

Procyanidins and HaCaT Cells: Exploring *In Vitro* Wound Healing Implications

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Abstract: Procyanidins, a subclass of flavonoids, were recognized as potent antioxidants derived from plant-based polyphenolic compounds. Due to their antioxidant properties, they were hypothesized to aid in wound healing by preventing cell damage. In this study, *in vitro* assays were conducted to evaluate the effect of procyanidins on human keratinocytes (HaCaT). Viability and proliferation of HaCaT cells were assessed using MTT assays, while scratch assays were employed to measure wound closure. The study revealed that procyanidins induced mild cytotoxicity in HaCaT cells, with both viability and proliferation decreasing significantly after 24 hours compared to untreated cells ($p < 0.05$). However, over prolonged incubation periods (48 and 72 hours), proliferation gradually increased, albeit not significantly compared to untreated cells. Migration assays revealed that procyanidins did not enhance wound closure compared to untreated cells, suggesting no positive effect on cell migration. In conclusion, despite being potent antioxidants, procyanidins showed no significant impact on wound healing activities in this study. The research identifies limitations in the effectiveness of procyanidins on wound closure, and further research is recommended to understand the cellular response to procyanidins by exploring signalling pathways and biological mechanisms involved.

Keywords: antioxidants; human keratinocytes; *in vitro*; procyanidins; wound healing.

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1. Introduction

Wound healing denotes the process by which a normal healthy skin, upon deviation from its typical state and functional integrity due to physical, chemical, or biological agents [1], undergoes a series of organized and sequential phases to restore its integrity. This intricate process involves haemostasis, inflammation, proliferation, and tissue remodelling [2]. Prompt management of skin injuries is essential to mitigate complications such as bleeding, infection, and potentially fatal inflammation [3].

Polyphenolic compounds, including procyanidins, garnered attention for their antioxidant and anti-inflammatory properties and were believed to facilitate wound healing [2,4]. Procyanidins, a subclass of flavonoids composed of monomeric subunits such as (+)-catechin and (-)-epicatechin, were renowned for their potent antioxidant activity, which was

believed to prevent cellular and tissue damage by scavenging free radicals [5,6]. These compounds were abundant in natural sources like fruits, nuts, legumes, and cereals, where they exhibited diverse biological activities, including wound healing properties [5,7,8].

Prior studies have explored the effects of procyanidins on cell viability and migration through both *in vitro* and *in vivo* assays, aiming to elucidate their cytotoxic or healing properties across various cell lines. While Zhang et al. [9] observed no significant cytotoxicity of grape seed-derived procyanidins on HepG2 cells, both Bak et al. [10] and Ziemlewska et al. [11] noted concentration-dependent effects on HaCaT cell survival and viability. This study aims to comprehensively evaluate the *in vitro* wound healing activities of procyanidins on immortalized human keratinocytes (HaCaT). The chosen *in-vitro* assays, including MTT assay for assessing cell viability and proliferation, and the scratch assay for evaluating wound closure capacity, were selected for their simplicity, speed, and effectiveness in monitoring interventions [2].

2. Materials and Methods

2.1. Preparation of experimental compound.

Pure synthetic procyanidins C1 were obtained from ChemFaces Biochemical Co., Ltd (Wuhan, China). A working solution of 1000 µg/mL was obtained by diluting the stock solution of pure synthetic procyanidins C1 (10 µg/mL) with sterile water. Subsequently, a serial dilution protocol was employed in Dulbecco's Modified Eagle's Medium (DMEM), resulting in concentrations of 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.63 µg/mL, 7.81 µg/mL, and 3.91 µg/mL for experimentation.

2.2. HaCaT cell culturing.

The human keratinocyte cell line (HaCaT) was obtained from the American Type Culture Collection (ATCC, United States of America). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) complete media supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution containing 100U/mL Penicillin and 1000 µg/mL Streptomycin. Cultures were maintained at 37°C in a 5% carbon dioxide (CO₂) humidified incubator. Prior to subculturing, cells were allowed to grow until reaching 70 – 80% confluency [11].

2.3. Cell viability and proliferation using MTT assay.

The experimental procedure closely followed the method outlined by Zhang et al. [9], with minor adjustments made to accommodate specific experimental requirements. HaCaT cells were seeded onto 96-well plates at a density of 6×10^3 cells per well after cell counting was conducted to determine the appropriate seeding density. The cells were then incubated at 37°C in a 5% carbon dioxide (CO₂) humidified incubator for 24 hours. Following incubation, the culture medium was replaced with the prepared experimental compound containing varying concentrations of procyanidins, and the cells were further incubated for 24 hours. For the proliferation assay, cells were treated with procyanidins for 24, 48, and 72 hours to assess proliferation over time.

After removing the treatment medium, MTT solution (0.5 mg/mL in DMEM) was added to each well and incubated for three hours. Upon the formation of insoluble purple

formazan, the supernatant was carefully removed, and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals with mild agitation to ensure thorough solubilization. The absorbance was then measured at OD 570 nm using a microplate reader (Tecan, Austria). Each experiment was performed in triplicate, with allantoin (30 µg/mL) serving as a positive control. After assessing viability (%) at 24 hours and proliferation rate (%) at each treatment time point (24, 48, and 72 hours), the data were analysed using GraphPad Prism 9, and the suitable non-toxic concentration was determined for use in the scratch assay.

2.4. Scratch assay.

The scratch assay involved seeding HaCaT cells in a 6-well plate and allowing them to reach at least 90% confluency at 37°C in 5% CO₂ atmosphere over 24 hours. The cell layer was then mechanically scratched using a sterile 200 µl plastic pipette tip to create a wound gap of approximately 1 mm wide. Treatment solutions at concentrations ranging from 7.81 – 62.5 µg/mL were applied to each well, with allantoin (20 µg/mL) used as the positive control. Phase-contrast images of the wounds were captured at 0, 8, and 24 hours using HAYEAR software to assess macroscopic migration effects [12]. Image J 1.54d software was utilized to quantify the scratch area, and relative migration (RM) was calculated by comparing the migration area between treated and untreated cells.

2.5. Statistical analysis.

The statistical analysis was conducted using GraphPad Prism 9.0 version 9. Results were presented as mean ± standard deviation (SD), with all analyses performed in triplicate. One-way ANOVA and Dunnett's multiple comparison tests were utilized to assess statistically significant differences between groups, with a *p*-value of <0.05 considered significant.

3. Results and Discussion

3.1. Cell viability.

The viability of HaCaT cells treated with procyanidins was analysed to determine the cytotoxic effects of different concentrations, as depicted in Figure 1. In this study, cell viability was assessed using the 3-[4,5- Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay, which measures viability based on the conversion of MTT to formazan crystals by living cells [13]. It was presumed that the quantity of formazan crystals produced correlates with the number of viable cells [13,14]. According to the classification by López-García et al. [15] based on ISO 109993-5 standards, cytotoxicity levels were determined by cell viability percentages: 80% and above indicated non-cytotoxicity, 60% - 80% indicated weak cytotoxicity, 40% -60% indicated moderate cytotoxicity, and below 40% indicated strong cytotoxicity.

The results demonstrated a significant decrease in viability of the treated HaCaT cells compared to both untreated cells (<100%) and allantoin (*p* = 0.0187). Notably, cells treated with procyanidins at 62.5 µg/mL exhibited the highest viability (82.68%), followed by those treated with 31.25 µg/mL (81.21%), both classified as non-cytotoxic. However, cells treated with lower concentrations (3.91, 7.81, 15.63, 125, and 250 µg/mL) showed viabilities ranging from 70% to 80%, indicating weak cytotoxicity. Conversely, the viability dropped to 53.71% at a concentration of 500 µg/mL, classified as moderately cytotoxic.

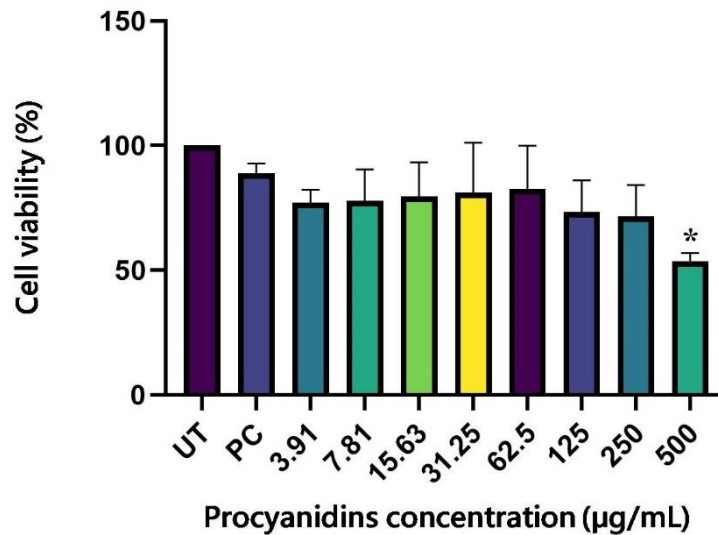


Figure 1. Cell viability (%) of human keratinocyte cells (HaCaT) treated with procyanidins at different concentrations (3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500 µg/mL) and Allantoin (30 µg/mL) as positive control (PC) for 24 hours at 37°C. *Significant differences against untreated cells (UT) as a normal control. Bars represent the mean ± S.E.M. of triplicate measurements (n=3).

Procyanidins, primarily composed of flavan-3-ol monomers and oligomers, are natural polyphenolic compounds known for their antioxidant properties [5]. Despite their documented health benefits, including antioxidant, antibacterial, anticancer, and anti-inflammatory properties [16–20], HaCaT cells treated with procyanidins at all concentrations exhibited decreased viability compared to untreated cells, which seems contradictory given their antioxidant-rich nature.

Previous studies on similar compounds, particularly epicatechin conjugates, have reported a concentration and time-dependent effect on cell viability [21, 22]. These studies found that while epicatechin conjugates might not significantly impact cell viability, proliferation, or differentiation, other compounds like procyanidins B2 strongly inhibited cellular proliferation at higher concentrations [17]. This variation indicates that the responses of flavonoid compounds are influenced by the molecular structure of polyphenols, with different structures eliciting different cellular reactions.

Given that procyanidins contain both epicatechins and catechins, these findings are particularly relevant. This suggests that while procyanidins have potential cytotoxic effects, adherence to a safe and proven non-toxic concentration could positively influence cell viability. This concentration and time-dependent effect underscores the importance of carefully determining the appropriate dosage to harness the benefits of procyanidins while minimizing their cytotoxic risks. Therefore, despite the potential cytotoxicity, procyanidins may play a role in promoting cell viability if used within safe concentration limits.

3.2. Cell proliferation.

In line with the cell viability assays, cell proliferation was conducted to assess the treated HaCaT cells' ability to reproduce and increase in number over time (Figure 2). The observation intervals were set at 24, 48, and 72 hours. During the initial 24-hour period, the treated cells exhibited a notable decrease in proliferation compared to the untreated cells, especially at higher concentrations starting from 31.25 µg/mL and above ($p < 0.001$), while lower concentrations showed a higher proliferation rate among treated cells alone. However, after 48 hours, higher concentrations showed a significant increase in proliferation rates

compared to the lower concentrations ($p < 0.001$), with lower concentrations exhibiting only a slight reduction in proliferation. Surprisingly, after 72 hours, there was an overall increase in proliferation for all concentrations, though not significantly different from untreated cells. Despite the increased rates, the positive control showed a decline in proliferation over time.

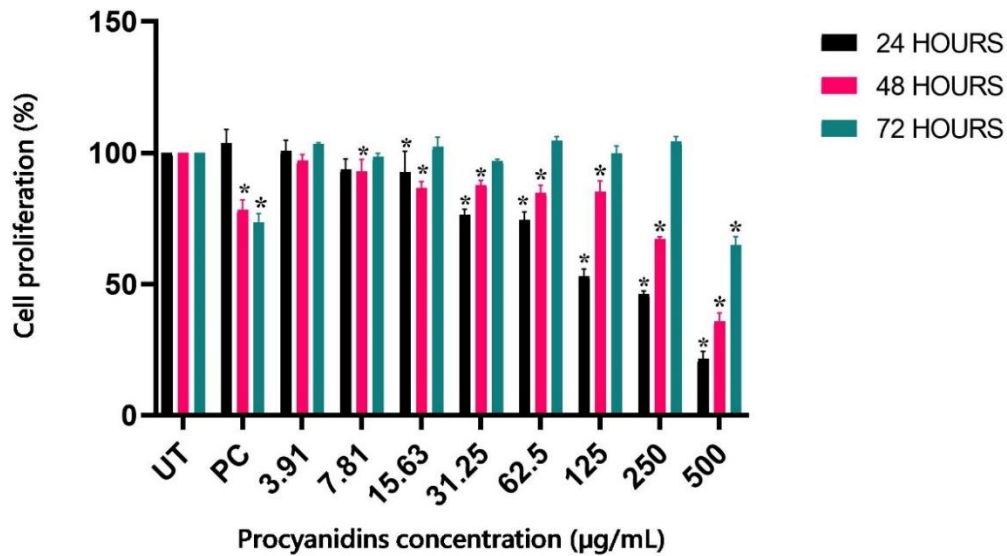


Figure 2. Cell proliferation (%) of human keratinocyte cells (HaCaT) treated with procyanidins at different concentrations (3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500 µg/mL) and Allantoin (30 µg/mL) as positive control (PC) for 24, 48 and 72 hours at 37°C. *Significant differences from untreated cells (UT) as a normal control. Bars represent the mean ± S.E.M. of triplicate measurements (n=3).

Table 1 presents the proliferation rates (%) for each time point, which were derived from the mean (average) of the triplicate measurements (n=3). It was observed that after 48 hours of incubation, lower concentrations (3.91, 7.81, 15.63 µg/mL) experienced a decrease in proliferation rate, while higher concentrations (31.25, 62.5, 125, 250, 500 µg/mL) appeared to be actively proliferating compared to the 24-hour mark. However, all concentrations exhibited a higher proliferation rate after 72 hours of incubation, irrespective of minor fluctuations observed within the 48-hour period.

Table 1. Rate of cell proliferation (%) in each incubation time (24 hr, 48 hr, and 72 hr).

Treatment (µg/mL)	Cell proliferation (%)		
	24 hours	48 hours	72 hours
Untreated	100	100	100
Allantoin (30)	103.65	78.32	73.52
3.91	100.75	97.03	103.32
7.81	93.75	92.87	98.5
15.63	92.61	86.6	102.18
31.25	76.35	87.72	96.85
62.5	74.48	84.63	104.5
125	53.0	85.16	99.74
250	46.24	67.13	104.3
500	21.47	35.91	64.96

These findings are consistent with the dual nature of antioxidants, which can both scavenge and generate free radicals, affecting cellular processes such as the cell cycle and apoptosis. While compounds with strong antioxidant activity may exhibit high toxicity [22], their ability to induce apoptosis in cancer cells while being non-cytotoxic to HaCaT cells suggests potential therapeutic applications, particularly for antioxidants like procyanidins B2 [23]. This dual role of antioxidants as both antioxidants and prooxidants, depending on the

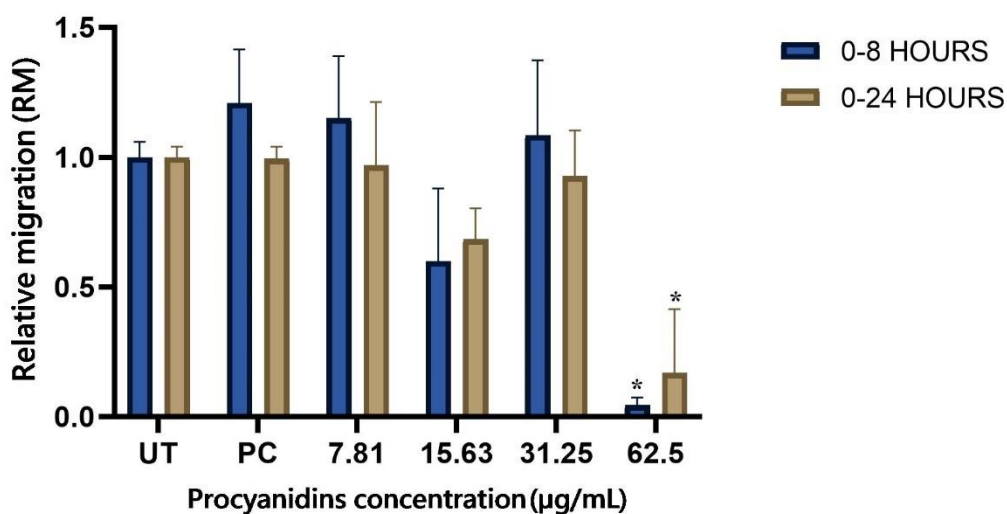
cellular environment, highlights their complex nature. Furthermore, variations in study outcomes may arise from complex interactions between compounds and cells, influenced by structural characteristics and compound types [24].

The observed reduction in proliferation is consistent with findings from research on wound healing with leaf extracts, where higher extract concentrations inhibited proliferation without significantly affecting cell energy levels [17]. The increase in proliferation over time may be attributed to changes in cellular behaviour due to frequent exposure to plant-derived polyphenols, potentially mitigating cytotoxicity tolerance [22]. However, the steady decline in proliferation observed in the positive control may be influenced by factors such as inappropriate concentrations and compound stability, along with differences in cell culture conditions and metabolic pathways.

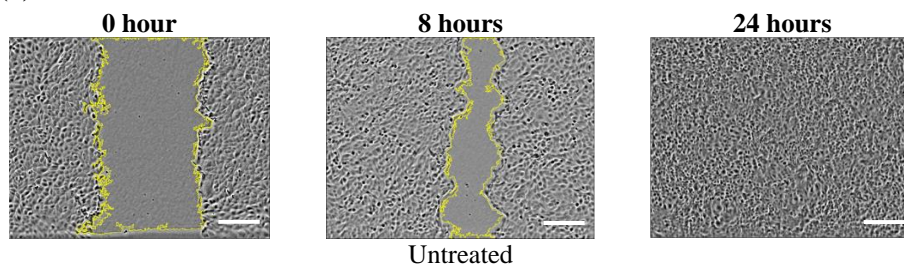
3.3. Cell migration.

Four concentrations of procyanidins (7.81, 15.63, 31.25, and 62.5 $\mu\text{g}/\text{mL}$) were tested, alongside untreated cells as a normal control and allantoin (20 $\mu\text{g}/\text{mL}$) as a positive control. Relative migration (RM) was calculated to assess migration effects, as graphically presented in Figure 3 (a), comparing both 8-hour and 24-hour periods. Lower concentrations, particularly 7.81 $\mu\text{g}/\text{mL}$ and 31.25 $\mu\text{g}/\text{mL}$, exhibited more effective migration, while higher concentrations, such as 62.5 $\mu\text{g}/\text{mL}$, displayed slower migration rates.

3 (a)



3 (b)



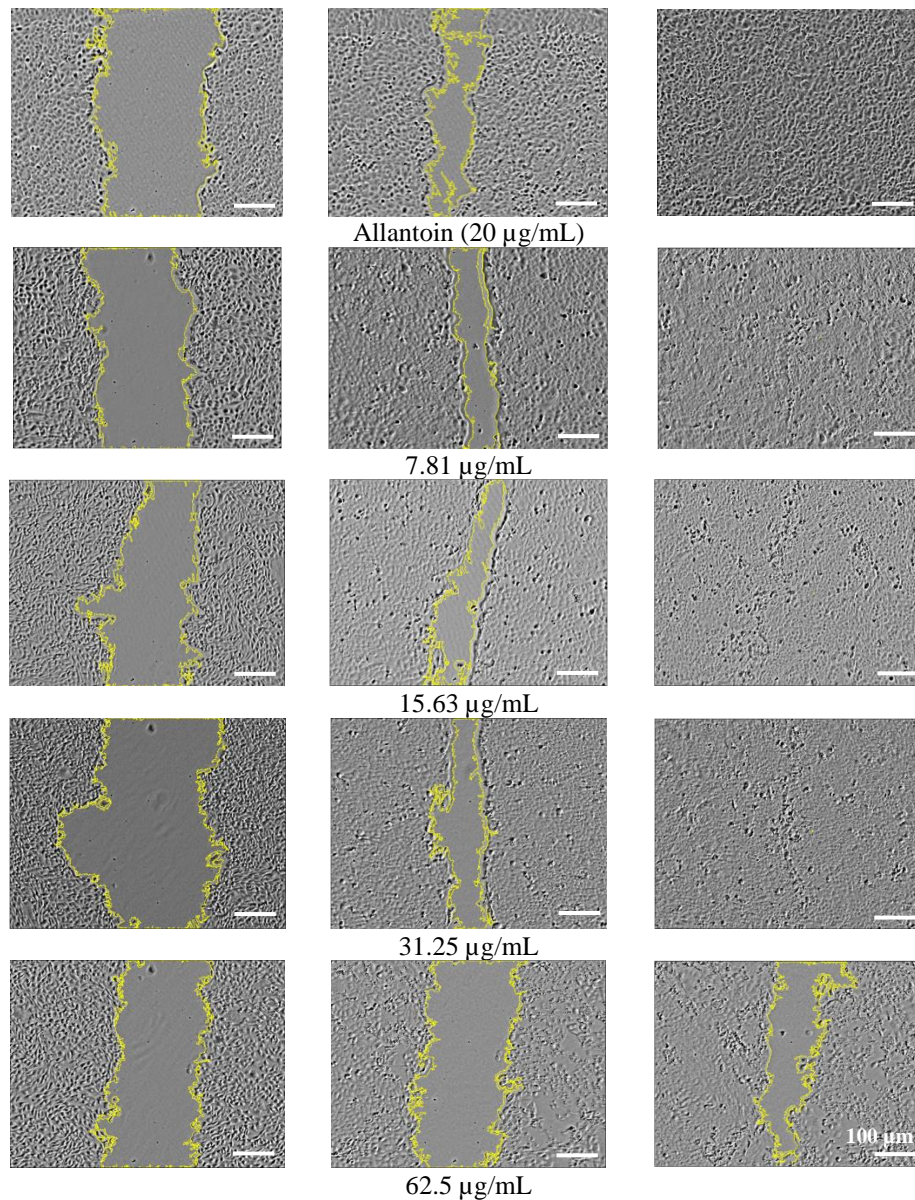


Figure 3. (a) Comparison of the relative migration (RM) of wounded HaCaT cells treated with procyanidins at 7.81, 15.63, 31.25 and 62.5 $\mu\text{g/mL}$, and Allantoin (20 $\mu\text{g/mL}$) as positive control (PC) between 0-8 hrs and 0-24 hrs at 37°C. *Significant differences from untreated cells (UT) as a normal control. Bars represent the mean \pm S.E.M. of triplicate measurements (n=3). **(b)** Phase contrast images of wounded human keratinocyte cells (HaCaT) after treatment with procyanidins at 7.81, 15.63, 31.25, and 62.5 $\mu\text{g/mL}$, allantoin (20 $\mu\text{g/mL}$) as positive control, and untreated cells (UT) as normal control. The images were captured at each respective interval time at 0 h, 8h, and 24 h after incubation at 37°C.

These differences were visually observed in Figure 3 (b), where most treatments showed positive migration over 8 hours, suggesting potential wound healing. However, 62.5 $\mu\text{g/mL}$ procyanidins resulted in areas remaining unclosed after 24 hours of treatment. Importantly, these differences were not statistically significant compared to the untreated cells ($p>0.05$).

The wound healing process involves several phases, including haemostasis or inflammation, migration and proliferation, and remodelling [25]. In this study, the scratch assay was employed to evaluate cell migration, a critical aspect of wound healing that involves re-epithelialization, facilitated by keratinocyte migration [26,27]. HaCaT cells were selected for their reproducibility and ability to mimic epidermal cell behaviour without becoming cancerous [22]. These findings contrast with *in vivo* studies demonstrating procyanidins' promotion of

wound healing and angiogenesis, suggesting potential differences in effectiveness between *in vitro* and *in vivo* settings [28]. Other factors, such as testing the compound independently without interference from other bioactive compounds, may also contribute to these differences. The study encountered several limitations, including the use of extensive cell passage (p58), which may lead to diminished responses to treatment. Variations in cell culture conditions, including changes in temperature, pH, media composition, cell seeding density, and the presence of contaminants, may also contribute to the outcomes, emphasizing the need for rigorous experimental control and optimization of culture conditions for reliable results.

4. Conclusions

Procyanidins, renowned for their antioxidant properties, were investigated for their potential in wound healing using immortalized human keratinocytes (HaCaT), focusing on viability, proliferation, and migration. While procyanidins exhibited mild cytotoxicity and a slight decrease in viability after 24 hours, proliferation improved over time. However, migration effects resembled untreated cells, suggesting a limited impact on wound healing. These findings underscore the complexity of cellular responses to procyanidins and highlight the need for further research to elucidate their mechanisms of action and explore potential health benefits beyond wound healing.

Author Contributions

All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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