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The Evaluation of Wound Healing Potential of Curcumin Dendrosomes on Fibroblast Cell Line-Focus on Anti-Bacterial and Anti-Inflammatory Properties

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Abstract

Background: It is essential to find natural compounds with wound-healing properties that are accompanied by antibacterial effects. Therefore, in the present study, the wound-healing potential of curcumin dendrosomes on fibroblast cell line was evaluated.

Methods: The HFF1 cell proliferation was evaluated by MTT test after 48 hours of curcumin (Cur) and curcumin dendrosomes (Cur.den) treatments. Scratching test was used to check cell migration and the expression levels of TNF-αand TGF-βgenes in cells were studied by Real-Time PCR technique. To investigate the antibacterial activity of Cur and Cur.den against S. epidermidis, well diffusion and microdilution tests were used.

Results: Cur.den improved significantly HFF1 cell proliferation and migration compared to both untreated control and Cur-treated cells. Moreover, this nanoformulation significantly overexpressed TGF-βand downregulated TNF-α genes compared to control cells. Both Cur and Cur.den reduced S. epidermidis growth in well diffusion assay and their MICs were measured at 200 and 100 µM, respectively.

Conclusion: The Cur.den has a high potential to be used as a wound-healing drug. However, studies in animal models and clinical settings are recommended.

Keywords: Curcumin, Dendrosome, Expression, Gene, Migration, Proliferation.

1. Introduction

The destruction of the anatomical and functional structure of the skin is considered a wound (1), and its healing includes inflammatory, cell proliferation, healing, and regeneration phases (2). Factors such as the size of the wound, local blood supply, presence of foreign bodies and microorganisms, age, comorbidities, and nutritional status affect the speed of wound healing (2).

Different drugs and dressings are used for wound healing, which are associated with different effectiveness (3). However, due to society's desire for natural compounds or medicinal plants, researchers are seeking to discover these compounds with wound-healing properties (4, 5). Curcumin (Cur) is a natural compound extracted from the *Curcuma longa*, which showed extensive pharmacological activity (6, 7). This polyphenolic compound belongs to the curcuminoids and affects various cellular, molecular, and biochemical pathways including inflammatory, antioxidant, cell proliferation cycle, and apoptosis, which proves its wide range of biological effects (6). The wound-healing properties of Cur have been reported in various studies. For example, Panchatcharam et al. (2006) showed that Cur led to the acceleration of wound healing, and the mechanism of action was attributed to increasing collagen synthesis and reducing oxidative stress and reactive oxygen species (ROS) (8). The deposition of collagen in the wound site due to the migration of fibroblast cells and the creation of granulation tissue is one of the most important stages of wound healing (9), and it has been shown in various studies that curcumin improves the infiltration of fibroblasts into the wound site and increases the speed of wound healing (10, 11). On the other hand, curcumin showed the ability to reduce the time of the inflammatory phase of wound healing (11), which indicates the high potential of this compound in wound

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healing. Despite the high potential of this compound in wound healing, Cur has low bioavailability and a short half-life, which limits its use (12). To overcome this problem, the development of formulations based on nanotechnology is recommended. For example, Krausz et al. (2015) showed that nanoparticles encapsulated with curcumin had antibacterial properties against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* and improved wound healing in animal models (12). The development of Cur-loaded nanoformulations has been introduced as a suitable approach to improve wound healing effects. Therefore, in the present study, the wound healing potential of curcumin dendrosomes (Cur-den) was evaluated by emphasizing the expressions of tumor necrosis factors α (TNF-α) and transforming growth factor β (TGF-β) genes. Also, the antibacterial effects of Cur and Cur.den were evaluated on *the Staphylococcus epidermidis* strain (PTCC 1435).

2. Materials and Methods

2.1. Preparations of Curcumin dendrosomes and HFF-1 cell line

Cur-den (100 mg/ml) was prepared by the research team of Dr. Majid Sadeghizadeh's laboratory, a scientific member of Tarbiat Modares University, Tehran-Iran. The HFF 1 cell line was obtained from the cell bank of the Pasteur Institute of Iran-Tehran. Cells were cultured in DMEM medium supplemented with 10% FBS (GIBCO, UK) and penicillin and streptomycin antibiotics (Sigma, USA) for 3 days at 37°C and 5% CO2.

2.2. HFF-1 cells' treatment

Cells were counted using Trypan blue dye exclusion method. Then, the cells (3×10^4 cells/well) were treated with different concentrations of curcumin dendrosomes (Cur.den), free curcumin (Cur), and dendrosome carrier (den) (0, 12.5, 25, 50, and 100 μM).

2.3. Cell viability

The cell viability was measured via MTT methods: Briefly, HFF-1 cells (3 × 104) were cultured in 96-well plates and then treated with Cur.den and Cur at the aforementioned concentrations for 48 hours. The plates were incubated at 37°C and 5% CO2. Then, 0.005 mg/mL of MTT solution (100 μL) was added to each well and incubated for 4 hours in the aforementioned conditions. Finally, Dimethyl sulfoxide (DMSO) was added to each well. After 2 hours, the absorption of samples was read by an enzyme-linked immunosorbent assay (ELISA) reader (Eppendorf, Germany) at a wavelength of 570 nm.

2.5. Cell migration assay

First, cells (4 × 105) were cultured in a 12-well plate. Then, scratches with the same diameter were made on the bottom of each well. Then, the treatments were applied and cell migration for 24 hours was evaluated using the optical microscope (Nikon, Japan).

2.6. Gene expression analysis

2.6.1. RNA extraction and cDNA synthesis

In this study, we used the RNA extraction kit (DenaZist, Iran) to extract RNA from HFF-1 cells according to the manufacturer's instructions. Nanodrop device and UV spectrophotometry were used for quantitative and qualitative evaluation of extracted RNA, respectively. For cDNA synthesis based on extracted RNA templates, we used a cDNA synthesis kit (Parstos, Iran) based on manufacturer instructions. The temperature-time program of the device included 25°C for 5 minutes, 85°C for 60 minutes, and 95°C for 5 minutes. The samples were stored at -20°C.

2.6.2. Primer design

Primer design was done through the NCBI database and Gene Runner software. Also, after primer designing, the sequences were blasted on the NCBI website to ensure the position of the primer on the exons. *The GADPH* gene was considered as a control, and the sequences of primers are given in Table 1.

Table 1. The sequences of primers for *TNF-α*, *TGF-β* and *GADPH* genes for studying their expression levels by RT-PCR technique

Genes	Sequences [5'-3']	Length (bp)
TGF-β	F- ACAATTCCTGGCGATACCTCA R- TGAACCCGTTGATGTCCACTTG	192
TNF-α	F- TCTCTCGAACCCCGAGTGA R- TATCTCTCAGCTCCACGCCA	128
GADPH	F- GTGGTCTCCTCTGACTTCAAC R- GGAAATGAGCTTGACAAAGTGG	96

2.6.3. RT-PCR

The RT-PCR reaction was performed using the Cyber Green method using Rotor-Gene Q (Qiagen, Germany). The reaction mixture included 7 μL of master mix, 0.5 μL of each forward and reverse primer, 1 μL of cDNA, and 6 μL of deionized water in a final volume of 15 μL. The RT-PCR temperature-time program includes one cycle of 95°C for 12 minutes, 35 cycles of 95°C for 15 seconds, 62°C for 20 seconds, and 72°C for 30 seconds, and the final cycle at 60-95°C. $2^{-\Delta CT}$ method was used to analyze gene expression data.

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2.7. Antibacterial assay

S. epidermidis strain (PTCC 1435) was obtained from the Iran Scientific and Industrial Research Organization. The strains were transferred to LB broth medium and kept at 32°C for 24 hours. Then, the bacteria were separated by centrifugation at 4000 rpm and the bacterial population was determined by the McFarland method and obtained by diluting the population to 0.1% of physiological serum (1.5×10^6 bacteria/mL).

For the antibacterial effects of Cur and Cur.den, we used the well diffusion method on an LB agar medium. Briefly, after culturing bacteria in LB agar culture medium, a well was created by a sterile Pasteur pipette on the medium, and different concentrations of Cur, Cur.den and nanocarrier were poured into each well. Then, the plates were incubated for 24 hours at 37°C. The diameter of the inhibition zone was measured using a ruler. Gentamicin (5 mg) was used as a positive control and DMSO was used as a negative control.

The microdilution method was used to determine the minimum inhibitory concentration (MIC) of Cur and Cur.den. For this purpose, 100 µL of LB broth was poured into 96-well plates and serial dilutions of Cur.den and Cur (400, 200, 100, 50, and 25 µM) were added to the wells. Bacteria were added to each well at a concentration of 1 McFarland (125 µL). The well contains 100 µL of culture medium and 100 µL of Cur.den were considered as negative control and the well containing 100 µL of culture medium and 100 µL of bacterial suspension were considered as positive control. The absorbance of the samples was read at a wavelength of 600 nm by ELISA Reader during 1, 2, 3, 4, 5, 24, and 48 hours.

10 µL from the last well where no bacterial growth was observed and so on were removed and placed on LB agar culture medium and incubated at 37°C for 24 hours. The lowest concentration at which the bacteria did not grow was considered the minimum bactericidal concentration (MBC).

2.8. Statistical analysis

$2^{-\Delta\Delta CT}$ method was used to analyze gene expression data. Data were expressed as mean ± SEM. $P<0.05$ was considered as a significant level. GraphPad Prism software was used for data analysis.

3. Results

3.1. Cell viability

The proliferation of HFF1 cells exposed to different concentrations of Cur and Cur.den was significantly increased in a concentration-dependent manner compared to the control (0 µM). Moreover, comparing the proliferation of HFF1 cells treated with Cur and Cur.den indicated a significant increase in the intensity of proliferation of cells exposed to Cur-den compared to Cur (Figure 1). Thus, cells treated with 25, 50, and 100 µM Cur.den showed 20 (P=0.048), 42 (P=0.009), and 40% (P=0.007) more cell proliferation compared to HFF1 cells exposed to Cur. These results show the improvement of fibroblast cell proliferation by Cur.den, which shows its therapeutic potential in healing wounds.

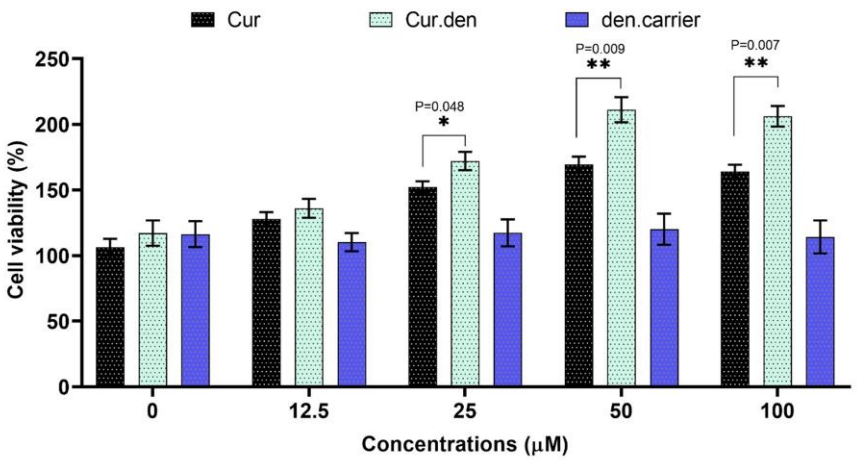


Figure 1. The viability of the HFF1 cell line was treated with different concentrations of curcumin (Cur) and curcumin dendrosomes (Cur.den) (0, 12.5, 25, 50, and 100 µM) for 48 hours (n=3).

3.2. Cell migration assay

One of the important characteristics of fibroblast cells is their ability to migrate to the wound site. Therefore, in the present study, the migration speed of HFF1 cells treated with Cur and Cur.den was studied in the scratching test (Figure 2). The results showed that HFF1 cells exposed to Cur.den had a greater ability to migrate, which indicates the higher potential of this nanoformulation in wound healing than free curcumin.

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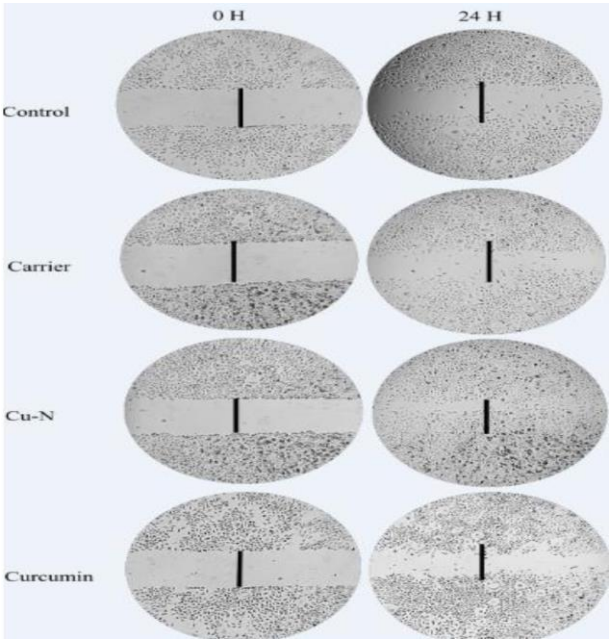


Figure 2. The scratching test for evaluating of HFF1 cell line migration speed was treated with 50 µM free curcumin and nano curcumin.

3.3. Gene expression analysis

The quality of the extracted RNA was evaluated by gel electrophoresis technique, and the results indicated the high quality of the extracted RNA (Figure 3a). Also, RT-PCR products were run on gel electrophoresis and its image is shown in Figure 3b. *TNF-α* gene was significantly down-regulated in both HFF1 cells treated with Cur (P=0.009) and Cur.den (P<0.0001) compared to the untreated control cells (Figure 3c). Nevertheless, the comparison of *TNF-a* gene expression levels in HFF1 cells treated with Cur and Cur.den indicated a greater decrease in the latter treatment (P=0.029). In contrast, *TGF-β* overexpressed significantly in both Cur (P=0.49) or Cur.den (P<0.001) treated HFF1 cell line compared control cell (Figure 3d). Also, the overexpression of this gene was higher in Cur.den treated HFF1 cells compared to free Cur ones (P=0.003).

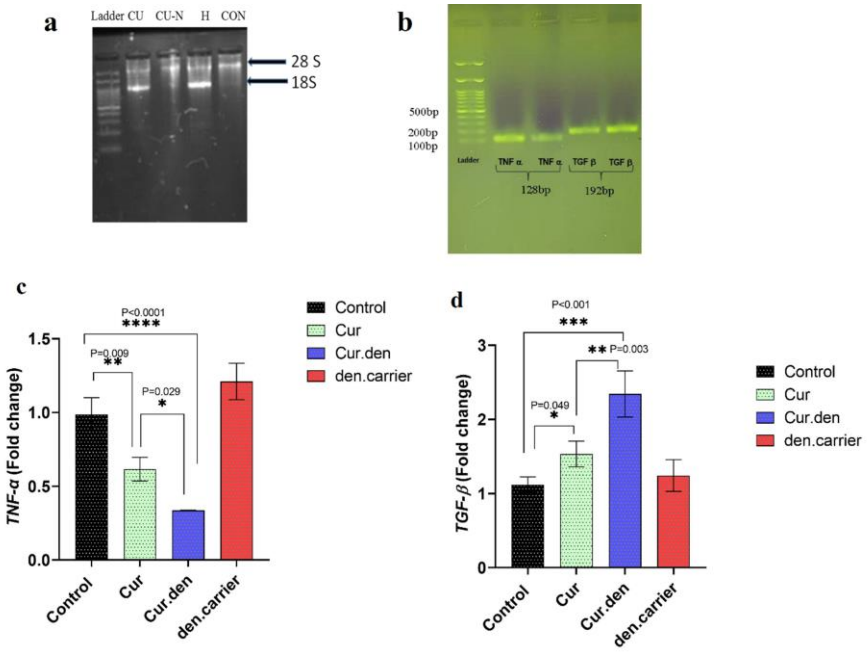


Figure 3. The extracted RNA from treated HFF1 cells and its running on gel electrophoresis (a); Running the RT-PCR products on gel electrophoresis (b). The expression levels of TNF-α(c) and TGF-β(d) in the HFF1 cell line treated with 50 µM curcumin (Cur) and curcumin dendrosomes (Cur.den).

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3.4. Antibacterial assay

We investigated the antibacterial effects of both curcumin and curcumin dendrosomes against S. epidermidis (PTCC 1435) using the well diffusion method and the results indicated the antibacterial effects of both compounds in a concentration-dependent manner (Table 2). The antibacterial effects of Cur.den against S. epidermidis (PTCC 1435) were stronger than free Cur. The zone inhibition diameter in bacteria treated with 100 µM Cur and Cur.den was 6.48±0.78mm and 8.98±0.53mm, respectively, which show the strong antibacterial effects of Cur.den.

Table 2. The zone inhibition diameter in S. epidermidis (PTCC 1435) treated with different concentrations of curcumin (Cur) and Curcumin dendrosomes (Cur.den) in well diffusion assay

concentration	Cur (mm)	Cur.den (mm)	den.carrier (mm)
0	-	-	-
25	1.44±0.2	1.66±0.14	-
50	2.63±0.41	3.1±0.34	-
100	6.48±0.78	8.98±0.53	-
200	12.28±1.22	14.96±0.28	-

Microdilution method was used to determine the MIC, and the MICs of Cur and Cur.den were calculated at 200 and 100 µM, respectively. Also, MBC of Cur was 400 µM and MBC of Cur.den against *S. epidermidis* (PTCC 1435) was 200 µM, indicating stronger antibacterial effects of Cur.den compared to Cur.

Table 3. The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of curcumin (Cur) and Curcumin dendrosomes (Cur.den) against *S. epidermidis* (PTCC 1435)

concentration	Cur	Cur.den	den.carrier
MIC (µM)	200	100	-
MBC (µM)	400	200	-

4. Discussion

The of the present study indicated that Cur.den and Cur improved the proliferation and migration of HFF1 cells, which indicates the high potential of these compounds in healing wounds. Also, Cur and Cur.den significantly decreased *TNF-α* gene expression and overexpressed *TGF-β*. Finally, the antimicrobial effects of both Cur and Cur.den against *S. epidermidis* (PTCC 1435) were confirmed.

One of the obstacles to the use of curcumin in the treatment of many diseases is its low bioavailability (13), which can be overcome by the design of curcumin-loaded nanoformulations (14). Therefore, in the present study, curcumin dendrosomes were used to investigate the wound healing potential *in vitro*. Dendrosomes are a family of drug carriers with spherical, granular, and self-assembled structures that have advantages such as stability, non-toxicity, and biodegradability (15). The non-toxicity of this drug carrier was also confirmed in the present study, and not only did not prevent the growth of HFF1 cells but also improved cell proliferation. This finding is in line with the findings of the study by Kostandova and Pamula (16), who stated that low doses of curcumin stimulate the growth and proliferation of fibroblast cells. It is believed that this compound leads to cell survival and has beneficial effects on the homeostatic response and therefore leads to improved cell growth and proliferation (17). Also, the migration of HFF1 cells treated with Cur.den was improved in the present study. In a study, it was shown that nanofiber loaded with curcumin and gelatin accelerated wound healing by improving cell mobility and migration, as well as reducing inflammation (18). Nevertheless, it has been shown in studies that free curcumin does not have a significant effect on the migration of fibroblast cells (19), and this difference can be attributed to the type of formulation designed in this research. Nanocomposite loaded with curcumin led to accelerated wound healing in animal models (20). Therefore, the development of nanoformulations loaded with curcumin, including curcumin dendrosomes, which were developed in the present study, is a suitable therapeutic solution for wound healing.

The inflammatory phase of the wound is accompanied by increased expression of inflammatory cytokines (21). TNF-α is one of the inflammatory cytokines that overexpresses during this stage, which is accompanied by apoptosis in fibroblast cells, keratinocytes, and endothelial cells, and leads to a decrease in collagen levels as a result lead to slow wound healing (22). Therefore, the reduction of *TNF-α* gene expression in fibroblast cells treated with Cur.den and Cur, which was observed in the present study, indicates the anti-inflammatory effects of this compound, which shows their potential in wound healing. TGF-β plays a role in the proliferation of fibroblasts and the initiation of the wound-healing process, which accelerates fibroplasia, angiogenesis, and the formation of granulation tissue and epithelial tissue (23). This factor stimulates fibroblasts to produce fibronectin and thus leads to the facilitation of cell attachment and fibroblast migration (24). The results of the present research showed that HFF1 cells treated with Cur and Cur.den have high levels of *TGF-β* gene expression, which can justify the improvement of the migration of these cells in the scratching test.

In most cases, wounds are accompanied by bacterial infections (25), for which antibiotics are prescribed. However, the emergence of antibiotic-resistant strains has led to a decrease in the effectiveness of these drugs in wound healing (26). Therefore, finding natural compounds with antibacterial properties is of great importance. In the present study, the strong antibacterial effects of Cur.den against *S. epidermidis* (PTCC 1435) were reported, which indicates the high potential of this compound in healing wounds. In various studies, the antibacterial effect of curcumin and curcumin-loaded nanoformulations against a wide range of pathogens has been reported (27-29). Interestingly, in most studies, nanoformulations loaded with curcumin have shown

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stronger effects than free curcumin, which is similar to the findings of the present research (30, 31). Nevertheless, it seems that gram-positive bacteria are more sensitive to curcumin nanoparticles than gram-negative bacteria. In addition, curcumin and curcumin nanoparticles have shown anti-biofilm effects in various studies, which indicates the high potential of this compound in bacterial infections.

5. Conclusion

In general, according to the findings of the present research, it can be concluded that dendrosomes loaded with curcumin have a high potential to improve wound healing. This can be attributed to increased proliferation and migration of fibroblast cells, decreased levels of TNF-α, and increased levels of TGF-β and its antibacterial effects. Nevertheless, studies in animal models and clinical settings are recommended.

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Conflict of Interest Statement

One of the authors of this article is a member of the committee board of the Journal of Human Genetics and Genomics.

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