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The antitumoral effect of the esculetin in HeLa cells through endoplasmic reticulum stress

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Abstract. – OBJECTIVE: Despite the multiple available treatment modalities, cervical cancer is one of the leading causes of mortality and morbidity among female gynecological cancers. Endoplasmic Reticulum (ER) is an effective organelle in ensuring cell homeostasis and is closely related to the development of cancer. Esculetin is a coumarin derivative that has anticancer, anti-inflammatory, antioxidant, and neuroprotective effects. Esculetin may have an anticancer effect by inducting apoptosis and ER stress. In this study, we evaluate that esculetin has an anti-tumor effect on human cervical cancer-derived (HeLa) cells *via* ER stress.

MATERIALS AND METHODS: Esculetin was applied to the HeLa cells, and a viability test was performed using the methyl thiazolyl tetrazolium proliferation (MTT) assay. Expression levels of apoptotic genes and anti-apoptotic genes were determined by real-time polymerase chain reaction. The results were statistically evaluated.

RESULTS: Analysis of the MTT assay detected that esculetin inhibited HeLa cell viability development. Based on Western blot and quantitative real-time polymerase chain reaction (qPCR) analyses, esculetin destroyed cervical cancer cells *via* the ER stress pathway.

CONCLUSIONS: The results showed that esculetin may have a potent antitumoral effect. It can potentially be utilized in the pharmacological therapy of cervical cancer.

Key Words:
Esculetin, Natural product, Coumarin derive, Cancer treatment, Anticancer effect.

Abbreviations

ATF3: Activating transcription factor 3; ATF4: Activating transcription factor 4; ATF-6: Activating transcription factor 6; CANX: Calnexin; CHOP: C/EBP homologous protein; DMEM: Dulbecco's modified Eagle's medium; EDEM: Endoplasmic reticulum degradation-enhancing alpha-mannosidase-like 1; ER: Endoplasmic reticulum; ERO1: Endoplasmic reticulum Oxidoreductase 1 alpha; FBS: Fetal bovine serum; GAPDH: Glyceric acid phosphate dehydrogenase; GRP78: Glucose-regulated protein 78; HEK293: Human embryonic kidney cell line; HeLa: Human cervical cancer-derived cells; IC_{50}: Inhibitory concentration 50%; IRE-1: Serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme; MTT: Methyl-thiazolyl-tetrazolium proliferation assay; UPR: Unfolded protein response; PERK: Proteins like protein kinase R-like endoplasmic reticulum kinase; PDI: Protein disulfide isomerase; QPCR: Quantitative real-time polymerase chain reaction; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; XBP1: X-box binding protein 1.

Introduction

Despite HPV screening programs and effective vaccination, cervical cancer continues to be detected more than expected worldwide. Traditionally, cervical cancer treatment consists of surgery, radiotherapy, chemotherapy, or their combinations, but treatment with natural substances with fewer side-effect profiles is under investigation. It has been reported[1,2] that natural compositions possess therapeutic potential in alternative treatments of cervical cancer. Many of these natural products work through different pathways to kill cancer cells, such as stimulating Endoplasmic Reticulum (ER) stress and ER stress-related apoptosis[3].

ER supports proper folding to synthase functional proteins. Tumor microcirculation could destroy the function of ER, therefore resulting in the gathering accumulation of unfolded proteins into the ER, called ER stress. This ER stress may have a relationship with cancer. While ER stress induces an unfolded protein response (UPR) to restore homeostasis, sustained ER stress can trigger apoptosis[4,5].

The high rates of protein translation are associated with tumor metabolism and cancer cell proliferation, and it consequently can induce ER

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stress and UPR. An active UPR creates a stress response *via* ER sensor proteins, such as the activation of transcription factor 6 (ATF-6), serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme (IRE-1), and protein kinase R-like endoplasmic reticulum kinase (PERK). Interestingly, on the other hand, if the stress is severe, constant, and cannot be resolved, the UPR actuates an ER stress-related cell death pathway by up-regulating the downstream target pro-apoptotic C/EBP homologous protein (CHOP) *via* activating transcription factor 4 (ATF4)[6].

Esculetin is a coumarin derivative natural product with anti-inflammatory, anti-apoptotic, and antioxidant effects[7]. Many studies[8-10] have revealed that esculetin has anticancer effects in human colon cancer, cervical cancer, leukemia, and breast cancer. The anticancer effect of esculetin may be related to the inhibition of cell viability and apoptosis by acting multiple signaling cascades through the activation of specific genes[1]. We aimed to show the antitumoral effect of esculetin in human cervical cancer-derived cell lines *via* ER stress.

Materials and Methods

In order to analyze the anti-tumor effects of esculetin on ER stress in human cervical cancer-derived (HeLa) cells, the effective dose of esculetin in cell culture with an inhibitory concentration of 50% (IC_{50}) was determined using methyl thiazolyl tetrazolium (MTT) assay. The effective dose of esculetin (IC_{50}) was applied to HeLa cells, and a quantitative real-time polymerase chain reaction (qPCR) was implemented to investigate gene expression levels; also, Western blotting was conducted to examine protein expression levels.

Cell Culture

In this study, a HeLa cell line and a human embryonic kidney cell line (HEK293) were used as controls. After obtaining the HeLa cell line from the American Type Culture Collection (ATCC, Manassas, Virginia, USA), it was cultured in Dulbecco's Modified Eagle Medium (DMEM, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, Hamburg, Germany) and 1% penicillin/streptomycin antibiotics (Waltham, Massachusetts, USA) in a humidified incubator at 37°C with 5% CO_2. When the cells covered 80-90% of the flask surface, they were passaged and stored.

MTT Proliferation Assay

The viability and effective dose (IC_{50}) of esculetin on HeLa cells were measured by a standard method, the MTT proliferation assay. The HeLa cells were seeded with 100 µl fresh culture medium per well in 96-well plates (~4.5-5x10^3 cells). After the cells were cultured for 48 h, the esculetin was applied to the wells at concentrations of 2.5 µM, 5 µM, 25 µM, 50 µM, 100 µM, 200 µM, 400 µM, 800 µM, and 1,000 µM at adjusted doses for 24, 48, and 72 h. The esculetin-treated and untreated control cells were treated with 10 µl of a 12 mM MTT solution (Sigma-Aldrich, Hamburg, Germany) and incubated at 37°C for 4 hours. The medium was then removed in a dark cell culture room.

Dimethyl sulfoxide (DMSO, 50 µl, Sigma-Aldrich, Hamburg, Germany) was added to the wells, which were gently shaken for 20 min to dissolve the crystal blue melts. The absorbance at 575 nm was recorded using the Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the IC_{50} dose of esculetin was determined to be 800 µM.

The Application of IC_{50} Value to Cells, Total RNA Extraction and Synthesis cDNA

The HeLa and HEK293 cells were again treated 48 hours after seeding with the IC_{50} dose (800 µM) of esculetin in a cell culture medium. All cell groups, including the control group, were subjected to the classical RNA isolation procedure by applying the TRIzol (Sigma-Aldrich, Hamburg, Germany), chloroform, and isoamyl alcohol method. The RNA pellet was precipitated with 75% absolute ethanol (96%, v/v) and dissolved in nuclease-free water. cDNAs were synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) of total RNA equalized to 1 µg according to the manufacturer's instructions.

qPCR

A qPCR analysis was performed for all genes in triplicate with the QuantStudio™3 Real-Time PCR system (Applied Biosystems, Waltham, MA, USA). The nucleotide sequences of primer pairs ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1), activating transcription factor-3 (ATF3), ATF4, ATF6, X-box binding protein-1 (XBP-1), CHOP, glucose-regulated protein 78 (GRP78) and IRE1 in the ER stress pathway used for quantitative gene expression are

given in Figure 1. A thermal profile followed by melting curve analysis steps was performed for 15 minutes at 95°C, 40 cycles at 95°C for 15 seconds, 56-60°C for 30 seconds, and 72°C for 15 seconds. The comparative ΔCT method and glyceraldehyde 3-phosphate dehydrogenase (GAPHD) as a housekeeping gene were used to compute the relative quantification of gene expression.

Western Blotting

Dose-treated (IC_{50}) and untreated (control) HeLa and HEK293 (esculetin-treated and untreated) cells were homogenized in a RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% nonidet P-40) containing a protease inhibitor cocktail (Sigma-Aldrich, Hamburg, Germany). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 6-10% acrylamide/bisacrylamide gels and transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were probed with the following primary ER stress pathway antibodies: PERK (D11A8) Rabbit mAb 5683, PDI (C81H6) Rabbit mAb 3501, BIP (C50B12) Rabbit mAb 3177, GRP78/HSPA5 antibody, Ero1-La antibody 3264, IRE1α (14C10) Rabbit mAb 3294, Calnexin (CANX) (C5C9) Rabbit mAb 2679, CHOP (L63F7) Mouse mAb 2895 and HRP-linked antibody (Cell Signaling Technology, Danvers, MA, USA).

The presence of the antigens was determined using an enhanced chemiluminescence detection kit (Amersham Bioscience, Piscataway, NJ, USA). Their Western blot profiles were exposure recorded and captured on a RAS-4000 image reader (Fujifilm, Minato City, Tokyo, Japan). Band profiles were further analyzed using Image J (Bethesda, MD, USA) imaging and the analysis program.

Statistical Analysis

SPSS version 21.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Based on the Student's *t*-test results, comparisons were made between the control and treated groups for the genes analyzed. The levels of ATF4, ATF6, ATF3, EDEM1, CHOP, XBP1, GRP78, and IRE1 expressions were analyzed by the $2^{-\Delta\Delta CT}$ method developed by Livak and Schmittgen[11]. Statistical significance was accepted for $p<0.05$.

Results

Esculetin Inhibited Cell Proliferation in HeLa Cells

Esculetin inhibits the proliferation of HeLa cell lines *in vitro via* ER stress. To analyze the impact of esculetin on cell viability, HeLa cells were exposed to different concentrations of esculetin (2.5, 5, 25, 50, 100, 200, 400, 800, and 1,000 µM) for 24, 48, and 72 h, and the cell viability was evaluated by MTT. The results indicate that esculetin significantly blocked the growth of HeLa

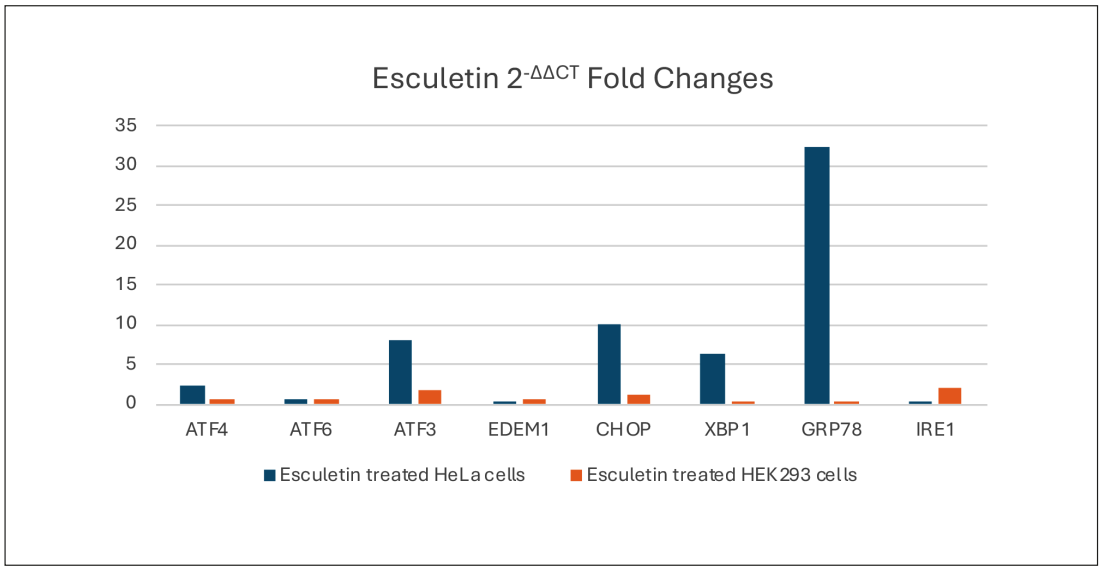

Figure 1. Expression of results of genes associated with ER stress pathway analyzed using the delta CT method.

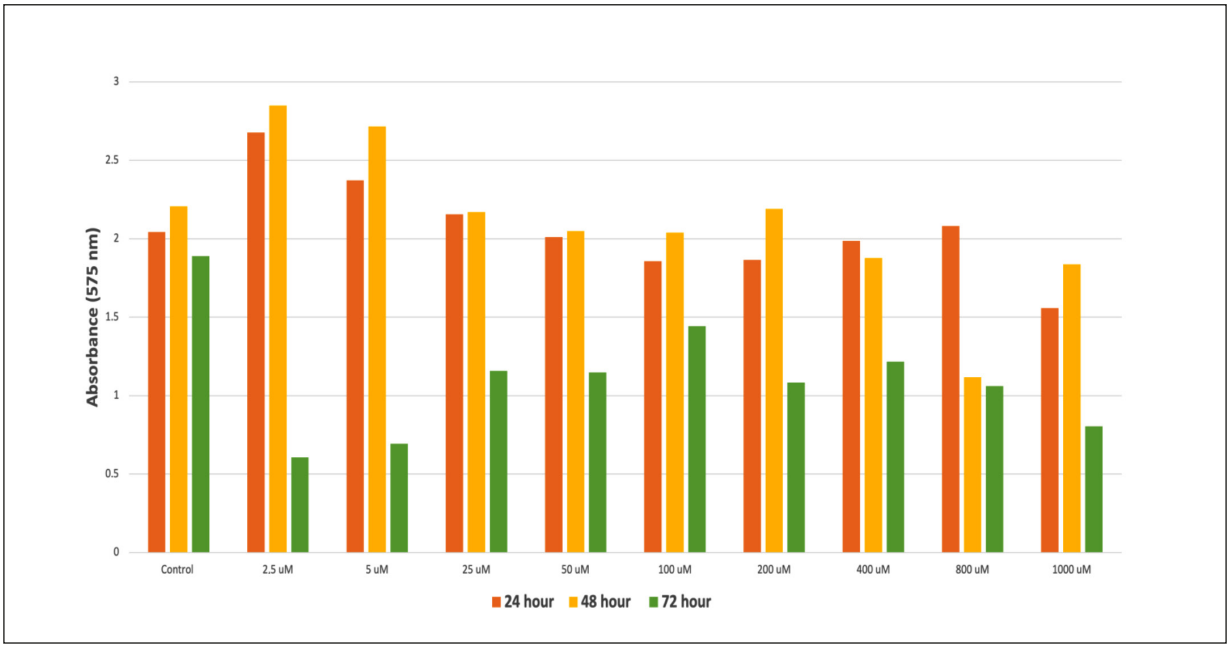

Figure 2. HeLa cells viability, MTT results. Esculetin after the administration for 0, 24, 48, and 72 hours of treatment. The results were represented as the percentage of viable cells.

cells in a dose-dependent manner (Figure 2). The results also show that esculetin clearly exhibits an anti-proliferative effect on HeLa cells. The IC_{50} dose of esculetin was determined as 800 µM for 48 hours using the results of this analysis, indicating that esculetin has a strong effect against the proliferation of HeLa cells at this dose.

Esculetin-Induced ER Stress in HeLa Cells

The expression analysis of genes associated with ER stress was carried out by a real-time PCR analysis involving the treated groups of HeLa and HEK293 cells. We evaluated the levels of ATF3, ATF4, ATF6, EDEM-1, CHOP, XBP-1, GRP78, and IRE-1 genes to analyze ER stress (Figure 1). The real-time PCR analysis demonstrated that esculetin increased the mRNA levels of ATF4, ATF3, CHOP, XBP-1, and GRP78 but decreased ATF6, EDEM-1, and IRE1 in a dose-dependent manner in HeLa cells.

At this point, to obtain the true fold change, the log base of this value was taken to even out the scales of up-regulated and down-regulated genes. Otherwise, the up-regulated genes have a scale of 1-infinity, while the down-regulated ones have a scale of 0-1. An increase of 2 times in ATF4, 8 times in ATF3, 6 times in XBP1, 32 times in GRP78, and 10 times in CHOP was observed. The values were all significant, $p<0.05$ (Figure 1).

ER stress has been observed in cells treated with esculetin in cervical carcinomas. Western blot analysis was then used to identify the impact of esculetin on GRP78; PERK; Protein Disulfide-isomerase (PDI); IRE1; ER oxidoreductase 1 alpha (ERO1); CANX, a molecular marker of ER stress; and CHOP, a transcription factor involved in ER stress-induced apoptosis (Figure 3). The analysis demonstrated that esculetin increased the protein levels of GRP78, ERO1, CANX, CHOP, and PDI and decreased PERK and IRE1 in a dose-dependent manner in HeLa cells (Figure 4). The reduction of PERK and IRE1 may potentially cause the inhibition of tumor development. A further finding of this study is that the increase in GRP78, CHOP, and ERO1 levels may support the inhibition of cancer cell proliferation *via* ER stress-induced apoptosis.

Discussion

Cervical cancer remains common in gynecological cancers despite effective screening methods and vaccination. Treatment strategies that have fewer side effects and are less toxic are being studied[12] in the treatment of cervical cancer. The use of natural compounds in the treatment of cervical cancer was investigated by Dasari et al[1]. Various plant-derived bioactive agents have been shown[13,14]

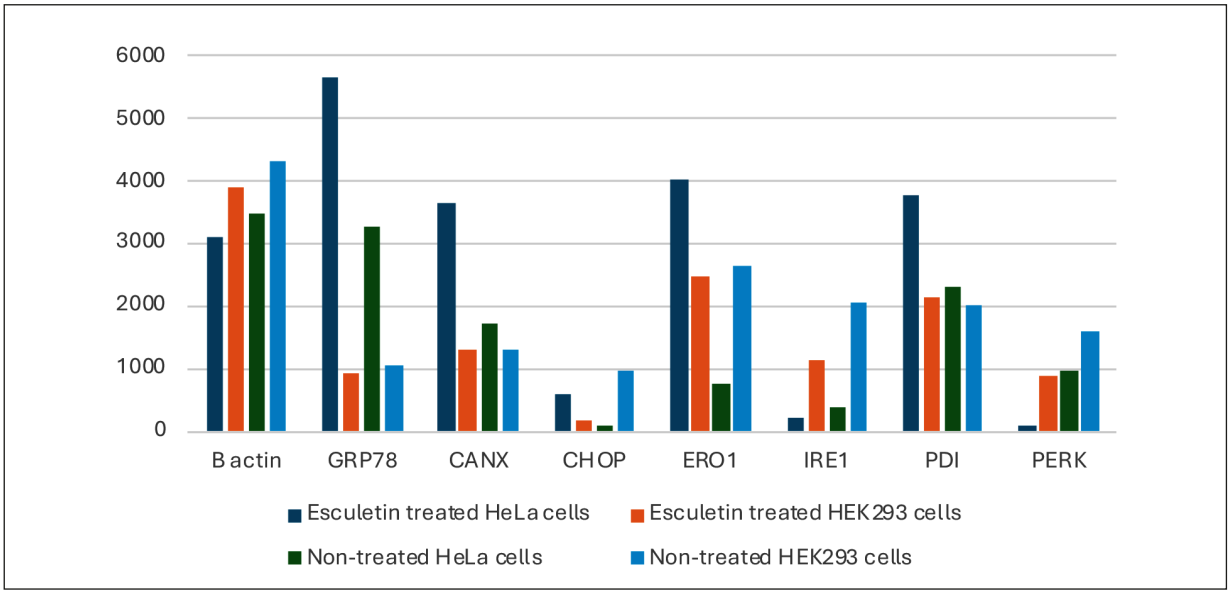

Figure 3. Western blot analysis of the levels of GRP78, ERO1, IRE1, CANX, PDI, PERK, and CHOP after esculetin treatment.

to have anti-tumor activity. Natural compounds or their bioactive agents have been reported[3] to increase apoptosis and decrease resistance to chemotherapies by inducing ER stress. Coumarins are substances known to have anticancer activity. A coumarin derivative called esculetin is an herbal substance used in traditional Chinese medicine. Studies[15] have indicated that esculetin has an inhibitory effect on free radicals and has anticancer and chemopreventive effects. Esculetin has been found[16] to have anti-tumor activities in leukemia, colon cancer, gastric cancer, and lung cancer through various cellular mechanisms.

ER is an organelle that provides the calcium balance necessary for cell homeostasis and is responsible for protein synthesis. A dysfunctional ER leads to the accumulation of unfolded proteins, thereby triggering the ER stress, which activates GRP78 and UPR and the induction of PERK, IRE1, and ATF6. Nevertheless, when ER stress is violent and permanent, the UPR mediators that maintain the cell viability may initiate apoptosis and, ultimately, termination of damaged cells[5,17]. It is still debated whether the UPR increases or decreases tumor development. Moreover, increases in UPR-connected factors have been found[18] in patients with various cancer types, and their excessive increase is related to poor prognosis and treatment resistance.

There are fewer studies in the literature showing the antitumor effectiveness of esculetin on cervical cancer. Therefore, the role of esculetin-induced ER stress in cervical cancer is unclear. GRP78 acts as a chaperone in the ER lumen and is found bound to PERK, IRE1, and ATF6 on the ER membrane. Increased levels of unfolded proteins cause GRP78 to sequester from membrane proteins and bind to unfolded proteins. GRP78 levels are maintained at relatively low levels within the cell. Its levels increase significantly under stresses that affect ER homeostasis[19]. Transcription factors produced under ER stress, such as CHOP, are an important mediator of apoptosis. If CHOP is stimulated when protein folding is suppressed, proapoptotic signals are exacerbated. It plays a role in ER stress-induced apoptosis by reducing B-cell lymphoma/leukemia-2 (Bcl-2) expression, increasing proapoptotic proteins such as ERO1, and initiating cytochrome c release[20]. Regarding esculetin-activated ER stress, in this study, we demonstrated that the expression of ER stress markers like ERO1, GRP78, and CHOP was increased by using Western blotting. On the other hand, esculetin decreased the expression of CHOP, GRP78, and ERO1 in the control group. Furthermore, the mRNA expression of GRP78, XBP1, CHOP, ATF 3, and ATF 4 were more elevated than the control group. This finding may show that esculetin affects normal cells less than tumoral cells.

Studies have identified the role of the PERK, ATF6, and IRE1α pathways in tumor development *in vivo*. They are associated with tumor growth and aggressiveness. PERK signal protein has been determined[21] to play a role in the de-

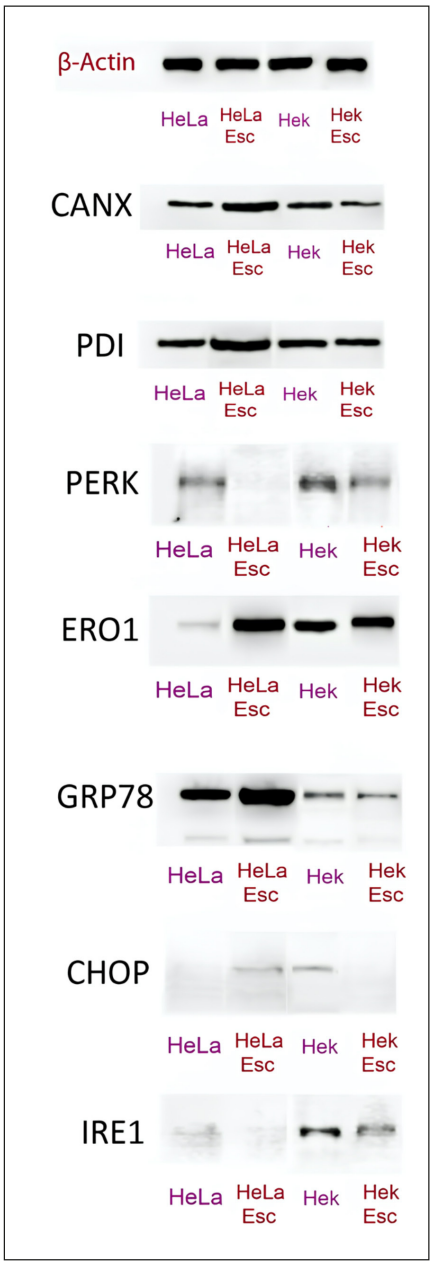

Figure 4. The protein bands for GRP78, ERO1, IRE1, CANX, PDI, PERK, and CHOP. Western blotting to probe for changes in protein abundance with differentiation.

velopment and progress of different tumors, and PERK protein deficiency has been associated with increased survival. Also, the IRE1 signaling protein has been associated with tumor progression, metastasis, and resistance to chemotherapy. The incorporation of cancer cells deleted PERK or IRE1α in mice caused tumor growth arrest despite the increased sensibility of cancer cells to ER stress upregulating agents[22]. The decrement of PERK signaling in malignant cells makes them highly vulnerable to cell demise induced after exposure to hypoxia, nutrient deprivation, and DNA damage[23]. Thus, blocking PERK results in cancer cell apoptosis and obvious antitumor influences[24]. ATF6 is expressed at high levels in recurrent tumors while also reducing tumor growth through inhibition of the mTOR pathway. In this study, Western blot analysis detected that PERK and IRE1 levels decreased, and PCR analysis detected that ATF6 and IRE1 decreased after esculetin application in HeLa cells.

The anticancer activity of anticancer agents is frequently associated with their capability to trigger tumor cell apoptosis. If ER stress is serious and permanent, ER cannot provide cell homeostasis, and pro-apoptotic pathways are activated. The ER induces and regulates apoptosis. The induction of apoptosis by natural compounds through heat stress is being investigated. In various cancer cells, ER stress can block the cell cycle and cause apoptosis by application of natural compounds. Increased apoptosis by ER stress can be used as an anticancer treatment[25,26]. On the other hand, in this study, CHOP, an important mediator of ER stress-induced apoptosis, and ERO1, a pro-apoptotic protein, increased in Western Blot analysis. ATF4, XBP1, and CHOP increased in PCR analysis. Therefore, the significant increase in CHOP and ERO1 levels may support the inhibition of cancer cell proliferation *via* ER stress-induced apoptosis.

Conclusions

As a result, it can be concluded indirectly that ER stress plays a role in the esculetin-induced apoptosis of cervical cancer cells. The study showed that esculetin can activate ER stress and apoptosis in HeLa cells based on the following findings: esculetin meaningfully raised the ER stress markers expression like GRP78, CHOP, ERO1; esculetin strongly increased the expression of CHOP, ERO1 and ATF4, important mediators of ER stress-activated apoptosis; and esculetin decreased the expression of prosurvival pathways like IRE-1 and PERK in HeLa cells. Antitumor activity of natural compounds through ER stress should be supported by further studies.

Conflict of Interest

The authors declare that they have no conflict of interest.

Informed Consent
Not applicable due to the design of the study.

Ethics Approval
This experimental study was carried out with decision number 25, dated 16/01/2019, from the Clinical Research Ethics Committee of Selcuk University Faculty of Medicine.

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Availability of Data and Materials
All data generated or analyzed during this study are included in this published article; the datasets are available from the corresponding author upon reasonable request.

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