치의과학석사 학위논문

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정 민 정

정민정의 석사학위논문을 인준함

2021 년 1 월

위 원 장 홍 성 두 (인)

부 위 원 장 조 성 대 (인)

위 원 윤 혜 정 (인)

# ABSTRACT

## *In vitro* induction of mitotic catastrophe as a therapeutic approach for oral cancer with *Juniperus squamata*

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***Objective:*** Mitotic catastrophe, a cell death mechanism characterized by abnormal mitosis, has been regarded as a therapeutic approach for the development of anti-cancer drug candidates. In this study, we investigated the potential effect of the ethanolic extract of *Juniperus squamata* (EEJS) on the occurrence of mitotic catastrophe in human oral cancer cell lines.

***Materials and Methods:*** The effect of EEJS on the occurrence of mitotic catastrophe was evaluated by measuring cytotoxicity, observing phase-contrast or transmission electron microscope findings, evaluating the appearance of microtubule or chromosome abnormalities, and detecting the phosphorylation of histone H3 ($Ser^{10}$). The apoptotic effect of EEJS was assessed by detecting cleaved

PARP, analyzing the sub-$G_1$ population, annexin V-FITC/PI double staining, western blotting, and the transient transfection of myeloid cell leukemia-1 (Mcl-1) overexpression vectors.

***Results***: EEJS treatment was effective at inhibiting cell proliferation in human oral cancer cell lines. EEJS resulted in the enrichment of enlarged multinucleated cells, the disturbance of microtubule formation, and increased phosphorylation of histone H3 ($Ser^{10}$), which demonstrates the occurrence of mitotic catastrophe. Additionally, the multinucleated cells underwent apoptotic cell death in a cell context-dependent manner, which was associated with the reduction of Mcl-1 protein levels.

***Conclusion***: This study indicates that EEJS could be effective for treating human oral cancer by promoting mitotic catastrophe linked to apoptotic cell death.

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**Keywords:** Mitotic catastrophe, Apoptotis, Phospho-Histone H3, *Juniperus squamata*, Mcl-1, Oral cancer

**Student Number:** 2018-27675

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# 1. Introduction

Genome integrity within normal cells is preserved by operating surveillance mechanisms including DNA damage checkpoints, DNA repair systems, and mitotic checkpoints [1]. However, defects in genome integrity cause impaired cellular responses, leading to genome instability. Genome instability, which is an enabling characteristic for the acquisition of the hallmarks of cancer, allows normal cells to undergo genetic alterations including gene mutations, which contribute to tumor progression [2]. In particular, the elimination of mitotically defective or failed cells has been regarded as a gateway to avoid genomic instability, including mitotic catastrophe [3]. Chromosome missegregation resulting from failed mitosis, which has been extensively characterized as the typical feature of mitotic catastrophe that is accompanied by mitotic arrest, leads to nuclear alterations such as micro nucleation and multinucleation [4]. Furthermore, the disturbance of mitotic spindle formation induced by the depletion of centrosomal proteins contributes to the occurrence of mitotic catastrophe because the mitotic spindle is responsible for perfect chromosome segregation during mitosis [5]. Although the eventual fate of cells following mitotic catastrophe remains indefinite, the cells appear to undergo senescence, apoptosis, and necrosis [3, 6, 7]. Several microtubule or non-microtubule targeting agents that act as mitotic catastrophe inducers have been evaluated in preclinical and clinical trials [8]. Natural compounds have been particularly noted to

cause mitotic catastrophe followed by apoptotic cell death in various types of cancer [9, 10]. The induction of mitotic catastrophe in tumor cells endows therapeutic advantages for the development of novel anti-cancer agents given the high susceptibility to mitotic aberrations in aneuploidy or tetraploid tumor cells and their usage at lower doses before the occurrence of cell death [4]. Therefore, inducing mitotic catastrophe is an attractive strategy for successful therapeutic outcomes.

Active compounds from natural products have consistently been considered for the discovery and development of potential anti-cancer drug candidates because of their various pharmacological functions with high safety profiles. Hence, natural product-derived agents are currently being investigated in clinical trials to identify valuable agents that can be developed as anti-cancer drugs [11]. The *Juniperus* genus, which contains approximately 75 species, is mainly distributed in large parts of the Northern Hemisphere and has been used as a traditional medicine for treating several symptoms including abdominal spasms, asthma, or diarrhea [12, 13]. The biological and pharmacological activities of *Juniperus* species seem to be caused by secondary metabolites including five terpenoids, two flavonoids, and one lignan [13]. Among these species, *Juniperus communis* has shown a remarkable ability to induce cell cycle arrest or apoptotic cell death in various types of cancer cell lines via regulating Bcl-2 family proteins, p53 signaling, or the Akt pathway [14, 15]. Likewise, the cytotoxic properties of bioactive compounds derived from

*Juniperus phoenicea* have been evaluated in several cancer cell lines [16]. However, unlike other *Juniperus* species, the biological activity of *Juniperus squamata (J. squamata)* against cancer has not yet been fully studied. Therefore, this study aimed to explore the potential effect of the ethanolic extract of *J. squamata* on mitotic catastrophe followed by apoptotic cell death in human oral cancer cell lines.

# 2. Materials and Methods

## 2.1 Preparation of plant extracts

The ethanolic extracts were provided by the International Biological Material Research Center at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea). All extracts were dissolved with dimethyl sulfoxide (DMSO), aliquoted, maintained at −20 °C, and brought to the final doses just before use. The final concentration of DMSO did not exceed 0.1%.

## 2.2 Cell cultures

Human oral cancer cell lines HSC-3 and HSC-4 were obtained from Hokkaido University (Hokkaido, Japan), and the HN22 cell line was provided by Dankook University (Cheonan, Republic of Korea). The SCC-9 cell line was supplied by the American Type Culture Collection (ATCC, Manassas, VA, USA), and the MC3 cell line was kindly provided by Fourth Military Medical University (Xi'an, China). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin as an antibiotic at 37 °C with 5% $CO_2$ in a humidified incubator. All experiments were held after the cells reached approximately 50% confluency.

## 2.3 Cytotoxicity measurements

### *A. Manual cell counting*

After being treated with EEJS for 24 h, the cells were washed

twice with ice-cold PBS and trypsinized. A hemocytometer was used to count the number of viable cells. Each experiment was performed thrice, and the results are expressed as the percentage of surviving cells compared with the DMSO-treated control group.

### *B. Cell Counting Kit-8 (CCK-8) assay*

The cytotoxic effect of EEJS on the human oral cancer cells was determined via a CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, the cells were seeded in 96-well plates and treated and incubated with various doses of EEJS for 24 h. 10 $\mu$L of CCK-8 solution was added to each well of the 96-well plates and incubated for 1~2 h at 37 °C with 5% $CO_2$ in a humidified incubator. The absorbance was measured at 450 nm using a Chameleon microplate reader (Hidex, Turku, Finland).

## 2.4 Transmission electron microscopy (TEM)

Following 24 h of treatment with either DMSO or EEJS, the cells were detached from the cell culture plate using 2X trypsin, resuspended in DMEM/F-12 media containing 10% FBS, and centrifuged at 800 rpm for 2 min. After discarding the media, the cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide, and embedded in Spurr low viscosity resin. Sections (1 μm thick) were prepared and stained with toluidine blue O. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate. TEM was performed by the

Electron Microscopy Core Facility in Seoul National University Hospital Biomedical Research Institute using JEOL JEM-1400 (Peabody, MA, USA).

## 2.5 Immunofluorescence staining

The cells were seeded on a 4-well chamber slide. After the cell confluency reached approximately 50%, the cells were treated with DMSO or EEJS. After 24 h, the cells were washed thrice with PBS and fixed in 4% paraformaldehyde for 20 min on ice and then washed three times with 0.1% BSA in PBS. The fixed cells were permeabilized in buffer containing 0.3% Triton X-100 and 1% BSA for 1 h at RT. The cells were incubated with α-tubulin (1:300, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4 °C in the dark. After being washed with 0.1% BSA in PBS three times, the cells were further incubated with Alexa Fluor® 488 anti-mouse antibodies (1:400, Jackson ImmunoResearch Inc., West Grove, PA, USA). The nuclei were further stained with DAPI (1:25, 50 μg/ml, Sigma-Aldrich, St. Louis, MO, USA), and the stained cells were examined by confocal microscopy (LSM700, Carl Zeiss, Germany).

## 2.6 Western blotting analysis

Protein was extracted from the EEJS-treated or untreated cells with RIPA lysis buffer (EMD Millipore, Billerica, CA, USA) along with phosphatase inhibitor tablets (Thermo Scientific Inc., Rockford, IL, USA) and protease inhibitor cocktails (Roche, Mannheim,

Germany). The protein concentration of each sample was quantified using a *DC* Protein Assay Kit (BIO-RAD Laboratories, Madison, WI, USA). After normalization, the protein lysates containing approximately 30 - 50 μg of protein were boiled with 5X protein sample buffer at 95 °C for 5 min and separated by SDS-PAGE, after which they were transferred to Immun-Blot PVDF membranes. The membranes were blocked with 5% skim milk in tris-buffered saline with Tween20 (TBST) at RT for 2 h and incubated with primary antibodies and corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. The primary antibodies that detect phospho-Histone H3 (Ser10), cleaved PARP, myeloid cell leukemia-1 (Mcl-1), and Bcl-xL were purchased from Cell Signaling Technology (Charlottesville, VA, USA). Bcl-2 and β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The immunoreactive bands were visualized with ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Piscataway, NJ, USA).

## 2.7 Analysis of sub-$G_1$ population

The trypsinized and floating cells were pooled, washed with PBS, and fixed in 70% ethanol at least overnight at −20 °C. The cells were incubated with propidium iodide solution (20 μg/ml) and RNase A (20 μg/ml) for 15 min at 37 °C after PBS washing. The cell cycle distribution was analyzed with a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA), and a minimum of 10 000 cells in each sample was analyzed with BD CellQuest™ Pro software. The

percentage of sub-$G_1$ fractions was quantified by FlowJo software version 9/10 (FlowJo LLC, Ashland, OR, USA).

## 2.8 Annexin V-FITC/PI double staining

Apoptosis induction was measured using an FITC-Annexin V Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's protocol. Briefly, floating and adherent cells were collected, washed twice with ice-cold PBS, and pelleted by centrifugation. Then, the cells were resuspended in annexin V binding buffer containing 3 μL of annexin V-FITC and 1 μL of PI and incubated for 15 min at RT in the dark. Subsequently, the cells were transferred to a FACS tube and analyzed by flow cytometry using a FACSCalibur flow cytometer. The cells showing Annexin V (+)/PI (−) staining indicate early-stage apoptosis whereas the cells showing Annexin V (+)/PI (+) staining indicate late-stage apoptosis. The percentage of Annexin V/PI stained cells was quantified from a minimum of 10 000 cells by BD CellQuest™ Pro software. The flow cytometry data were reanalyzed with FlowJo software version 9/10.

## 2.9 Construction of Mcl-1 overexpression vector and transient transfection

The Mcl-1 overexpression vector was constructed as described previously [17]. The HSC-3 cells were transfected with either empty pcDNA3.1 or pcDNA3.1-Mcl-1 using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the

manufacturer's instructions.

## 2.10 Statistical Analysis

Statistical significance was calculated using a two-tailed Student's t-test for comparisons between two groups. For multiple-group comparisons, one-way ANOVA analyses were applied to determine the significance of differences between the control and treatment groups; values of $p < 0.05$ were considered statistically significant (*).

# 3. Results

## 3.1 EEJS exhibits cytotoxic effect on human oral cancer cell lines

To discover a novel anti-cancer drug candidate based on natural products that have a potential chemotherapeutic effect on human oral cancer cell lines, we screened 49 plant extracts at doses of 20 μg/ml for 48 h to investigate their cytotoxicity on the MC3 cell line. The results indicated that the ethanolic extract of *J. squamata* (hereafter referred to EEJS) displayed a growth inhibitory effect of more than 80% in MC3 cells (Table 1 and Fig. 1). We further examined the cytotoxic effect of EEJS on other human oral cancer cell lines. HSC-3 and HSC-4 cells were treated with various concentrations of EEJS for 24 h. As shown in Fig. 2A, EEJS remarkably decreased the viability of human oral cancer cells in a dose-dependent manner. Consistently, EEJS significantly suppressed cell proliferation in HSC-3 and HSC-4 cells according to the CCK-8 assay (Fig. 2B). Similar results were observed in HN22 and SCC-9 cells, as evidenced by the reduced cell survival (Fig. 3A). These results indicate that EEJS manifests a cytotoxic effect in human oral cancer cell lines.

Table 1. *In vitro* screening for cytotoxicity of plant extracts in MC3 cell line

| No. | Name | Family | Viability (%) |
|---|---|---|---|
| 1 | *Ficus oligodon Miq.* | Moraceae | 94.93 |
| 2 | *Malpighia glabra L.* | Malpighiaceae | 94.08 |
| 3 | *Schefflera elegantissima (Veitch ex Mast.) Lowry & Frodin* | Araliaceae | 77.59 |
| 4 | *Syzygium malaccense (L.) Merr. & L.M. Perry* | Rosaceae | 79.28 |
| 5 | *Acer prolificum W.P. Fang & M.Y. Fang* | Aceraceae | 100.74 |
| 6 | *Dipterocarpus tuberculatus Roxb.* | Dipterocarpaceae | 99.26 |
| 7 | *Terminalia argyrophylla King & Prain* | Combretaceae | 98.77 |
| 8 | *Sida cordifolia L.* | Malvaceae | 107.37 |
| 9 | *Glochidion sphaerogynum (Mull. Arg.) Kurz* | Phyllanthaceae | 98.03 |
| 10 | *Glochidion sphaerogynum (Mull. Arg.) Kurz* | Myrtaceae | 89.68 |
| 11 | *Erythroxylum coca Lam.* | Erythroxylaceae | 98.28 |
| 12 | *Citrus medica var. sarcodactylis (Hoola van Nooten) Swingle* | Rutaceae | 96.81 |
| 13 | *Syzygium odoratum (Lour.) DC.* | Rutaceae | 98.77 |
| 14 | *Smilacina henryi (Baker) H.Hara* | Asparagaceae | 98.28 |
| 15 | *Callistemon citrinus (Curtis) Skeels* | Myrtaceae | 89.19 |
| 16 | *Phaeanthus saccopetaloides W. T. Wang* | Annonaceae | 96.56 |
| 17 | *Rubus pirifolius var. cordatus T.T. Yu & L.T. Lu* | Rosaceae | 99.75 |
| 18 | *Rhodiola fastigiata (Hook. f. & Thomson) S.H. Fu* | Crassulaceae | 100.25 |
| 19 | *Pueraria edulis Pamp.* | Fabaceae | 99.75 |
| 20 | *Rhododendron telmateium Balf. F. & W.W. Sm.* | Ericaceae | 97.54 |
| 21 | *Dysoxylum mollissimum Blume* | Meliaceae | 98.03 |
| 22 | *Acacia farnesiana (L.)Willd.* | Fabaceae | 97.00 |
| 23 | *Olea europaea L.* | Oleaceae | 109.81 |
| 24 | *Orophea hainanensis Merr.* | Annonaceae | 98.64 |
| 25 | *Sida alnifolia var. obovata (Wall. Ex Mast.) S.Y. Hu* | Malvaceae | 100.54 |
| 26 | *Piptanthus nepalensis (Hook.) Sweet* | Fabaceae | 96.73 |
| 27 | *Claoxylon subsessiliflorum Croizat* | Euphorbiaceae | 100.54 |
| 28 | *Ilex polyneura (Hand.-Mazz.) S.Y. Hu* | Aquifoliaceae | 97.28 |
| 29 | *Turnera ulmifolia L.* | Turneraceae | 102.45 |
| 30 | *Livistona chinensis (Jacq.) R.Br. ex Mart.* | Arecaceae | 99.46 |
| 31 | *Vitex glabrata R. Br.* | Verbenaceae | 92.92 |
| 32 | *Duranta repens L.* | Verbenaceae | 98.64 |
| 33 | *Trichosanthes cucumerina L.* | Cucurbitaceae | 100.00 |
| 34 | *Lindernia montana (Blume) Koord.* | Scrophulariaceae | 96.46 |
| 35 | *Piper mullesua Buch.-Ham. ex D. Don* | Piperaceae | 95.91 |
| 36 | *Sabina squamata cv. Meyeri* | Cupressaceae | 13.08 |
| 37 | *Callicarpa bodinieri var. iteophylla C.Y. Wu* | Lamiaceae | 102.45 |
| 38 | *Polygonum barbatum L.* | Polygonaceae | 95.10 |
| 39 | *Ligustrum delavayanum Har.* | Oleaceae | 91.87 |
| 40 | *Pleurospermum astrantioideum (H. Boissieu) K.T. Fu &* | Apiaceae | 88.92 |

| | | | |
|---|---|---|---|
| | *Y.C. Ho* | | |
| **41** | *Swertia yunnanensis Burkill* | Gentianaceae | 96.31 |
| **42** | *Lonicera hispida Pall. Ex Roem. & Schult.* | Caprifoliaceae | 90.64 |
| **43** | *Rhododendron rupicola var. chryseum (Balf. F. & Kingdon-Ward) Philipson & M.N. Philipson* | Ericaceae | 91.63 |
| **44** | *Caltha palustris L.* | Ranunculaceae | 101.23 |
| **45** | *Primula secundiflora Franch.* | Primulaceae | 100.00 |
| **46** | *Amomum villosum var. xanthioides (Wall. ex Baker) T.L. Wu & S.J. Chen* | Zingiberaceae | 94.58 |
| **47** | *Picris divaricata Vaniot* | Asteraceae | 97.29 |
| **48** | *Cardamine tangutorum O.E. Schulz* | Brassicaceae | 93.10 |
| **49** | *Rhodomyrtus tomentosa (Aiton) Hassk.Rhodomyrtus tomentosa (Aiton) Hassk.* | Myrtaceae | 94.83 |

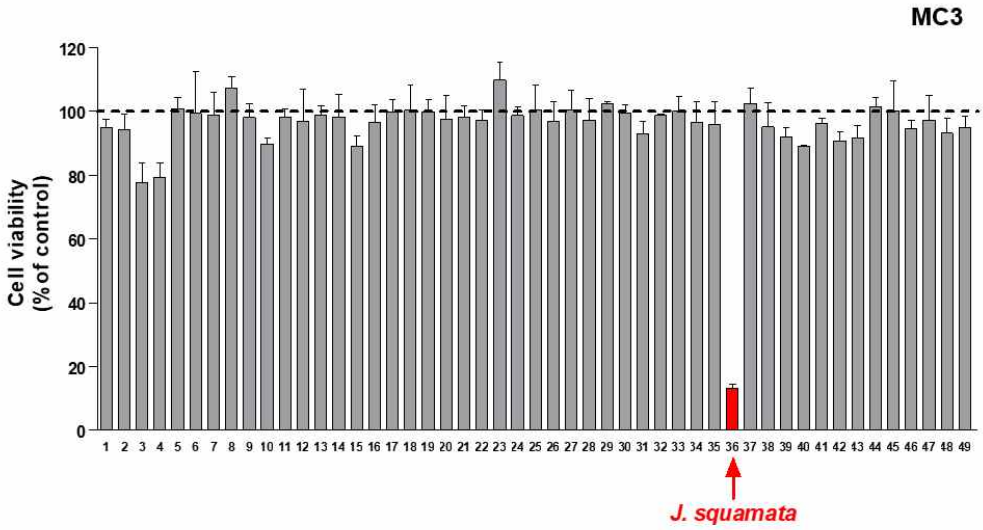


**Fig. 1.** Cytotoxic effects of plant extracts in MC3 cells. The cells were treated with 49 plant extracts at 20 $\mu$g/ml for 48 h to examine the cytotoxicity on MC3 cells. Bar graphs represent the mean ± SD of triplicate experiments. $*p < 0.05$.

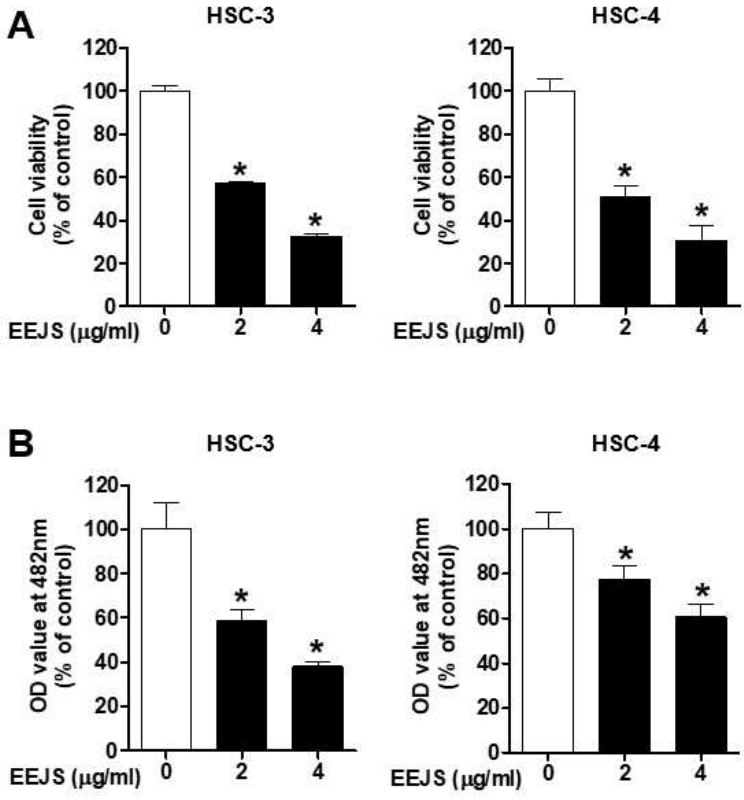


**Fig. 2.** EEJS induces a cytotoxic effect on human oral cancer cells. HSC-3 and HSC-4 cells were treated with DMSO or EEJS for 24 h. (A) Cell viability was manually examined at different EEJS doses in the indicated cell lines. (B) The cytotoxicity of EEJS on both cell lines was measured using a CCK-8 assay. All bar graphs represent the mean ± SD of three independent experiments. $*p < 0.05$.

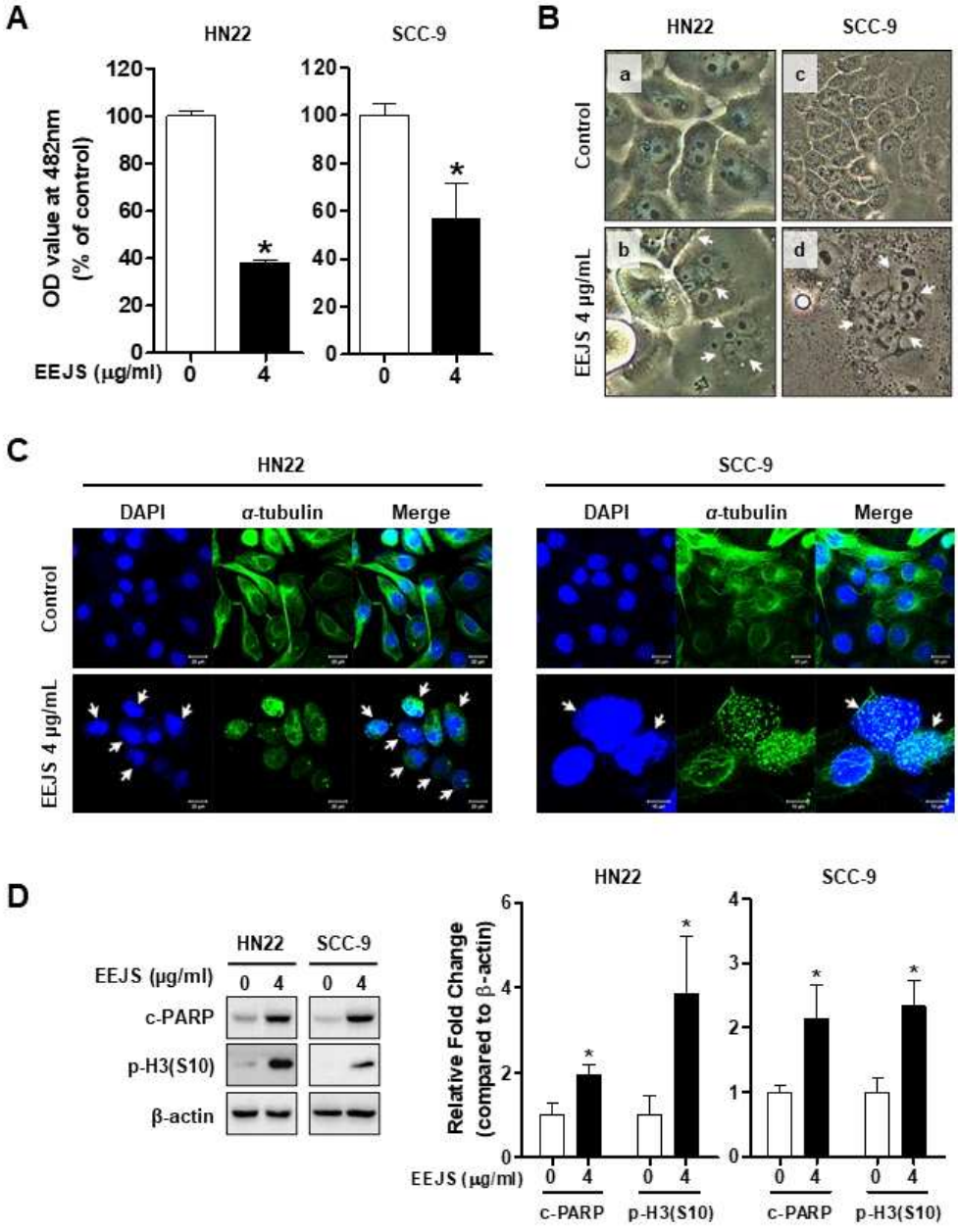
A
HN22
SCC-9
OD value at 482nm (% of control)
120
100
80
60
40
20
0
*
EEJS (μg/ml)
0
4
B
HN22
SCC-9
Control
EEJS 4 μg/mL
a
b
c
d
C
HN22
SCC-9
DAPI
α-tubulin
Merge
Control
EEJS 4 μg/mL
D
HN22
SCC-9
EEJS (μg/ml)
0 4 0 4
c-PARP
p-H3(S10)
β-actin
Relative Fold Change (compared to β-actin)
6
4
2
0
3
1
EEJS (μg/ml)
c-PARP
p-H3(S10)

**Fig. 3.** EEJS evokes mitotic catastrophe, leading to apoptosis induction in other human oral cancer cell lines, HN22 and SCC-9. (A) HN22 and SCC-9 cells were treated with DMSO or 4 μg/ml EEJS for 24 h, after which cell viability was measured using a CCK-8 assay. (B) Representative images of PCM in the absence or presence of EEJS. The white arrows indicate multinucleated cells. (C) Immunofluorescence staining of α-tubulin (magnification, 400X). The white arrows indicate multinucleated cells. *Scale bar*, 20 μm. (D) Western blot analysis showing the expression levels of the indicated proteins. β-actin was used as a loading control. The results are shown as the mean ± SD of three independent experiments. $*p < 0.05$.

## 3.2 EEJS provokes mitotic catastrophe in human oral cancer cell lines

Morphologic changes were observed under a phase-contrast microscope (PCM) and a transmission electron microscope (TEM) to examine the effect of EEJS on the occurrence of mitotic catastrophe. Following treatment with 4 μg/ml EEJS for 24 h, enlarged multinucleated cells with multiple micronuclei were observed in human oral cancer cells compared with the control group (Fig. 3B and Fig. 4). Immunofluorescence staining was performed to confirm whether the enrichment of enlarged multinucleated cells upon EEJS treatment was accompanied by disturbances in microtubule formation. As shown in Fig. 3C and Fig. 5A, EEJS disrupted the microtubule fibers and triggered abnormal chromosome segregation. To clarify the biological function of EEJS, we analyzed the phosphorylation of histone H3 ($Ser^{10}$), a specific marker of mitosis. Western blotting analysis revealed that the phosphorylation of histone H3 at the $Ser^{10}$ residue was notably increased upon EEJS treatment in a dose-dependent manner (Fig. 3D and Fig. 5B). These data indicate that EEJS facilitates mitotic catastrophe by disturbing microtubule formation and chromosome segregation.

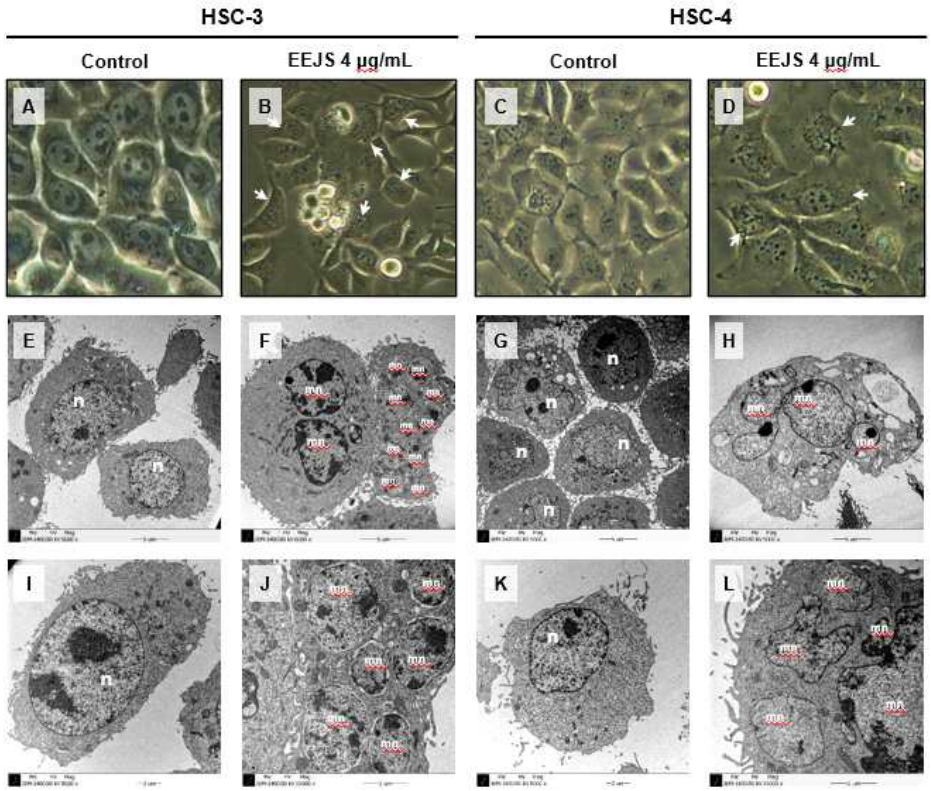


**Fig. 4.** EEJS promotes multinucleation of human oral cancer cells. HSC-3 and HSC-4 cells were treated with DMSO or 4 μg/ml EEJS for 24 h. (A-D) Representative images of PCM in the absence or presence of EEJS. The white arrows indicate multinucleated cells. (E-H) Representative images of TEM. *Scale bar*, 5 μm. (I-L) The images are magnified inserts of mononucleated cells (n) or multinucleated cells (mn). *Scale bar*, 2 μm.

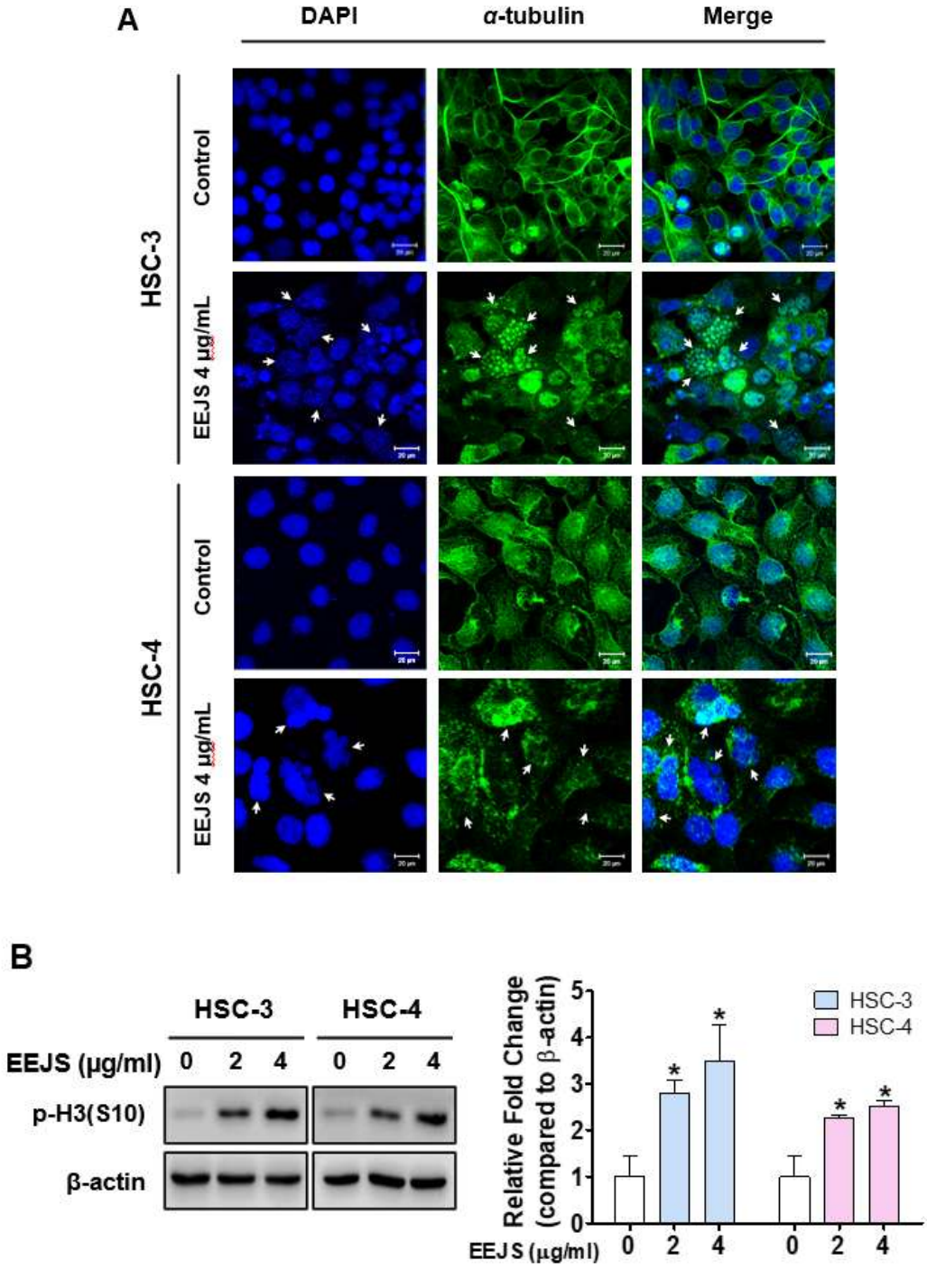
A
DAPI
α-tubulin
Merge
HSC-3
Control
EEJS 4 μg/mL
HSC-4
Control
EEJS 4 μg/mL
B
HSC-3
HSC-4
EEJS (μg/ml)
0
2
4
0
2
4
p-H3(S10)
β-actin
Relative Fold Change (compared to β-actin)
HSC-3
HSC-4
EEJS (μg/ml)
0
2
4
0
2
4

**Fig. 5.** EEJS triggers mitotic catastrophe by accumulating phosphorylated histone H3 ($Ser^{10}$) in human oral cancer cells. (A) Immunofluorescence staining of α-tubulin in the absence or presence of EEJS. Representative images for the staining of α-tubulin (green) and nuclei counterstained with DAPI (blue). The merged panels combine the two images (magnification, 400X). The white arrows indicate multinucleated cells. *Scale bar*, 20 μm. (B) Representative western blot images in both cell lines treated with DMSO or EEJS. β-actin was used as the loading control. All bar graphs represent the mean ± SD of three independent experiments. $*p < 0.05$.

## 3.3 Mitotic catastrophe induced by EEJS treatment results in apoptotic cell death in human oral cancer cell lines

To determine whether the mitotic catastrophe induced by EEJS treatment was accompanied by apoptotic cell death in human oral cancer cells, we examined the expression of cleaved PARP, a hallmark of apoptosis. As shown in Fig. 3D and Fig. 6A, EEJS clearly increased the expression of cleaved PARP in three human oral cancer cell lines; conversely, no sign of an apoptotic effect from EEJS was observed in the HSC-4 cells. The flow cytometry analysis results revealed that the percentage of HSC-3 cells in the sub-$G_1$ phase following EEJS treatment increased significantly up to 6-fold compared with the vehicle control group (Fig. 6B). Consistently, the

rate of annexin V-positive HSC-3 cells was increased from 5.77% in the vehicle control group to 7.24% or 10.35% in the EEJS treatment group (Fig. 6C). These data indicate that mitotic catastrophe induced by EEJS treatment leads to the induction of apoptotic cell death in human oral cancer cells in a cell context-dependent manner.

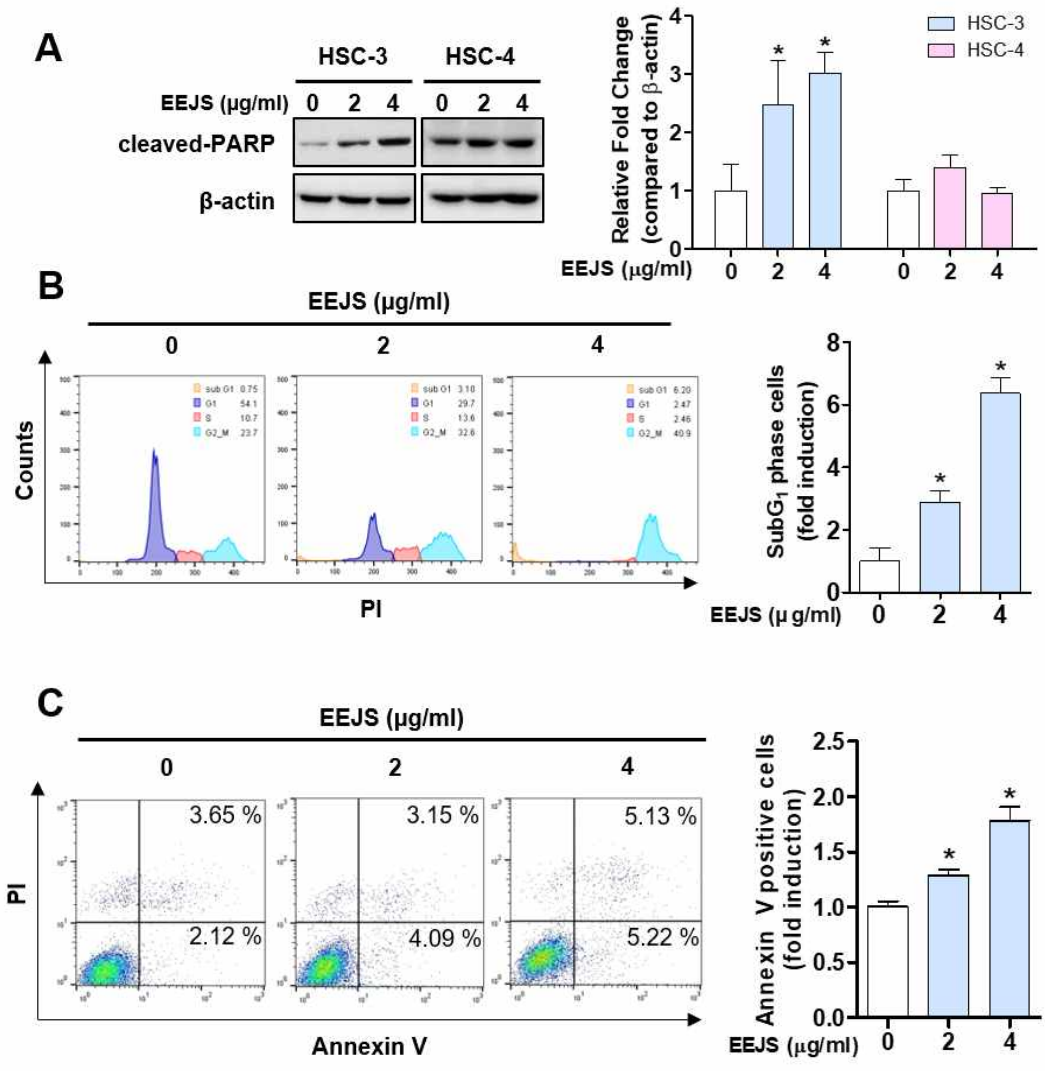


**Fig. 6.** EEJS enhances apoptotic cell death in human oral cancer cells in a cell context-dependent manner. (A) Western blot analysis showing the expression levels of cleaved PARP. β-actin was used as a loading control. Data are shown as the mean ± SD of three independent experiments. $*p < 0.05$. (B) The sub-$G_1$ population in HSC-3 cells treated with EEJS was measured by a FACS analysis as described in the materials and methods section. All bar graphs

represent the mean ± SD of three independent experiments. $*p < 0.05$. (C) Annexin V/PI double staining of HSC-3 cells treated with EEJS. All bar graphs represent the mean ± SD of three independent experiments. $*p < 0.05$.

## 3.4 Suppression of Mcl-1 following EEJS treatment determines the susceptibility to apoptotic cell death in human oral cancer cell lines

To determine whether EEJS-induced apoptotic cell death is a consequence of regulating anti-apoptotic Bcl-2 family proteins, we analyzed Mcl-1, Bcl-xL, and Bcl-2 protein expressions. As shown in Fig. 7A, EEJS caused a pronounced reduction of Mcl-1 protein expression in a dose-dependent manner in HSC-3 cells only; no significant differences in Bcl-xL or Bcl-2 protein levels were observed in either cell line. To clarify the biological role of Mcl-1 in EEJS-induced apoptotic cell death, we overexpressed the Mcl-1 protein in HSC-3 cells. Compared with the control vector (pcDNA3.1), Mcl-1 overexpression (pcDNA3.1-Mcl-1) partly abolished the expression of cleaved PARP after EEJS treatment (Fig. 7B). These data indicate that Mcl-1 may act as a determinant for EEJS-induced apoptotic cell death in human oral cancer cells.

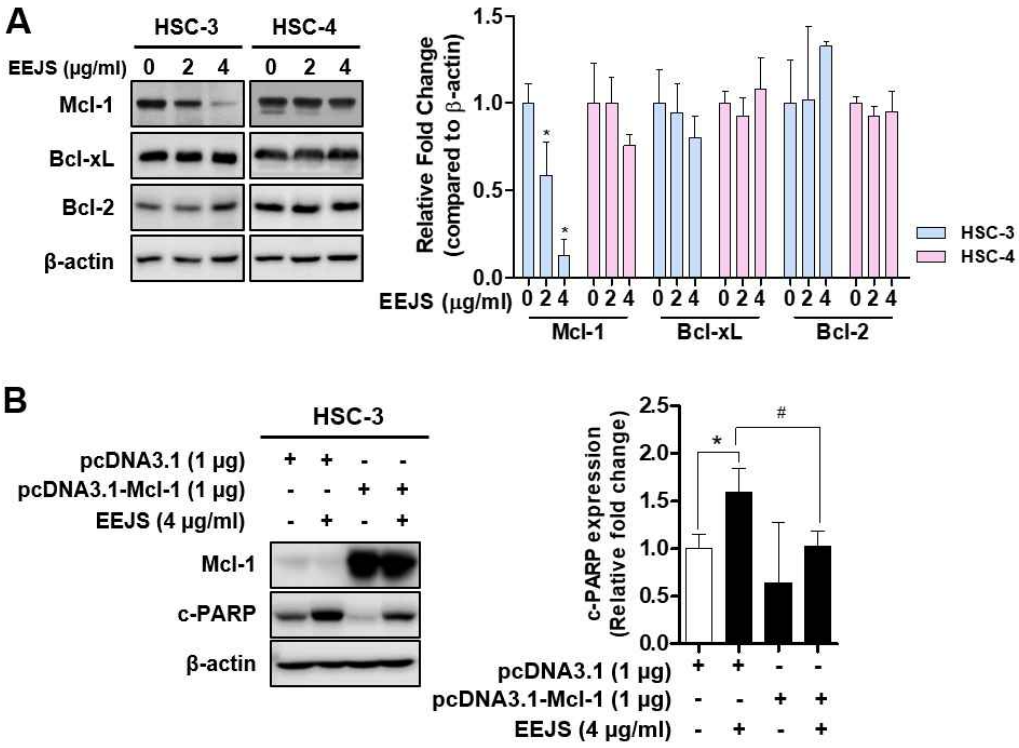


**Fig. 7.** EEJS reduces Mcl-1 expression in HSC-3 cells but not in HSC-4 cells. (A) Western blot analysis showing the expression levels of anti-apoptotic Bcl-2 family proteins. β-actin was used as a loading control. The results are shown as the mean ± SD of three independent experiments. $*p < 0.05$. (B) HSC-3 cells were transiently transfected with the empty vector or Mcl-1 overexpressing vector for 6 h and then treated with DMSO or 4 μg/ml EEJS for 24 h. All bar graphs represent the mean ± SD of three independent experiments. $*p < 0.05$; $^{\#}p < 0.05$.

# 4. Discussion

Cancer in the oral cavity and pharynx is the eighth most prevalent type of neoplasm and was responsible for approximately 4% of all cancer cases in males in the United States in 2020 [18]. The five-year survival rate of patients with oral cavity cancer (hereafter referred to as oral cancer) is 41.7%, and survival seems to be significantly correlated with local invasion and distant metastasis [19]. Although surgical management has been considered the standard treatment strategy for oral cancer patients, adjuvant therapy remains necessary for treating advanced oral cancer before or after surgery [20]. However, the anti-cancer efficacy of conventional chemotherapy using platinum-based agents is not sufficient to improve survival in oral cancer patients, which leads to several therapeutic attempts including targeted therapy and immunotherapy [21]. Natural products have been proposed as promising anti-cancer drug candidates given their remarkable ability to prevent oral cancer belonging to head and neck cancer [22]. Studies on oral cancer therapy in our laboratory have demonstrated that natural products function as chemotherapeutic agents by promoting pro-apoptotic Bcl-2 family proteins and DNA damage responses [23, 24]. Thus, a chemotherapeutic approach using natural products could be a promising therapeutic option for favorable prognosis in oral cancer patients. In this study, our data revealed the chemotherapeutic effect of *J. squamata* on human oral cancer cells.

Mitotic catastrophe is defined as an oncosuppressive cell death mechanism for the evasion of genomic instability, which can precede

senescence, apoptotic cell death, or necrotic cell death [4]. It is characterized by morphological features such as enlarged multinucleated cells that arise from missegregated chromosomes and microtubule formation disruption [3]. Hence, microtubule targeting agents have been proposed as effective anti-cancer drugs in various types of cancer given their obstruction of mitotic progression. For example, docetaxel treatment of breast cancer cells primarily leads to mitotic catastrophe, which is considered the cell death mode [25]. The use of nanocarriers as a drug delivery system for paclitaxel and cetuximab has resulted in anti-tumor activity in colorectal cancer *in vitro* and *in vivo* by enhancing mitotic catastrophe followed by apoptotic cell death [26]. Notably, several natural products seem to cause mitotic catastrophe. Bioactive compounds such as gallic acid, pseudolaric acid B, or thalicthuberine result in arrest at the $G_2$/M phase of cancer cells by disturbing centrosomal clustering, microtubule formation, or chromosome segregation, which contributes to undergo mitotic catastrophe [9, 27, 28]. Chelidonine and glyfoline can effectively cause the phosphorylation of histone H3 ($Ser^{10}$), which is an indicator of mitotic catastrophe, and thereby induce apoptotic cell death [10, 29]. Similar to previous studies, we noted that EEJS treatment clearly led to the enrichment of enlarged multinucleated cells, disturbances of the mitotic spindle, and increased phosphorylation of histone H3 ($Ser^{10}$) in human oral cancer cells. These observations suggest that EEJS could be potentially effective against human oral cancer by facilitating the occurrence of mitotic

catastrophe. Encouraging evidence has shown that mitotic catastrophe can determine the cell death mode [6]. Furthermore, the occurrence of mitotic catastrophe induced by viriditoxin leads to both apoptotic cell death and autophagic cell death [30]. The current study sought to investigate which type of cell death is the endpoint of EEJS-induced mitotic catastrophe in human oral cancer cells, and our findings indicated that EEJS treatment generally promoted apoptotic cell death in three human oral cancer cell lines (HSC-3, HN22, and SCC-9). Conversely, there was no significant change in the HSC-4 cells, which suggests that apoptotic cell death from EEJS treatment is cell context-dependent. Thus, our findings provide a possibility that the mitotic catastrophe induced by EEJS treatment predominantly causes apoptotic cell death in human oral cancer cells, even if further studies are necessary to clarify the causality between mitotic catastrophe and apoptotic cell death.

The Bcl-2 family proteins, which can be divided into three groups based by their function (*e.g.*, anti-apoptotic, pro-apoptotic, and BH3-only proteins), orchestrate the balance of survival and death in cells. Mcl-1 proteins, which are anti-apoptotic Bcl-2 family proteins, bind and sequester the pro-apoptotic Bcl-2 family proteins Bax/Bak, resulting in the disturbance of mitochondria-dependent apoptotic cell death [31]. Mcl-1 is highly expressed in various types of cancers and is associated with poor prognosis in cancer patients [31-33]. Hence, several Mcl-1 inhibitors have entered preclinical or clinical trials [32, 34]. Natural products have been regarded as one of the most

promising therapeutic strategies given their abilities to regulate Mcl-1 proteins at the transcriptional, post-transcriptional, and post-translational levels [35]. Our previous findings have demonstrated that bioactive compounds derived from natural products serve as apoptosis-inducing agents via modulating the protein stability of Mcl-1 [17, 36]. Based on these observations, Mcl-1 is considered a crucial molecular target for cancer treatment. In this study, we investigated whether EEJS-induced apoptotic cell death is accompanied by anti-apoptotic Bcl-2 family proteins and noted that EEJS treatment suppressed Mcl-1 expression in HSC-3 cells; however, there was no significant change in Bcl-xL and Bcl-2 expressions. These results caused us to further investigate the biological role of Mcl-1 in EEJS-induced apoptotic cell death. As we expected, the ectopic expression of Mcl-1 almost abrogated the expression of cleaved PARP in HSC-3 cells following EEJS treatment. This highlights the possibility that the suppression of Mcl-1 upon EEJS treatment plays a decisive role in the high susceptibility of HSC-3 cells to apoptotic cell death. Conversely, Mcl-1, as well as Bcl-2 and Bcl-xL, are likely key regulators during mitosis. Mitotic arrest always precedes mitotic catastrophe in cells exhibiting defective or failed mitosis [4], and the sustained CDK1-mediated phosphorylation of Mcl-1 during mitotic arrest results in cell death by suppressing Mcl-1 levels [3]. However, the mitotic catastrophe induced by EEJS treatment is not likely a consequence of reduced Mcl-1 expression levels because there was significant

enrichment of mitotic catastrophe in all human oral cancer cell lines regardless of Mcl-1 expression levels. Therefore, reduced Mcl-1 expression levels upon EEJS treatment may be a determinant for apoptotic cell death after mitotic catastrophe.

Based on our observations, the results revealed an unexpected phenomenon in which EEJS-induced mitotic catastrophe was insufficient to induce apoptotic cell death in HSC-4 cells only. Based on this result, we speculate that EEJS may enable HSC-4 cells to evade apoptotic cell death due to the maintenance of Mcl-1 expression. Although we must continue to elucidate the reasons why EEJS-induced mitotic catastrophe resulted in the induction of apoptotic cell death in a cell context-dependent manner, we cannot exclude the possibility that there are different cell death mechanisms following EEJS treatment. A previous study showed that the final endpoints of mitotic catastrophe could be accompanied by apoptotic cell death or necrotic cell death, depending on the genetic background of the cells [6]. Thus, we cautiously speculate that EEJS-induced mitotic catastrophe in HSC-3 and HSC-4 cells is likely to decide their endpoints based on their different genetic backgrounds.

In conclusion, the results of the present study provide evidence that EEJS may function as a promising therapeutic agent that can induce mitotic catastrophe in human oral cancer *in vitro*.

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# 국문초록

## *Juniperus squamata*를 활용한 구강암 치료로서의 mitotic catastrophe 유도에 관한 연구

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**1. 목적:** 비정상적인 유사 분열을 특징으로하는 세포 사멸의 기전인 mitotic catstrophe는 항암제 후보 개발을 위한 치료적 접근법으로 여겨져왔다. 이 연구에서 우리는 인간 구강암 세포주에서 mitotic catastrophe 발생에 대한 *Juniperus squamata* 에탄올 추출물 (EEJS)의 자재적 효과를 조사했다.

**2. 재료 및 방법:** Mitotic catastrophe 발생에 대한 EEJS의 영향은 세포 독성 측정, 위상차 또는 투과 전자 현미경 관찰, 미세소관 또는 염색체의 기형, histone H3 ($Ser^{10}$)의 인산화 검출을 통해 평가되었다. 또한, EEJS의 세포 사멸 효과는 cleaved PARP의 검출, sub-G1 분석, Annexin V-FITC/PI 이중 염색, 웨스턴 블롯팅 및 Mcl-1 과발현 벡터의 transient transfection을 수행하여 평가되었다.

**3. 결과:** EEJS는 인간 구강암 세포주에서 세포 증식을 억제하는 효과를 나타냈다. EEJS는 세포의 다핵화, 미세소관 형성 장애 및 histone H3

(Ser$^{10}$)의 인산화 증가를 가져와 mitotic catastrophe의 발생을 야기했다. 또한, 다핵 세포는 Mcl-1 단백질의 감소에 따라 세포사멸 활성이 나타났다.

**4. 고찰:** 이 연구를 통해 EEJS가 세포 사멸과 관련된 mitotic catastrophe를 촉진함으로써 인간 구강암 치료에 대한 효과적인 후보물질이 될 수 있다는 것을 제시한다.

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**주요어:** Mitotic catastrophe, Apoptotis, Phospho-Histone H3, *Juniperus squamata*, Mcl-1, Oral cancer
**학번:** 2018-27675