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Esculetin Inhibits the Survival of Human Prostate Cancer Cells by Inducing Apoptosis and Arresting the Cell Cycle

ORIGINAL ARTICLE

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Background: Prostate cancer (PCa) is one of the most important causes of death in men and thus new therapeutic approaches are needed. In this study, antiproliferative and anti-migration properties of a coumarin derivative esculetin were evaluated.
Methods: Human PCa cell lines PC3, DU145, and LNCaP were treated with various concentrations of esculetin for 24 to 72 hours, and cell viability was determined by the MTT test. Cell cycle and apoptosis were analyzed by using cell-based cytometer. Gene expression levels were assessed by reverse transcription and quantitative real-time PCR, cell migration was determined by the wound healing assay. The protein expression was measured by Western blotting.
Results: Esculetin inhibited cell proliferation in a dose- and time-dependent manner. Cell migration was inhibited by esculetin treatment. Administration of esculetin significantly reduced the cells survival, induced apoptosis and caused the G1 phase cell cycle arrest shown by image-based cytometer. The induced expression of cytochrome *c*, p53, p21 and p27, and down-regulated CDK2 and CDK4 may be the underlying molecular mechanisms of esculetin effect. Esculetin suppressed phosphorylation of Akt and enhanced protein expression of tumor-suppressor phosphatase and tensin homologue.
Conclusions: Our findings showed that the coumarin derivative esculetin could be used in the management of PCa. However, further in vivo research is needed.
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Key Words: Esculetin, Apoptosis, Cell cycle, Prostate cancer, Cancer prevention

INTRODUCTION

Prostate cancer (PCa) is the most common cancer type of men in the world, and unfortunately, 10% of the disease results in death.[1] Though widely investigated, there has been no precise therapy of PCa especially androgen-independent metastatic PCa challenging. Therefore, novel treatment strategies are required. Phytochemicals are widely used in the folk medicine, and epidemiological investigating shows that regular consumption of natural flavonoids may be associated with a decreased risk of selected cancers.[2]

Coumarin-derived compounds have been shown to exhibit various therapeutic effects, such as anti-inflammation, antioxidant, and antitumor activities.[3,4] Esculentin (6,7-dihydroxy coumarin) is a naturally occurring coumarin compound that is mostly obtained from *Aesculus hippocastanum*, *Cichorium intybus, Artemisia scoparia*, and in the leaves of *Citrus limonia*.[5] Structurally, it consists of a pyron and a benzene ring conjugated structure, with electron and charge transport properties which is leading toxicity in cancer cells [3] with low toxicity and limited side effects.[6] Pharmacokinetic studies have shown that after oral administration, the plasma level of esculetin reaches the highest level within 5 minutes and the half-life in plasma is 45 minutes.[7] Due to a very rapid absorption and tissue distribution, esculetin might be an important potential in therapy. Esculetin inhibits cell proliferation by targeting β-catenin pathway in HCT116 human

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colon cancer cells,[8] induces apoptosis via mitochondrial pathway in HT-29 colon cancer cells,[9] increases reactive oxygen species production and initiates mitogen-activated protein kinase activation in HL-60 leukemia cells, which causes the G1 phase cell cycle to arrest.[10]

Although survival rates have increased significantly over the years, PCa is an important health problem for men around the world. The molecular mechanisms underlying the progression of PCa have not yet been fully elucidated, but are known to be the major risk factors for genetics, aging, and western diet. Phosphatase and tensin homologue (PTEN) plays an important role in proliferation and survival of PCa cells as a tumor suppressor gene, primarily through its negative regulation of the phosphatidylinositol-4,5-biphosphate-3-kinase (PI3K), which in turn, results in decreased Akt activity.[11,12] In the early stages of the disease, most patients respond to existing treatment strategies, but an androgen-independent stage of malignancy in aggressive and spreading of PCa is challenging to treat.[13]

Considering present data, esculetin has an effect on cellular pathways preventing cancer development. However its effect on PCa has not yet been investigated. Here, we have examined the potential therapeutic properties of esculetin and its underlying molecular mechanisms in human LNCaP, DU145, and PC3 PCa cells. LNCaP cells respond to androgens (androgen-dependent) by endogenously exerting androgen receptor (AR), and these cells therefore represent the initial phase of PCa. However, since PC3 and DU145 cells do not carry AR, they are not susceptible to androgens (androgen-independent) and represent the metastatic stage of the disease.

MATERIALS AND METHODS

1. Cell culture

Human PCa cell lines were obtained from ATCC (Manassas, VA, USA). The cells were seeded in complete DMEM/F-12K medium (DMEM/Ham's F-12 Mix 50/50 (Winsent; St-Bruno-de-Montarville, QC, Canada) with 2 mM L-glutamine and 1,500 mg/L sodium bicarbonate, 10% FBS, 1% penicillin-streptomycin and maintained at 37°C in a humidified incubator at 5% CO_2. Esculetin (Santa Cruz Biotechnology, Dallas, TX, USA) was dissolved in 100% dimethyl sulfoxide (DMSO) (Hybri-Max; Sigma Aldrich, Steinheim, Germany) and stored at −20°C until use, and did not exceed the final concentration of DMSO in culture. The final concentration of DMSO did not exceed 0.1%, and the controls received the same volume of vehicle.

2. Cell viability assay

Cells were cultured in 96-well plates and treated with esculetin at various concentrations (9.3-600 μM) after 16 hours of incubation. After the incubation period the cells were incubated with 1 mg/mL of MTT (Sigma-Aldrich, St. Louis, MO, USA) solution were added to each well. The culture was incubated at 37°C for 3 hours to form formazan crystals. A total of 200 μL of DMSO was added to each well to dissolve formazan crystals, and then the absorbance of each well was measured at 570 nm in plate reader (Multiskan GO; Thermo Scientific, Vantaa, Finland). Percentage of cell viability was calculated according to control.

3. Annexin V/propidium iodide staining assay

After the incubations, PC3, DU145, and LNCaP cells at a density of 2.5×10^5 were cultured in a 6-well plate for 16 hours and then treated with 25 μM, 75 μM, and 150 μM esculetin for 48 hours. The cells were then washed twice with ice-cold PBS, suspended in annexin V binding buffer (ABB) and incubated with Annexin V Alexa Fluor 488 (Invitrogen/Life Technologies, Carlsbad, CA, USA)

Table 1. Primer sequences used in this study for reverse transcription and quantitative real-time PCR analysis

Genes and accession number	Primers
TNF-R1	F: 5′-AGGGGACAGGGAGAAGAGAG-3′
NM_001346092.1	R: 5′-GTGCACACGGTGTTCTGTTT-3′
Bcl-2	F: 5′-ATGTGTGTGGAGAGCGTCAA-3′
NM_000633.2	R: 5′-ACAGTTCCACAAAGGCATCC-3′
Caspase 3	F: 5′-GGCATTGAGACAGACAGTGG-3′
NM_001354779.1	R: 5′-CATGGAATCTGTTTCTTTGC-3′
Caspase 8	F: 5′-CTGCTGGGGATGGCCACTGTG-3′
XM_005246894.3	R: 5′-TCGCCTCGAGGACATCGCTCTC-3′
Bax	F: 5′-TTGCTTCAGGGTTTCATCCA-3′
XM_017027077.1	R: 5′-CAGCCTTGAGCACCAGTTTG-3′
Cytochrome *c*	F: 5′-AGTGGCTAGAGTGGTCATTCATTTACA-3′
NM_018947.5	R: 5′-TCATGATCTGAATTCTGGTGTATGAGA-3′
$p21^{Cip1}$	F: 5′-GGCGTTTGGAGTGGTAGAAA-3′
NM_001291549.1	R: 5′-GACTCTCAGGGTCGAAAACG-3′
$p27^{Kip1}$	F: 5′-CCGGCTAACTCTGAGGACAC-3′
NM_004064.4	R: 5′-TTGCAGGTCGCTTCCTTATT-3′
p53	F: 5′-GAGGTTGGCTCTGACTGTACC-3′
NM_001126118.1	R: 5′-TCCGTCCCAGTAGATTACCAC-3′
CDK2	F: 5′-GTACCTCCCCTGGATGAAGAT-3′
NM_001290230.1	R: 5′-CGAAATCCGCTTGTTAGGGTC-3′
CDK4	F: 5′-CTGGTGTTTGAGCATGTAGACC-3′
NM_000075.3	R: 5′-GATCCTTGATCGTTTCGGCTG-3′
Cyclin D1	F: 5′-CGTCCATGCGGAAGATC-3′
NM_053056.2	R: 5′-CAGAGGGCAACGAAGGT-3′
Tubulin	F: 5′- ATGAGTATGCCTCCCCTGTG-3′
NM_004048.2	F: 5′-CAAACCTCCATGATGCTGCTTAC-3′

at room temperature (RT) in the dark for 15 minutes. Following centrifugation at 300 ×*g* for 5 minutes, the cells were again resuspended in ABB and allowed to incubate with propidium iodide (PI) in the dark for 5 minutes at RT. Annexin V/PI was evaluated by Tali image-based cytometer within 30 minutes (Invitrogen/Life Technologies).

4. Cell cycle analysis

PC3, DU145, and LNCaP cells were cultured and treated as described in above section (3. Annexin VI/PI staining assay). Subsequently, cells were trypsinized and fixed in 70% ethanol and incubated at −20°C, overnight. Next, the cells were washed in PBS, and incubated with propidium iodide kit (Tali Cell Cycle Kit; Life Technologies, Carlsbad, CA, USA) at RT for 30 minutes. Analysis were performed according to the manufacturer's instructions using image-based cytometer. The results were stated as percentage of G0/G1, S, and G2/M cells.

5. Reverse transcription and quantitative real-time PCR

Briefly, total RNA was extracted from PC3 cells treated with various concentrations of esculetin for 48 hours using commercial isolation kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, cDNA samples were synthesized from mRNA template by using cDNA synthesis kit (Thermo Fisher Scientific). Quantitative mRNA expression analyses were achieved on a Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix (Thermo Fisher Scientific). The PCR cycling conditions included an initial denaturation at 95°C for 5 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 45 seconds and 72°C for 15 seconds. The fold enrichments of the targets were calculated using the relative quantification according to the $2^{-\Delta\Delta Ct}$ methods. Oligonucleotide primers (Table 1) were synthesized by PRZ Biotech (Ankara, Turkey). Tubulin expressions were used as an internal reference. Each sample was amplified in triplicate wells. The experiments were performed two or three times on separate occasions using different RNA.

6. Wound healing assays

Cell motility was evaluated using wound-healing assay. PC3 cells were plated in 6-well plates with serum-free medium to

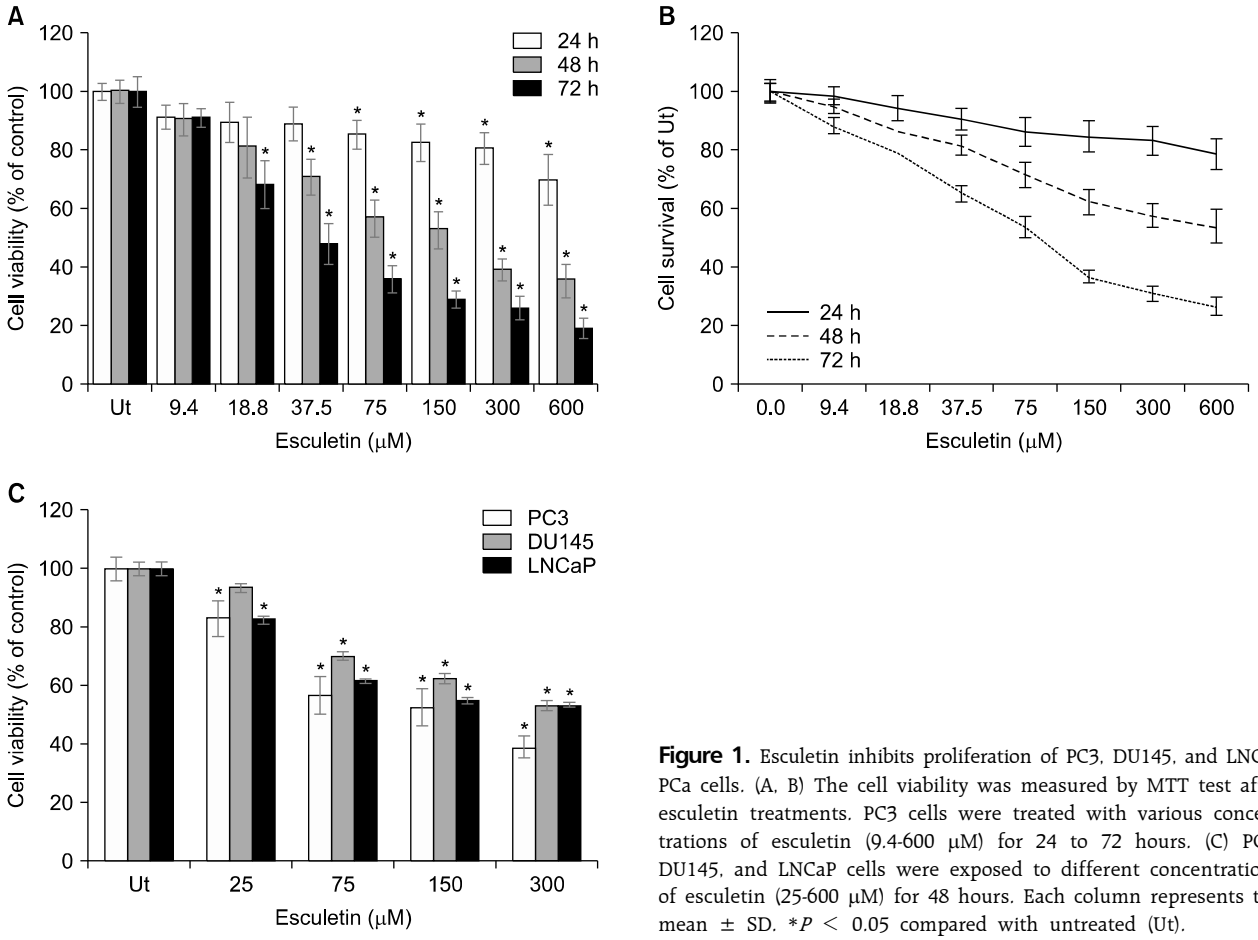

Figure 1. Esculetin inhibits proliferation of PC3, DU145, and LNCaP PCa cells. (A, B) The cell viability was measured by MTT test after esculetin treatments. PC3 cells were treated with various concentrations of esculetin (9.4-600 μM) for 24 to 72 hours. (C) PC3, DU145, and LNCaP cells were exposed to different concentrations of esculetin (25-600 μM) for 48 hours. Each column represents the mean ± SD. **P* < 0.05 compared with untreated (Ut).

reach 80% to 90% confluence. The cell monolayers were scratched with a 200 μL micropipette tip across the center of each well, and treated with 25 μM, 75 μM, and 150 μM esculetin and allowed to migrate. The resulting wounded healing were monitored at indicated time-points by phase contrast microscopy with a 20× objective and determined by an inverted microscope. The experiments were repeated twice.

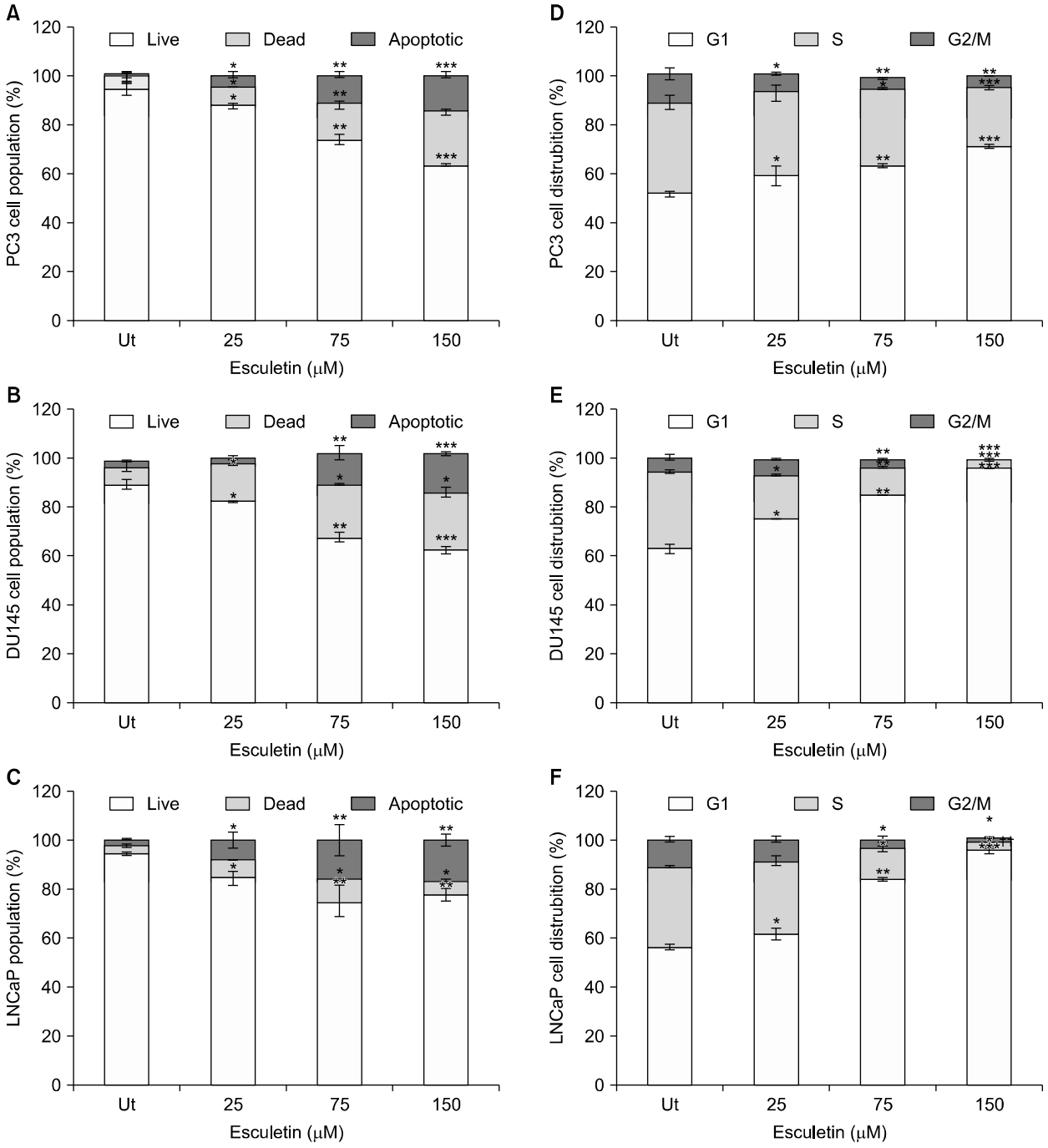

Figure 2. Esculetin dose-dependently induces apoptosis and cell cycle arrest of PC3, DU145, and LNCaP cells. The cells were treated with 25 μM, 50 μM, and 150 μM esculetin for 48 hours, then apoptosis rates and cell cycle analyses were determined by image-based cytometer. Live, dead, and apoptotic populations of (A) PC3, (B) DU145, and (C) LNCaP cells. Esculetin administration caused cell cycle arrest at G1 phase in (D) PC3, (E) DU145, and (F) LNCaP cells. *$P < 0.05$ vs. untreated (Ut), **$P < 0.01$ vs. Ut and 25 μM esculetin, ***$P < 0.01$ vs. Ut, 25 and 75 μM esculetin groups.

7. Protein expression analysis

Total protein was isolated from PC3 cells treated with 25 μM, 75 μM, and 150 μM of esculetin for 48 hours, using radioimmunoprecipitation assay lysis buffer (25 mM Tris/HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS with protease inhibitors). Proteins (50 μg) were separated by 8% to 12% polyacrylamide gel, and then proteins were transferred onto polyvinylidene difluoride membrane (Technologies, Carlsbad, CA, USA). Membranes were incubated overnight at 4°C with primary cytochrome *c* and β-actin (Novus Biologicals, Littleton, CO, USA), PTEN, and pAkt1/2/3 (Thr 308) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) antibodies followed by incubations with secondary antibodies for 1 hour at RT. The immunoreactive bands were detected using an enhanced chemiluminescence Western blot substrate kit (Thermo Fisher Scientific) by gel imaging system (Bio-Rad ChemiDoc MP System, Carlsbad, CA, USA).

8. Data analysis

The data obtained from treated and untreated cells were analyzed using one-way ANOVA, followed by Duncan's multiple range tests for multiple comparisons using the statistic software IBM SPSS for Windows ver. 19.0 (IBM Co., Armonk, NY, USA). $P \leq 0.05$ was considered statistically significant. Each experiment was performed in triplicate and was repeated at least twice; each data point represents the mean ± SD.

RESULTS

1. Effects of esculetin on prostate cancer cell survival

In this study, PC3 PCa cells were treated with various concentrations of esculetin (9.4-600 μM) for 24 hours, 48 hours, and 72 hours. The results of MTT assay showed that esculetin significantly inhibited PC3 cell proliferation in a dose- and time-dependent manner (Fig. 1A and 1B). Esculetin suppressed DU145 and LNCaP cell proliferation similar to that of PC3 cells (Fig. 1C).

2. Esculetin induces apoptosis of prostate cancer cells

In consistence to the data of MTT and Annexin V/PI assay confirmed the induction of apoptosis of PC3 (Fig. 2A), DU145 (Fig. 2B), and LNCaP cells (Fig. 2C). PCa cells undergo apoptosis after the incubations of 25 μM, 75 μM, 150 μM esculetin for 48 hours and more apoptotic cells were identified with higher concentrations. For instance, PC3 cells treated with 25 μM, 75 μM, and 150 μM esculetin induced apoptosis by 0.5%, 6.25%, and 20%, respectively.

The underlying molecular mechanism of apoptosis induced by esculetin in PC3 cells was evaluated by using reverse transcription and quantitative real-time PCR (RT-qPCR). After treatment with esculetin (75 μM, 150 μM, and 300 μM) for 48 hours, significant increase in the mRNA expressions of *TNF-R1*, *caspase-8*, *-3*, *Bax*, and cytochrome *c* were detected ($P < 0.05$), while there was a marked decrease of *Bcl-2* mRNA (Fig. 3A). Western blotting result has also revealed that the level of

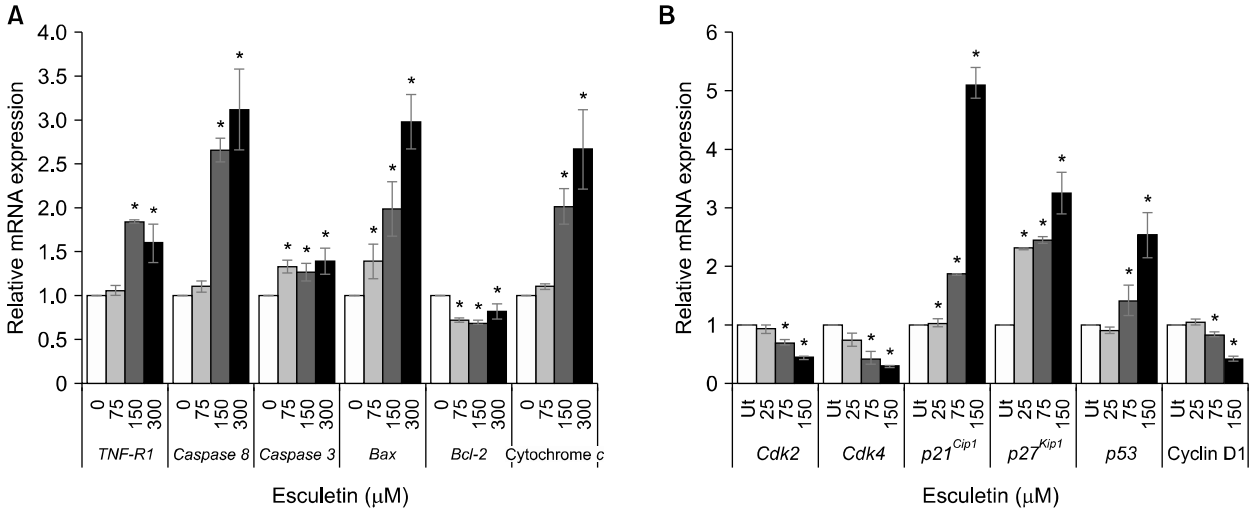

Figure 3. Effect of esculetin on apoptosis- and cell cycle-related gene expression. The cells were treated with 25 μM, 75 μM, 150 μM, and 300 μM of esculetin for 48 hours, and then targeted mRNA expressions were analyzed by reverse transcription and quantitative real-time PCR. (A) The fold changes of apoptosis-associated genes and (B) cell cycle related genes were plotted on graphs. For each group, two different experiments were performed in triplicate (mean ± SD). *$P < 0.05$ vs. untreated (Ut) and 0.

cytochrome *c* protein is dose-dependently increased by esculetin treatment in PC3 cells (Fig. 4C and 4D).

3. Esculetin causes G1 phase cell cycle arrest in prostate cancer cells

In order to determine whether esculetin regulates the cell cycle phases, we used cell-based cytometry and RT-qPCR techniques. Esculetin triggered significant G1-phase cell cycle arrest in PC3, DU145, and LNCaP cells (Fig. 2D-2F). Incubation with 75 μM of esculetin for 48 hours significantly elevated the percentage of cells in G1 phase increased from 72.5% to 82.8%, 63% to 85%, and 56.5% to 83.7% in PC3, DU145, and LNCaP, respectively (Fig. 2D-2E). Esculetin therapy decreased the population at the S and G2/M phase in all cell lines.

The quantification of mRNA expression showed that esculetin significantly induced $p21^{Cip1}$ and $p27^{Kip1}$, while down-regulated cyclin dependent kinase 2 (*CDK2*), *CDK4*, and cyclin D1 (Fig. 3B). Esculetin significantly up-regulated *p53* mRNA, which regulates the *p21* gene, mediated cell cycle G1 phase arrest (Fig. 3B).

4. Esculetin inhibits PC3 cell migration

The effect of esculetin on migratory capacity of PC3 cells was determined by wound healing assay. Esculetin treatment resulted in prevention of wound gap closure in a concentration-

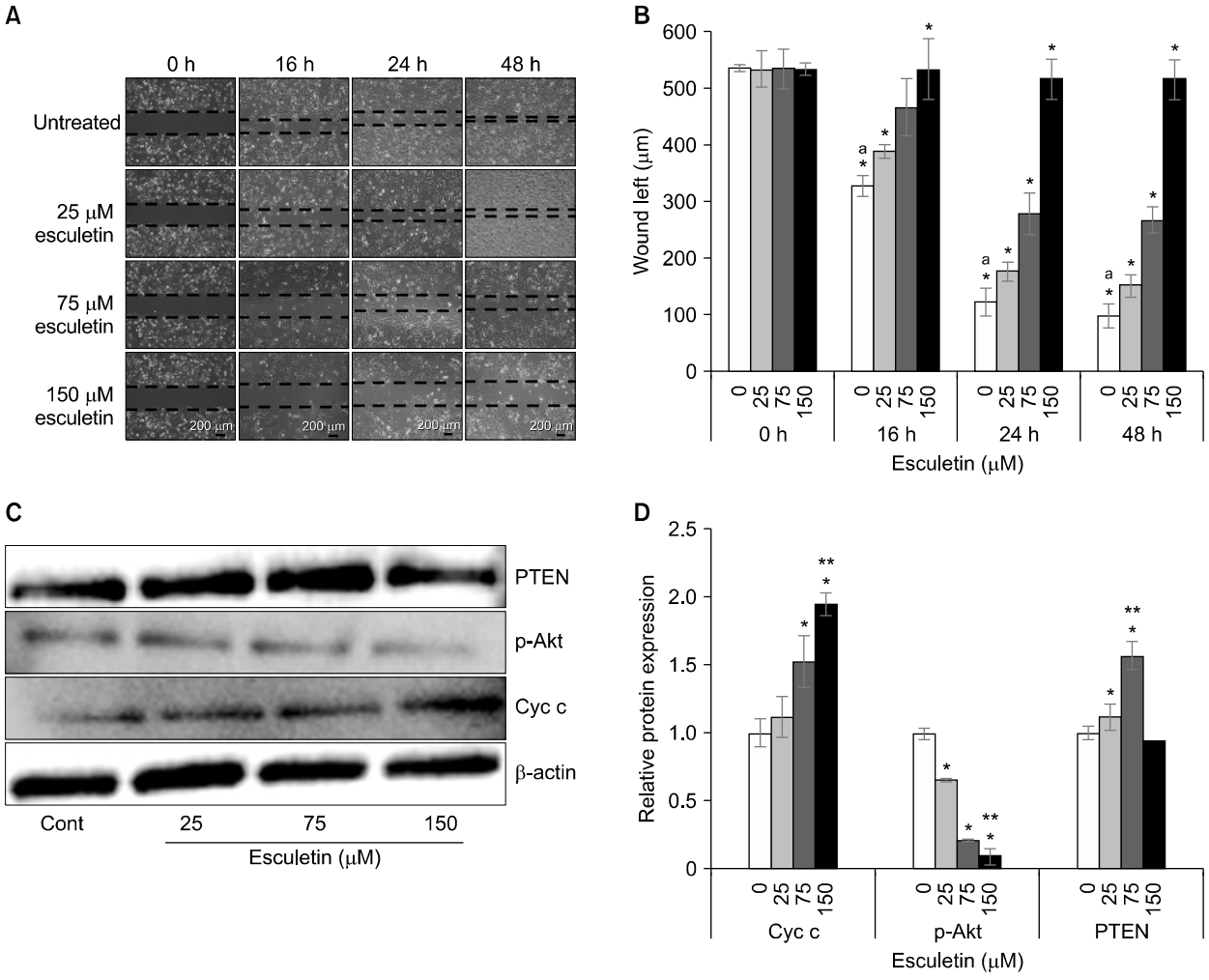

Figure 4. Effect of esculetin on cell migration and protein expression. (A) PC3 cell migration was determined by wound healing assay during treatment with 25 μM, 75 μM, and 150 μM esculetin during 48 hours in serum-free medium. Esculetin dose-dependently reduced cell migration (magnifications of all fictures: ×10). (B) The migration ratio (%) was calculated by measuring the distance between two sides. (C) Protein lysates were analyzed by Western blotting. Beta-actin was used as loading control. Protein expression levels of phosphatase and tensin homologue (PTEN) and cytochrome *c* (Cyc c), and phosphorylation status of Akt are given as fold changes of each protein compared to loading control (cont). (D) The intensities of immunoreactive bands were quantified by densitometric analysis. $*P < 0.01$ vs. 0 hour, $**P < 0.01$ vs. 25 μM and 75 μM esculetin, $^{a}P < 0.01$ vs. all groups at the same time period.

dependent fashion at concentrations of 75-150 μM (Fig. 4A and 4B), though lower dose did not significant effect.

5. Esculetin up-regulates phosphatin and tensin homologue and down-regulates p-Akt

Tumor suppressor PTEN acts as a lipid phosphatase, modulates PI3K/Akt signaling pathway and cell cycle progression and cell survival. Thus, we have analyzed the potential regulatory role of esculetin on PTEN protein expression and p-Akt phosphorylation in PC3 cells treated for 48 hours. It was shown that esculetin markedly up-regulated the expression levels of PTEN and suppressed the phosphorylation of p-Akt (Fig. 4C and 4D).

DISCUSSION

This study was undertaken to evaluate the potential value of esculetin, a compound of coumarin, on PCa therapy and to reveal underlying mechanisms. Coumarin derivatives such as esculetin may play a role in the prevention of lung,[14] colon,[8] hepatocellular,[15] and breast cancers.[16] However, the role of esculetin on PCa cells has not been characterized yet. Thus, we have investigated the mechanisms of action, potency on apoptosis, effects on cell cycle and migration of PC3, DU145, and LNCaP human PCa cells.

Esculetin treatment inhibits tumor growth through inducing apoptosis,[9] G1 phase cell cycle arrest,[15,16] targeting β-catenin,[8] and suppressing NF-κB signaling.[14] The Cip/Kip family of proteins bind to both cyclins and the CDK subunits and can modulate the activities of cyclin D-, E-, A, and CDKs,[17] which inhibits cell survival. The $p21^{Cip1}$ controls the rate of progression through the G1 phase in response to a number of stimuli and is an important transcriptional target of p53 and mediates G1 or G2 to arrest cell cycle arrest due to DNA damage.[18] The p27 plays a key role in down-regulating CDK2 activity before and after peak DNA synthesis.[19] In the present study, we have found that esculetin treatment could inhibit the proliferation of PC3 cells in a dose-/time-dependent manner, as is evident by cell viability and annexin V/PI tests. There was similar effect in DU145 and LNCaP cells to PC3 where only dose escalation was tested. Previous data and our results are in good agreement that esculetin up-regulates *p21* and *p27* while down-regulates *CDK2*, *CDK4*, and *cyclin D1*. Therefore, induced cell cycle arrest at G1 phase may contribute to the decreased cell proliferation. It can be argued that down-regulation of CDK2, CDK4, *cyclin D1*, and the up-regulation of *p53*, $p21^{Cip1}$, and $p27^{Kip1}$ by esculetin acted as a tumor suppressor that regulates the cell cycle and thus protects against PCa cells.

PCa metastasis accounts for the majority of deaths from PCa, and suppression of metastasis at the earliest stage is important for the progression of PCa.[20] Thus, we analyzed whether esculetin treatment would inhibit PC3 cell motility. Although lower levels of esculetin did not inhibit cell migration, higher concentrations significantly reduced PC3 cell migration. Esculetin therapy did up-regulate PTEN expression and down-regulated the phosphorylation of Akt. Inhibition of Akt signaling by esculetin treatment may lead to attenuation of Snail and Twist expressions, which cause suppression of the migration.[21] This result demonstrates that esculetin may provide a promising strategy to control the metastasis and invasiveness of the tumor.

It has been revealed that esculetin induces apoptotic cell death by activation of JNK in human leukemia cells [22] and up-regulation of ERK1/2.[23] Esculetin also induces the rate of apoptosis through production of reactive oxygen species in human colon cancer cells,[9] hepatocellular carcinoma,[24] and pancreatic cancer cells through activation of caspases-3, -8, and -9.[25] We found that esculetin induces apoptosis by down-regulating Akt phosphorylation and up-regulating PTEN expression in PC3 cells. This led to modulate the extrinsic and intrinsic apoptotic pathways in both castration-dependent and castration-independent PCa cells. PTEN is one of the important regulatory factors in the DNA repair process and is increased to allow for the prevention of cancer development.[26] As expected, PTEN acts as a negative regulator for PI3K/Akt pathway ,[27] and the pAkt acts as a negative regulator for p53 expression.[28] Aberrant methylation and consequent silencing of the PTEN gene cause the activation of the PI3K/Akt and ERK pathways and subsequently promotes cancer cell proliferation and progression of tumor.[29]

In summary, the results of this study highlight the therapeutic potential of esculetin in PCa cells. Esculetin significantly inhibits the proliferation of PCa cells by inducing apoptosis, promoting cell cycle arrest and modulating the PTEN/Akt signaling pathway. Reduced cell motility by esculetin also contributes to its therapeutic properties. Although it is expected that esculetin may be useful as an effective anti-tumor agent in the prevention and treatment of androgen-dependent and androgen-independent PCa, additional in vitro and in vivo studies are needed.

CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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