracted proteins were quantified using the BCA protein assay kit (Pierce, IL, USA). A total of 40 μg of protein samples were separated by 10-15% SDS-PAGE electrophoresis (Bio-Rad, CA, USA) and transferred to a PVDF membrane using a semidry transfer system (Bio-Rad). The membranes were blocked with 5% nonfat milk in PBS-T solution (PBS with 0.1% Tween-20) and incubated overnight with primary antibodies. After three washes with PBS-T solution, the membranes were cultured with HRP-conjugated secondary antibodies for 2 h at 4°C. Subsequently, the membranes were washed three times with PBS-T. Finally, the expression of target proteins was visualized by exposing the membranes to an X-ray film using an enhanced chemiluminescence detection kit obtained from Santa Cruz (CA, USA). Intensity analysis of the bands was performed using the ImageJ software.

Statistical analysis

All statistical analyses were performed using Prism v5.01 GraphPad (San Diego, CA, USA). Differences between the control and experimental groups were assessed using Student's *t*-test, and statistical significance was considered at $P < 0.05$. The results are presented as mean ± SD. Statistical significance was considered as $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS

The EtOAc fraction of O. japonicus inhibited the survival of tumor cells

In previous studies, we employed various organic solvents to extract *O. japonicus*, compared the anticancer activities of each extract, and discovered that the ethyl acetate-soluble fraction (E-OJ) exhibited the most potent anticancer effects among all fractions [1,8,16]. Additionally, gas chromatography-mass spectrometry (GC-MS) confirmed the presence of kaempferol, quercetin, and gallic acid in E-OJ [16]. Next, we sought to analyze the anticancer effects of E-OJ by comparing it with the specific components of *O. japonicus.*

To evaluate the effects on HeLa cell survival rates, cells were treated with various concentrations of E-OJ (2.5, 5, 7.5, and 10 μg/mL) for 12 or 24 h, and the cell survival rates were assessed using MTS analysis. As shown in **Fig. 1**, cells treated with E-OJ for 12 or 24 h showed a significant decrease in cell survival rates compared to untreated control cells. Overall, the survival rates of the cells after E-OJ treatment decreased in a time- and dose-dependent manner.

O. japonicus and its constituents induce apoptosis

Apoptosis mediated by the caspase pathway is an essential mechanism that inhibits cell growth, promotes tissue homeostasis, and eliminates damaged cells [22-25]. To investigate HeLa cell apoptosis induced by E-OJ and its constituents (kaempferol, quercetin, and gallic acid), we examined caspase dependence and assessed cell survival rates in the presence of caspase inhibitors. HeLa cells were pre-treated with the general caspase inhibitor, Z-VAD-FMK, in the culture medium and subsequently exposed to E-OJ (10 μg/mL) for 12 h (**Fig. 2**). |Consistent with previous experiments, E-OJ significantly reduced the HeLa cell survival

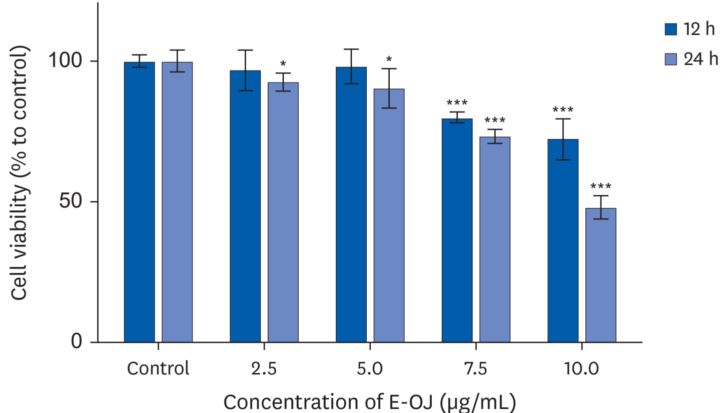

Fig. 1. Effect of *O. japonicus* on HeLa cell viability. HeLa cells were treated with the various concentrations of the EtOAc fraction of *O. japonicus* (E-OJ) for 12 and 24 h. Cell viability was measured using MTS assay. The results are presented as mean ± SD.
E-OJ, the ethyl acetate fraction of *O. japonicus* extract.
*$P < 0.05$; ***$P < 0.001$.

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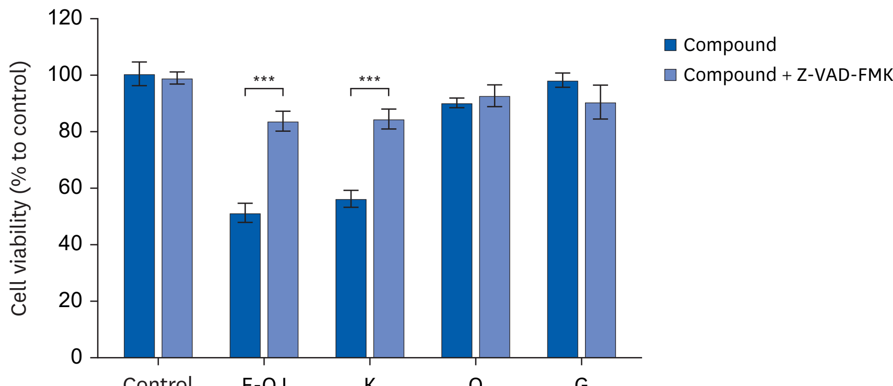

Fig. 2. Effect of general caspase inhibitor on viability in HeLa cells after treatment with E-OJ. HeLa cells were treated with 10 μg/mL E-OJ, 80 μM kaempferol (K), quercetin (Q), and gallic acid (G) for 12 h, followed by treatment with or without Z-VAD-FMK (a general caspase inhibitor). Cell viability was determined using the MTS assay. Values are expressed as mean ± SD.
E-OJ, the ethyl acetate fraction of *O. japonicus* extract.
***$P < 0.001$.

rates, which was partially reversed by caspase inhibition with Z-VAD-FMK. Examination of the cell survival rates induced by the constituents of E-OJ revealed that kaempferol had the most pronounced effect on HeLa cell death. The kaempferol-induced decrease in the survival rate was substantially restored by caspase inhibition. Quercetin exhibited a milder effect, but still showed a significant decrease in survival rates compared to the control group, while the effects of gallic acid were not evident in the MTS assay.

O. japonicus regulates the Bax/Bcl-2 ratio

The permeabilization of the mitochondrial outer membrane, accompanied by the release of cytochrome c and the formation of apoptosomes, is a well-known major pathway leading to cell death [26,27]. To investigate the effect of E-OJ and its extracts on the mitochondrial apoptosis pathway, we measured the changes in cell death-related Bcl-2 family proteins in HeLa cells treated with E-OJ, kaempferol, quercetin, and gallic acid. Bcl-2 family proteins can be classified as either anti-apoptotic or pro-apoptotic, the ratio of which determines the sensitivity or resistance of cells to apoptosis stimulation. In particular, the Bax/Bcl-2 ratio is a crucial indicator of cell death sensitivity [27-29].

Upon examining changes in the Bcl-2 and Bax protein levels in HeLa cells after E-OJ treatment, a dose-dependent increase in Bax expression was observed (**Fig. 3A**). Kaempferol showed the highest Bax/Bcl-2 ratio, quercetin exhibited approximately half the Bax/Bcl-2 ratio compared to kaempferol, and gallic acid did not show a significant difference compared to the control (**Fig. 3B**). These results confirm that kaempferol, quercetin, and gallic acid induce apoptosis via the mitochondrial pathway at varying intensities. Additionally, the higher the concentration of E-OJ, the higher the sensitivity to cell death.

O. japonicus induces caspase-mediated apoptosis

The regulation of apoptosis by caspases involves both extrinsic pathways triggered by death receptor signaling and intrinsic pathways induced by cellular stress [30]. Both pathways are controlled by various types of caspases, with each caspase known to undergo activation through cleavage and operate in an activated form [31]. For instance, the executioner caspase caspase-3 plays a crucial role in the final stages of apoptosis, breaking down essential proteins and inducing cell death through cleavage. Caspase-8 is involved in the

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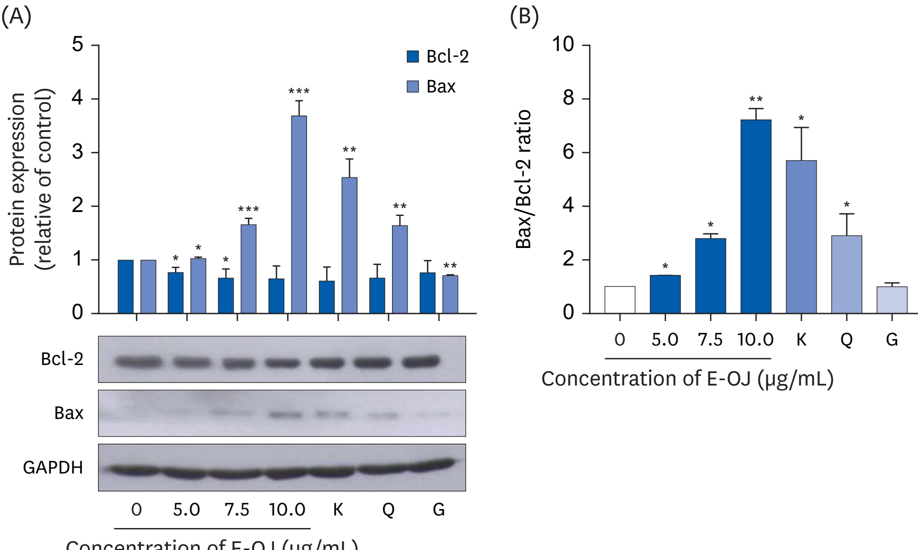

Fig. 3. Alterations in the expression of Bcl-2 and Bax proteins upon treatment with E-OJ, kaempferol, quercetin, and gallic acid. HeLa cells were treated with E-OJ, kaempferol (K), quercetin (Q), or gallic acid (G) for 12 h, respectively. (A) Western blotting analysis was performed to analyze the expression of Bcl-2 and Bax proteins, with GAPDH serving as an internal control. (B) The ratio of Bax/Bcl-2 was calculated. The band intensities were measured using densitometry in three separate experiments with comparable results. The data are expressed as mean ± SD.

E-OJ, the ethyl acetate fraction of *O. japonicus* extract.

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

extrinsic death receptor pathway (extrinsic pathway), whereas caspase-9 is associated with the mitochondrial pathway (intrinsic mitochondrial pathway). To elucidate the apoptotic signaling pathways, we assessed the protein levels of pro-caspase-3, cleaved caspase-3, pro-caspase-8, cleaved caspase-8, pro-caspase-9, and cleaved caspase-9, and examined both pathways of apoptosis.

For the ultimate apoptosis trigger, caspase-3, we observed a dose-dependent increase in the cleavage ratio induced by E-OJ (**Fig. 4A**). However, when treated with constituents of *O. japonicus*, a lower caspase-3 cleavage ratio was induced. The activation of caspase-8 by kaempferol, quercetin, and gallic acid was less than that induced by E-OJ (**Fig. 4B**). Interestingly, for caspase-9 associated with the intrinsic pathway, cleavage occurred at levels similar to those of E-OJ for all three compounds (kaempferol, quercetin, and gallic acid) (**Fig. 4C**). These results did not align with the MTS assay results (**Fig. 2**) or with caspase-3 activation (**Fig. 4A**).

To determine whether the extrinsic or intrinsic apoptotic pathways affect E-OJ-induced apoptosis, general caspase inhibitors (e.g. Z-VAD-FMK) and narrow-spectrum caspase inhibitors (e.g. Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor), and Z-LEHD-FMK (caspase-9 inhibitor)) were used. As shown in **Fig. 5A-C**, upon pre-treatment with a caspase inhibitor followed by E-OJ treatment, an increase in the pro-apoptotic forms of caspases and a decrease in the expression of the active forms (cleaved caspases) was observed.

These results indicate that caspase-3, -8, and -9 play critical roles in inducing the apoptotic effects of E-OJ in HeLa cells. Consequently, E-OJ appears to function through both the extrinsic and intrinsic apoptotic pathways.

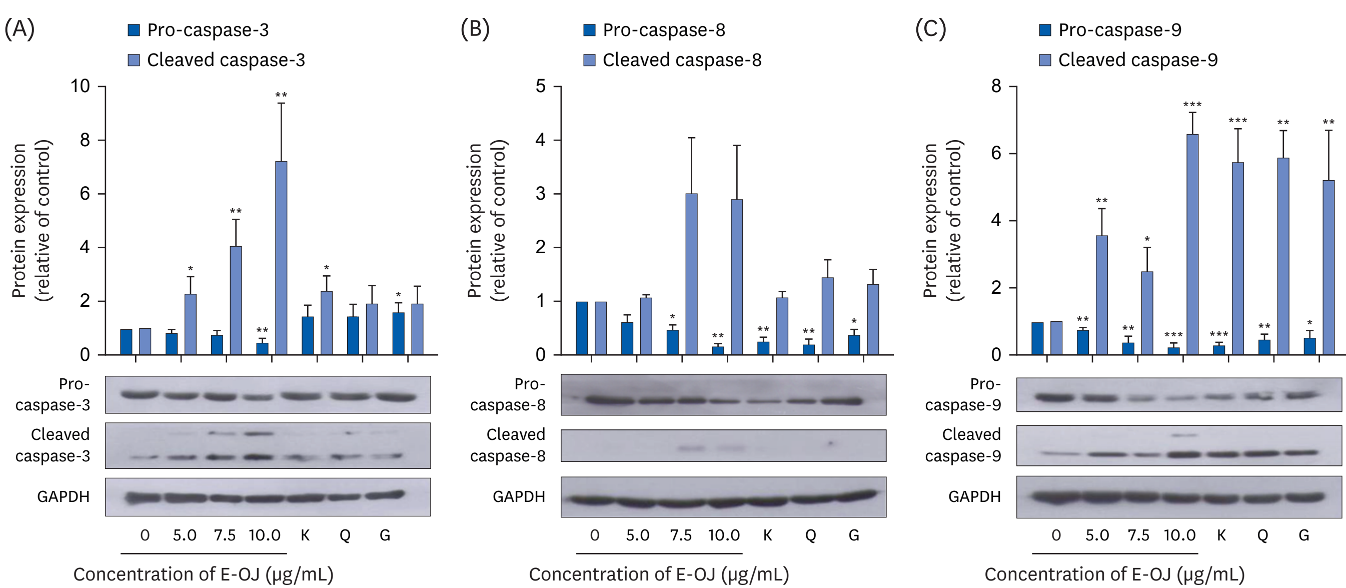

Fig. 4. Regulation of caspase-3, -8 and -9 levels in HeLa cells following treatment with E-OJ. HeLa cells were treated with different concentration of E-OJ, 80 μM of kaempferol (K), quercetin (Q), or gallic acid (G) for 12 h. The level of pro-caspase-3, cleaved caspase-3 (A), pro-caspase-8, cleaved caspase-8 (B), pro-caspase-9, and cleaved caspase-9 (C) were analyzed using western blotting analysis. The band density was quantified and presented in a bar graph with GAPDH as an internal control. The results were obtained from three separate experiments and expressed as mean ± SD.
E-OJ, the ethyl acetate fraction of *O. japonicus* extract.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

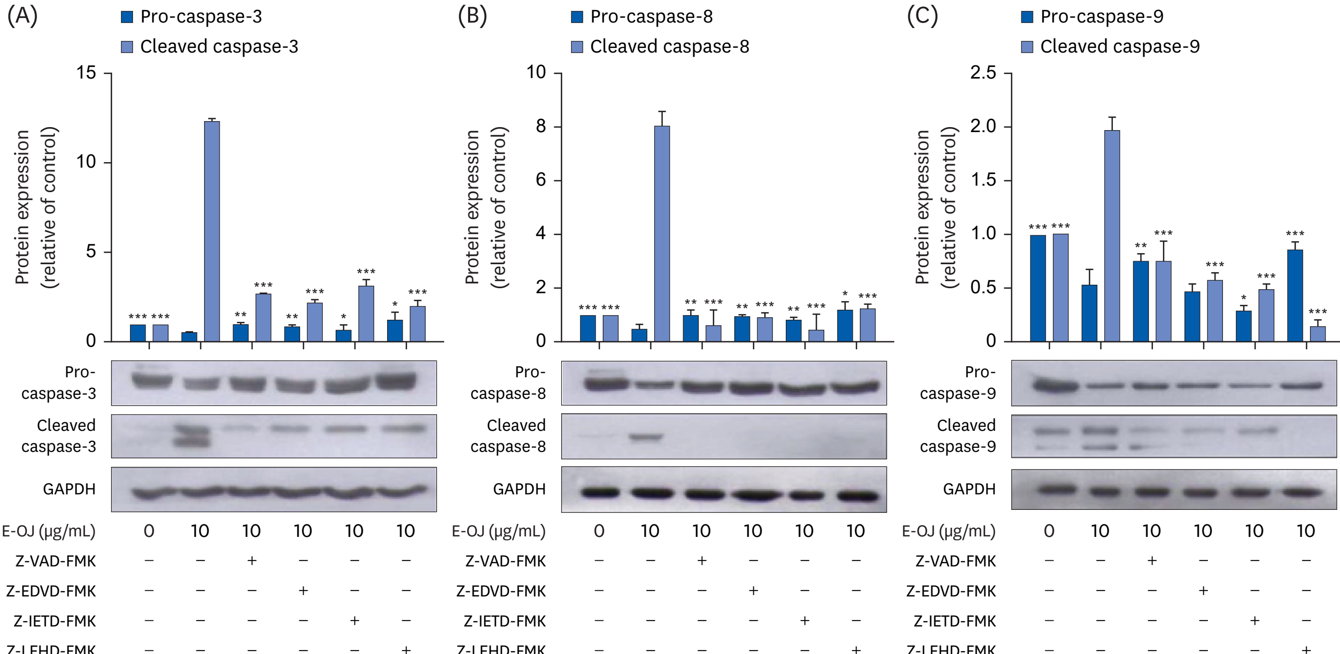

Fig. 5. Effect of caspase inhibitors on apoptosis of HeLa cells treated with E-OJ. Hela cells were pretreated with Z-VAD-FMK (pan-caspase inhibitor), Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor), and Z-LEHD-FMK (caspase-9 inhibitor) and then treated with 10 μg/mL E-OJ, 80 μM kaempferol (K), quercetin (Q), and gallic acid (G) for 12 h. The levels of pro-caspase-3, cleaved caspase-3 (A), pro-caspase-8, cleaved caspase-8 (B), pro-caspase-9, and cleaved caspase-9 (C) were analyzed using western blotting analysis with GAPDH as an internal control. Values are expressed as mean ± SD. Significance compared to the cells treated with E-OJ only.
E-OJ, the ethyl acetate fraction of *O. japonicus* extract.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

DISCUSSION

In this study, we investigated the effect of the ethyl acetate fraction of *Orostachys japonicus* (E-OJ) on the induction of apoptosis in HeLa cervical cancer cells. Compared to known constituents of *O. japonicus*, such as kaempferol, quercetin, and gallic acid, we elucidated the apoptotic mechanisms triggered by E-OJ. The activation of caspase-9 cleavage by *O. japonicus* constituents and E-OJ was indicative of the occurrence of apoptosis through the mitochondrial pathway. This was further confirmed by the increased Bax/Bcl-2 ratio following E-OJ and kaempferol treatment. Additionally, the induction of apoptosis through the death receptor pathway involving caspase-8 was also observed (**Fig. 6**). This study aimed to elucidate the mechanism underlying the induction of apoptosis by E-OJ in cancer cells and its triggering pathways.

HPV has a double-stranded DNA genome encoding eight proteins, namely, E1, E2, E4, E5, E6, and E7 (early), as well as L1 and L2 (late) [32,33]. Among these, E5, E6, and E7 affect the signal transduction in the death receptor pathway of apoptosis. E5 disrupts the formation of the death-inducing signaling complex triggered by FasL and TRAIL. E6 induces the inactivation of pro-apoptotic proteins, such as p53, Bak, and FADD. Furthermore, it hinders Fas-triggered apoptosis by preventing the activation of caspase-3 and -8 [34]. E7 acts as an oncoprotein by inducing the degradation of the anti-apoptotic protein pRb via the ubiquitin pathway [35,36]. Additionally, E7 increases the expression of the cellular inhibitor of apoptosis protein 2 (c-IAP2), which is involved in the degradation of caspase and DISC proteins, leading to resistance against apoptosis [32,37]. Therefore, HeLa cells, an HPV-induced human cervical

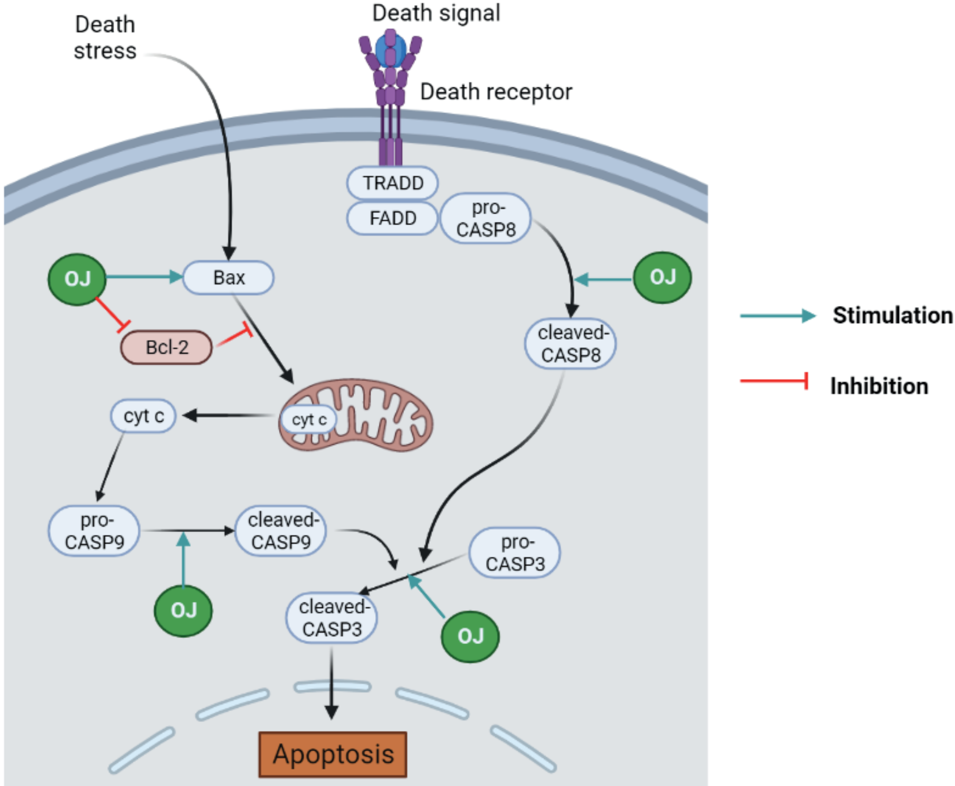

Fig. 6. Mechanisms of caspase-dependent apoptosis induced OJ.
OJ, *Orostachys japonicus*.

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cancer cell line, exhibit resistance to apoptosis, which is distinct from other cancer cell lines. The activity of caspases, which play crucial roles in apoptosis, differs from that in other cancer cells. In this study, we confirmed that E-OJ and *O. japonicus* extracts primarily induced apoptosis through the mitochondrial pathway (**Fig. 3**). These results suggest that owing to the resistance of HPV-derived proteins to death receptor-triggered apoptosis, cell death proceeds through an alternative mitochondrial pathway. However, the significant decrease in caspase-8 protein expression and the increase in cleaved caspase-8 observed in **Fig. 4B** indicate that E-OJ overcame the resistance to death receptor-triggered apoptosis. Nevertheless, this study did not elucidate the direct or indirect interactions between E-OJ and HPV E5, E6, and E7 proteins, warranting further investigation in future studies.

In this study, we investigated the caspase-mediated apoptotic pathway induced by E-OJ components. Previous studies have extensively explored the induction of apoptosis by these compounds. Kaempferol has been reported to inhibit proliferation and induce G2/M phase cell cycle arrest in the triple-negative breast cancer cell line, MDA-MB-231. Additionally, it increases the expression of cleaved caspase-9 and -3, leading to apoptosis [38,39]. Apoptosis has also been reported in human acute leukemia Jurkat T-cell clones and human umbilical vein endothelial cells through the activation of caspase-9, -8, and -3 [40,41]. Quercetin induces cell cycle arrest in the S and G2/M phases, and promotes apoptosis by modifying Foxo3a signaling in MDA-MB-231 cells [42]. Studies have shown that quercetin induces DNA damage, p53 upregulation, loss of mitochondrial membrane potential, and sequential cleavage of caspase-9 and -3, ultimately leading to apoptosis. Quercetin mediates apoptosis via the mitochondrial pathway [43]. Gallic acid is known to induce apoptosis through mitochondria-dependent pathways, involving the cleavage of caspase-9, in various cancer cell lines, such as small cell lung cancer, leukemia, and pancreatic cancer [44-47]. Zeng *et al.* [48] reported that gallic acid induces apoptosis in Bladder Cancer T24 Cells via mitochondrial dysfunction, which is characterized by increased mitochondrial ROS levels, cytochrome c release, and an increased expression of cleaved caspase-3.

Previous analyses of E-OJ using GC-MS have revealed that kaempferol, quercetin, and gallic acid constitute approximately 16.13% of E-OJ (kaempferol 6.81%, quercetin 5.08%, and gallic acid 4.24%), while the remaining 83.88%, represented by 12 peaks, was unidentified [16]. In this study, we confirmed that E-OJ, which contains kaempferol, quercetin, and gallic acid, induces apoptosis through the mitochondrial pathway rather than the death receptor pathway (**Fig. 5**). This aligns with previous research indicating that kaempferol, quercetin, and gallic acid induce apoptosis via the mitochondrial pathway. Therefore, the anticancer efficacy observed in HeLa cells treated with E-OJ is likely derived from the constituents of *O. japonicus*, and appears to exhibit higher anticancer efficacy than the individual components.

This study has limitations in that it only investigated the effects on a single cancer cell line, and the evaluation was limited to virus-derived cancer cells. Additionally, protein expression pattern analysis using western blotting was conducted at a single time point (12 h). Furthermore, we focused on analyzing specific molecules within the signaling pathways that induce cell death. However, further studies are needed for a comprehensive understanding of the entire pathway. Although this study analyzed apoptosis based on the activity of specific caspases, identifying changes in a broader range of signaling molecules is necessary to accurately distinguish between the intrinsic and extrinsic pathways. Therefore, additional research is required to address these limitations and to provide a more comprehensive understanding of the observed effects.

In this study, *O. japonicus* extract was analyzed in comparison with its individual components. As a result, *O. japonicus* extract was found to be more effective in inhibiting cancer cell proliferation and inducing apoptosis. The apoptotic response induced by E-OJ operates via the pathways depicted in **Fig. 6**. These findings highlight the potential of *O. japonicus* extract as an anticancer therapeutic agent, particularly for the treatment of cervical cancer.

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