Clinical and Experimental Health Sciences

Modulation of MMP9 and AKT by Escin in Retinal Pigment Epithelial Cells: Exploring Novel Therapeutic Approaches for Proliferative Vitreoretinopathy

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ABSTRACT

Objective: The aim of this study was to investigate the anti-inflammatory and antioxidant effects of Escin molecule obtained from horse chestnut seed extract on retinal pigment epithelial cell lines (ARPE-19).

Methods: In this research, the ARPE-19 cell line, which is a commercially available retinal pigment epithelial cell line derived from the normal eyes of a 19-year-old male, was utilized. Escin was administered to the cells in varying concentrations of 100, 50, 10, 5, and 1 micromolar throughout a 48-hour timeframe. The IC50 concentration was subsequently determined through MTT cell viability assays. To determine cell migration, a wound healing assay was executed. To quantify MMP9 and AKT protein levels, analysis was conducted using Western blot. Additionally, the mRNA expression levels of *EGF, EGFR, PDGF-β, PDGFβ-R*, and *HIF1A* were analyzed using RT-PCR.

Results: Escin inhibited cell migration in RPE cells. Western blot analysis showed that escin decreased the levels of AKT and MMP9 proteins. Furthermore, it was found that the mRNA expression levels of *PDGFß, PDGFβ-R,* and *HIF1A* were suppressed following escin administration.

Conclusion: Escin has the potential to slow disease progression by suppressing cell migration in retinal pigment epithelial cells. With its anti-angiogenic properties, escin shows promise for developing new therapeutic approaches for the treatment of retinal diseases.

Keywords: Escin, cell migration, retinal pigment epithelial cell, MMP9, AKT

1. INTRODUCTION

The retinal pigment epithelium (RPE) is integral to the retina, carrying out important functions necessary for vision. This monolayer consists of regular polygonal cells situated in the retina's outermost layer. On its outer side, the RPE is linked to Bruch's membrane and the choroid, while its internal face interacts pertaining to the outer segments of photoreceptor cells. The RPE handles a diverse array of functions that are crucial for preserving visual health. These include light absorption, formation of the outer blood-retina barrier, continuity of the visual cycle, regulation of ion and fluid flow, as well as providing protection against oxidative damage, immunomodulation, and phagocytosis of the outer segments of photoreceptors (1).

Retinal diseases can arise from various causes and often have serious clinical consequences. The most common include retinal detachment (2), macular degeneration (3), diabetic retinopathy (4) and retinal vein occlusions (5). These conditions can lead to vision loss and even blindness. Although treatment options vary depending on the patient's condition and severity of the disease, these clinical conditions can be effectively managed with appropriate treatment and care.

Proliferative vitreoretinopathy (PVR) is one of the serious consequences of retinal detachment, characterized by abnormal proliferation, migration, and extracellular matrix (ECM) production of retinal cells. The surgical success rate in PVR cases is very low (6,7). Most other diseases that cause permanent visual damage, such as PVR, are characterized by excessive proliferation. All of these conditions can be referred to as proliferative retinopathy (PR).

Escin is a triterpenoid saponin obtained from the grains of the buckeye tree (*Aesculus hippocastanum*). It is acknowledged in modern medicine for its anti-inflammatory, antioxidant, and vascular-protective properties, which make it useful in treating a number of conditions, such as wound healing and venous insufficiency (8). Research indicates that escin as a favorable impact on the wound healing workflow and enhances the quality of healing (9). Specifically, it has been

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noted that escin facilitates fibroblast proliferation and collagen synthesis, thus speeding up wound healing (10). Moreover, escin's anti-inflammatory properties aid the healing process by mitigating inflammation in affected wounds (11). In light of this, further investigation is essential to gain a deeper understanding of escin's effects on wound healing and to assess its clinical effectiveness. These potential therapeutic benefits of escin may mark a significant advancement in developing new treatment strategies, particularly for wound healing and chronic wounds.

Matrix metalloproteinases (MMPs) are enzymes that are essential for tissue remodeling and wound healing. Among them, matrix metalloproteinase-9 (MMP-9) is particularly important for cell migration, angiogenesis, and the reorganization of the extracellular matrix (ECM), especially during the wound healing process (12). In the inflammatory phase of wound healing, MMP-9 aids the migration of inflammatory cells to the injury site and assists in reorganizing wound tissue by breaking down ECM components (13). Additionally, MMP-9's effects on angiogenesis contribute for the growth of novel blood vessels, which enhances the distribution of oxygen and nutrients to the wound area, thus speeding up the healing process (14). On the other hand, an overexpression of MMP-9 can result in tissue damage and the formation of chronic wounds, emphasizing the necessity for careful regulation of its levels (15). These dual roles of MMP-9 underline its potential as both a therapeutic target and a biomarker in the wound healing process.

AKT (protein kinase B) is a pivotal element of a signaling pathway that significantly influences cellular growth, proliferation, and survival. The stimulation of the AKT pathway can result in the enhanced expression of MMP-9, initiating a variety of cellular processes. Once activated, AKT alters gene expression in the nucleus by phosphorylating transcription factors that govern intracellular signaling pathways (16). Specifically, transcription factors like NF-κB and AP-1 bind to the regulatory domain of the MMP-9 gene, leading to increased levels of MMP-9 mRNA (17). This regulatory influence of AKT on MMP-9 is essential for processes such as cell migration, invasion, and tissue remodeling. Notably, in cancer cells and during wound healing, the AKT-mediated upregulation of MMP9 aids the healing process by promoting the breakdown of the ECM and enhancing cellular migration (18). Therefore, gaining a deeper understanding of the AKT-MMP-9 interaction may be crucial for developing novel therapeutic strategies for wound healing.

This study focused on examining how escin influences cell migration in RPE cells, specifically regarding the roles of MMP-9 and AKT.

2. METHODS

Escin was obtained from Sigma-Aldrich (Cat. No: 6805-41-0). The ARPE-19 cell line was sourced from the ATCC (CRL-2302, Manassas, VA, USA). The primer antibodies for MMP-9 (Cat No: 10176-2-AP), AKT (Cat. No: 10375-2-AP), and beta actin (Cat. No: 20536-1-AP) were purchased from Proteintech (IL, USA). All PCR primers were synthesized by Bioligo Biotechnology (Ankara, Turkey).

2.1. Cell Culture

Complete medium was prepared by adding 10 ml of fetal bovine serum, 1 ml of L-Glutamine, and 1 ml of penicillin-streptomycin to 100 ml of DMEM. Cells were then incubated in this medium at 37°C with 5% CO2. All experiments were conducted using commercially available ARPE-19 cell lines to represent RPE cells.

2.2. Determination of IC50 Doses Using the MTT Cell Viability Assay

Cells were seeded into 96-well plates at a density of 10^4 cells per well and exposed to different concentrations of escin (100, 50, 10, 5, and 1 micromolar) for 48 hours. Following the incubation period, 10 µl of MTT solution (5 mg/ml, SERVA, Heidelberg, Germany) was added to each well and the plates were incubated for another 4 hours at 37°C in a 5% CO2 environment. Upon completion of the incubation, 100 µl of dimethyl sulfoxide (DMSO) was introduced to each well, and the absorbance was assessed using a multiplate reader (Epoch, Biotek, USA) at a wavelength of 572 nm. IC50 values were determined using GraphPad Prism version 8.0.1 (GraphPad Software, Inc., CA, USA). All analyses were conducted in triplicate.

2.3. Protein assessment from cell lysates

Following two washes with PBS, they were lysed with RIPA buffer. The lysate obtained was centrifuged at 16,000 g for 15 minutes at 4°C and the supernatant was transferred to a clean Eppendorf tube. After protein isolation, quantification was performed with the BCA assay (TaKaRa, Shiga, Japan).

2.4. Western Bloting

Thirty micrograms of the isolated protein were combined with 5X SDS PAGE Sample Loading Buffer (ABP Biosciences, Cat. No: P013), 10X Sample Reducing Agent (Novex Bolt, Ref: B0009), and water to achieve a final volume of 20 µl. This mixture was heated at 95°C for 10 minutes using a BIORAD T100 Thermal Cycler and then rapidly cooled to +4°C. Separation of denatured proteins was achieved through SDS-PAGE on a 4-12% Bis-Tris gradient gel (Invitrogen NuPAGE, Cat. No: NP0323BOX) and transferred to a nitrocellulose membrane using the iBLOT2 Gel Transfer System (Invitrogen, Ref. No: IB23001). The membrane was treated with a 5% BCA-PBST solution at room temperature for 1 hour. Primary antibodies utilized included Beta Actin (Ptglab, Cat. No: 20536-1-AP), MMP9 (Ptglab, Cat No: 10176-2-AP), and AKT (Ptglab, Cat. No: 10375-2-AP), while IgG (Ptglab, Cat. No: SA000001-2) served as the secondary antibody. For chemiluminescence imaging, the membrane was treated with ECL buffer and

photographed using a Syngene chemiluminescence imager, with bands quantified using the GeneTools software.

2.5. Isolation of Total RNA, cDNA Synthesis, and Quantitative PCR

After 48 hours of incubation, cells were harvested and rinsed with cold PBS. Total RNA was purified using the RNA Purification Kit (Thermo Scientific, Catalog No: K0731), and its amount and quality were assessed with the Epoch Take3 plate system (Agilent, USA). Following the manufacturer's instructions (Biorad Catalog No: BR1708891), cDNA was synthesized from 1 µg of RNA using reverse transcriptase. Subsequently, Each 20 µl PCR reaction mixture contained 1 µl of cDNA, 10 µl of 2X SYBR Green PCR Master Mix (diluted to 1X as per the manufacturer's instructions), and specific primers at a final concentration of approximately 750 nM per reaction. All procedures were performed under cold chain and sterile conditions Target gene expression was normalized against the housekeeping gene GAPDH. The gene expression values were calculated using the RQ = $2^{-\Delta\Delta Ct}$ formula, based on the ΔΔCt method, with analysis performed using the REST2009 program. Table 1 provides the details of the primer sequences used in the PCR reactions and their respective conditions. Each analysis was conducted in quadruplicate.

Table 1. *Oligonucleotide Primer Sequences and PCR Programs*

Genes	Primer sequences (5' → 3')	RT-PCR Programs	Cycle
GAPDH	F-5'GATTTGGTCGTATTGGGCGC 3' R-5'AGTGATGGCATGGACTGTGG 3'	95˚C-30s/59˚C-1m/72˚C-30s	35
EGF	F-5'CTGAATGTCCCCTGTCCCAC-3' R-5 CTCGGTACTGACATCGCTCC-3'	95˚C-30s/59˚C-1m/72˚C-30s	35
EGFR	F-5'CGCAAAGTGTGTAACGGAATAGG-3' R-5'GGCTGACGACTGCAAGAGAA-3'	95˚C-30s/58˚C-1m/72˚C-30s	35
PDGF-ß	F-5'CTCGTCCGTCTGTCTCGATG-3' R-5' CACACCCACCAAGAGGAGTC-3'	95˚C-30s/59˚C-1m/72˚C-30s	35
PDGFß-R	F – 5'CACCAACGTGGCTTTTCTGG-3' R – 5' GGTGCGGTTGTCTTTGAACC-3'	95˚C-30s/57˚C-1m/72˚C-30s	35
HIF1A	F – 5'TGCTGGGGCAATCAATGGAT-3' R – 5'CTACCACGTACTGCTGGCAA-3'	95˚C-30s/60˚C-1m/72˚C-30s	35

2.6. Wound Healing Assay

6-well plates were used for the assay, and cells were seeded at a density of 300,000 cells per well and incubated overnight. After creating scratches in the monolayer using a sterile 200 µl pipette tip, the cells were rinsed twice with PBS. Following washing, escin at the previously determined IC50 dose from the MTT assay was applied to the wells for 48 hours. Control wells contained 0.5% DMSO in the culture medium. The culture medium was standardized to 5% FBS for all groups. Wound images were taken immediately after scratching and after 48 hours, with three measurements taken at different points across the wound area for each sample. Wound closure rates were analyzed using the ImageJ program. Each experiment was repeated in triplicate (n = 3) to ensure statistical significance.

2.7. Statistical Analysis

Data analysis for the study was performed using GraphPad Prism version 8.0.1 (GraphPad Software, Inc., CA, USA). To assess whether the data followed a normal curve, the Shapiro-Wilk normality test was applied. After confirming normal distribution, a two-tailed t-test, which is a parametric test, was used to compare the two groups. A p-value of <0.05 was considered statistically significant.

3. RESULTS

3.1. IC50 doses of bioactive compounds

The IC50 value of escin for a 48-hour treatment was determined to be 18.99 µM (Figure 1).

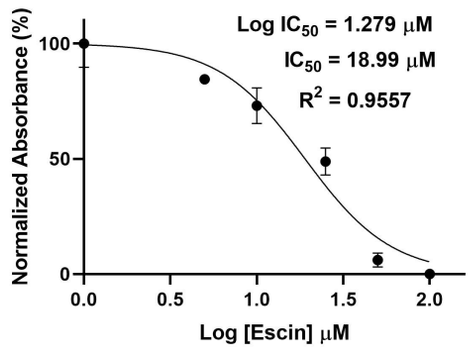

Figure 1. *IC50 dose of Escin*

3.2. The IC50 dose of escin slowed down cell migration

According to the results of the wound healing model, wound closure was observed at 71.16 ± 5.511% in the control group, while it was 51.81 ± 4.374% in the escin group (Table 2, Figure 2). Wound healing was notably slower in the group treated with escin compared to the control group, with a p-value of .0015.

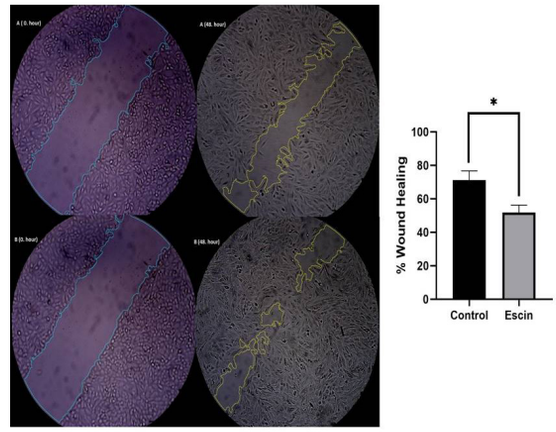

Figure 2. *The effect of Escin at IC50 dose (18.99 µM) on wound healing. A: Escin group, B: Control group, 0th hour on the left, 48th hour on the right in all groups. Wound healing measurements were made using ImageJ software.*

Table 2. *Comparison of % wound healing assay, western blot findings and RT-PCR data between groups.*

% Wound Healing Assay			
	Control	Escin	*p* value
% Wound Healing	71.16±5.511[a]	51.81±4.374[b]	.0015
Normalized Band Intensity			
MMP9	1.095 ± 0.1267[a]	0.4556 ± 0.01944[b]	.0010
AKT	1.001 ± 0.02690[a]	0.4878 ± 0.04081[b]	<.0001
mRNA Expression Levels			
EGF	1[a]	0.917[a]	.604
EGFR	1[a]	0.959[a]	.824
PDGFβ	1[a]	0.710[b]	.044
PDGFβ-R	1[a]	0.645[b]	.025
HIF1A	1[a]	0.684[b]	.036

There is a statistical difference between the values represented by different superscript the rows. (p < .05). All expression levels were compared to the control group. The expression level data represent fold increases or decreases. P < .05 was considered statistically significant. Different superscripts between groups indicate that there is a statistical difference between these groups. MMP9: Matrix metalloproteinase 9, AKT: Protein kinase B, PDGF-β: Platelet Derived Growth Factor Beta, PDGFβ-R: Platelet Derived Growth Factor Beta Receptor, HIF1A: Hypoxia-inducible factor-1α

3.3. Escin decreased MMP9 levels via AKT

As a result of the Western blot analysis, AKT protein levels were suppressed to 0.4878 ± 0.04081 fold ($p < .0001$), while MMP9 protein levels were suppressed to 0.4556 ± 0.01944 fold ($p = .001$) in the escin-treated group compared to the control group (Table 2, Figure 3). This result suggests that escin may impair migration pathways critical for wound closure, potentially through its suppressive effects on AKT signaling and MMP9 protein levels. These findings align with previous studies indicating that MMP9 suppression is associated with reduced cellular migration in cancer models.

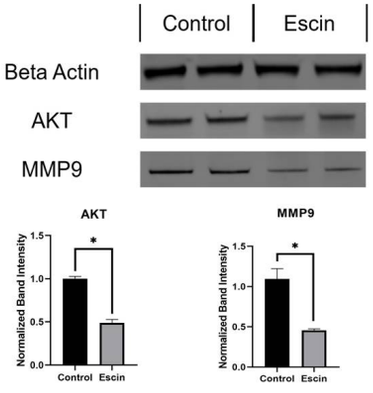

Figure 3. *Western blot band ve grafikleri. MMP9: Matrix metalloproteinase 9, AKT: Protein kinase B. *p≤0.0001*

3.4. Escin had a suppressive effect on the mRNA expression levels of PDGFβ, PDGFβ-R and HIF1A.

In the escin-treated group, the mRNA expression levels of *PDGFβ*, *PDGFβ-R*, and *HIF1A* were significantly suppressed compared to the control group ($p = .044$, $p = .025$, and $p = .036$, respectively)(Figure 4). These results suggest that escin exerts a regulatory effect on genes associated with angiogenesis and cell proliferation. The observed suppression of *HIF1A*, a key transcription factor activated under hypoxia, supports the notion that escin may interfere with hypoxia-induced signaling pathways, thereby limiting the cellular responses to low oxygen conditions.

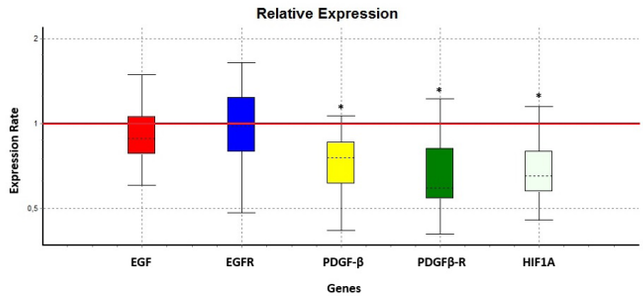

Figure 4. *Relative mRNA expression levels of PDGF-β, PDGFβ-R and HIF1A. Values are expressed as the mean ± SD. All groups were compared with the control group, and the results were given as fold increase/ decrease. The REST 2009 software (Qiagen) was used for statistical analysis and graphing. The red line parallel to the x-axis shows the position of the control group. p < 0.05 was considered statistically significant. EGF: Epidermal Growth Factor, EGFR: Epidermal Growth Factor Receptor, PDGF-β: Platelet Derived Growth Factor Beta, PDGFβ-R: Platelet Derived Growth Factor Beta Receptor, HIF1A: Hypoxia-inducible factor-1α*

Interestingly, no significant changes were detected in the mRNA expression levels of *EGF* and *EGFR* ($p = .604$ and $p = .824$, respectively), indicating that escin's effects might be more specific to the *PDGFβ* signaling axis rather than broadly targeting all growth factor pathways. This specificity may reflect a targeted mechanism of action that distinguishes escin from other agents with broader, less selective effects. Similarly, the selective downregulation of *PDGFβ* signaling has been implicated in reduced cell migration and proliferation.

Taken together, these results highlight escin's potential as a modulator of angiogenic and hypoxia-related signaling pathways, making it a promising candidate for therapeutic applications targeting tumor progression or pathological angiogenesis.

4. DISCUSSION

Oxidative stress has been shown to contribute to various acquired and hereditary diseases in the retinal pigment epithelial (RPE) layer. Factors such as aging, exposure to sunlight, and inflammation significantly increase the risk of conditions like macular degeneration, proliferative vitreoretinopathy (PVR), and Stargardt disease (19). A critical aspect of PVR is the migration and proliferation of RPE cells, which transforms into fibroblast-like cells that contribute to the formation of epiretinal membranes. Therefore,

suppressing cell migration represents a promising therapeutic strategy to prevent the progression of PVR (20,21).

Recent studies have focused on the role of various bioactive agents and molecular targets in modulating RPE cell behavior, particularly in suppressing migration. Biological molecules and antibodies that inhibit cell migration have shown potential for use in treating PR, with promising results in preventing recurrence following surgical treatments (22,23). However, further research is essential to establish the safety and efficacy of these therapeutic approaches in clinical settings.

A growing body of work has explored the use of bioactive compounds to target key biochemical pathways in RPE cells. For example, Wang et al. (24) reported that escin exhibited both cytoprotective and anti-protective effects against oxidative stress in RPE cells, specifically activating AKT-Nrf2 signaling to shield RPE cells from oxidative damage. In addition, escin has been shown to mitigate ischemic damage in the brain by enhancing antioxidant enzyme activities, including superoxide dismutase and glutathione peroxidase.

Furthermore, studies such as those by Hollborn et al. (25) have analyzed the impact of matrix metalloproteinases (MMPs) like MMP-2 and MMP-9, along with vascular endothelial growth factor (VEGF), in RPE cell proliferation and migration. Their findings indicate that hypoxic conditions elevate the expression of MMP-2 and MMP-9, with MMP-9 subsequently enhancing VEGF expression in RPE cells. In a similar vein, Sen et al. (26) examined the protective effects of rosmarinic acid (RA) and thymoquinone (TQ) against retinal damage, noting that overdose of these compounds hindered the wound healing process, emphasizing the need for optimal dosing.

In this study, the effects of escin on cell migration in RPE cells were investigated, leading to significant findings. The results indicate that escin suppresses the wound healing process by decreasing cell migration. This finding is consistent with previous studies and supports escin's properties in suppressing cell migration (27).

Escin treatment was shown to suppress the mRNA expression levels of key signaling molecules, including PDGF-β, PDGFβ-R, and HIF1A in RPE cells, which are critical for regulating cell migration. PDGF-β is involved in cell proliferation, migration, and angiogenesis, while PDGFβ-R plays an essential role in the signaling pathway, enhancing cell motility (28). Our findings suggest that escin's suppression of these molecules may contribute to the inhibition of RPE cell migration.

We also assessed the expression of epidermal growth factor (EGF) and its receptor EGFR in RPE cells. Interestingly, escin treatment did not significantly alter the expression levels of EGF or EGFR compared to controls, with relative expression changes of 0.917 ($p = 0.604$) for EGF and 0.959 ($p = 0.824$) for EGFR. These results imply that escin may not directly influence the EGF/EGFR signaling axis in RPE cells, which distinguishes it from other agents that act through this pathway.

The role of EGF and EGFR in angiogenesis and pathological cell behaviors has been well established. Studies by Keller and Schmidt (29) demonstrated that EGFR and its variant EGFRvIII significantly contribute to tumor progression, promoting angiogenesis and cell invasion, particularly in glioblastoma. In retinal diseases such as diabetic retinopathy and age-related macular degeneration, EGFR activation also promotes pathological neovascularization, as demonstrated by Deng et al. (30). However, our study suggests that escin's mechanism of action does not primarily involve this pathway, highlighting its potential to avoid EGFR-mediated toxicity in normal tissues.

The lack of significant changes in EGF and EGFR expression suggests that escin may exert its effects through alternative pathways, such as PDGFβ, PDGFβ-R, and HIF1A. This specificity of action could be advantageous in contexts where broad EGFR inhibition is undesirable. Furthermore, escin's ability to suppress cell migration by targeting these signaling pathways may be particularly beneficial in treating retinal diseases, including PVR.

Additionally, escin's suppression of PDGF-β, PDGFβ-R, and HIF1A expression may contribute to the inhibition of cell migration under both normoxic and hypoxic conditions. This could be valuable in preventing pathological migration and proliferation of RPE cells, a hallmark of PVR. MMP9, a crucial player in wound healing and extracellular matrix remodeling, was also significantly downregulated in response to escin, consistent with its known regulation by the AKT pathway (16,17). The reduction in MMP9 expression may explain the observed inhibition of wound healing and cell migration, reinforcing escin's role in modulating these processes.

While the findings of this study suggest that escin may be a promising therapeutic agent for treating proliferative retinal diseases, further in vivo studies are necessary to confirm the clinical applicability of these results. In addition, this study focused on a limited number of cell signaling pathways and proteins, and future investigations should explore a broader range of molecular pathways and cell types to better understand the full spectrum of escin's effects on RPE cell migration.

Our study was conducted under in vitro conditions, and the effects of escin on RPE cells in in vivo models remain uncertain. Further research involving in vivo models is needed to validate the potential of escin as a therapeutic agent. Additionally, this study examined only a limited subset of molecular pathways, and a more comprehensive analysis of other signaling pathways is required to fully characterize escin's effects.

5. CONCLUSION

In this study, we investigated the effects of escin on retinal pigment epithelial (RPE) cells, focusing on its potential to modulate key signaling pathways involved in cell migration and angiogenesis. Our findings demonstrated that escin significantly suppresses the wound healing process by

reducing cell migration, likely through the downregulation of PDGFβ, PDGFβ-R, and HIF1A mRNA expression levels. Additionally, escin was shown to decrease MMP9 and AKT protein levels, suggesting a potential mechanism for its inhibitory effects on cell migration. These results align with existing literature and support the hypothesis that escin may impair cellular pathways critical for RPE cell migration, particularly in pathological conditions like proliferative vitreoretinopathy (PVR).

Despite these promising results, the study acknowledges certain limitations, including its in vitro nature and the focus on a limited set of molecular pathways. Further in vivo research is essential to confirm the therapeutic potential of escin in retinal diseases and to explore its broader impacts on other signaling pathways. Overall, escin shows promise as a potential therapeutic agent for managing proliferative retinal conditions, but comprehensive studies are required to validate its efficacy and safety in clinical settings.

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Author Contributions:

Research idea: SŞ and MK
Design of the study: SŞ, MK, SS and OT
Acquisition of data for the study: SŞ
Analysis of data for the study: SŞ, MK and SS
Interpretation of data for the study: MK, SŞ and OT
Drafting the manuscript: SŞ, MK, SS and OT
Revising it critically for important intellectual content: SŞ, MK, SS and OT
Final approval of the version to be published: SŞ, MK, SS and OT

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