Research Article / Araştırma Makalesi

Esculin-Induced Apoptosis and Suppression of Leukemia Surface Markers in HL-60 and THP-1 Cells: A Potential Selective Anticancer Agent

HL-60 ve THP-1 Hücrelerinde Eskulin'in İndüklediği Apoptoz ve Lösemi Yüzey Belirteçlerinin Baskılanması: Potansiyel Seçici Bir Antikanser Ajan

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Abstract: Esculin, a natural coumarin compound primarily derived from Cortex fraxini, is known for its anti-inflammatory and antioxidant properties. Leukemia, a type of hematological cancer, is characterized by the uncontrolled proliferation of white blood cells and has high mortality rates. In this study, we aimed to investigate the potential anticancer effects of esculin (Esculetin-6-Glucoside) on leukemia cell lines, focusing on how this compound could be utilized in cancer treatment through apoptotic pathways. Our experiments used acute promyelocytic leukemia (HL-60) and acute monocytic leukemia (THP-1) cell lines. Cancer cell counting and viability analyses were conducted using the MTS assay(5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazol)-3-(4-sulfophenyl) tetrazolium inner salt assay). Apoptosis was assessed using FITC-labeled Annexin V and propidium iodide. Caspase-3 activation, cytochrome C release, leukemia cell surface markers, and mitochondrial membrane potential (MMP) were analyzed via flow cytometry. Our results demonstrated that esculin can induce apoptosis in leukemia cell lines. Additionally, leukemia surface markers post-treatment were statistically significantly reduced post-treatment in both cell lines. HL-60 and THP-1 cells exhibited different cellular responses in terms of MMP, Caspase-3, and Cytochrome C activities; HL-60 cells were more resistant to esculin treatment, while THP-1 cells were more sensitive. These findings suggest that esculin could become a potential agent in cancer treatment by targeting apoptotic pathways. However, more in vivo studies and preclinical modeling are needed to understand the anticancer effects of esculin fully. Evaluating its efficacy against different cancer types could further expand the therapeutic potential of this compound.
Keywords: Esculin, Leukemic Cells, Apoptosis, Surface Markers

Özet: Esas olarak Cortex fraxini'den türetilen doğal bir kumarin bileşiği olan eskülin, anti-inflamatuar ve antioksidan özellikleriyle bilinir. Hematolojik kanser türlerinden biri olan lösemi, beyaz kan hücrelerinin kontrolsüz çoğalmasıyla karakterizedir ve yüksek ölüm oranlarına sahiptir. Bu çalışmada, eskülinin (Eskületin-6-Glukozid) lösemi hücre hatları üzerindeki potansiyel antikanser etkilerini araştırmayı amaçladık ve bu bileşiğin apoptotik yollarla kanser tedavisinde nasıl kullanılabileceğine odaklandık. Deneylerimizde akut promyelositik lösemi (HL-60) ve akut monositik lösemi (THP-1) hücre hatları kullanıldı. Kanser hücresi sayımı ve canlılık analizleri MTS testi (5-(3-karboksimetoksifenil)-2-(4,5-dimetiltiazol)-3-(4-sülfofenil) tetrazolyum iç tuz testi) kullanılarak gerçekleştirildi. Apoptozis, FITC etiketli Annexin V ve propidyum iyodür kullanılarak değerlendirildi. Kaspaz-3 aktivasyonu, sitokrom C salınımı, lösemi hücre yüzey belirteçleri ve mitokondriyal membran potansiyeli (MMP) akış sitometrisi ile analiz edildi. Sonuçlarımız eskülinin lösemi hücre hatlarında apoptozu indükleyebileceğini gösterdi. Ek olarak, lösemi yüzey belirteçleri tedavi sonrası her iki hücre hattında da tedavi sonrası istatistiksel olarak anlamlı şekilde azaldı. HL-60 ve THP-1 hücreleri MMP, Kaspaz-3 ve Sitokrom C aktiviteleri açısından farklı hücresel tepkiler gösterdi; HL-60 hücreleri eskülin tedavisine daha dirençliyken, THP-1 hücreleri daha duyarlıydı. Bu bulgular eskülinin apoptotik yolları hedefleyerek kanser tedavisinde potansiyel bir ajan olabileceğini düşündürmektedir. Ancak eskülinin antikanser etkilerini tam olarak anlamak için daha fazla in vivo çalışma ve klinik öncesi modellemeye ihtiyaç vardır. Farklı kanser tiplerine karşı etkinliğinin değerlendirilmesi bu bileşiğin terapötik potansiyelini daha da genişletebilir.
Anahtar Kelimeler: Esculin, Lösemik Hücreler, Apoptozis, Yüzey İşaretleyicileri

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1. Introduction

Esculin (6,7-dihydroxy coumarin), a natural coumarin compound, is known as one of the main components of Cortex fraxini **(Figure 1)** (1). In addition to its pharmacological effects, esculin has also garnered attention for its potential to inhibit cancer cell proliferation and induce apoptosis (2). Research highlights that the chemical structure and cell line specificity of coumarins, such as esculin, play a critical role in determining their anticancer properties. Esculin has been shown to reduce the growth, adhesion, and migration of glioblastoma U87 cells in a dose-dependent manner (3). Furthermore, esculin has been reported to significantly inhibit the proliferation of MDA-MB-231 breast cancer cells through the regulation of the p53-p21 signaling pathway (4).

These mechanisms reflect p53's function in halting the cell cycle and eliminating abnormal cells (5), while p21 is a key negative regulator of the cell cycle transcriptionally controlled by p53 (6). Esculin also inhibits the migration and invasion of nasopharyngeal carcinoma HNE-3 cells by reducing MMP-2 and MMP-9 protein levels (7). The apoptosis induced by esculin is mediated by the early activation of Caspase-3 (8), while Caspase-9 plays a pivotal role in the mitochondrial apoptosis pathway (9).

Studies on the anticancer effects of esculin also involve the downregulation of pathways

such as the ERK signaling pathway (10).In glioblastoma and anaplastic astrocytoma cell lines, esculin induces apoptosis and autophagy by inhibiting MEK/ERK and PI3K activation, as well as promoting mitochondrial apoptosis through increased expression of Caspase-3 and Bcl-2 (2).

Esculin and its oligomeric fractions have also been observed to reduce tumor size and weight in mice by activating the immune response, supporting the potential of esculin in cancer therapy (11). Moreover, the ability of esculin to inhibit oxidative DNA damage suggests its possible use in cancer prevention (12,13). These effects have also been noted in studies showing its protective role against mutagenic damage (3,14)

These studies demonstrate that esculin, in addition to its other anti-cancer effects, can suppress cell migration, invasion, and proliferation, induce apoptosis and autophagy, and stimulate the immune system. To date, limited studies have been conducted on the anticancer effects of coumarin derivatives on leukemia. In this study, the anticancer effects of esculin on leukemia cell lines (HL-60, THP-1) were analyzed through changes in mitochondrial membrane activity, apoptosis, and leukemia markers (CD34, CD45, CD123, CD33).

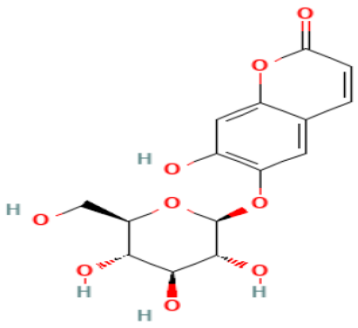

Figure 1. Chemical structure of Esculin (C15H16O9)

2. Materials and Methods

2.1. Propagation of Leukemia Cell Line

HL60 and THP-1 cell lines, sourced from the American Type Culture Collection (ATCC®) in Manassas, VA, USA, were cultured in a growth medium enriched with 10% fetal bovine serum and 2 mM L-glutamine. The medium was further supplemented with 1% PSA, containing 10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin, and 25 µg/ml of amphotericin B. The cells were incubated at 37°C in a humidified atmosphere with 5% CO2. Appropriate culture containers were utilized based on the cell volume, with daily monitoring to ensure optimal growth conditions.

2.2. Analysis of Cell Viability in Leukemia Cells

Esculin stock solution was prepared by initial dissolution in DMSO, followed by subsequent dilution with RPMI medium to achieve the required concentrations. HL60 and THP-1 cells were seeded in 96-well plates at a density of 2 x 10^3 cells per well in 200 µl of RPMI medium, which was supplemented with 10% fetal bovine serum, 1% PSA (penicillin at 10,000 units/ml, and streptomycin at 10,000 µg/ml, and 25 µg/ml of Amphotericin B). The cells were then treated with various concentrations of esculin (800,1000, 2000, 3000, 4000 µM), with each concentration tested in quadruplicate. 0.1% DMSO solution served as the solvent control, while untreated cells were used as the negative control. Cell viability was assessed at 48, 72, and 96 hours post-treatment by adding a cell viability reagent (Promega's CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS), Cat. No: G3580) to each well, following the manufacturer's instructions. The cells were incubated for 2,5 hours at 37°C in darkness, after which the absorbance was measured at a wavelength of 490 nm. The IC50 value, the concentration at which esculin reduced the absorbance of treated cells by 50% relative to the negative control, was determined from these measurements.

2.3. Analysis of Cell Viability in Healthy PBMNC Cells After Esculin Treatment

The isolation of mononuclear cells (MNCs) from healthy peripheral blood (PB) samples was performed using the Ficoll-Paque density gradient centrifugation method, as previously described (15,16). Briefly, 15 ml of patient blood was diluted 1:1 with DPBS in a 50 ml Falcon tube and gently mixed by slow inversion. The diluted blood was then layered over 10 ml of Ficoll-Paque and centrifuged at 300g for 30 minutes without a brake. Following centrifugation, the supernatant was carefully removed, and the cloudy interphase containing the MNCs was transferred to a new 50 ml Falcon tube. The MNCs were washed three times with DPBS, each time by gentle inversion. Cells were then centrifuged at 1500 rpm for 5 minutes with a brake, and the supernatant was discarded. The MNC pellet was resuspended to a density of $5x10^3$ cells per well in 200 µl of RPMI medium supplemented with 10% fetal bovine serum and 1% PSA (penicillin at 10,000 units/ml, streptomycin at 10,000 µg/ml, and amphotericin B at 25 µg/ml) and seeded into 96-well plates. Cells were treated with various concentrations of esculin (800, 1000, 2000, 3000, 4000 µM), with each concentration tested in quadruplicate. A 0.1% DMSO solution served as the solvent control, and untreated cells were used as a negative control. Cell viability was assessed at 48, 72, and 96 hours post-treatment using Promega's CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Cat. No: G3580), following the manufacturer's instructions. Cells were incubated with the MTS reagent for 2.5 hours at 37°C in the dark, and absorbance was measured at 490 nm to determine cell viability.

2.4 Analysis of the Effect of Esculin on Cancer Cell Number and Viability

2.4.1. Apoptosis Analysis

HL-60 and THP-1 cells were seeded into 6-well plates at a concentration of 500,000 cells per well. These cells were treated with esculin

at the IC50 concentrations previously established, as well as with 0.1% DMSO as a control, with each condition tested in triplicate. The plates were then incubated in a humidified environment at 37°C and 5% CO2 for three days. Following the incubation, cells were collected from the plates and centrifuged at 1500 rpm for 5 minutes. The resulting cell pellet was resuspended in 1X binding buffer to prepare for apoptosis evaluation. For the detection of apoptotic cells, staining was performed using FITC Annexin V and propidium iodide (PI), according to the manufacturer's protocol (ABP, FITC Annexin V and PI Apoptosis Kit, Cat. No: A026). Flow cytometric analysis was conducted on a Cytoflex S Flow Cytometer (Beckman, Cat. No: B47903, USA), following methodologies outlined in (15,16).

2.4.2. Assessing Leukemia Marker Modulations in Monoclonal Antibodies

The quantification and percentage of leukemia cells in HL-60 and THP-1 cultures treated with 1% DMSO and the IC50 concentration of esculin were assessed approximately 72-96 hours post-treatment. This evaluation involved analyzing surface markers—specifically, CD34, CD45, CD33, and CD123—using a flow cytometer. The cells were first stained with fluorochrome-conjugated antibodies, following methodologies consistent with prior research. For this purpose, leukemia cells were seeded in a 96-well plate at a density of 50,000 cells/well. The cells were then labeled with leukemia surface antibodies according to the manufacturer's instructions (1:1000 dilution ratio). Labeled cells were analyzed by flow cytometry (16,17).

2.4.3. Evaluation of Mitochondrial Membrane Potential (ΔΨm) Dynamics

HL-60 and THP-1 cells underwent treatment with esculin at the previously established IC50 values. At 72-96 hours post-treatment, the mitochondrial membrane potential (MMP) was assessed using the Elabscience Mitochondrial Membrane Potential Assay Kit (with JC-1), Cat. No: E-CK-A301, following the manufacturer's instructions. For the positive control, 10 mM CCCP was diluted 1000 times in the cell culture medium to achieve a final concentration of 10 µM CCCP. The cells were incubated with 10 µM CCCP for 20 minutes. Other cells were collected (5×10^5 - 1×10^6 cells), centrifuged at 300×g for 5 minutes, and the supernatant was discarded. The cells were resuspended in 500 µL of JC-1 working solution and incubated at 37°C for 20 minutes. After incubation, the cells were centrifuged at 300×g for 5 minutes, and the supernatant was discarded. The cells were washed once with pre-cooled 1×JC-1 assay buffer (300×g, 5 minutes), and the supernatant was discarded. The cells were resuspended in an appropriate amount of pre-cooled 1×JC-1 assay buffer and analyzed by flow cytometry.

2.4.4. Assessment of Caspase-3 Activation and Cytochrome c Release

HL-60 and THP-1 cells received treatment with esculin at the established IC50 concentrations. 72-96 hours later, Caspase-3 activity was evaluated using the "BD Pharmingen™ FITC Active Caspase-3 Apoptosis Kit" (Cat. No: 550480), adhering to the provided guidelines. Additionally, to assess the release of Cytochrome-C after 72 hours of treatment, the procedure outlined for the "GMP FITC anti-Cytochrome c Antibody" (Cat. No: 260100) was employed. Leukemia cells were collected (5×10^5 - 1×10^6 cells), centrifuged at 300×g for 5 minutes, and the supernatant was discarded. To the pellet, 0.5 ml of 4% PFA was added and incubated at room temperature for 10 minutes. The cells were centrifuged at 300×g for 5 minutes, and the supernatant was discarded. To the pellet, 0.5 ml of 0.1% Triton X-100 permeabilizing solution was added and incubated at room temperature for 10 minutes. The cells were centrifuged at 300×g for 5 minutes, and the supernatant was discarded. The pellet was resuspended in 200 µl of PBS. The cells were then labeled with intracellular antibodies (Caspase-3 and Cytochrome-C) according to the manufacturer's manual (1:1000 dilution ratio) and incubated in the dark at room temperature for 20 minutes. Labeled cells were analyzed by flow cytometry.

2.5. Statistical Analysis

All data were statistically analyzed using one-way ANOVA or a two-tailed Student's t-test. GraphPad Prism (version 8.0.1) software was utilized for performing the statistical analyses and plotting the graphs. Error bars represent the standard error of the mean (SEM) from a minimum of three independent experiments. Statistical significance was defined as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

3. Results

3.1. Evaluation of Cell Survival and Growth in HL-60 Cells

Figure 2 illustrates the dose-response curves and half-maximal inhibitory concentration (IC50) values for HL-60 and THP-1 cells treated with esculin for 96 hours. In **Figure 2A**, the dose-response curve for HL-60 cells shows a linear decrease in cell viability with increasing esculin concentrations, resulting in an IC50 value of 1873 µM. The trendline equation, $y = -0.0027x + 55.059$, with an R^2 value of 0.8624, indicates a strong negative correlation between esculin dose and cell viability. Similarly, **Figure 2B** presents the dose-response curve for THP-1 cells, demonstrating a similar linear decrease in viability, but with a higher IC50 value of 2675 µM. The trendline equation, $y = -0.0055x + 64.715$, with an R^2 value of 0.9505, suggests an even stronger negative correlation for THP-1 cells compared to HL-60 cells.

These results highlight the cytotoxic effects of esculin on both leukemia cell lines, with THP-1 cells being more resistant to esculin treatment than HL-60 cells. The IC50 values calculated through nonlinear regression analysis further indicate a statistically significant difference in sensitivity between the two cell lines. The strong R^2 values for both cell lines reinforce the reliability of the dose-response relationship, particularly in THP-1 cells, which exhibit a more pronounced correlation between esculin concentration and viability reduction.

3.2. Esculin's Impact on Healthy PBMNC Viability and Growth

Figure 3 shows that esculin significantly reduces cell viability in leukemia cell lines (HL-60 and THP-1) at their respective IC50 values (**** $p < 0.0001$, *** $p < 0.001$), while no toxic effects are observed in healthy PBMNCs at the same concentrations. In fact, PBMNC proliferation appears to increase compared to the control group ($p < 0.05$). These results suggest that esculin selectively targets cancer cells, exerting cytotoxic effects on leukemia cells without affecting the viability of healthy cells. The statistical significance of these findings, confirmed by two-way ANOVA, further supports esculin's potential as a selective anticancer agent.

3.3. Evaluation of the Impact of Esculin on Cancer Cell Proliferation and Viability

3.3.1. Assessment of Apoptosis in Cancer Cells

Figure 4 demonstrates that esculin treatment significantly reduces the percentage of viable cells while increasing the percentages of pre-apoptotic and post-apoptotic cells in both HL-60 and THP-1 cell lines compared to the untreated control. In **Figure 4B**, HL-60 cells treated with 1873 µM esculin show a notable increase in pre-apoptotic and post-apoptotic cell populations (**** $p < 0.0001$, *** $p < 0.001$). Similarly, in **Figure 4C**, THP-1 cells treated with 2675 µM esculin exhibit a significant rise in apoptotic cells (**** $p < 0.0001$). These results provide strong evidence that esculin effectively induces apoptosis in both leukemia cell lines in a dose-dependent manner.

The morphological changes accompanying these apoptotic effects are further illustrated in **Figure 5**. **Figure 5A** shows untreated HL-60 cells, which appear dense and healthy with no signs of apoptosis. In contrast, **Figure 5B** shows a significant decrease in cell density in HL-60 cells treated with 1873 µM esculin, indicating apoptosis. Similarly, **Figure 5C** displays untreated THP-1 cells with typical healthy morphology, while **Figure 5D** shows a noticeable reduction in cell density in THP-1

cells treated with 2675 µM esculin, further supporting the occurrence of apoptotic death.

3.3.2. Evaluation of Leukemia Marker Modulations Induced by Monoclonal Antibodies

Figure 6 illustrates the significant reduction in the expression of leukemia surface markers CD34+, CD45+, and CD123+ in both HL-60 and THP-1 cells following esculin treatment. In **Figure 6A**, for HL-60 cells, CD34+ (1.2 ± 0.07) and CD33+ (0.2 ± 0.02) expression almost completely disappears post-treatment, while CD45+ decreases significantly from 94.1 ± 0.8 to 79.8 ± 1.6 (***$p < 0.001$) and CD123+ drops from 96.5 ± 4.2 to 23.3 ± 6.3 (****$p < 0.0001$). **Figure 6B** shows a similar trend in THP-1 cells, where esculin treatment causes a significant reduction in CD34+ (48.9%, $p < 0.0001$), CD45+ (82.77%, $p < 0.0001$), CD33+ (29.75%, $p = 0.0002$), and CD123+ (32.4%, $p < 0.0001$). These statistically significant reductions in marker expression were confirmed using flow cytometry and analyzed through repeated-measures ANOVA.

3.3.3. Analysis of Mitochondrial Membrane Potential (MMP) Dynamics

Figure 7 illustrates the MMP dynamics following esculin treatment in both HL-60 and THP-1 cells. In **Figure 7A**, HL-60 cells treated with 1873 µM esculin show a significantly lower percentage of JC-1 (+) post-apoptotic cells compared to the untreated and DMSO-treated groups (*$p < 0.05$), indicating that esculin helps preserve MMP in these cells. No significant changes were observed in pre-apoptotic cells. In contrast, **Figure 7B** shows that in THP-1 cells treated with 2675 µM esculin, there is a significant increase in the percentage of JC-1 (+) post-apoptotic cells and a decrease in JC-1 (+) pre-apoptotic cells (***$p < 0.0001$), suggesting a loss of MMP and progression to apoptosis. These findings, analyzed using two-way ANOVA, indicate that esculin exhibits different effects on MMP dynamics in HL-60 and THP-1 cells, preserving MMP in the former while inducing MMP loss in the latter.

3.3.4. Evaluation of Caspase-3 Activation and Cytochrome-C Release

The analysis of Caspase-3 and Cytochrome-C activation, as depicted in **Figures 8** and **9**, further demonstrates the apoptotic effects of esculin. In **Figure 8A**, HL-60 cells treated with 1873 µM esculin show a slight reduction in Caspase-3 positive cells, decreasing by 0.7-fold compared to the untreated group. In contrast, **Figure 8B** shows a significant 9-fold increase in Caspase-3 positive cells in THP-1 cells treated with 2675 µM esculin. Similarly, **Figure 9A** illustrates that Cytochrome-C positive cells in HL-60 decrease by approximately 1.5-fold after treatment, while **Figure 9B** demonstrates a 3-fold increase in Cytochrome-C positive cells in THP-1 cells under the same conditions. These findings indicate that esculin induces a much stronger apoptotic response in THP-1 cells compared to HL-60 cells. The statistical significance of these results was confirmed using one-way ANOVA, with data obtained from ELISA and flow cytometric assays.

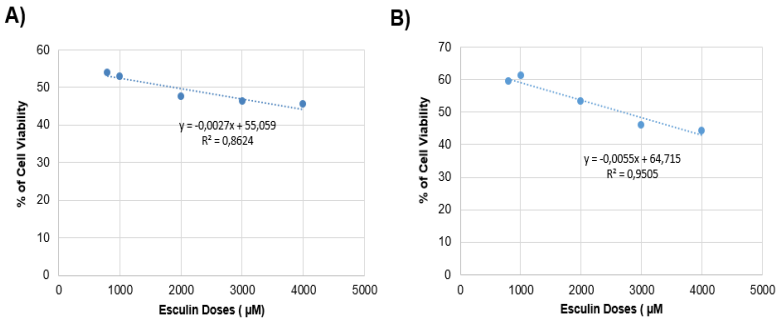

Figure 2. Dose-response curve and half maximal inhibitory concentration (IC50) values in **A)** HL-60 (IC50:1873 µM), **B)** THP-1 (IC50: 2675 µM) Cells after 96 hours of treatment.

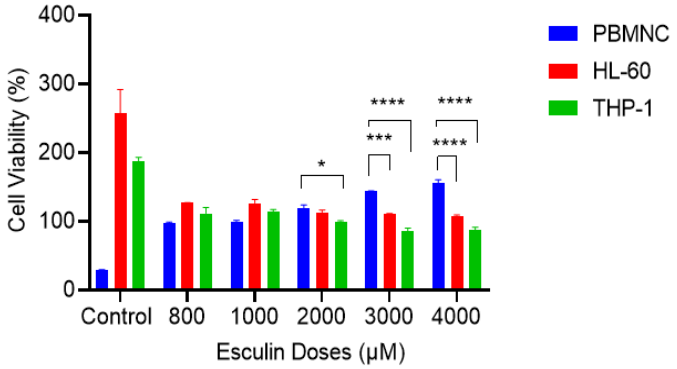

Figure 3. Comparative analysis of cell viability between leukemia cell lines and healthy peripheral blood mononuclear cells (PBMNCs) following 96 hours of esculin treatment (**** $p < 0.0001$, *** $p < 0.001$ and * $p < 0.05$).

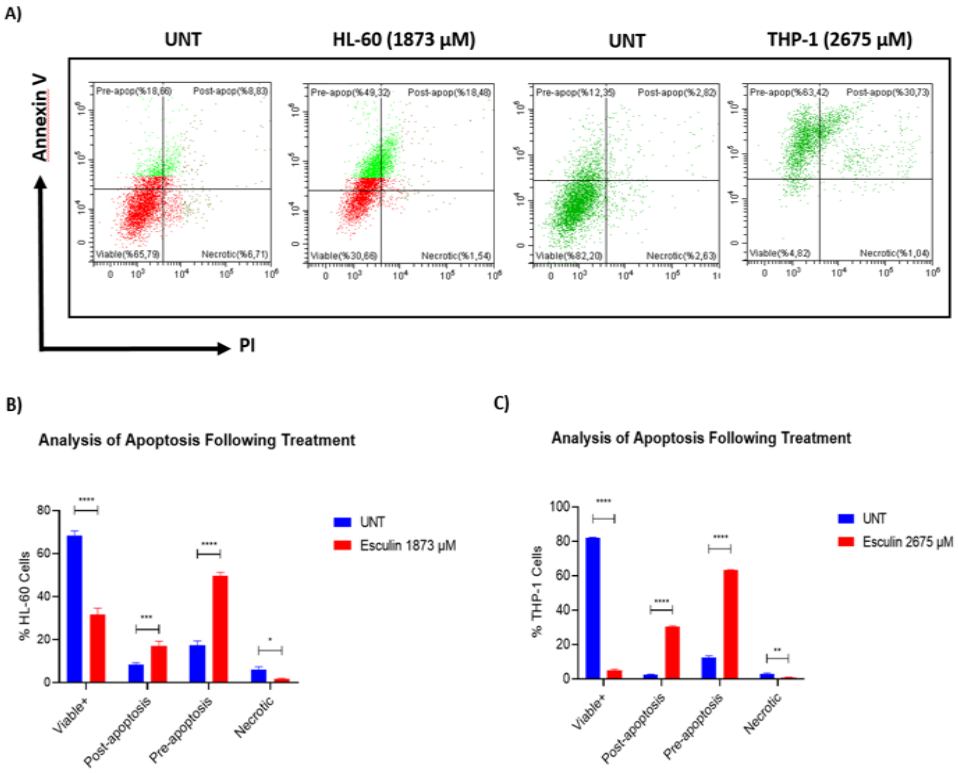

Figure 4. Analysis of apoptosis following esculin treatment in HL-60 and THP-1 cells. **A)** Flow cytometry plots illustrating apoptosis in HL-60 and THP-1 cells, **B)** Quantification of apoptosis in HL-60 cells, **C)** Quantification of apoptosis in THP-1 cells. The data indicate that esculin treatment significantly reduces the percentage of viable cells and increases the percentage of pre and post apoptotic cells in both cell lines compared to the control. Statistical significance is denoted as follows: untreated (UNT), **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. Error bars represent the standard error of the mean (SEM).

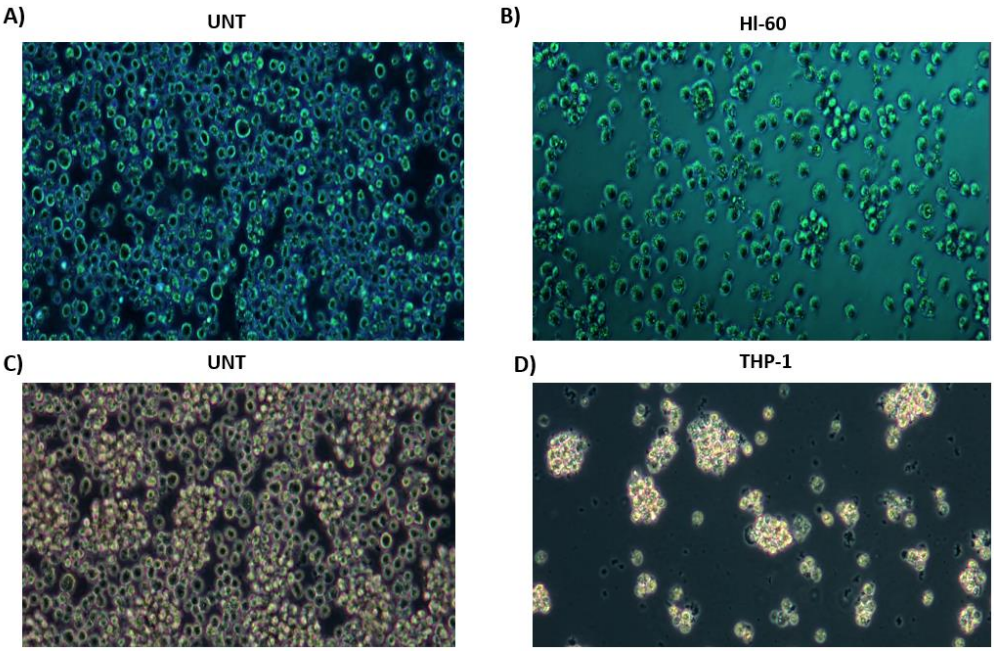

Figure 5. Microscopic images of HL-60 and THP-1 cells under different esculin treatments (10X, % 39 zoom) **A)** Untreated HL-60 cells showing dense and healthy morphology with no signs of apoptosis, **B)** HL-60 cells treated with 1873 µM esculin, **C)** Untreated THP-1 cells with dense and healthy morphology, **D)** THP-1 cells treated with 2675 µM esculin. Images were captured using the ZEISS microscopy system with the ZEN 3.4 (Blue Edition) software application.

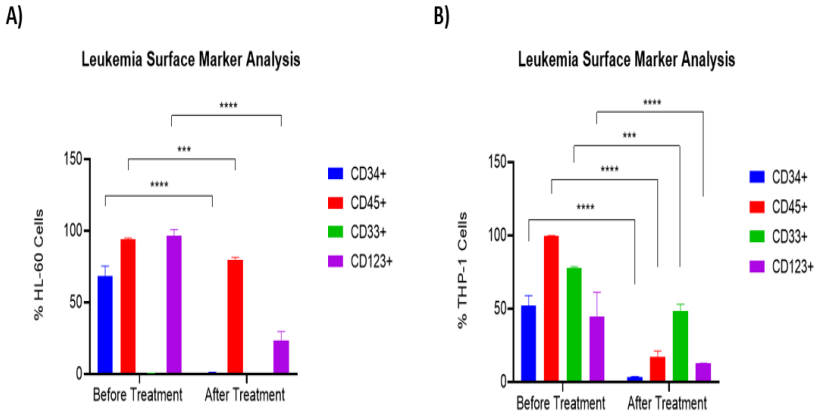

Figure 6. Evaluation of leukemia surface markers in HL-60 and THP-1 cells pre- and post-esculin treatment. The bar charts depict the percentage of **A)** HL-60 and, **B)** THP-1 cells expressing various surface markers (CD34+, CD45+, CD33+, and CD123+) before and after esculin treatment (***$p < 0.001$ and ****$p < 0.0001$).

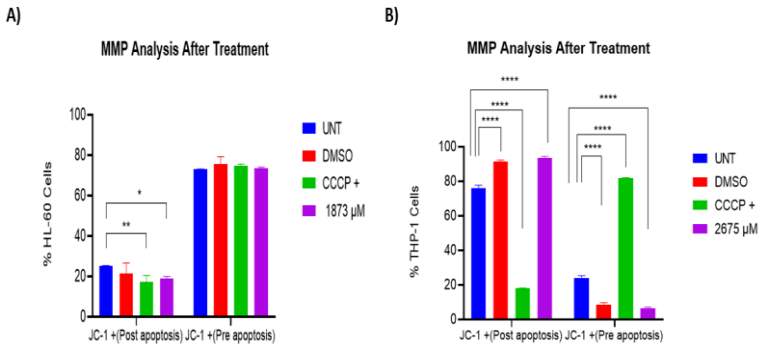

Figure 7. Effects of Esculin on MMP in **A)** HL-60, **B)** THP-1 Cells**.** The error bars represent the standard error of the mean (Untreated (UNT), solvent control (DMSO), pozitive control (CCCP+), **** $p < 0.0001$, ** $p < 0.01$, and * $p < 0.05$).

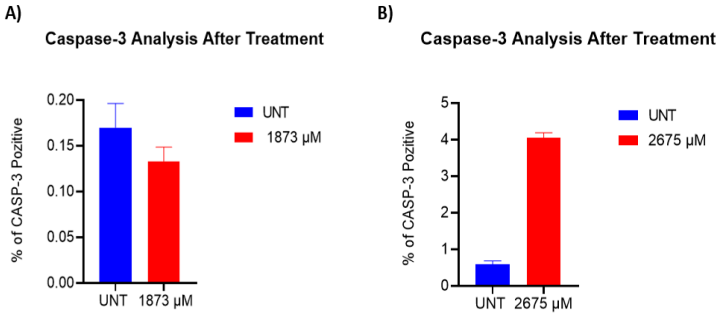

Figure 8. Caspase-3 Analysis After Treatment with Esculin in **A)** HL60, **B)** THP-1 Cells (Untreated (UNT)).

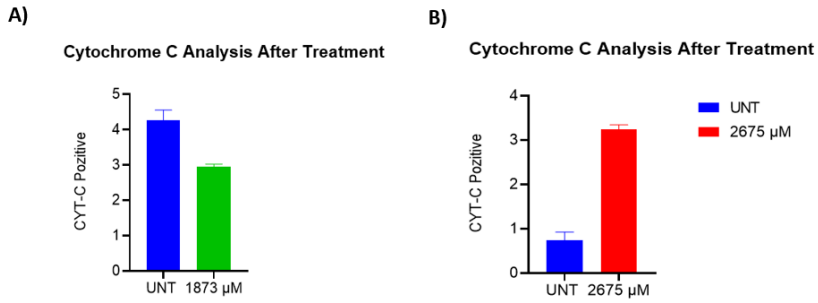

Figure 9. Ctyochrome-C Analysis After Treatment with Esculin in **A)** HL60, **B)** THP-1 Cells (Untreated (UNT)).

4. Discussion

Various studies have demonstrated that esculin inhibits proliferation and induces apoptosis in human cancer cells, positioning it as a promising chemotherapeutic agent. (4) showed that esculin inhibits the proliferation of triple-negative breast cancer cells (MDA-MB-231) by targeting the p53 signaling pathway. (12) similarly reported that esculetin,

a compound related to esculin, inhibited the proliferation of TMT-081 rat breast tumor cells in a concentration-dependent manner. Moreover, combinations of coumarins, including esculin, with other anticancer agents have shown potential in enhancing efficacy against cancer cells (2). Specifically, esculin and its oligomeric fractions have been observed to inhibit proliferation, adhesion, and migration, all key processes in tumor metastasis, in glioblastoma cells (U87) (3).

These findings align with our results, where esculin significantly reduced cell viability in leukemia cell lines (HL-60 and THP-1) at specific IC50 doses while exhibiting no significant toxicity to healthy PBMNCs. PBMNC proliferation increased compared to the control group. This suggests that esculin exerts cytotoxic effects on leukemia cells while sparing healthy cells, reinforcing its potential as a selective anticancer agent.

Our study shows that esculin induces apoptosis in both leukemia cell lines, as evidenced by a significant reduction in viable cells and an increase in both pre-apoptotic and post-apoptotic cells. Apoptosis, a crucial biological process for maintaining tissue homeostasis and eliminating damaged cells, is a key target in cancer therapy (18). Coumarin derivatives, including esculin, exhibit various antitumor mechanisms, such as inhibiting kinases, affecting cell cycle phases, and inducing apoptosis in cancer cells (19). These mechanisms involve interactions with signaling pathways that regulate apoptosis (20).

Our findings regarding the apoptotic effect of esculin are consistent with those from Rubio et al. (2017), where esculetin induced apoptosis in NB4 human leukemia cells through ROS modulation (21). While we primarily observed mitochondrial apoptotic pathways involving Caspase-3 and Cytochrome-C activation, Rubio et al. demonstrated that esculetin enhanced apoptosis by regulating ROS levels, indicating a different yet complementary mechanism of action. This highlights the versatility of esculin and its derivatives in triggering apoptosis through multiple pathways depending on the experimental conditions and cell type.

Additionally, Wang et al. found that esculetin could induce both apoptosis and autophagy in HL-60 cells via G0/G1 phase cell cycle arrest and downregulation of Cyclin D1 and D3 (22). While our study focused on apoptosis, these findings suggest that esculin may have broader effects on cell cycle regulation, which warrants further investigation. Exploring whether esculin also induces autophagy in HL-60 and THP-1 cells could provide deeper insights into its antitumor mechanisms.

Our study is the first to demonstrate that esculin affects the expression of certain surface markers in leukemia cells. In HL-60 cells, CD34+ and CD33+ expression nearly disappeared after esculin treatment, suggesting an effect on stem cell properties and myeloid differentiation. Additionally, the reduction in CD45+ and CD123+ markers in both cell lines indicates that esculin suppresses the proliferation capacity of leukemia cells. These results suggest that esculin could be a promising agent in leukemia treatment, paving the way for novel therapeutic strategies.

Loss of mitochondrial membrane potential (MMP) is often an early indicator of apoptotic cell death. In THP-1 cells, esculin treatment resulted in a strong pro-apoptotic effect, as observed by increased Caspase-3 and Cytochrome-C activity. However, no changes were observed in the MMP of pre-apoptotic cells, suggesting that esculin may induce apoptosis through mitochondrial-independent pathways. This observation contrasts with the findings of Rubio et al., where ROS modulation played a significant role in esculetin-induced apoptosis (21). This difference may be due to variations in the experimental conditions, such as the type of oxidative stress or cell line used, suggesting that esculin's apoptotic effects may be context-dependent.

Furthermore, while our study observed IC50 values of 1873 µM for HL-60 cells and 2675 µM for THP-1 cells, Wang et al. reported significantly lower IC50 values (20 µM) for

esculetin in HL-60 cells (22). This discrepancy could be attributed to differences in treatment durations or cell line sensitivities, highlighting the need for standardized protocols when evaluating the cytotoxicity of coumarin derivatives.

In conclusion, this study demonstrates that esculin has significant potential as a selective anticancer agent, particularly in targeting leukemia cells through apoptotic pathways. Esculin significantly reduced cell viability in HL-60 and THP-1 cells while sparing healthy PBMNCs, indicating its therapeutic promise. However, given the in vitro nature of this study, further in vivo research is needed to confirm these findings. Future studies should focus on animal models, exploring detailed molecular mechanisms of esculin-induced apoptosis, assessing its effects across a broader range of cancer types, and eventually designing clinical trials to evaluate its efficacy and safety in human patients. These steps will be essential for developing esculin as a novel therapeutic agent in cancer treatment.

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Ethics

Ethics Committee Approval: Bu çalışma için *in vitro* hücre kültürü çalışması olduğu için etik kurul iznine ihtiyaç yoktur. Çalışmada kullanılan hücre hatları ilgili firmadan satın alınmıştır.
Informed Consent: This study did not require informed consent.
Authorship Contributions: NM designed and executed the experiments, collected and analyzed the data, formulated the study designs, and contributed to writing the manuscript. CÖ conducted data analysis and composed the manuscript.
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