Esculetin has therapeutic potential via the proapoptotic signaling pathway in A253 human submandibular salivary gland tumor cells

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Abstract. Esculetin is a natural lactone that is commonly derived from coumarins. According to previous experiments using human cancer cells, esculetin has potent antitumor activity; it also inhibits proliferation and induces the apoptosis of cancer cells. In the present study, the anti-proliferative effect of esculetin on the submandibular salivary gland tumor cell line, A253, was evaluated via *in vitro* and *in vivo* analyses. Furthermore, the anti-cancer effects of esculetin in A253 cells and a xenograft model of salivary gland tumors were determined using 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide and TUNEL assay, apoptosis protein array, quantitative polymerase chain reaction and western blot analysis. Esculetin (50-150 μM) was demonstrated to have an anti-proliferative effect in the A253 cell line *in vitro*; this observed effect was dependent on the dose and duration of treatment. Esculetin also increased the levels of Bax, cleaved caspase-3, cleaved-9 and cleaved poly (ADP-ribose) polymerase apoptosis-related proteins, and decreased the expression levels of the Bcl-2 anti-apoptotic protein. With respect to apoptosis regulation, esculetin significantly decreased the proliferation of tumor cells in a xenograft model (100 mg/kg/day) for 18 days. Overall, esculetin could be a potential oral anticancer drug against salivary gland cancer.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common non-skin-related cancer worldwide. Each year, 600,000 patients are diagnosed with this cancer, and 50% succumb (1). Salivary gland carcinoma is a relatively rare malignant tumor, accounting for <5% of HNSCC cases (2). Patients with high-grade salivary carcinoma have a 5-year survival rate of ~40%, whereas those with low-grade salivary carcinoma have a 5-year survival rate of 85-90% (3,4). The standard treatments for major and minor salivary gland tumors include surgical excision, radiotherapy and chemotherapy. Cisplatin, 5-fluorouracil and methotrexate are common drugs used to treat squamous cell carcinoma of the salivary glands (5). However, doxorubicin hydrochloride (Adriamycin), cisplatin, cyclophosphamide or cisplatin with 5-fluorouracil can be used for the primary treatment of patients with recurrent, metastatic or unresectable salivary gland tumors (6,7). Only a few effective treatment options are available for patients with recurrent or unresectable tumors. Despite the effective treatment features of cisplatin, various studies have revealed only a 10-month survival period when this drug is used alone or combined with other medicines. Chemotherapy causes speech/swallowing defects, chronic pain, muscle atrophy and other side effects (8). Therefore, novel chemotherapy and treatment regimens are required for salivary carcinoma (9).

Esculetin, also known as 6,7-dihydroxy coumarin, is derived from coumarin and can be obtained from various plants, such as *Citrus limonia*, *Artemisia capillaries* and *Euphorbia lathyris* (10,11). Pleiotropic biological activity is a well-known characteristic of esculetin. Moreover, esculetin has various advantages, including antioxidant and xanthine oxidase inhibitory activities, platelet aggregation and anticancer behavior (12-16). Esculetin also induces the apoptosis of oral squamous cell carcinoma (OSCC), resulting in the suppression of cancer cell proliferation (13,17). To date, no study has evaluated the effect of esculetin on human salivary gland tumors. Thus, in the present study, *in vitro* and *in vivo* experiments were performed to assess the induction of apoptosis and the antiproliferative effect of esculetin on the human submandibular carcinoma A253 cell line.

Materials and methods

Materials. Esculetin with a purity >98% was obtained from Tokyo Chemical Industry. DMSO, cisplatin and 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich; Merck KGaA. The apoptosis detection kit for TUNEL-FITC and the human apoptosis proteome profiler kit were supplied by Promega Corporation and R&D Systems, Inc., respectively. All the required antibodies, including Bax (cat. no. ab53154), Bcl-2

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(cat. no. ab196495), poly (ADP-ribose) polymerase (PARP; cat. no. ab32139), caspase-3 (cat. no. ab184786), caspase-9 (cat. no. ab184786), and β-actin (cat. no. ab8227), were purchased from Abcam. The A253 cells were obtained from the American Type Culture Collection.

Cell culture. A253 cells were cultured in modified RPMI 1640 medium (Welgene, Inc.) supplemented with 10% fetal bovine serum (Welgene, Inc.), streptomycin (100 μg/ml), and penicillin (100 U/ml), and maintained in an incubator containing 5% CO_2 at 37˚C.

Cell viability and apoptosis assay. The MTT assay was used to estimate the effect of esculetin on cell viability. The A253 cells were seeded in a 96-well cell culture plate at a density of $5x10^4$ cells per well. Thereafter, the cultured cells were treated with different doses of esculetin (0, 50, 100 and 150 μM in 0.1% DMSO) for 24 and 48 h. MTT (5 mg/ml; 10 μl) was then added to the cells, which were further incubated at 37˚C for 4 h. After MTT removal, the violet formazan crystals were dissolved in 1 ml DMSO. A microplate reader (540 nm; Tecan Group, Ltd.) was used to obtain the MTT assay results. The cells were cultured on autoclaved coverslips to determine esculetin-treated apoptotic cells. TUNEL staining was conducted to detect apoptotic cells, after 24 and 48 h of pre-incubation with esculetin. Cells were then washed with PBS, and fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. Fixed cells were washed with PBS and treated with the rTdT reaction mix at 37˚C for 1 h; reactions were terminated by immersing in 2X SSC solution for 15 min at room temperature. Cells were stained with 0.2 mg/ml of DAPI in PBS at room temperature for 15 mi and mounted onto glass slides with the mounting medium (Vectashield, Vector Laboratories, Inc.) and analyzed under a fluorescent microscope (Olympus Corporation). Images of at least five random fluorescent fields were captured, and TUNEL-positive cells were counted. Data were expressed as the percentage of apoptotic cells per field.

Western blot analysis. A protein extraction RIPA lysis buffer (Elpis Biotech) containing a Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, Inc.) was used to lyse the treated cells. Thereafter, Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc.) was used to quantify the extracted protein. Equal amounts of proteins (50 μg/lane) were separated by 12.5% SDS-PAGE gel and transferred to a PVDF membrane (MilliporeSigma). The membranes were then blocked with 5% skimmed milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) at room temperature for 2 h. The primary antibodies used were anti-Bax (1:500), anti-Bcl-2 (1:500), anti-PARP (1:500), anti-caspase-3 (1:500), anti-caspase-9 (1:500), and β-actin (1:1,000). The membranes were incubated with primary antibodies diluted in TBST overnight at 4˚C, washed three times with TBST. Finally, a western blotting detection kit (ECL Western blotting substrate kit; Abcam) was used to observe the protein bands after treatment with a horseradish peroxidase-linked secondary antibody (1:1,000, Advansta) at room temperature for 1 h.

Proteome profiling of human apoptosis array. Different doses of esculetin (0, 50, 100, and 150 μM) were used to treat A253 cells for 48 h. A proteome profiler human apoptosis antibody array kit (cat. no. ARY009, R&D systems) was used to analyze the lysed cells according to the manufacturer's instructions. ImageJ software version 1.52a (National Institutes of Health) was used to desensitize the obtained signals, where the average pixel density was expressed by normalizing the pixel density to untreated samples.

Quantitative polymerase chain reaction (qPCR). TRIzol® reagent (Thermo Fisher Scientific, Inc.) was used to extract total RNA from the collected cells based on the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed in a final volume of 30 μl using the ImProm-II reverse transcriptase system kit (Promega Corporation) according to the manufacturer's instructions. cDNAs were used for PCR. The primer sequences are summarized in Table I. The AniQ5 Continuous Fluorescence Detector System (Bio-Rad Laboratories, Inc.) and a 2X SYBR® Green PCR Master Mix (cat. no. RR420A, Takara Bio, Inc.) were used to perform the qPCR at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec and a single cycle of 95˚C for 15 sec, 60˚C for 60 sec, and 95˚C for 15 sec to generate dissociation curves. All PCR reactions were performed in triplicate, and the specificity of the reaction was determined by melting curve analysis. Comparative quantification of each target gene was performed based on cycle threshold (Ct) normalized to β-actin using the $2^{-\Delta\Delta Cq}$ method (18).

Tumor xenograft model. A total of 18 male BALB/c nude mice (age, 6 weeks old; weight, 15-20 g) were obtained from Central Lab Animal Inc. Mice were housed under a 12-h light/dark cycle at a temperature of 23±1˚C and 55±5% humidity and were provided with standard rodent pellets and filtered water *ad libitum*. The protocols approved by the Institutional Animal Care and Use Committee of the Jeonbuk National University Hospital (Jeonju, South Korea; approval no. JBNUH 2021-019) were used for the assessment. Briefly, A253 cells were suspended in RPMI-1640 culture medium containing 10% FBS, and maintained in a humidified atmosphere containing 5% CO_2 at a controlled temperature of 37.6˚C. Before tumor inoculation, all mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). To obtain a xenograft model, mouse shoulders were inoculated with A253 cells at a total of $2x10^6$ cells in 50 μl Dulbecco's modified Eagle's medium (Welgene, Inc.) mixed with 50 μl Matrigel (Becton, Dickinson and Company) for a total volume of 100 μl per injection site. After 7 days, mice were randomly divided into three groups, each containing six mice. Esculetin and cisplatin were dissolved in 0.5% carboxymethylcellulose. Thereafter, esculetin (100 mg/kg per day) or cisplatin (7.5 mg/kg per day), as a positive control, was orally administered for 18 days (19,20). Mice in the negative control group were orally administered the vehicle (0.5% carboxymethylcellulose). The *in vivo* antitumor activity of esculetin was assessed by measuring tumor size at 3-day intervals. Esculetin was administered for 18 days until the tumor size in the negative control group reached 200 mm^3. Tumor volume was calculated using the following equation: $\pi/6 \times (a)^2 \times (b)$, where 'a' and 'b', correspond to the shortest and longest tumor diameters, respectively (21). No adverse reactions or compound-related side effects were observed in the

Table I. List of primer sequences used in this study.

Gene	Forward primer	Reverse primer
Caspase-3	5'-AATGGACCTGTTGACCTGAAA-3'	5'-ATAATAACCAGGTGCTGTGGAG-3'
Bcl-2	5'-GGAGGATTGTGGCCTTCTTT-3'	5'-GAGACAGCCAGGAGAAATCAA-3'
Bax	5'-TTGCTTCAGGGTTTCATCCA-3'	5'-AGTTGAAGTTGCCGTCAGAA-3'
β-actin	5'-TGACGATATCGCTGCGCTC-3'	5'-CAGTTGGTGACAATGCCGTG-3'

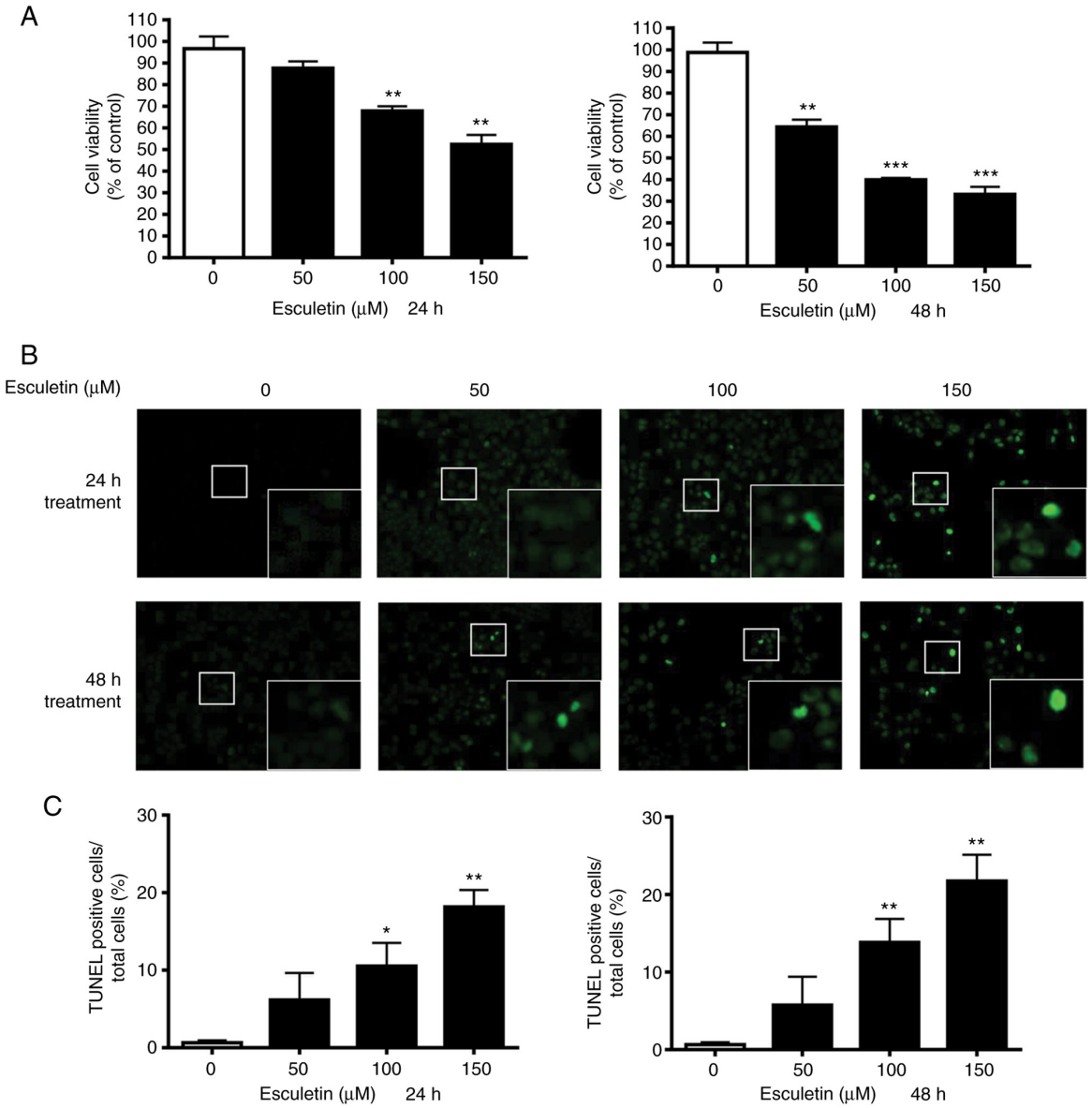

Figure 1. Effect of esculetin on A253 cells. (A) Effect of various doses of esculetin on A253 cell viability. (B) Fluorescence images of TUNEL staining showing the role of esculetin on A253 cell apoptosis (original magnification x100; inset x400). (C) Diagrams showing the apoptotic rate of A253 cells. Data are presented as the mean ± standard deviation (n=3; *P<0.05, **P<0.01 and ***P<0.001 vs. untreated control group).

mice. Body weight was measured and the mice were sacrificed with ketamine (500 mg/kg) and xylazine (50 mg/kg) to excise the tumor xenografts at necropsy.

H&E and TUNEL staining. For evaluation of the tissue structures, the tumor tissues were fixed in formaldehyde solution (4%) at room temperature for 24 h, dehydrated using gradient ethanol, embedded in paraffin, incised into smaller sections (4 μm) and then stained with H&E. The TUNEL assay was also performed using the one-step TUNEL kit, according to the defined guidelines. After paraffin removal, the sections were incubated in 100 μl Proteinase K (20 μg/ml) for 10 min at

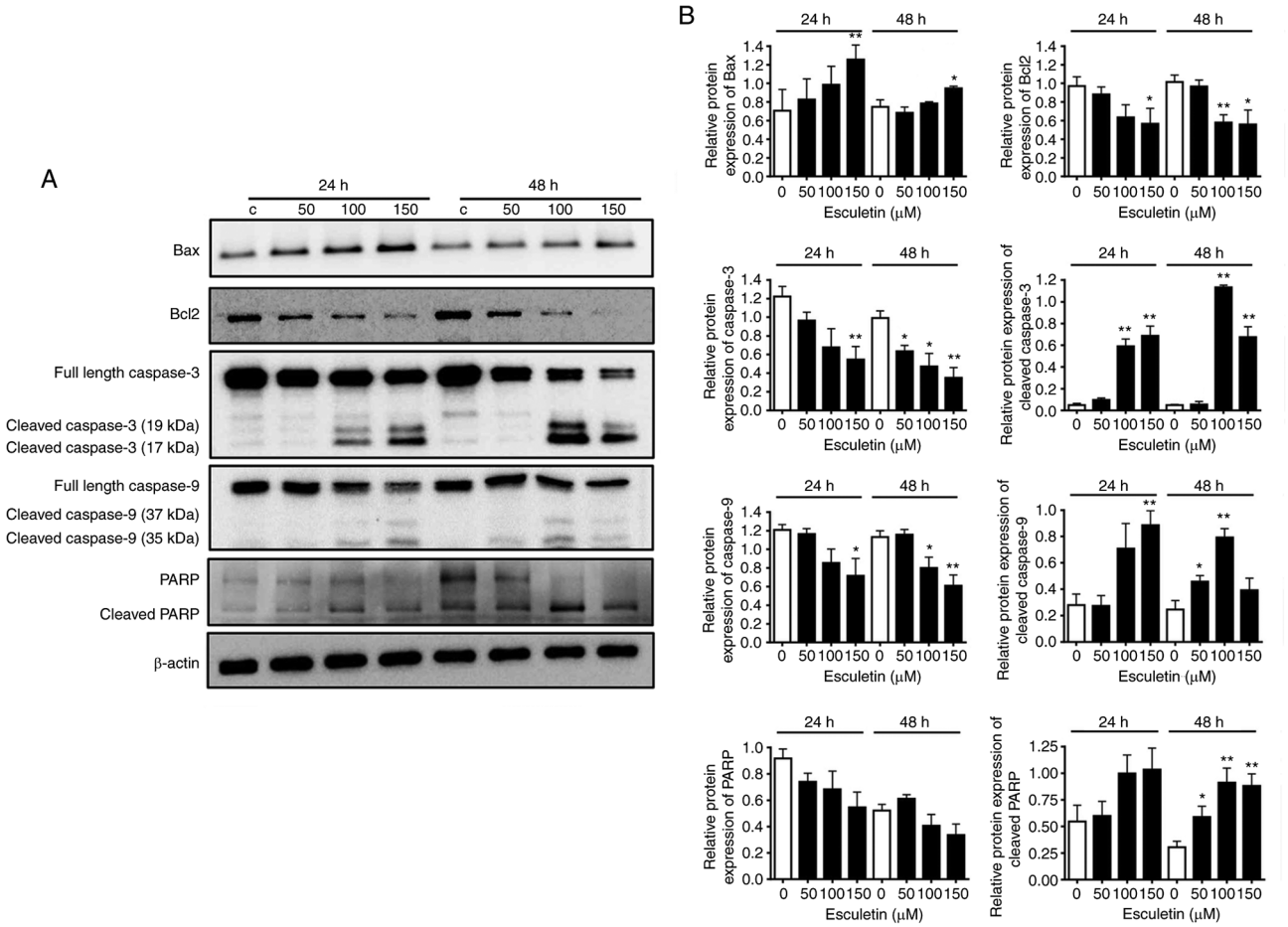

Figure 2. Esculetin induces the apoptosis of A253 cells by regulating the expression levels of apoptosis-related proteins. (A) Relative protein levels were assessed using western blot analysis. Treatment of A253 cells with different doses of esculetin (0, 50, 100 and 150 μM) for 24 and 48 h. (B) Relative expression of apoptotic proteins to β-actin. Data are presented as the mean ± standard deviation (n=3; *P<0.05 and **P<0.01 vs. untreated control group). PARP, poly (ADP-ribose) polymerase.

room temperature, transferred into a TUNEL reaction mixture with a volume of 50 μl, and placed in a dark incubator for 1 h at 37˚C. A solution of Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc.) was used to stain the prepared sections. Subsequently, a fluorescence microscope was used to capture images of the prepared slices. Images of at ≥5 random fluorescent fields were captured. In this evaluation, the number of TUNEL-positive apoptotic cells per 100 cells was determined using green fluorescence exerted by the cells.

Statistical analysis. The results are presented as the mean ± standard deviation. One-way analysis of variance was used to analyze the data, followed by Tukey's multiple comparison test. The assessments were conducted using GraphPad Prism 6.0 (GraphPad Prism Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of esculetin on the proliferation and apoptosis of A253 cells. Several concentrations of esculetin were used to treat A253 cells for 24 and 48 h to explore the therapeutic potential of esculetin. Further, MTT assays were performed to determine the viability of the cells. The proliferation of A253 cells was inhibited by esculetin after both 24 and 47 h of treatment in a concentration-dependent manner (Fig. 1A). The 50% maximal inhibitory concentration (IC_{50}) values of esculetin at 24 and 48 h were 157.4±30.0 and 78.5±5.3 μM, respectively. TUNEL staining was performed to determine the effect of esculetin on apoptosis. As shown in Fig. 1B and C, the apoptotic cell ratio was enhanced in the treated cancer cells compared with that in the control cells in a concentration-dependent manner. Based on the observed trend, apoptosis was the main cause of the decrease in cell viability of cancer cells treated with esculetin.

Role of esculetin in apoptosis induction via the regulation of apoptosis regulatory factors in A253 cells. The role of esculetin in apoptosis induction through the regulation of apoptosis regulatory proteins in tumor cells has been widely reported (22-24). To determine the expression level of apoptosis-related proteins, A253 cells were treated with various concentrations of esculetin (0, 50, 100 and 150 μM) for 24 or 48 h. The levels of Bax, cleaved caspase-3, cleaved caspase-9 and cleaved-PARP apoptosis-related proteins increased by esculetin after both 24 and 47 h of treatment. However, the level of the anti-apoptotic protein, Bcl-2, decreased in an esculetin

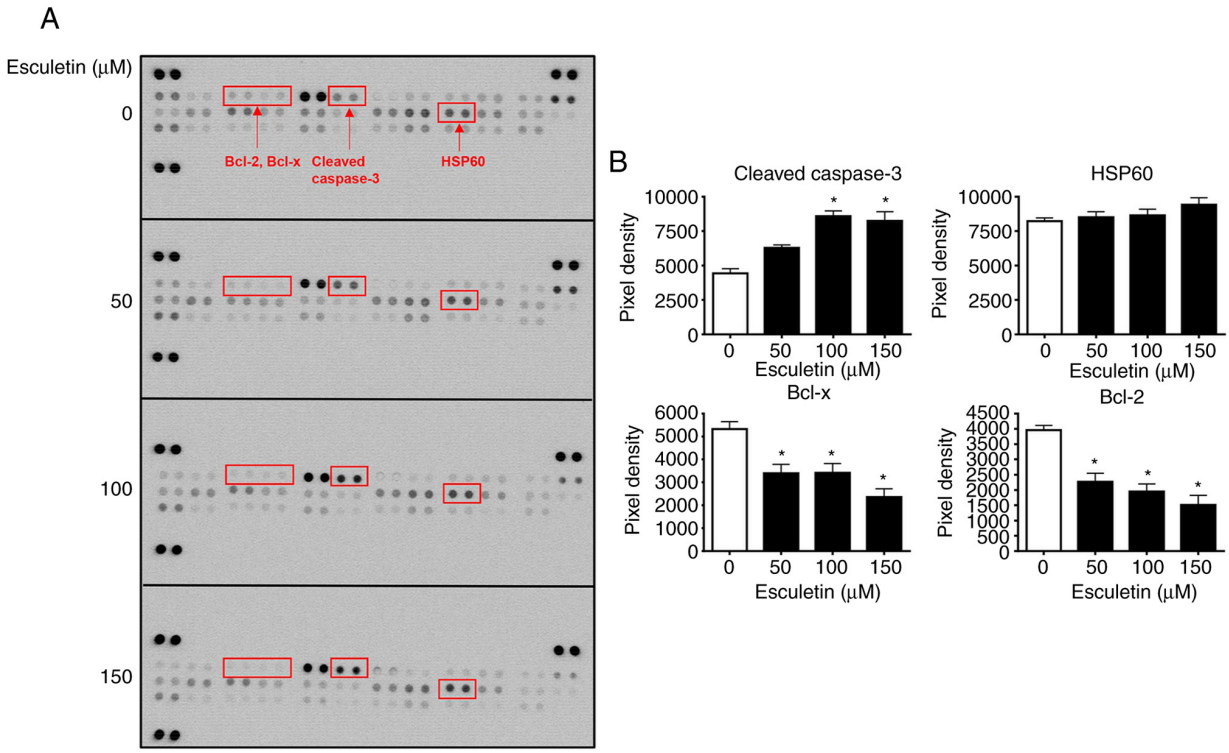

Figure 3. Effect of esculetin on the expression of pro- and anti-apoptotic proteins. (A) Apoptosis proteome profile array of A253 cells with various esculetin concentrations (0, 50, 100, and 150 μM) for 48 h. (B) Data are presented as the mean ± standard deviation (n=4; *P<0.05 vs. untreated control group). HSP60, heat-shock protein 60.

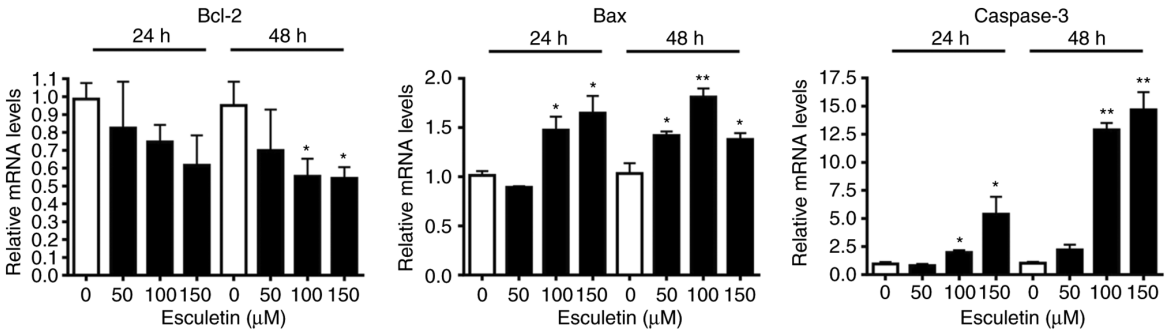

Figure 4. Effect of esculetin on the mRNA expression level of apoptosis-related factors in A253 cells. Relative mRNA levels of Bax, Bcl-2 and Caspase-3 were assessed using quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation (n=3; *P<0.05 and **P<0.01 vs. untreated control group).

dose-dependent manner (Fig. 2). These results indicated that esculetin induced apoptosis in the A253 cell line. To further elucidate the effect of esculetin on the differential expression of pro- and anti-apoptotic proteins, 56 proteins were identified using a human apoptosis proteome profiler array kit (Fig. 3A). As predicted, cleaved caspase-3 was significantly upregulated in cells treated with esculetin compared with untreated control cells. Further, HSP60 expression was found to be raised, whereas the expression levels of the anti-apoptotic proteins, including Bcl-2 and Bcl-x, was decreased in esculetin-treated cancer cells (Fig. 3A and B). Therefore, esculetin may promote A253 cell death through pro-apoptotic protein induction and anti-apoptotic protein reduction. To confirm the aforementioned results, the mRNA expression levels of pro- and anti-apoptotic genes in esculetin-treated A253 cells were evaluated. Caspase-3 and Bax mRNA expression levels were significantly increased, while those of Bcl-2 were decreased in a dose-dependent manner (Fig. 4).

Effect of esculetin on the proliferation and apoptosis of A253 cells. An *in vivo* examination was performed using a xenograft nude mouse model of A253 cells to determine the role of esculetin in the inhibition of tumor growth. The data revealed the inhibitory effect of esculetin on the growth of tumors (Fig. 5). Esculetin was found to reduce tumor weight and size. In fact, at necropsy, tumor sizes in the vehicle, esculetin and cisplatin-treated groups were 197.9±66.0, 49.8±29.8, and 140±72.0 mm^3, respectively. Thus, esculetin suppressed xenograft tumor development in mice by 74% relative to that of vehicle-treated mice. Nevertheless, the body weight of xenograft nude mice was not significantly affected by esculetin. Cisplatin did not significantly suppress A253 tumor growth

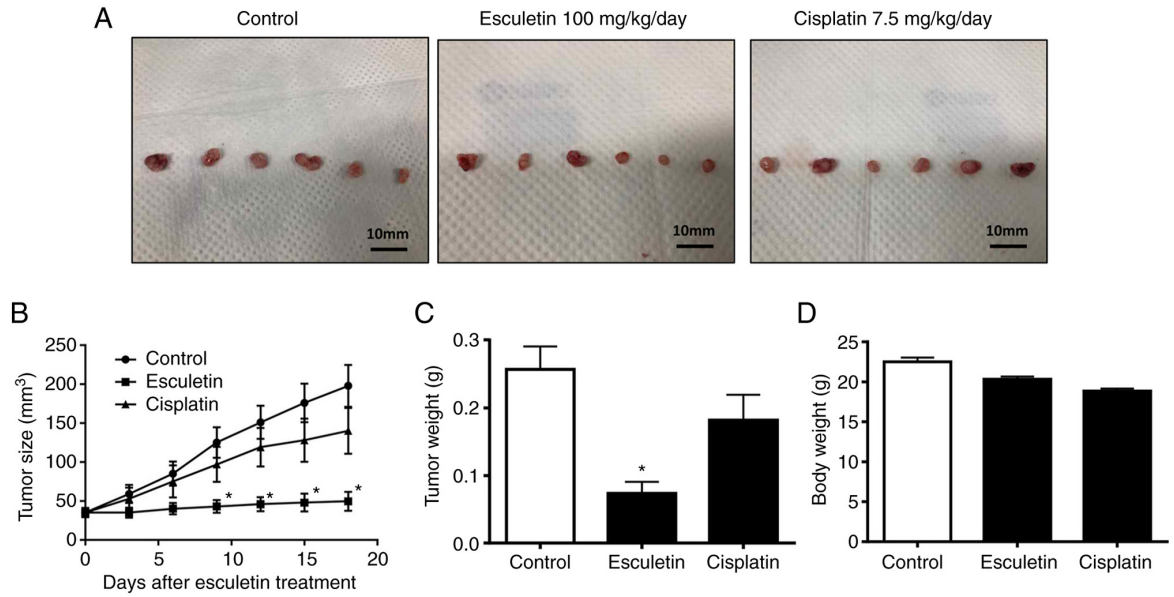

Figure 5. Esculetin antitumor effect on a BALB/c nude mouse xenograft model. (A) Tumor images from each group, (B) calculated tumor sizes, (C) average tumor weight and (D) changes in body weight. Data are presented as the mean ± standard deviation (n=6; *P<0.05 vs. vehicle-treated control group).

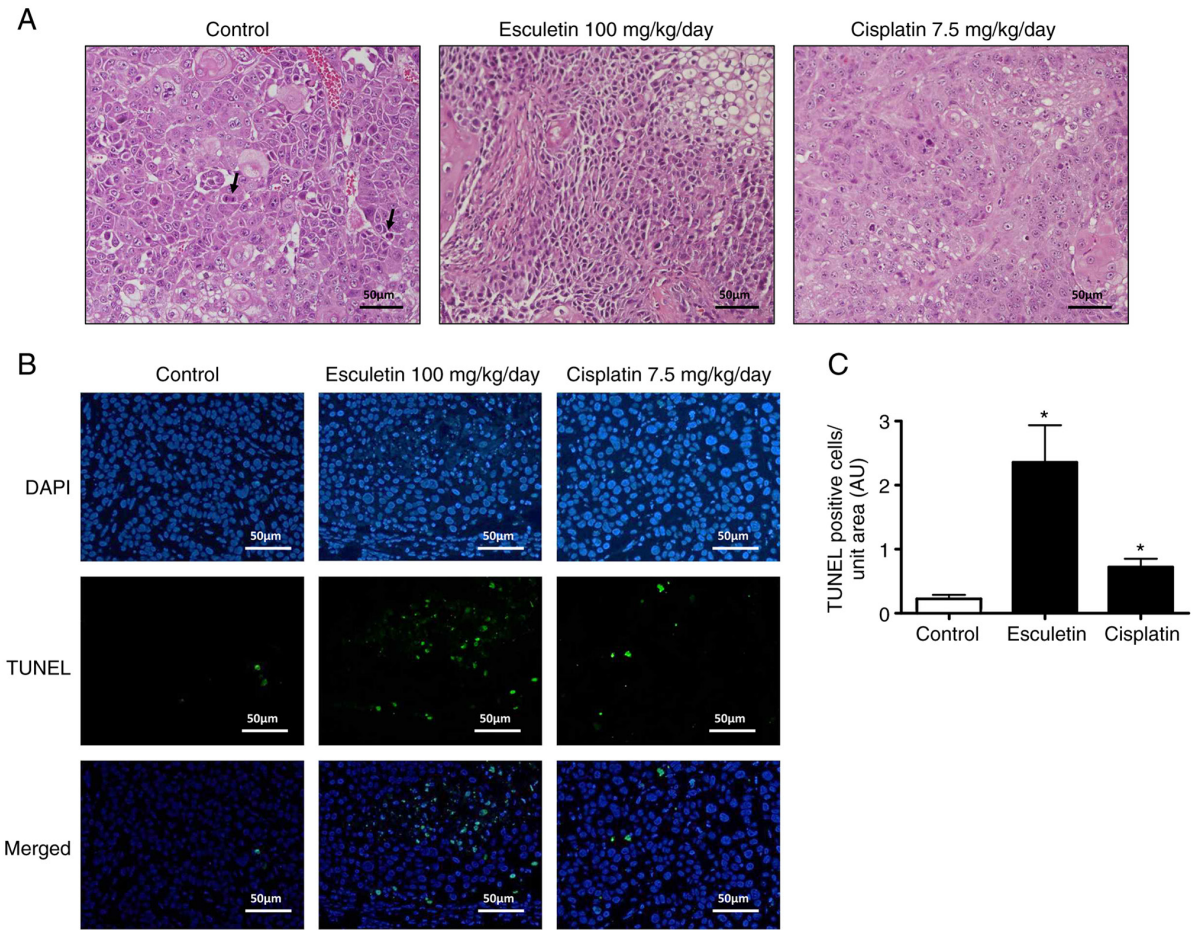

Figure 6. Esculetin-induced apoptotic changes in A253 xenograft tumors. (A) Histological analysis of esculetin in tumors of the A253 cell xenograft model. (B) Images of the TUNEL assay. (C) Quantification of TUNEL-positive cells. Data are presented as the mean ± standard deviation (n=6; *P<0.05 vs. vehicle-treated control group). AU, arbitary units.

(P>0.05). Thus, esculetin has more potent antitumor activity than cisplatin. These findings suggest that the growth of human submandibular salivary gland tumors can be suppressed by the application of esculetin.

Effect of esculetin on apoptosis induction in the A253 cell xenograft model. Based on H&E staining, the xenograft tumor tissues of the control group showed vigorous growth, irregular morphology, nuclear hypertrophy, numerous abnormal mitoses, overt dysplasia, giant tumor cells and increased tumor angiogenesis (Fig. 6A). However, the esculetin group rarely showed necrotic lesions, with small nuclei, decreased dysplasia and reduced tumor angiogenesis. Notably, these tumor-related histopathological changes were not suppressed in the cisplatin-treated group. To detect apoptotic cells in tumor tissues, TUNEL analysis was performed, as shown in Fig. 6B and C. The treated group exhibited more TUNEL-positive cells than the control group, which aligns with the *in vitro* results. Hence, induction of apoptotic cells and inhibition of cell proliferation could occur in an A253 cell xenograft model using esculetin. Overall, both *in vitro* and *in vivo* analyses suggested that esculetin induces apoptosis in submandibular salivary gland tumors.

Discussion

The proliferation, migration and survival of carcinoma cells can occur through several pathways, such as the RAS/RAF/ERK, PI3K and JAK/STAT pathways (25). Some chemotherapy drugs, including cisplatin, 5-fluorouracil, docetaxel, methotrexate, bleomycin and cetuximab, are considered targeted agents. In addition, various signals, such as those of VEGFR, EGFR, tyrosine protein kinase Met and insulin-like growth factor 1 (IGF-1) receptor, have been considered as therapeutic molecular targets (26). Cetuximab is the only EGFR-targeting agent approved by the Food and Drug Administration in the United States, but has insignificant effects due to intrinsic or acquired resistance, despite ubiquitous EGFR expression in HNSCC tumors (26).

In the present study, the antiproliferative and pro-apoptotic activities of esculetin against an HNSCC tumor cell line were investigated using *in vitro* and *in vivo* examinations. Based on recent studies, esculetin exerts antitumor activity in several cancer types. For example, esculetin (20-100 μM) inhibits the proliferation and expression of cyclin D1, cyclin-dependent kinase 4 and matrix metalloproteinase-2, and prevents the production of transforming and vascular endothelial growth factors in osteosarcoma LM8 cells (27). Esculetin also induces gastric cancer MGC-803 cell apoptosis by triggering the activation of the mitochondrial apoptotic pathway; this is linked to the mitochondrial membrane potential reduction, Bax/Bcl-2 ratio increase, agitation of the activities of caspase-3 and caspase-9, and enhancement of released cytochrome *c* from the mitochondria (28). Esculetin prevents cancer cell proliferation and induces the apoptosis of gastric cancer cells, which is mediated by the mitochondrial apoptosis pathway involving IGF-1/PI3K/Akt (28). Similarly, esculetin enhances the activities of caspase-3 and caspase-9, and promotes Bax expression, Bcl-2 expression reduction, mitochondrial membrane potential collapse, cytochrome *c* release and IGF-1/PI3K/Akt inactivation in SMMC-7721 cells (29). Lee *et al* (30) reported that the Wnt pathway could be a versatile therapeutic and suppressive target for various human cancer types. The β-catenin-T-cell complex factor, a key canonical Wnt signaling mediator, has been implicated in the development of human colon cancer. Esculetin, with its small molecular structure, is considered an effective Wnt/β-catenin pathway inhibitor. In another study, esculetin was reported to act as an effective lead chemotherapy agent for human metastatic colorectal cancer management; this can be linked to the ability of esculetin to prevent E-cadherin-mediated Wnt signaling via Axin2 inhibitors (31).

In several cancer cell lines, esculetin is known to inhibit cell proliferation and induce apoptosis or cell cycle arrest. Several studies have shown that esculetin inhibits lung cancer cell growth by adversely affecting c-Myc, cyclin D1 and NF-κB (32). Esculetin also causes Akt phosphorylation suppression and enhances protein expression of tumor suppressor phosphatase and tensin homologs by arresting the G_1 phase of the cell cycle (33). Esculetin markedly inhibits STAT3 phosphorylation, blocks translocation of STAT3 to the nucleus and restricts the G_1/S phase of the cell cycle in laryngeal cancer (34). A Bax/Bcl-2 ratio increase, caspase-3 and -9 activation, and mitochondria-mediated apoptosis pathway induction have been observed in hepatocellular carcinoma cells following the application of this therapy (35). Furthermore, 2-aminoethoxydiphenyl borate-sensitive store-operated $Ca2^+$ entry, $Ca2^+$ release from the endoplasmic reticulum and activation of the mitochondrial apoptosis pathway result in $Ca2^+$ influx via esculetin, ultimately leading to cell cycle arrest in ZR-75-1 human breast cancer (36).

Previously, esculetin was revealed to play an anti-proliferative role in head and neck cancer (13). The induction of apoptosis was caused by the inhibition of the specific protein 1 transcription factor (Sp1) (22), as well as modulation regulation of the p27, p21 and cyclin D1 target genes in OSCC, HSC-4 and HN22, and in human malignant melanoma cell lines (37). Sp1 is an essential transcription factor for a number of genes required for the regulation of multiple aspects of tumor cell survival, growth and angiogenesis (38). Previous studies have demonstrated that Sp1 is a drug target, and several antineoplastic agents have been shown to inhibit Sp1 expression (39). Sp1 is involved in the regulation of apoptosis. In fact, the promoters of a number of anti-apoptotic genes (bcl-2, bcl-x and survivin) and pro-apoptotic genes (bax, trail, fas, fas-ligand, caspase-9 and caspase-3) contain Sp1-binding sites (40). The inhibition of Sp1 expression regulates these genes. Indeed, inhibition of Sp1-DNA binding was shown to induce caspase 9-dependent apoptosis in bone marrow stromal cells (41). The inhibition of Sp1 by esculetin may lead to an increase in levels of Bax, cleaved caspase-3, cleaved-9 and cleaved PARP, and a decrease in Bcl-2 anti-apoptotic protein levels in A253 cells. Although the present study did not provide concrete scientific evidence of the effect of esculetin on the tumorigenesis of A263 cells, the observed results were consistent with its reported effects on various tumors.

The antitumor activity of esculetin was compared with that of cisplatin, which is a well-known anticancer drug. As previously reported, cisplatin is a positive control drug that reduces tumor size (42). In the present study, esculetin displayed more potent antitumor activity than cisplatin. In fact, cisplatin did not suppress A253 tumor growth ($P>0.05$). Similarly, in previous studies, the tumor growth of Cal-27 human head and neck squamous cell carcinoma cells did not show any growth delay when treated with cisplatin ($P>0.05$), whereas that of FaDu human head and neck squamous cell carcinoma cells

showed a slight but significant growth delay when treated with cisplatin ($P<0.01$) (43). These results suggest that the antitumor activity of cisplatin varies depending on the head and neck carcinoma cell line used. Notably, the clinical use of cisplatin is often limited by undesirable side effects, such as severe nephrotoxicity and hepatotoxicity (44). Although the precise mechanism for this cisplatin-induced toxicity is not well understood, cisplatin is preferentially taken up and accumulates in the human liver and kidney cells, resulting in enhanced production of reactive oxygen species and a decrease in levels of antioxidant enzymes (45,46).

In the *in vitro* system of the present study, the effective dose of esculetin to induce apoptosis in A253 cells was as low as 50 μM, whereas the maximum dose was 150 μM. In several previous reports, esculetin (50 and 100 mg/kg/day) dose-dependently inhibited xenograft tumor growth in laryngeal cancer cells (33). Zhu *et al* (32) reported that esculetin (100 mg/kg/day) suppressed tumor growth in a mouse model of lung cancer. Based on these reports, the oral dosage of esculetin (100 mg/kg/day) in mice was determined. In the *in vivo* investigation, 150 mg/kg/day of esculetin was identified as the most influential dose. In the present study, there was no difference in body weight between the esculetin-treated and vehicle-treated mice. Injecting 700 mg/kg esculetin per day was suggested to have no adverse effects on the body weight of the mice (34). Esculetin had a median lethal dose (LD_{50}) of 1,450 mg/kg via intraperitoneal injection and >2,000 mg/kg by oral gavage (47). Based on the guidelines of the Organization for Economic Cooperation and Development, an LD_{50} >2,000 mg/kg indicates the safety of the applied compound (48). Collectively, these results suggest that esculetin has relatively low toxicity in mice, highlighting the safety of esculetin for medical applications.

In the present study, the antiproliferative and pro-apoptotic activities of esculetin against human submandibular salivary gland tumor cells were evaluated. However, the antiproliferative activity of cisplatin was not evaluated *in vitro*, thereby serving as a study limitation. Previously, Lee *et al* (49) reported that the IC_{50} value of cisplatin on the proliferation of A253 cells was ~6.7 μM. In the present study, multiple oral doses of esculetin were required to determine the effective dose range in the animal experiment, which served as another limitation of the study. Therefore, the detailed beneficial role of esculetin in submandibular salivary gland tumor cells requires further study.

Overall, the *in vitro* and *in vivo* experiments of the present study confirmed the apoptosis and cell proliferation inhibitory effects of esculetin on human submandibular salivary gland tumors. These findings clearly indicate that esculetin may be a promising treatment option for HSNCC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SBP, WKJ, HRK, HYY and YHK performed the experiments, collected data and wrote the manuscript. SBP and JK analyzed the data and wrote the manuscript. SBP and JK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Institutional Animal Care and Use Committee of the Jeonbuk National University Hospital, Jeonju, South Korea (approval no. JBNUH 2021-019).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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