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Evaluation of *In Vitro* Wound Healing Activity of Thymoquinone

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ABSTRACT

Objective: *Nigella sativa* has been extensively investigated as an important potential agent for the healing of wounds and there have been numerous studies regarding its effect. Although thymoquinone (TQ) is a well-known active constituent of *Nigella sativa*, studies in to the usability of TQ on wound healing are still insufficient. In this study, we aimed to evaluate the *in vitro* wound healing potential of TQ.

Materials and Methods: NIH/3T3 mouse embryonic fibroblast cells were used to evaluate the wound healing effect of TQ. Different concentrations of TQ (0.1, 1 and 10 μM) were applied to the cells and their cytotoxic effect on cells after 24- and 48- hours was measured by MTT assay. Its effect on wound healing after 18- and 24- hours recovery was examined by *in vitro* scratch assay. Also, the level of β-catenin, an effective protein in the process of healing wounds, was determined by Western blot assay.

Results: MTT analysis indicated that 0.1, 1 and 10 μM doses of TQ had increased the cell numbers. *In vitro* scratch assay data showed that treatment with 1 and 10 μM TQ resulted in a statistically significant wound closure activity (91.35% and 90.84%, respectively) compared to the control. Additionally, we observed a statistically significant increase in the β-catenin protein level which supported our data.

Conclusion: Our results demonstrated that TQ increases both the viability of NIH/3T3 cells and its wound closure activity *in vitro*, and that it has the effect of increasing crucial protein β-catenin. This study suggests that TQ may be a valuable substance for the healing of wounds and that its usability should be investigated.

Keywords: Thymoquinone, wound healing, NIH/3T3

INTRODUCTION

Wounds are physical injuries that cause disruption to the normal structure and function of the skin (1). Different types of wounds may occur in humans. The most difficult wounds to heal are chronic wounds and delayed acute wounds. Current studies demonstrate that nearly 6 million people in the world have chronic wounds. Annual health care expenditure is extremely high for these types of wounds, at more than $3 billion (2,3). In addition, it is estimated that 1% of the European population suffers from recurrent and chronic ulceration such as leg and foot ulcers (2,4). Wounds related

to diabetics, burns, and ulcers still have significant impact on the population. Therefore, to develop new strategies is important and recent studies have increasingly focused on natural products to treat wounds.

The current synthetic therapeutics used in wound healing still remains insufficient. They may cause adverse reactions and allergic problems that limit the use of these drugs. In order to eliminate these conditions, the development of wound healing agents taken from sources such as medicinal plants and natural products is highly important. For this reason, numerous medicinal plants have been described in the literature such as *Curcuma*

longa, Moringa oleifera, Aegle marmelos, Phyllanthus muelleri-anus and so on (5). One of these plants is *Nigella sativa,* an herbaceous plant, which belongs to family of the Ranunculaceae (6). It has been used traditionally for the treatment of diseases. It has antimicrobial, antiviral, antifungal, antiparasitic properties (7). *In vitro* and *in vivo* studies have proved that it might be useful for wound healing. In studies performed on *in vivo* burn models, it was demonstrated that *N. sativa* cream and *N. sativa* extract showed significant wound healing effects compared with a commercially available cream (6,8). In the *in vivo* diabetic model, it was shown that the wound area decreased as a result of the treatment of *N. sativa* extract (9). It is also reported that *N. sativa* extract reduces radiation-related delayed wound healing in mice (10). When human gingival fibroblast cells are used as an *in vitro* wound healing model, aqueous extract of *N. sativa* induced fibroblast proliferation and accelerated wound closure activity (1). All these healing effects and properties originate from bioactive compounds of *N. sativa* such as thymoquinone (TQ). It is known that Thymoquinone has antimicrobial (11,12), antiallergic (13), antidiabetic (14), anti-inflammatory (15), and antioxidant (16) properties . These properties make TQ an important potential agent for the healing of wounds. Interestingly, the wound healing potential of TQ has not been clearly investigated. Moreover, most of the studies are based on the *in vivo* models. However, *in vitro* studies are needed to highlight the wound healing mechanism at molecular level.

Thus far, there have been numerous studies on the wound healing effect of *N. sativa*, but studies on TQ are still insufficient. The purpose of this study is to evaluate the role of TQ on the wound healing process and on β- catenin expression in NIH/3T3 fibroblast cell line.

MATERIALS AND METHODS

Experimental Reagents
TQ was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA) and dissolved in dimethyl sulfoxide (DMSO). Tissue culture reagents were obtained from Gibco (Carlsbad, CA, USA). SMART™ BCA Protein Assay Kit was from iNtRON Biotechnology (Seongnam, Gyeonggi, Korea). Polyvinylidene fluoride (PVDF) membrane was obtained from Millipore (Darmstadt, Germany). Mouse anti-β-catenin monoclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG were obtained from Enzo Life Sciences (Farmingdale, NY, USA). HRP conjugated GAPDH Loading Control Monoclonal Antibody and Pierce™ ECL Western Blotting Substrate was from ThermoFisher Scientific (Kwartsweg, Bleiswijk, Holland). All other chemicals and reagents were from Sigma (St. Louis, MO, USA).

Cell Culture
Mouse embryonic fibroblast cells (NIH/3T3) were kindly provided by Istanbul University Faculty of Science, Department of Biology. NIH/3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM/F12) which included 1% *(v/v)* antimycotic-antibiotic solution (100 μg/mL streptomycin and 0.25 μg/mL amfoterisin B and 100 U/mL penicillin) and 10% *(v/v)* heat-inac-

tivated fetal bovine serum. The cells were incubated at 37℃ in a humidified atmosphere with 5% CO_2. The cells were passaged before they reached full confluence.

Cell Viability Assay
MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) cell viability assay was used in order to investigate the proliferative and cytotoxic effect of TQ on NIH/3T3 fibroblast cells. Briefly, the cells were seeded in a 96-well plate at a confluency of 3×10^4 per well. Adherent cells were then treated with increasing concentrations of TQ (0.01-1000 μM) which was diluted with DMEM/F12. After 24 and 48 h incubation at 37℃, the medium was removed. 30 μL of MTT (5 mg/ml) was added to the cells. After a 4 h incubation at 37℃, 150 μL of DMSO was added to solubilize formazan crystals and the absorbance was measured at 540 nm using a microplate reader. Experiments were performed in triplicate.

Thymoquinone Treatment
NIH/3T3 cells were seeded in 24 well-plates at a density of 1.8×10^5 per well for cell treatment with TQ. According to MTT assay results, final TQ concentrations of 0.1, 1 and 10 μM were applied to the cells incubated for 24 h. Experiments were performed in triplicate.

In vitro Scratch Assay
The capability of TQ on migration of NIH/3T3 cells was determined by *in vitro* scratch assay. The cells were seeded at a confluency of 18×10^4 per well into a 24-well plate containing DMEM/F12 medium and incubated overnight. After incubation, the medium was removed and the adherent cells were scratched with a sterile p1000 pipette tip. The scratched cell layers were washed with phosphate buffered saline (PBS) to remove cell debris. After that, the cells were treated with 0.1, 1, 10 μM of TQ that was diluted in DMEM/F12. Fresh medium was applied for the control group. Photographs of the scratched area were recorded by inverted light microscope equipped with a camera (Nikon Eclipse Ti-E) under 10X magnification at 0, 18 and 24 h. Data were analyzed with Image J software (NIH, USA) in order to determine the width of the scratch and the rate of migration of cells. Experiments were performed in triplicate.

Immunoblotting Assay
Western blotting assay was performed to analyze samples, as described by Şengelen and Önay-Uçar (17). In short, the cells were trypsinized and centrifugated at 700×g for 10 min. Lysis buffer [20 mM Tris-HCl (pH 6.8), 0.04% (w/v) EDTA, 1% (v/v) Triton X-100, and EDTA-free PIC (protease inhibitor cocktail), 1 mM PMSF] was used to resuspend the pellets. The extracts were centrifuged at 20,000×g for 20 min at 4℃. Protein concentrations were quantified by bicinchoninic acid protein assay. Thirty μg of protein was separated by SDS-PAGE gel and transferred onto PVDF membranes. 5% non-fat dry milk in Tris-buffered saline/Tween 20 (TBST) was used to block membranes for 1 h. After that, the membranes were incubated overnight with anti-β-catenin primer antibody (1:1000) at 4℃. The following day, the membranes were washed five times with TBST, and then incu-

bated with IgG-HRP secondary antibody (1:5000) for 2 h at 37°C, and washed with TBST again. Protein bands were visualized using an ECL kit. GAPDH (antibody diluted 1:2000) was used for data normalization. ImageLab 5.2.1 software (Bio-Rad) was used to determine protein expression levels. Experiments were performed in triplicate.

Statistical Analysis

One-way or two-way ANOVA was used to analysis data followed by Tukey post-hoc-test. The statistically significance was taken to be $P<0.05$. The results were presented as mean ± standard deviation (SD) and the number of experiments were indicated with n. Statistical analysis and graph generation were carried out in GraphPad Prism Software (San Diego, CA, USA) version 7.0.

RESULTS

Thymoquinone Effects Viability of NIH/3T3 Cells

The effect of increasing concentrations of TQ on cell viability was evaluated in NIH/3T3 after 24 h and 48 h incubation using MTT assay. The data is shown in Figure 1. The cells were treated with 0.01–1000 µM of TQ. MTT results showed that 1 and 10 µM of TQ was able to promote cell viability. However, 50 µM and above of TQ showed decreased cell viability after 24 h incubation. In addition, the data showed that TQ decreased cell viability of NIH/3T3 cells in a dose-dependent manner after 48 h incubation. The half-maximal inhibitory concentration (IC_{50}) was determined 51.43 µM after 24 h and 47.71 µM after 48 h incubation. According to the MTT results, different concentration of TQ was used and selected according to significantly increased cell viability compared to thecontrol. Therefore, the final concentrations of thymoquinone which are 0.1, 1 and 10 µM were used in the experiments.
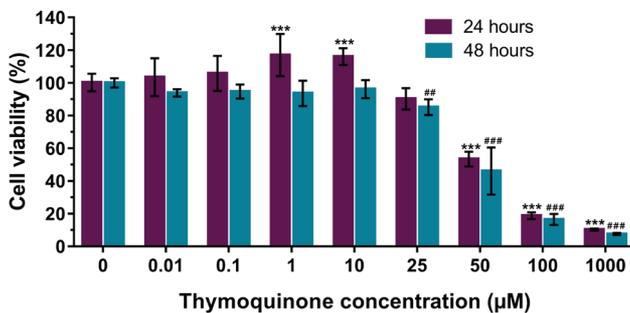
Figure 1. Determination of TQ effect on cell viability after 24 h and 48 h incubation. The graph represents the mean±SD of three independent experiments analyzed together (n=3). ***$P<0.001$ compared to control for 24 h, ###$P<0.001$, ##$P<0.01$ compared to control for 48 h determined by one-way ANOVA using Tukey post-hoc-test.

The Effect of Thymoquinone on NIH/3T3 Cells Wound Healing

In vitro scratch assay was used to determine wound closure activity of TQ on NIH/3T3 cells after 18 and 24 h recovery. *In vitro* scratch assay data showed that, although there was no statisti-

cally significant difference between 0.1 µM TQ and the control groups, treatment with 1 and 10 µM TQ resulted in a statistically significant recovery in the wound area compared to the control after 18 and 24 h (Figures 2 and 3). The closure of the control was 57.9%, 64.98% for 0.1 µM, 75.38% for 1 µM and 71.82% for 10 µM after 18 h of incubation. After 24 h of incubation, the clo-
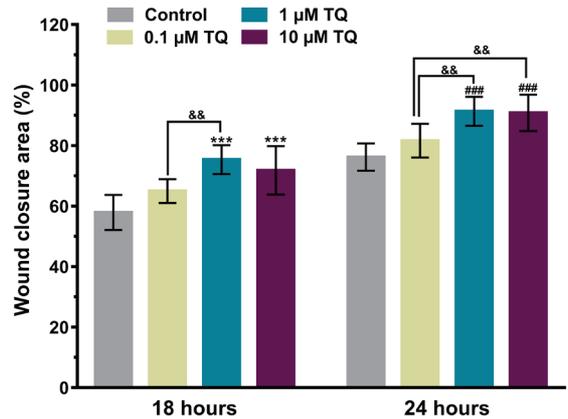
Figure 2. Percentage of wound closure area of NIH/3T3 fibroblast cells treated with TQ determined by *in vitro* scratch assay after 18 hours and 24 h incubation. The graph represents the mean±SD of three independent experiments analyzed together (n=3). ***$P<0.001$ compared to control for 18 h, ###$P<0.001$ for 24 h. &&$P<0.01$ shows multiple comparisons between different groups. P values were determined by one-way ANOVA using Tukey post hoc-test.
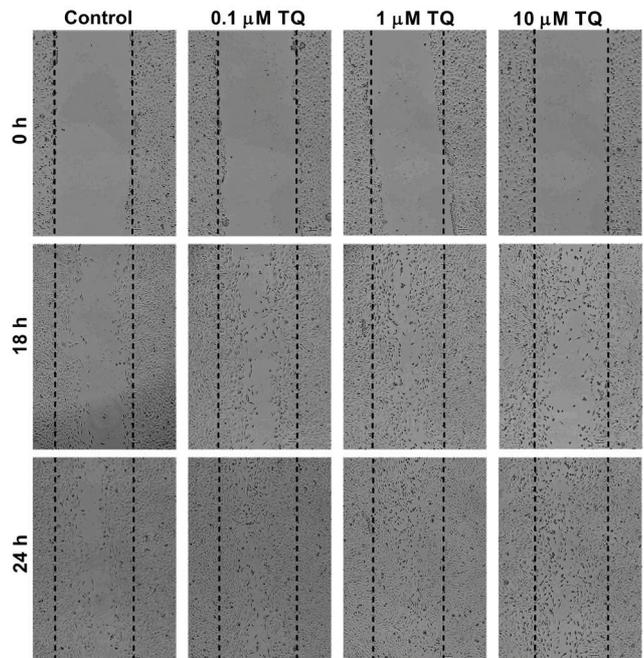
Figure 3. Microscopy images of NIH/3T3 fibroblast cells migration after scratch at 0, 18 and 24th h of 0.1, 1 and 10 µM TQ treatment under 10X magnification.

sure of the control was 76%, 81.64% for 0.1 µM, 91.35% for 1 µM and 90.84% for 10 µM. According to the results, 1 µM of TQ was observed as the most efficient concentration for *in vitro* wound healing activity on NIH/3T3 cells.

Determination of β-catenin Expression in NIH/3T3 Fibroblast Cells

Western blotting assay was performed to analyze the effect of TQ on β-catenin expression in NIH/3T3 fibroblast cells (Figure 4). We observed that 1 µM TQ treatment increased β-catenin expression. No statistically significant difference was observed between 0.1 and 10 µM TQ groups compared to the control ($P>0.05$). 1 µM TQ increased the expression of β-catenin by 35.2%. In contrast, 0.1 µM and 10 µM TQ decreased the level of β-catenin by 12.5% and 4.6% respectively.
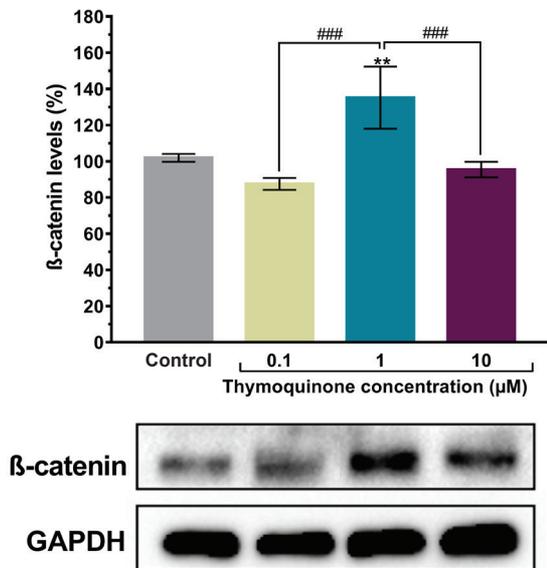
Figure 4. Expression of β-catenin in response to TQ (0.1, 1 and 10 µM) treatments for 24 h. All data were normalized to GAPDH. The graph represents the mean±SD (n=3). **$P<0.01$ versus control group. ###$P<0.001$ shows multiple comparisons between different groups. P values were determined by one-way ANOVA using Tukey post hoc-test.

DISCUSSION

Wounds are still a major global health challenge. Many people suffer from different types of wounds. Although synthetic therapeutics form the basis of wound care, they may cause side effects and allergic problems so the use of these drugs are limited. Current studies have also reported the positive effects of natural products and their bioactive constituents. Many medicinal plants are screened as a wound healing agent and one of these medicinal plants is *N. sativa*. Studies carried out to date have shown the significant wound healing effects of *N. sativa* extracts and oils *in vivo* burn models (6,8), diabetic wound models (9), radiation-related delayed wound models (10) and *in vitro* wound models (1). Although these studies suggest that

N. sativa oil or extract is a wound healing agent, none of them explained which active constituent provides a healing effect on wounds. As is well known, many bioactive compounds are found as a mixture in extracts or oils of medicinal plants. They may cause various side effects such as allergies or irritation. This limits the use of commercially available impure extracts and oils of *N. sativa* as a wound healing agent. In addition, it has been reported that *N. sativa* extract and oil cause irritation or allergic problems (18,19). Therefore, research should focus on active components *of N. sativa*.

Many studies have reported on and evaluated the wound healing effect of natural bioactive components. In diabetic wound healing models, treatment with curcumin provided acceleration of wound healing by its antioxidant effect (20). A study performed on uterine wounds treated with resveratrol showed an increasing activity of antioxidant enzymes and decreasing lipid peroxidation. That resulted in thickness of the uterine wall (21). Furthermore, rosmarinic acid demonstrated anti-inflammatory properties in thermal injury and liver ischaemia–reperfusion rat models (22). TQ is the main biologically active component of essential oil of *N. sativa*, also well known for its pharmacological activities such as antimicrobial (11,12), anti-inflammatory (15), antioxidant (16) effects. According to these known properties, TQ has been thought to have potential as a wound healing agent.

Herein, our results indicated that low concentrations of TQ significantly increased NIH/3T3 cell viability in both a dose and time dependent manner. The highest cell viability was observed after 24 h incubation with 1 and 10 µM TQ, but at high concentrations (>25 µM), cell viability significantly decreased. Additionally, all concentrations of TQ decreased cell viability after 48 h. According to these results, the study was carried out with 24 h of treatment. IC_{50} value of TQ was determined as 51.43 µM. These results suggest that higher doses (>25 µM) of TQ and longtime treatment are more toxic to NIH/3T3 fibroblast cells. Thus, low doses of TQ are more effective for cell viability in healthy fibroblast cells. One study showed that TQ accelerated the rate of wound closure by reducing inflammation and oxidative stress in burn models (23). Another study performed with alloxan-induced diabetic rats resulted in acceleration of wound healing after TQ treatment during the inflammatory phase which arose due to the antioxidant, anti-inflammatory and antimicrobial properties of TQ (24). Our study is consistent with these studies as well as valuable because more thorough investigation is needed *in vitro* with different cell types which act during the phases of wound healing to determine the process at cellular and molecular level and understand the role of TQ. Additionally, the results of treatments with potential agents will be predictable when *in vivo* studies are performed.

In *vitro* models, wound healing is indicated with migration of cells and explains the rate of wound closure. The *in vitro* scratch assay is an easy method to measure cell migration *in vitro* (25). Accordingly, we used *in vitro* scratch assay to create a wound area and to test TQ treatments *in vitro*. The photographs taken at 0, 18 and 24 h time intervals to observe the closure of the wound

area clearly indicated that all selected doses of TQ (0.1, 1 and 10 µM) have potential wound closure activity due to fibroblast migration. In addition, while 1 and 10 µM TQ showed a statistically significant effect in comparison with the control (P <0.001), the highest wound closure rate was observed in 1 µM TQ treatment. The rate of wound closure is increasingly thought to be related to the idea that TQ may also promote the proliferation of cells.

Following an injury, the wound healing response is controlled by many signaling pathways (26). Studies should be carried out to understand these molecular pathways and their components which are involved in the wound healing process in order to generate potential therapeutic agents and therapies. The canonical Wnt–wingless signaling pathway is well known which regulates many biologic processes by increasing the transcriptional activity and stability of β-catenin (27-29). Additionally, Wnt pathway is important for wound healing because its key mediator β-catenin has a pivotal role on the proliferation phase of wound healing. β-catenin also participates in some phases of wound repair. First the phosphorylation occurs and it accumulates in the cytoplasm and then migrates into the nucleus. In the nucleus the regulation of the target gene transcription occurs and this results in proliferation, migration and accumulation in the collagen of fibroblasts. Our western blot analysis data is showed that 1 µM TQ treatment resulted in an increase by 35.2% in β-catenin expression compared with other selected doses of TQ (P<0.001). It might be thought that TQ plays a role in the activity of β-catenin. To better understand the results of elevated β-catenin protein levels after TQ treatment on wound healing, more parameters should be investigated, and further studies should be performed *in vivo* physiological conditions.

CONCLUSION

Our results show that TQ may promote cell viability, accelerate wound healing and trigger protein levels which are effective in wound healing phases *in vitro*. Our results reveal promising data regarding the possibility of a TQ derived product being used in the treatment of wounds and other dermatological problems. TQ derived products and drugs may be developed combined with other active substances and/or administered topically. Although the study was performed in one of the important cell groups, fibroblasts, on wound healing, further research should be carried out with other cell lines to determine TQ usability on wound healing and understand its effect on the wound healing mechanism.

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Conflict of Interest: The authors declare that they have no conflicts of interest to disclose.

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