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PUNICA GRANATUM EXTRACT EXERTS DUAL ANTI-AGING ACTION BY ENHANCING EXTRACELLULAR MATRIX INTEGRITY AND INDUCING AUTOPHAGY IN HUMAN FIBROBLASTS

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ABSTRACT

Punica granatum has been extensively studied for its diverse biological activities, largely attributed to its rich content of polyphenols, flavonoids, and tannins. Beyond its well-documented antioxidant and anti-inflammatory properties, recent findings highlight that pomegranate extract (CEPG) demonstrates a dual anti-aging mechanism in human fibroblasts by enhancing extracellular matrix (ECM) components and inducing autophagy. CEPG increased total collagen and hyaluronic acid synthesis while reducing MMP-1, MMP-2, and MMP-9 secretion and collagenase activity. Mechanistically, CEPG upregulated LC3-II and reduced p-mTOR levels, indicating autophagy induction through mTOR pathway modulation. These results position CEPG as a promising dermocosmetic ingredient with dual ECM-protective and pro-autophagic activity.

Keywords: *Punica granatum*; Autophagy; Anti-aging; Collagen; Fibroblasts

INTRODUCTION

Skin aging involves degradation of the extracellular matrix (ECM), particularly collagen and hyaluronic acid (HA), driven by increased metalloproteinases (MMPs) and reduced fibroblast functionality. Natural bioactive compounds have gained attention as modulators of ECM integrity [1-3].

Punica granatum (pomegranate) is rich in phenolic compounds, especially punicalagins, with strong antioxidant and dermoprotective activity. Previous studies have shown its potential to stimulate collagen production and inhibit MMPs [1,4,5]. Based on this evidence, recent investigations have explored additional cellular mechanisms that may underlie its anti-aging effects. Declines in autophagy are linked to cellular aging, and because ECM synthesis and autophagy share regulatory pathways involving mTOR, we hypothesized that CEPG exerts a dual mechanism: ECM protection and autophagy induction [1,2,6]. The present short communication aimed to evaluate the effects of

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CEPG on the human fibroblast cell line CCD1072Sk, assessing its proliferative capacity and role in the synthesis of extracellular matrix elements and inhibition of collagenase activity. Furthermore, recognizing the importance of cellular recycling in skin aging, we investigated its effect on the autophagy pathway via LC3-II and its upstream regulator mTOR. This comprehensive approach seeks to demonstrate the dual-action anti-aging enhanced potential of CEPG, providing a novel mechanistic basis for its application in advanced dermo/cosmetic formulations.

MATERIALS AND METHODS

The crude dry extract of *Punica granatum* L. peel was prepared by percolation using 60% ethanol. The extract was characterized regarding its content of total phenols and flavonoids, and the concentration of α-punicalagin and β-punicalagin was determined by HPLC-DAD. The antioxidant capacity was assessed via the DPPH scavenging assay (EC50). Human fibroblasts (CCD-1072Sk) were cultured in ISCOVE'S medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics, and maintained at 37°C in a 5% CO_2 atmosphere. Prior to all treatments, cells were serum-starved for 6 hours. Fibroblasts were treated with CEPG at a concentration of 1 mg/mL (based on initial cytotoxicity screening by MTT) for 24 hours for signal transduction assays and 48 hours for matrix synthesis and degradation assays. Viability was confirmed using the MTT assay. Experiments included untreated control groups and positive controls (e.g., Ascorbic Acid). The modulation of the ECM was assessed by measuring total collagen synthesis using the Sirius Red method on cell layers, and HA synthesis quantified in the culture supernatant using a commercial ELISA kit. Furthermore, the degradation parameters were evaluated by quantifying collagenase activity and the secretion levels of MMP-1, MMP-2, and MMP-9 in the culture supernatant using commercially available ELISA kits. To investigate the proposed dual-action mechanism, the cellular renewal pathway (autophagy) was assessed. The formation of autophagosomes was tracked via the conversion of LC3-I to LC3-II using western blotting. Concurrently, the activation of the main negative regulator of autophagy, mTOR, was evaluated by quantifying the levels of phospho-mTOR (p-mTOR) and total mTOR using specific ELISA kits. Data are presented as means ± standard deviation (SD). Statistical significance was determined by ANOVA followed by Tukey's post-hoc test, with $p < 0.05$ considered significant. All analyses were performed using GraphPad Prism software.

RESULTS AND DISCUSSION

The CEPG extract was thoroughly characterized (**Figure 1A**) and presented high content of polyphenols, with punicalagins (α and β) quantified as the major components. The initial MTT screening confirmed that CEPG at 1 mg/mL was non-cytotoxic and promoted significant fibroblast proliferation (**Figure 1B**), establishing the ideal concentration for subsequent mechanistic studies. Furthermore, the extract showed potent antioxidant capacity. The antioxidant activity test using the DPPH method, whose curve points yielded the equation $y = -1.7934x + 98.899$ ($R^2 = 0.9758$), provided a value of 27.27 µg/mL as the EC_{50} concentration, capable of scavenging 50% of the DPPH radical. This value is higher than those found in the literature [8-10].

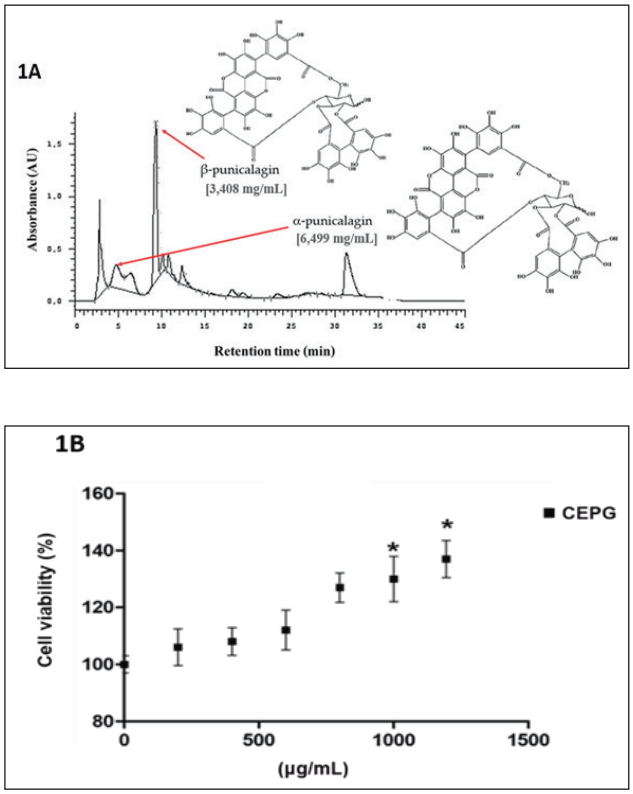

Figure 1. (**A**) Chromatogram in HPLC of the pomegranate extract. The retention time for α-punicalagin is approximately 6.5 minutes, and for β-punicalagin, it is approximately 9.5 minutes. Chemical structures are from Solakyıldırım (2019). (**B**) Proliferative effect of CEPG on CCD1072Sk fibroblasts by MTT assay after 24 hours exposure to different concentrations of CEPG (200–1.000 µg/mL). (*) $p<0.05$, significant compared to control (untreated cells). ANOVA, Tukey. Tests conducted in triplicate. GraphPad Prism v5.0.

CEPG promotes extracellular matrix integrity (action 1 of dual mechanism).

Treatment with CEPG significantly increased the synthesis of total collagen, HA content and collagenase activity in human fibroblasts (**Figure 2A, 2B,** and **2C** respectively). Concurrently, CEPG demonstrated a strong protective effect by reducing both overall collagenase activity and the secretion levels of key matrix-degrading enzymes, including MMP-1, MMP-2, and MMP-9 (**Figure 2D**, **2E**, and **2F**, respectively). These findings confirm the extract's primary role in enhancing and protecting the structural integrity of the dermal matrix [1,5].

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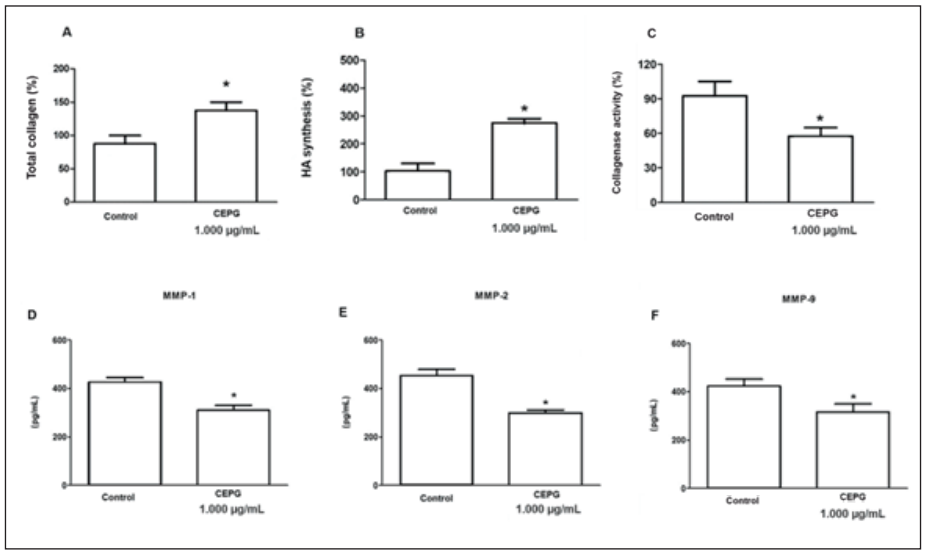

Figure 2. (**A**) Fibroblasts were treated with CEPG (1.000 µg/mL) for 24 hours, stained with Sirius Red, and absorbance was determined at 570 nm. (**B**) Hyaluronic acid synthesis was determined using an ELISA assay. (**C**) Fibroblasts were treated with CEPG (1 mg/mL) for 48 hours, and then the supernatant was collected for the ELISA test, with absorbance measured between 450 and 570 nm. (**D**, **E**, and **F**) The effects of CEPG on the secretion of MMP-1 (**D**), MMP-2 (**E**), and MMP-9 (**F**) in CCD1072Sk fibroblasts, were determined by ELISA assay. Assays were performed in triplicate and means ± standard deviations are shown as *p<0.05 compared to untreated cells. GraphPad Prism v8.0.

CEPG modulates the cellular renewal pathway (action 2 of dual mechanism).

To investigate the deep mechanism of action, the cellular signaling pathways linked to longevity were assessed. Western blot analysis showed an increase in LC3-II expression (**Figure 3A**), a hallmark of autophagosome formation. This increase was attributed to the induction of the autophagic process, supported by the observation that CEPG significantly decreased the levels of phospho-mTOR (p-mTOR) (**Figure 3B**), which is the major negative regulator of autophagy. The inhibition of the mTOR pathway suggests that CEPG acts as an autophagy inducer, promoting internal cellular cleaning and renewal [1,2,6,7].

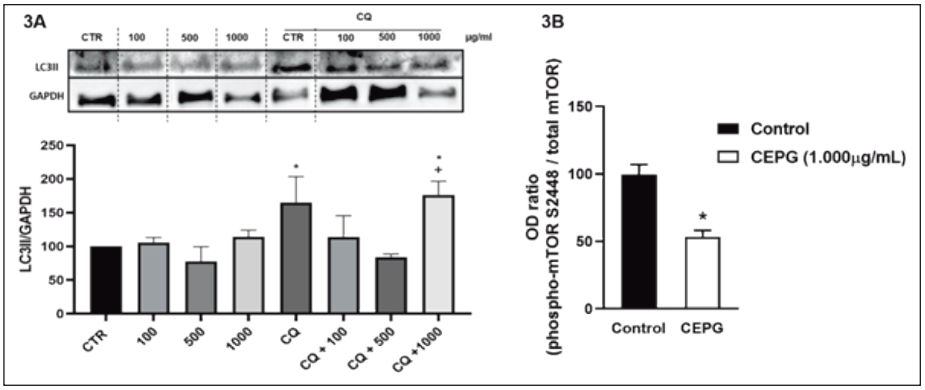

Figure 3. (**A**) Western blot analysis was performed to detect LC3B-II in fibroblasts treated with 100, 500, and 1.000 µg/mL concentrations, as well as a control (CTR-group). Cells were evaluated with or without the addition of 100 µM chloroquine (CQ) for 2 hours, introduced near the end of the experiment. Statistical analysis was conducted using ANOVA followed by Tukey's post hoc test. *P* < 0.05 vs. ctr; + P<0.05 *vs* 1.000 µg/ml extract. (**B**) Phospho-mTOR (S2448) and total mTOR levels were quantified by ELISA in fibroblasts treated with 1,000 µg/mL of extract. Results are expressed as the OD ratio (phospho-mTOR S2448 / total mTOR). Data represent mean ± SD of three independent experiments. Statistical significance was determined by unpaired t-test. P < 0.05 vs. control (untreated-cells).

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CONCLUSIONS

In conclusion, CEPG reinforces extracellular matrix integrity while modulating mTOR signaling and LC3-II expression in human dermal fibroblasts, corroborating its proposed dual anti-aging potential. While current results are consistent with the activation of autophagy-related pathways, further studies evaluating functional markers of autophagic flux, such as p62/SQSTM1, and upstream regulatory mechanisms contribute to a more comprehensive understanding of the signaling events involved. These future investigations will help to further delineate the molecular structure underlying the ECM-protective and pro-autophagic effects presented.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable.

INFORMED CONSENT STATEMENT

Not applicable.

DATA AVAILABILITY STATEMENT

The data presented in this study are available.

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