Dental Journal
Majalah Kedokteran Gigi

Dental Journal
(Majalah Kedokteran Gigi)
2025 September; 58(3): 273–279

Original article

Ethanol extracts of *Rhynchosia nulubilis* induce G2/M phase arrest by inducing deoxyribonucleic acid damage in human oral squamous cell carcinoma cells

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ABSTRACT

Background: *Early cancer diagnosis is very important for cancer treatment and improvement of patient prognosis, but the development of effective cancer treatment agents is also necessary to overcome cancer. Accordingly, research is being actively conducted to derive ingredients that control cancer progression.* ***Purpose:*** *The antioxidant and anti-inflammatory effects of Rhynchosia nulubilis have been reported in various human cells. We attempted to ascertain the underlying mechanism by which the ethanol extracts of Rhynchosia nulubilis (EERN) induced cytotoxicity and cell cycle arrest in human oral squamous cell carcinoma (OSCC) cells.* ***Methods:*** *The EERN was prepared from the whole Rhynchosia nulubilis. A 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed for cytotoxicity of EERN in YD38 OSCC cells. A Matrigel-coated Transwell was used for invasion assay. Changes in the cell cycle distribution were monitored using fluorescence-activated cell sorting analysis. A phosphorylated form of H2AX (γH2AX) foci formation was observed using a fluorescence microscope.* ***Results:*** *Invasion activity of YD38 cells in a Matrigel-coated Transwell was significantly decreased by EERN in a dose-dependent manner. Cytotoxicity was observed at a treatment concentration of 8–10 mg/ml EERN, which induced Transwell invasion inhibition. The YD38 cells were more sensitive to EERN cytotoxicity than immortalized gingival fibroblasts. The EERN treatment arrested the YD38 cell cycle in the G2/M phase, and DNA damage marker γH2AX formation was increased by the EERN treatment. The phosphorylation of ataxia telangiectasia mutated and Chk2 was also increased by EERN treatment.* ***Conclusion:*** *These results indicate that EERN inhibits YD38 cancer cell growth and invasion activity through DNA damage and cell cycle arrest.*

Keywords: *Cell cycle; deoxyribonucleic acid damage; ethanol extracts of Rhynchosia nulubilis*
Article history: *Received 28 April 2024; Revised 12 July 2024; Accepted 15 July 2024; Online 27 May 2025*

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INTRODUCTION

Cancer is the most common cause of death worldwide, and the number of cancer patients is expected to increase due to longevity, environmental pollution, and dietary changes.[1] Normal cells maintain cell number homeostasis through proper cell division and death; however, cancer cells become immortalized and proliferate due to cell cycle regulation disturbance and damage organ functions through invasion and metastasis, ultimately leading to death.[2] Cancer can be treated by surgical operations, radiation therapy, and immunotherapy, but problems due to side effects, recurrence, and complications after treatment have not been overcome. Oral cancer, for example, causes eating disorders, pronunciation disorders, and aesthetic disorders after treatment, and the incidence of patients is increasing despite the still-poor cancer prognosis.[3] Therefore, research for natural materials that have cancer-specific anti-cancer effects but with few side effects is steadily progressing.

Oral cancer is a disease that occurs anywhere in the oral cavity, such as the tongue, gums, and jawbone; it causes dysfunction after treatment and still has a poor treatment prognosis.[4–6] Oral cancer accounts for 0.2% of all cancers occurring in Korea, and pathologically, squamous cell carcinoma is the most common at 87%. Smoking and drinking are the main causes of severe carcinogenesis,

DOI: 10.20473/j.djmkg.v58.i3.p273–279

although poor oral hygiene, mechanical irritation caused by dentures or teeth, syphilis, and submucosal fibrosis of the oral cavity are also causes. In addition, an association with infection of human immunodeficiency virus and human papillomavirus has also been reported. According to data from the Surveillance, Epidemiology, and End Results Program of the National Cancer Institute, tongue cancer, which has the highest incidence among oral squamous cell carcinoma (OSCC), accounts for 1% of all cancers occurring in the past year and 0.5% of cancer-related deaths. The 5-year survival rate between 2014 and 2020 was 70.4%, and the incidence is 2–3 times higher in men than in women (https://seer.cancer.gov/statfacts/html/tongue.html). Therefore, early diagnosis is very important in preventing oral cancer and improving prognosis.

Several researchers have suggested the prevention effects of chronic degenerative diseases and cancer by isoflavones, which are physiologically active substances in soybeans.[7–9] Isoflavones are contained in 0.2%–0.4% of soybeans, three non-glycosides (aglycone; daizein, genistein, glycitin), and nine glycosides (β-glycosides [daidzein, genistine, glycitin], 6''-O-acetyl-glucosides [daidzine, genistein, glycitin], 6''-O-malonyl-glucosides [daidzine, genistein, glycitin]). Recent studies have highlighted the antioxidant, anti-inflammatory, and anti-obesity properties of *Rhynchosia nulubilis* extracts, along with their ability to inhibit the proliferation of certain cancer cells.[8–12] The potent antioxidant properties of *Rhynchosia nulubilis* help neutralize free radicals, leading to reduced oxidative stress, suppressed fat accumulation resulting in weight loss, and alleviated pain by reducing inflammation. These findings suggest the potential of *Rhynchosia nulubilis* as a health supplement or pharmaceutical ingredient, underscoring the need for further clinical research to ensure its safety. However, despite its multifunctional benefits, most research has primarily focused on its antioxidant properties, with limited exploration of the intracellular mechanisms underlying its anticancer effects. This study investigates the effects of ethanol extracts of *Rhynchosia nulubilis* (EERN) on inducing G2/M phase arrest and DNA damage in human OSCC cells, aiming to elucidate the molecular mechanisms underlying its anti-cancer properties.

MATERIALS AND METHODS

The reagents and antibodies used for cell culture and analysis were as follows: Dulbecco's modified Eagle's medium (DMEM), Ham's F12 nutrient mixture, fetal bovine serum, antibiotic (10,000 units/ml penicillin G, 10,000 μg/ml and streptomycin), phosphate-buffered saline (PBS), and 0.25% trypsin–EDTA were purchased from Gibco BRL (Rockville, MD, USA); 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, and propidium iodide (PI), 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA); phosphorylated histone H2AX Ser139 (γH2AX) was purchased from Upstate Biotechnology (Charlottesville, CA, USA); histone H2AX (D17A3), phosphorylated Chk2 (Thr68), Chk2, phosphorylated ataxia telangiectasia mutated (ATM) (Ser1981), ATM, phosphorylated p53, and p53 were purchased from Cell Signaling Technology (Denver, MA); β-actin was purchased from Sigma Chemical (St. Louis, MO, USA); FITC-goat anti-rabbit IgG (H+L) was purchased from Zymed (San Francisco, CA, USA); horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Amersham Life Science (Little Chalfont, UK).

The YD38 OSCC and immortalized gingival fibroblasts (IGF) were provided by the Oral Cancer Research Institute, Yonsei University College of Dentistry (Seoul, Korea), and were cultured in DMEM/F12 (3:1 ratio) medium supplemented with 10% fetal bovine serum, 1 x 10^{-10} M cholera toxin, 0.4 mg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2 x 10^{-11} M triiodothronine at 37°C in a 5% CO_2 incubator. The EERN was provided by COSMAX R&I Center (COSMAX Inc., Seong-Nam, Korea). The *Rhynchosia nulubilis* was roasted at 100°C–110°C for 20 min using a roaster (Proaster THCR-01, Taewhan Automation Industry Co., Gyeonggi, Korea). The roasted *Rhynchosia nulubilis* was ground with a grinder (FM-860T, Hanil Electric, Seoul, Korea) and extracted by stirring for 72 h in 70% ethanol. The extracts were then concentrated with a rotary evaporator under reduced pressure and stored in the refrigerator until used. All plant extraction processes were performed by a herbalist in COSMAX.

For the invasion assay, a Transwell inner chamber (pore size 8 mm) was coated with 30 mg/well Matrigel (Becton Dickinson, Lincoln Park, NJ, USA), and cells (5 x 10^3 cells/well) were loaded into the upper part of the Matrigel. Invasion was performed in the complete medium with or without EERN for 48 h. Invaded cells on the bottom of the chamber were fixed with ethanol, stained with hematoxylin, and counted under a microscope.

For the cell viability assay, cells were cultured with 10% medium and treated with various concentrations of EERN for 24 h and 48 h, respectively. A 5 mg/ml MTT solution was then treated for an additional 1 h at 37 °C, and 100 μl dimethyl sulfoxide was added to dissolve the formazan product. Absorbance was measured at 570 nm using a microplate reader (Synergy™ HTX Multi-Mode Microplate Reader; BioTek Instruments Inc., Winooski, VT, USA).

For cell cycle analysis, cells were treated in complete medium with or without EERN for 24 h. The cells were fixed in 70% ethanol at 4 °C for 2 h and stained with 50 μg/ml PI after treatment with 0.25 mg/ml RNase-A for 30 min. The DNA content was analyzed by a FACSCalibur using WinMDI 2.8 software (BD, Franklin Lakes, NJ, USA).

For immunofluorescence analysis, cells were cultured in complete medium with or without EERN for 8 h on a glass chamber slip, fixed with 4% paraformaldehyde, and

DOI: 10.20473/j.djmkg.v58.i3.p273–279

permeabilized with 0.5% Triton X-100. Fixed cells were then blocked with 5% bovine serum albumin for 2 h and incubated with monoclonal antibodies against γH2AX. The cells were washed with PBS containing 0.1% Triton X-100 and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibodies; DAPI was used for DNA counterstaining, and images were captured and analyzed using an LSM 510 laser scanning confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA).

For western blot analysis, whole cell lysates were prepared with lysis buffer containing protease inhibitor cocktail (Roche, Switzerland). Whole lysates (50 mg) were separated on SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk/PBS. The membranes were reacted with primary antibody (1:1,000) overnight and then incubated with a 1:3,000 dilution of HRP-conjugated anti-mouse or anti-rabbit secondary antibody for 2 h at room temperature. The targeted proteins were visualized using enhanced chemiluminescence reagents (Amersham Life Science) and exposure to Kodak BioMax XAR Film.

Statistical analyses were performed with InStat GraphPad Prism ver. 5.01 statistical software (GraphPad Software Inc., San Diego, CA, USA). The experiments were repeated three times, and representative results were shown for each experiment. The results are expressed as mean ± standard error. The non-parametric Kruskal–Wallis test

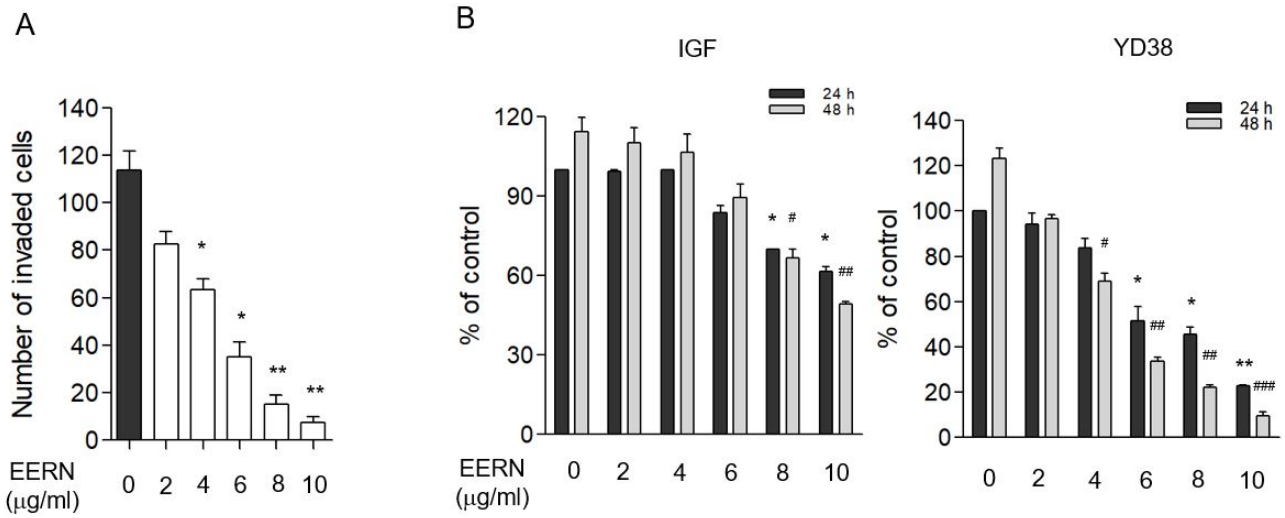

Figure 1. Effects of ethanol extracts of *Rhynchosia nulubilis* (EERN) on cancer cell invasion and viability of YD38 oral squamous cell carcinoma cells. (A) Matrigel-coated Transwell invasion assay. Cells were treated with or without EERN in a Matrigel-coated Transwell chamber, and invaded cells were counted in the lower chamber after 48 h using hematoxylin staining. $*p < 0.01$, $**p < 0.001$ versus without EERN control. (B) Cytotoxicity assay. Cells were treated with the indicated concentrations of EERN for 24 or 48 h, and viable cells were monitored by MTT assay. The percentage of viable cells was calculated as a ratio of control cells (without EERN). Data values are mean ± standard error of three independent experiments. $*p < 0.01$, $**p < 0.001$ versus without EERN control at 24 h. $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ versus without EERN control at 48 h. IGF; immortalized gingival fibroblasts.

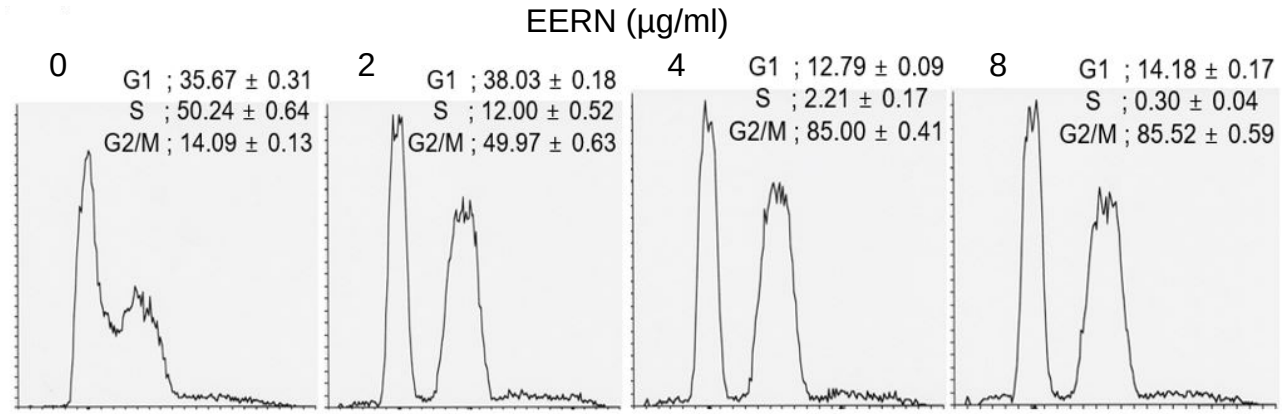

Figure 2. Effects of ethanol extracts of *Rhynchosia nulubilis* (EERN) on cell cycle distribution. Cells were cultured for 24 h in the presence or absence of indicated concentrations of EERN (0–8 mg/ml), and the cell cycle distribution was determined by propidium iodide staining for flow cytometric analysis.

DOI: 10.20473/j.djmkg.v58.i3.p273–279

with Dunn's post hoc analysis was employed for multiple comparisons, and *p*-values of <0.05 were considered statistically significant.

RESULTS

To observe the effect of EERN on YD38 OSCC cell invasion, a Matrigel-coated Transwell invasion assay was performed. Cancer cells were placed in a Matrigel-coated Transwell insert and cultured in the lower chamber with medium containing EERN. After 48 h, cells that passed through the Transwell insert and moved to the lower chamber were considered invaded cells. Compared with the control group without EERN, the invasion activity of YD38 cells was significantly reduced at ≥4 mg/ml EERN (Figure 1A). As a result of the MTT assay under the same conditions as the invasion assay, significant growth inhibition was observed at ≥6 mg/ml EERN during the 24 h reaction and at ≥4 mg/ml EERN during the 48 h reaction (Figure 1B). In IGF, growth inhibition was observed at a relatively high concentration of ≥8 mg/ml EERN.

To elucidate the reason for cell growth inhibition, cells were treated with different concentrations of EERN for 24 h, and the cell cycle phase distribution was monitored by flow cytometry. As shown in Figure 2, EERN-treated YD38 cells showed a different cell cycle phase proportion in the G2/M ($p < 0.01$) compared with the control without EERN. The 4 or 8 mg/ml EERN-treated cells had 85.00% ± 0.41% or 85.52% ± 0.59% of the cells distributed in the G2/M phase compared with 14.09% ± 0.13% of the control cells without EERN; EERN-induced G2/M arrest was observed with maximal arrest at a concentration of 4 mg/ml. There was also a significant difference in the G1 ($p < 0.01$ vs. control) and S ($p < 0.001$ vs. control) phases of the cell cycle.

Phosphorylation of Ser139 H2AX is an early cellular response to the induction of DNA double-strand breaks.[13] Therefore, γH2AX is a sensitive molecular marker for monitoring DNA damage. The EERN-treated YD38 cells were reacted with γH2AX antibody, and fluorescence was observed. To observe the γH2AX level of EERN-treated YD38 cells in fluorescence, cells cultured in a glass chamber slip were blocked with 5% bovine serum

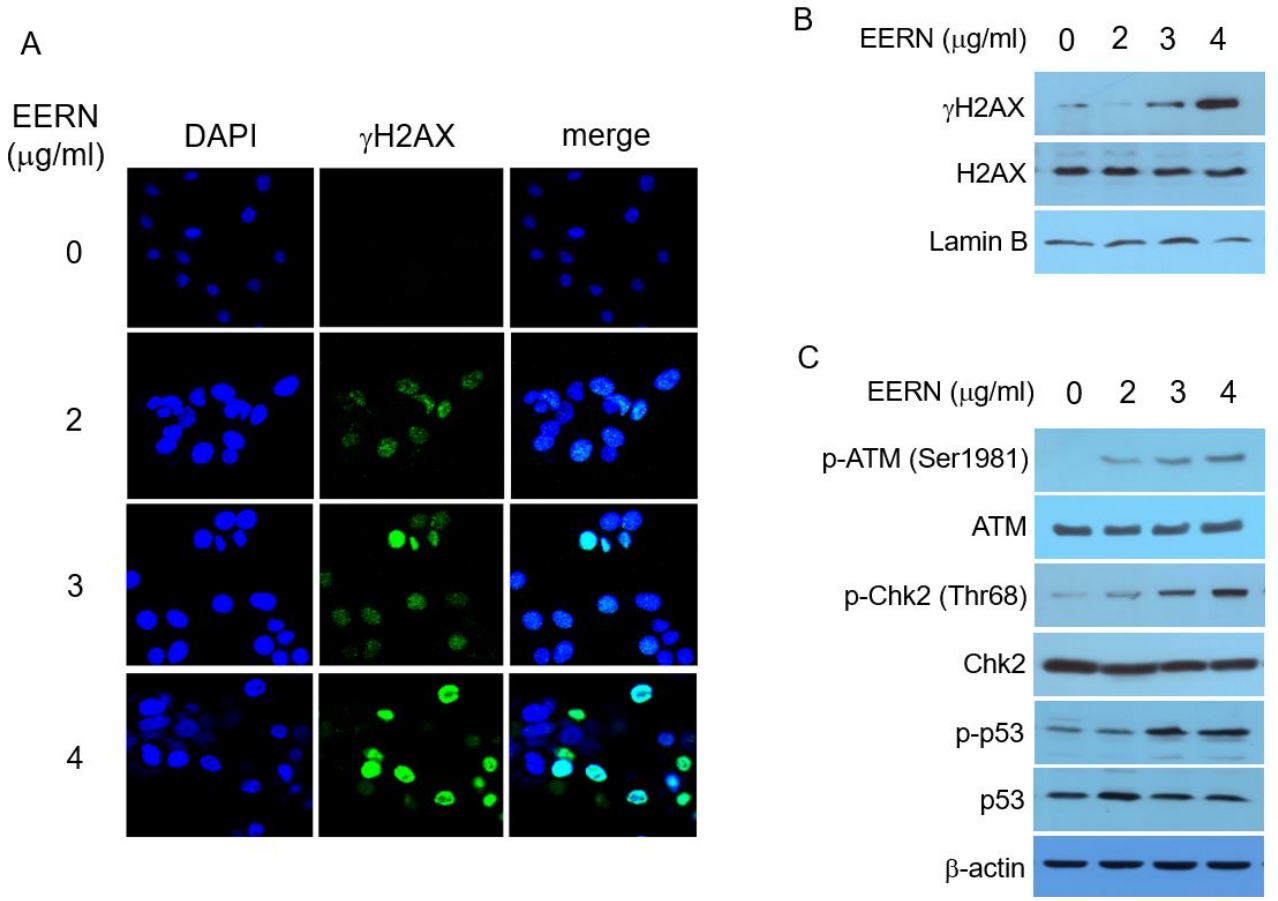

Figure 3. Effects of ethanol extracts of *Rhynchosia nulubilis* (EERN) on DNA damage and cell cycle-associated proteins. (A) Cells were cultured for 24 h in the presence or absence of indicated concentrations of EERN, and phosphorylated H2AX (γH2AX) was observed under a fluorescence microscope (20 original magnification). DAPI was used for nuclei detection. (B) The γH2AX protein level was detected by western blot analysis. Total H2AX was also detected. Lamin B was used as a nuclei control. (C) Phosphorylated ataxia telangiectasia mutated (ATM) (Ser1981), Chk2 (Thr68), and p53 levels were detected by western blot analysis. Total ATM, Chk2, and p53 were also detected; β-actin was used as a loading control.

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albumin. Cells treated with 0.5% Triton X-100 were reacted with γH2AX antibody and sequentially reacted with Alexa Fluor 488-conjugated IgG secondary antibody and observed under a fluorescence microscope. As shown in Figure 3A, significant γH2AX fluorescence in nuclei was monitored at ≥2 mg/ml EERN-treated cells. The protein level of γH2AX was also increased by EERN treatment in a dose-dependent manner (Figure 3B). To elucidate the protein related to the DNA damage signaling pathway in the EERN-induced G2/M phase arrest in YD38 cells, a western blot was then performed. Following treatment with 2, 3, and 4 mg/ml EERN, protein levels of p-ATM (Ser1981), p-Chk2 (Thr68), and p-p53 were increased (Figure 3C). Phosphorylation of ATM, Chk2, and p53 was significantly increased up to 3 mg/ml in EERN-treated cells, respectively.

DISCUSSION

Given the reported antioxidant, anti-inflammatory, and anti-obesity properties of *Rhynchosia nulubilis* extract, it is becoming recognized as a promising raw material for treating various diseases.[8–12] *In vitro* studies have demonstrated that EERN can inhibit the proliferation of OSCC by inducing cell cycle arrest and DNA damage. Consequently, we aimed to assess the potential of *Rhynchosia nulubilis* extracts as an effective natural agent for cancer management and to elucidate the intracellular mechanisms behind its anticancer effects. Flavonoids, useful plant components contained in soybeans, benefit cardiovascular health by lowering blood triglyceride and cholesterol levels, and their powerful antioxidant activity can reduce oxidative stress and reduce the risk of chronic diseases, such as diabetes. Additionally, flavonoids are similar in structure and function to estrogen, which not only helps maintain bone density but also mimics the activity of estrogen and helps regulate the balance of hormone levels in the body, helping to prevent hormone-dependent diseases, including menopausal syndrome and certain cancers. Black soybean seeds do not differ significantly in composition from yellow beans, although black bean skins contain more anthocyanins. Black soybean anthocyanins have been reported to have no side effects on the human body and have strong antioxidant and anti-aging effects.[14] *Rhynchosia nulubilis* (Seomoktae) is the smallest of the black beans and has high detoxification properties, so it is mainly used as a herbal medicine. *Rhynchosia nulubilis* hull is black, but the bean seed is blue. A small black soybean, *Rhynchosia nulubilis* is mainly reported to have strong antioxidant activity.[15]

Considering the lack of studies on the effect of *Rynchosia nulubilis* on cancer progression, we tested the anticancer activity of *Rynchosia nulubilis* in an invasion assay against highly invasive YD38 oral cancer cells. Ethanol extract from whole *Rhynchosia nulubilis* (EERN) was tested as a natural compound. As a result of the experiment, cancer cell invasion activity was significantly decreased by EERN dose-dependently. Using an MTT assay to confirm the effect of the EERN on cell proliferation, cytotoxicity was also observed with EERN. Cytotoxicity was increased with increasing EERN concentration. The cytotoxicity of the EERN is considered to be the cause of the decrease in cancer cell invasion activity. Inhibition of various cancer cell proliferation through induction of cancer cell differentiation and apoptosis by soy isoflavones has been reported.[16] According to fluorescence-activated cell sorting analysis to confirm the effect of EERN on the cell cycle of YD38 cells, EERN strongly arrests the cell cycle at the G2/M phase. These results indicate that EERN inhibits YD38 cancer cell growth and invasion activity through DNA damage and cell cycle arrest, suggesting that it is an effective natural material for cancer management.

The cell cycle is a series of processes in which one cell divides into two cells and is regulated by various factors *in vivo* and *in vitro*. Cells are programmed to enter pathological processes when certain phases of the cell cycle are abnormal. Uncontrolled cell growth following loss of cell cycle control is associated with cancer development.[17] Arrest of the G2/M cell cycle occurs when DNA is damaged, preventing cells from entering mitosis and providing an opportunity for DNA repair to maintain genomic integrity. To determine the cause of the G2/M phase arrest of the EERN-induced YD38 cell cycle, H2AX phosphorylation was monitored by fluorescence analysis. When DNA is damaged, the H2AX serine residue is rapidly phosphorylated and γH2AX is generated.[7] Compared with the control, increased γH2AX fluorescence was observed in EERN-treated cells, suggesting that cell cycle arrest is due to DNA damage by EERN treatment. Cells with damaged DNA activate ATM and cell cycle checkpoints to arrest the cell cycle and repair damaged DNA or, in severe cases, induce apoptosis. Therefore, they act as upper cell signal molecules that suppress genomic stability and tumorigenesis when DNA damage occurs.[8] Furthermore, p53 is also involved in cell cycle arrest to suppress genomic stability and tumorigenesis by receiving signals from activated ATM.[18] Western blot analysis revealed that γH2AX, p-ATM (Ser1981), p-Chk2 (Thr68), and p-p53 protein levels were increased in EERN-treated YD38 cells, suggesting that EERN induces cell cycle arrest through activation of the ATM pathway. Damaged DNA induces H2AX phosphorylation, which subsequently activates ATM through autophosphorylation of ATM at Ser1981; ATM (Ser1981) then induces cell cycle arrest by promoting phosphorylation and activation of Chk2 and p53. The G2/M phase checkpoint is an opportunity to repair DNA damage prior to entry into mitosis.[19] In this study, G2/M phase cells were significantly increased in an EERN dose-dependent manner. Therefore, it was revealed that EERN induces YD398 cell cycle arrest at the G2/M phase checkpoint and inhibits cancer cell proliferation by the suppression of mitotic entry.

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Black soybeans contain a higher amount of isoflavones than other soybeans. In particular, soybean isoflavone genistein has an antioxidant effect that removes reactive oxygen species.[18] The anti-cancer effect of genistein has been confirmed in previous studies on breast, rectal, ovarian, and prostate cancer and functions by inhibiting the production of heat shock protein, which helps evade cancer cells from the immune system[20] and the production of stress protein glucose-related protein.[21,22] Cytotoxicity in various cancer cells and cell cycle arrest by soy isoflavone genistein has also been reported.[23] Compared with adolescents who did not consume soy, adults who consumed soy regularly had a lower risk of breast cancer.[24] In addition, the breast cancer recurrence rate of women who consumed soy protein and isoflavones regularly was significantly lower than that of women who consumed less.[7] The major bioactive compounds contained in black soybeans have been reported as isoflavone, tocopherol, phenolic acids, and anthocyanin. Compared with the single activity, their mixture showed a synergistic physiological effect. In particular, the highest synergistic effect in antioxidant activity was observed when anthocyanins were mixed.[25,26] This research suggests that effective anticancer drugs can be developed from soybeans because the ingredients contained in soybeans have anticancer effects through various mechanisms; however, the research results on active ingredients other than genistein are insufficient. It is necessary for various researchers to reveal the functions and intracellular mechanisms of more diverse soybean active ingredients, and further research using combinations of these active ingredients will help develop soybean-derived anticancer drugs with higher pharmacological activity and fewer side effects. This will be beneficial in advancing cancer treatment options and reducing the adverse effects associated with current therapies.

In conclusion, the anti-proliferative effect of the ethanol extracts of small black soybean *Rhynchosia nulubilis* on OSCC cells was confirmed in this study. There is a limitation in not analyzing the anti-proliferative effect compared with the main physiologically active compounds of black soybeans. Therefore, further research is needed to isolate the pure compound from the ethanol extracts of small black soybean *Rhynchosia nulubilis* and to elucidate its functional ingredient. In addition, a study confirming the synergistic physiological effect of pure compounds is also required.

ACKNOWLEDMENT

This research was supported by Eulji University in 2025.

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DOI: 10.20473/j.djmkg.v58.i3.p273–279