RESEARCH ARTICLE

The effect of quercetin on phosphorylated p38, Smad7, Smad2/3 nuclear translocation and collagen type I of keloid fibroblast culture

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Keloid is connective skin tissue overgrowth resulting from abnormal wound healing activated by transforming growth factor-β (TGF-β) through particularly TGF-β/Smad signaling pathway which translocate Smad2/3 into nucleus to induce type I collagen synthesis and normally regulated by inhibitory Smad7. The expression of Smad2/3 are increased while Smad7 is decreased in keloid fibroblast. The keloid fibroblast phosphorylated p38 (p-p38) increased and inhibited Smad7 expression. Those biomolecular findings cause keloid recurrence. Therefore, studies on keloid recurrence prevention must be developed. Quercetin is a flavonoid found in many fruits and vegetables. Previous studies showed that quercetin inhibited p-p38 which provided opportunity increasing Smad7, decreasing nuclear Smad2/3, and preventing keloid recurrence. This study aimed to determine the quercetin effect on p-p38, Smad7, Smad2/3, and collagen type I levels in human keloid fibroblasts. Four replicates three-passages primary human keloid fibroblast culture incubated with 5, 10, 20 µg/mL quercetin were conducted with culture media as control for 48 hours. The expressions of p-p38, Smad7, and Smad2/3 were measured using western blotting. The level of collagen type I was measured using enzyme-linked immunosorbent assay (ELISA). Mean comparisons between multiple groups were analyzed using one-way analysis of variance (ANOVA) or Kruskal-Wallis test. Mean comparison between two groups were analyzed using independent T-test or Mann-Whitney test. The results showed that 10 µg/mL quercetin group showed lower p-p38 expression than that of control group (*P* < 0.05). The 10 and 20 µg/mL quercetin groups showed lower Smad7 expression than that of control (*P* < 0.05). The 20 µg/mL quercetin group showed lower nuclear Smad2/3 expression than that of control (*P* < 0.05). All quercetin groups demonstrated lower nuclear Smad2/3 compared to cytoplasmic Smad2/3 expression (*P* < 0.05), while control group did not show nuclear and cytoplasmic Smad2/3 expression significant difference. This study confirmed that quercetin could regulate keloid fibroblast TGF-β/Smad pathway by inhibiting p-p38 and Smad2/3 nuclear translocation and could be used, as biomolecular basis, for keloid prevention in wound healing process.

Keywords: quercetin; keloid fibroblast; p-p38; Smad7; Smad2/3; nuclear translocation; collagen type I.

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Introduction

Keloids are benign overgrowths of connective skin tissue caused by aberrant wound healing and an excess of extracellular matrix induced by transforming growth factor-β (TGF-β) *via* multiple signal transduction pathways, particularly the TGF-β/Smad pathway [1].

According to one study on keloid epidemiology, keloid incidence was 1.68% of all skin patient visits [2]. The keloid patient frequently had pain, itching, cosmetic impairment, and functional deformity, all of which reduced patient's quality of life [3]. Most single treatments for keloids have a high recurrence rate. The combination of surgery and medicinal therapy decreased the recurrence rate. Triamcinolone acetonide intralesional injection monotherapy is effective in reducing small size keloid volume. However, it is not effective and has undesirable side effects such as telangiectasia, acne lesions, and ulceration in large lesions [4]. Therapy to suppress recurrence after keloid surgery and prevent keloid formation after injury while the wound healing process is still maintained must developed.

Keloids occur because of the increased synthesis and decreased degradation of collagen, which is the major component of the extracellular matrix (ECM) in wound healing. Collagen synthesis is activated by TGF-β through various signal transduction pathways, particularly the TGF-β/Smad pathway. The binding of TGF-β to its receptor on fibroblasts causes the phosphorylation of Smad2/3 to combine with Smad4 to form the Smad2/3/4 complex and translocate to the cell nucleus, triggering the synthesis of collagen type I. The TGF-β/Smad pathway is inhibited by Smad7 as a Smad inhibitor by suppressing Smad2 and Smad3 phosphorylation. Smad7 expression in keloid fibroblasts is inhibited by p38, which is activated by TGF-β through the mitogen-activated protein kinase (MAPK) pathway [5]. Upregulation of Smad7 expression occurred in cultured keloid fibroblasts receiving radiation therapy. This increase in Smad7 expression occurred through the suppression of p38 phosphorylation, and then inhibition of collagen synthesis [6]. Inhibition of p38 phosphorylation also inhibited Smad2 and Smad3 phosphorylation. Inhibition of p38 phosphorylation also decreased the nuclear accumulation of Smad2/3 [7].

Quercetin is a flavonoid that is found in many fruits and vegetables. Quercetin can inhibit human keloid fibroblast proliferation [8]. Fibroblast proliferation, however, is still required for wound healing. A study showed that quercetin inhibited p38 phosphorylation through hydrogen bonding with the serine/threonine kinase portion of p38 [9]. Its effect on p38 phosphorylation provided the opportunity to regulate Smad7 as a negative regulator of the TGF-β/Smad pathway. The inhibition of p38 phosphorylation by quercetin also provided an opportunity to decrease the nuclear accumulation of Smad2/3.

This study aimed to determine the effects of quercetin on phosphorylated p38 (p-p38), Smad7, cytoplasmic Smad2/3 expression, nuclear Smad2/3 expression, and collagen type I levels in human keloid fibroblasts, which would show the regulation effect of quercetin to keloid development through TGF-β/Smad signaling pathway. The study result would show the biomolecular potencies of quercetin to inhibit recurrence of keloid. These quercetin potencies effect could be used as keloid recurrence prevention without impair wound healing process.

Materials and Methods

Cell culture and quercetin incubation

The primary keloid dermal fibroblast culture skin explant method was adopted in this study. Dermal fibroblasts were obtained from a 20 years old Asian woman who had undergone earlobe keloid excision without prior treatment. This study was approved by the Health Study Ethics Committee of the Dr. Saiful Anwar General Hospital, Malang, Indonesia, and followed the Declaration of Helsinki and required the signing of an informed consent form. Dermal keloid tissue was immediately placed into a 50 mL centrifuge tube filled with sterile phosphate-buffered saline (PBS). The tissue was cleaned by carefully shaking the tube for 10 minutes which was repeated thrice with new sterile PBS added

each time. The dermal tissue was used as a cell source of keloid fibroblast culture. The cell culture procedure was completed within 6 hours of excision surgery. The keloid tissue edge was punched, excised, and placed on petri dish before dissection to separate the dermis and epidermis. Dermal tissue was cut into 5 to 10 slices of 2-3 mm^2 size and placed in a tissue culture dish covered with 22 mm glass coverslip. The Dulbecco's Modified Eagle's media (DMEM) with 4.5 g/L D-Glucose, L-Glutamine, and antibiotic-antimycotic solution (ThermoFisher Scientific, Rockford, IL, USA) at 4°C were carefully placed over the tissue. The tissue culture dish was placed in an incubator at 37°C and 5% CO_2. Fibroblast development and medium changes were monitored every 3-4 days. The morphology of keloid dermal fibroblast was spindle, adherent, and growing from the edge. When fibroblast development reached 80-90% of the tissue culture plate, the subculture procedure was initiated. Third-passage keloid fibroblast primary cultures were applied. Cultured cells were divided into control, 5, 10, and 20 µg/mL quercetin groups. Keloid fibroblast cultures in the control group were supplemented with culture medium only and different concentrations of quercetin (Tokyo Chemical Industry, Tokyo, Japan) were added to the culture media of other cell groups. The fibroblast cultures were then incubated at 37°C and 5% CO_2 for 48 hours.

Cytoplasmic and nuclear protein extraction

Culture plates containing 5-10 x 10^6 cells per plate were prepared. The cell culture was centrifuged at 2,500 rpm for 3 minutes at room temperature and the supernatant was removed. Cell pellets were resuspended in 1 mL of ice-cold PBS buffer before centrifugation again at 6,000 rpm for 5 minutes in a cold room. The supernatant was discarded, and the cells were placed on ice. Cytoplasmic protein extraction was carried out by adding five times of the pellet volume of cytoplasmic extraction buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, protease inhibitor (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and non-ionic surfactant Triton X-100 (Promega, Madison, Wisconsin, USA)) to resuspend the cell pellet, incubate on ice for 5 minutes, and vortex. The cells were then centrifuged in a cold room at 3,000 rpm for 5 minutes. All supernatants were transferred into clean tubes as cytoplasmic extracts. The remaining cell pellets were resuspended carefully in 100 µL of cytoplasmic extraction buffer without surfactant Triton X-100 and centrifuged in a cold room at 3,000 rpm for 5 minutes. The nuclear protein extraction was performed by adding an equal volume of the nuclear extraction buffer (920 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 25% Gliserol, and protease inhibitor (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany)) to resuspend cell pellets, incubate on ice for 10 minutes, and vortex. The cells were then centrifuged at 14,000 rpm for 5 minutes at 4°C. All supernatants were transferred to clean tubes as nuclear extracts. All extracts were stored in -80°C for future use.

Western Blot

The supernatant containing 50 µg of protein which was quantified by using Qubit Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) was prepared for 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Solarbio Life Sciences, Beijing, China) using mini-PROTEAN vertical electrophoresis apparatus (Bio-Rad Laboratories, Hercules, California, USA). The SDS-PAGE gel was transferred to polyvinylidene fluoride (PVDF) western blotting membranes (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The membranes were blocked with Tris-Buffered Saline Tween 20 (TBST) containing 5% fat-free milk for 10 minutes at room temperature. Membranes were then incubated with the appropriate primary antibodies including p-p38 antibody (Invitrogen, ThermoFisher Scientific, Rockford, IL, USA), Smad7, Smad2/3, and β-actin antibodies (Santa Cruz Biotechnology, Dallas, Texas, USA) overnight at 4°C. The membranes were then incubated with a goat anti-mouse IgG secondary antibody (Rockland Immuno Chemicals, Boyertown, PA, USA) conjugated with peroxidase at a dilution of 1:2,000 for 1 hour at room temperature. The membranes were washed with TBST to remove

secondary antibodies. The membrane blots were visualized using ImageQuant LAS 500 (General Electric, Uppsala, Sweden). The ImageJ software (https://imagej.net/ij/) was used to assess the densities. β-Actin was used as the loading control for western blotting.

Enzyme-linked immunosorbent assay (ELISA)

Extracellular collagen type I levels were measured in the cell culture supernatant using enzyme-linked immunosorbent assay (ELISA). Human Collagen Type I ELISA kit (MyBiosource, San Diego, CA, USA) was used for this experiment following the manufacturer's instructions. The color absorbance was measured at wavelength of 450 nm using Zenix-320 microplate reader (Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, Guangdong, China).

Statistical analysis

SPSS version 27 (IBM, Armonk, New York, USA) was employed for statistical analysis of this study. Mean comparisons between multiple treatment groups were analyzed using one-way analysis of variance (ANOVA) or the Kruskal-Wallis test. Mean comparison between two treatments groups were analyzed using independent T-test or Mann-Whitney test. *P* value less than 0.05 was set as the significant difference.

Results

Quercetin effect on the expression levels of p-p38, Smad7, and Smad2/3

The expression levels of p-p38, Smad7, and Smad2/3 were measured using western blotting after incubation for 48 hours and were shown in membrane bands. The data of p-p38 were normally distributed and homogeneous. ANOVA revealed a significant effect of quercetin on p-p38 expression ($P < 0.05$). Tukey's post hoc analysis showed significant quercetin inhibition in the 10 µg/mL quercetin group ($P < 0.05$), although there was a significant increase in p-p38 expression in the 20 µg/mL quercetin group compared to that in the 10 µg/mL quercetin group (Figure 1). The distribution of Smad7 expression was not normal. Kruskal-Wallis test showed a significant inhibitory effect of quercetin on Smad7 expression ($P < 0.05$) with the significant inhibition observed in the 10 and 20 µg/mL quercetin groups ($P < 0.05$) (Figure 2). The Kruskal-Wallis test also showed a significant effect of quercetin on Smad2/3 expression in both the cytoplasm and nucleus ($P < 0.05$). Significant inhibition of cytoplasmic Smad2/3 expression was observed in the 20 µg/mL quercetin group compared to that in the 5 µg/mL and 10 µg/mL quercetin groups ($P < 0.05$). A significant inhibition of nuclear Smad2/3 expression was observed in the 20 µg/mL quercetin group compared to that in all other quercetin groups ($P < 0.05$) (Figure 3). The Mann-Whitney test showed significant inhibition of nuclear Smad2/3 expression compared to cytoplasmic Smad2/3 expression in all treatment groups ($P < 0,05$) except in control group (Figure 4).

Quercetin effect on collagen type I level

The level of collagen type I in the supernatant of the third passage culture of keloid fibroblasts was measured using ELISA. The Shapiro-Wilk test showed that the collagen type I level data were not normally distributed. The Kruskal-Wallis test did not show significant differences in collagen type I levels, although the 20 µg/mL quercetin group showed the lowest levels (Figure 5).

Discussion

The etiology of keloids remains unclear and requires further investigation. Primary dermal fibroblast culture as an *in vitro* model is essential for a better understanding of disease biology and the identification of novel drug targets. Keloid fibroblast culture allows detecting the mechanisms action of therapy on cellular signaling as target [10]. This study aimed to observe the effect of quercetin on keloid dermal fibroblast primary cultures to determine the mechanisms of action of therapy on cellular signaling. The quercetin concentration used in this study was less than 20 µg/mL, which was

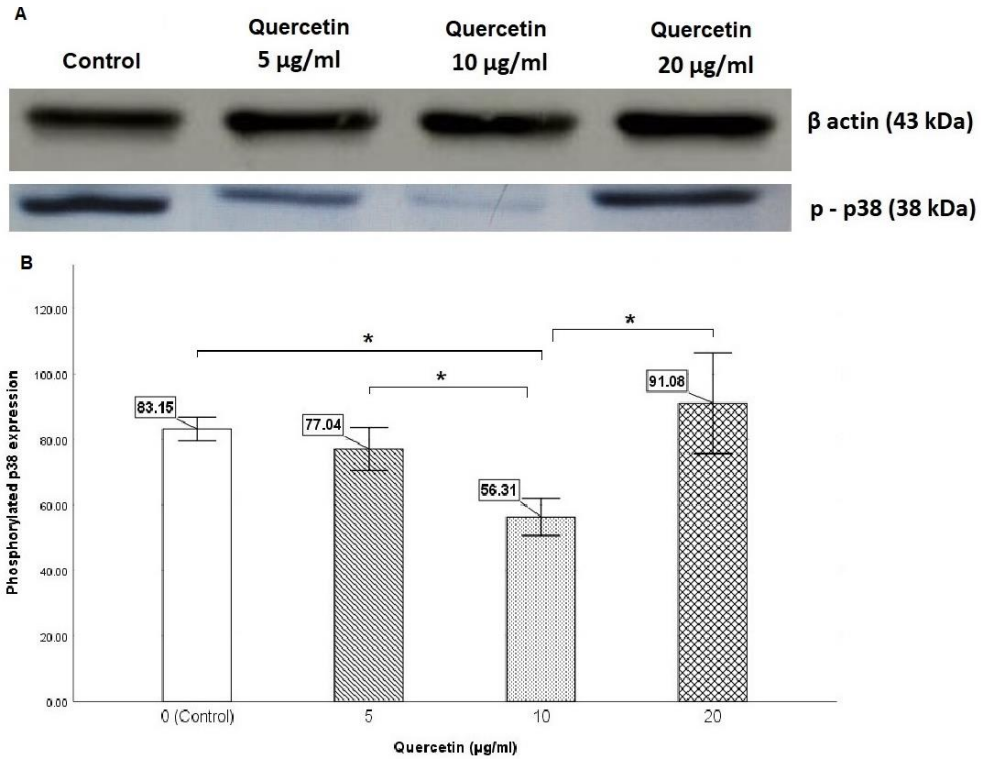

Figure 1. Western blot results of keloid fibroblasts p-p38 expression (**A**), and comparison of p-p38 expression levels on the treatments of different quercetin concentrations (**B**). (*: $P < 0.05$).

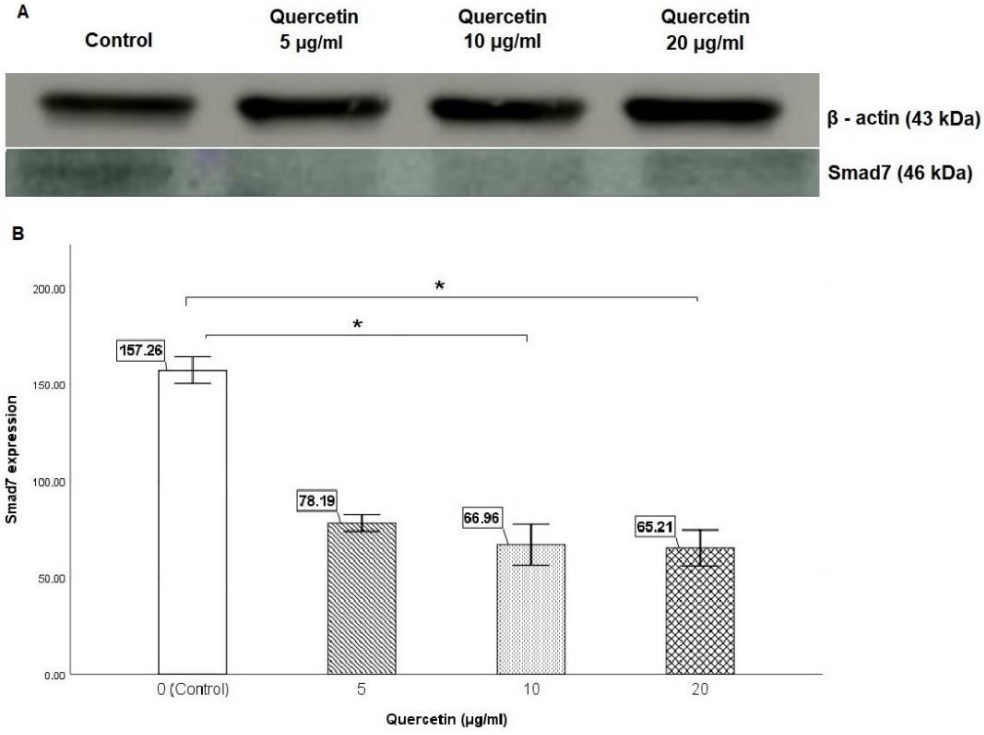

Figure 2. Western blot results of keloid fibroblasts Smad7 expression (**A**), and comparison of Smad7 expression levels on the treatments of different quercetin concentrations (**B**). (*: $P < 0.05$).

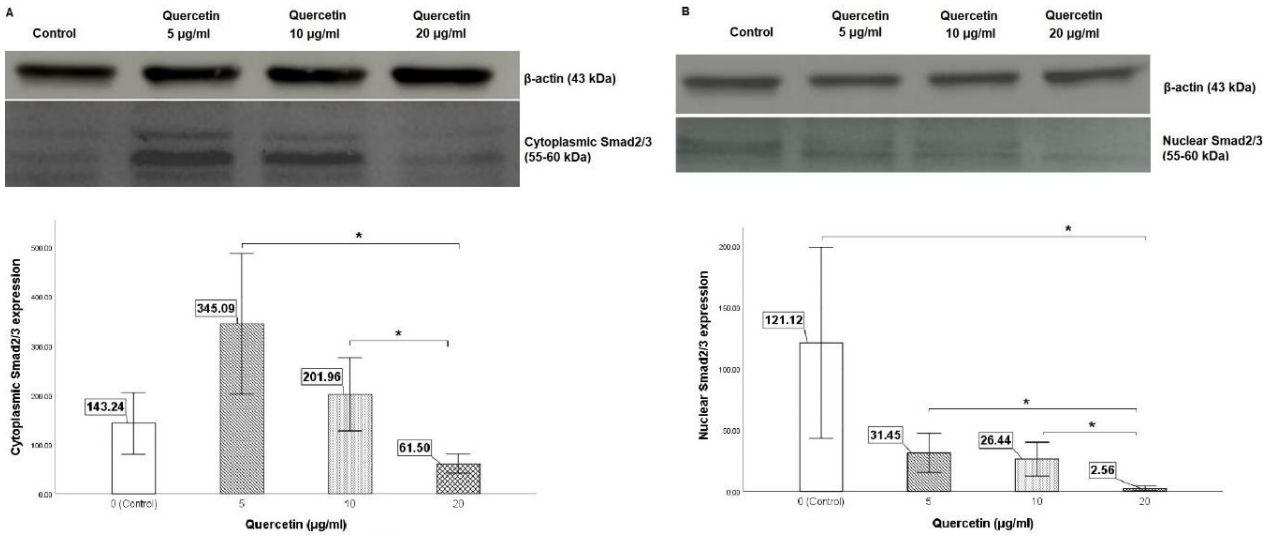

Figure 3. A. Western blot results of keloid fibroblasts cytoplasmic Smad2/3 expression and comparison of cytoplasmic Smad2/3 expression levels on the treatments of different quercetin concentrations. **B.** Western blot results of keloid fibroblasts nuclear Smad2/3 expression and comparison of nuclear Smad2/3 expression levels on the treatments of different quercetin concentrations. (*: $P < 0.05$).

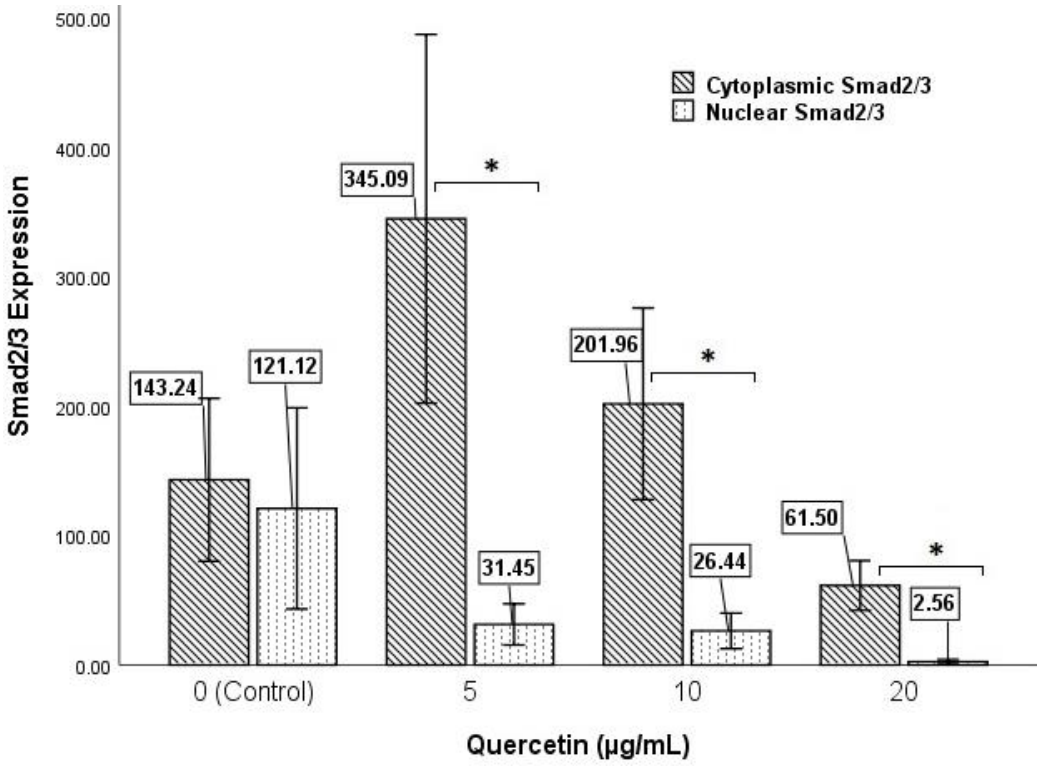

Figure 4. The nuclear Smad2/3 expressions were significantly lower than cytoplasmic Smad2/3 expressions in all treatment groups while control group did not show significant difference between nuclear and cytoplasmic Smad2/3 expression levels. (*: $P < 0.05$).

below the cytotoxic concentration. This study observed the effect of quercetin on p-p38, Smad7, and Smad2/3 expression, which were involved in the TGF-β/Smad signaling pathway, and collagen type I as an extracellular product in keloid pathogenesis. The involvement of p-p38,

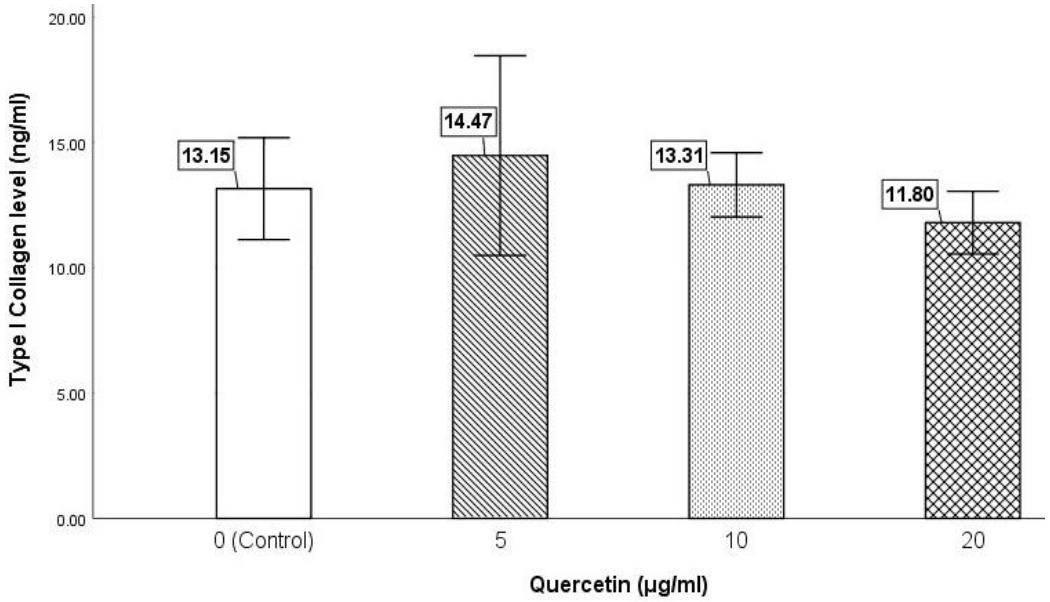

Figure 5. The levels of collagen type I on all experimental groups. No significant difference was observed.

Smad7, and Smad2/3 in keloid pathogenesis is well established. The increase in Smad2/3 and p-p38 levels and suppression of Smad7 in the TGF-β/Smad signaling pathway have been shown to be involved in keloid pathogenesis. Phosphorylation of Smad2/3 is an important factor in TGF-β/Smad signaling pathway-mediated overproduction of collagen type I in keloid pathogenesis [5]. The phosphorylation of Smad2/3 is also activated by the p38 mitogen-activated protein kinase (MAPK) pathway. Phosphorylation of p38 also inhibits Smad7 expression, which in turn inhibits Smad2/3 phosphorylation as a negative regulator of collagen type I synthesis [11].

The effect of quercetin on fibrosis has been investigated previously. Quercetin inhibits Smad2/3 phosphorylation by binding to activin receptor-like kinase 5 (ALK5) [12]. A previous study showed that Smad2/3 phosphorylation in keloid pathogenesis could be activated by p38 phosphorylation [11]. The study of quercetin on p38 of dermal fibroblast is limited. The effect of quercetin on neonatal dermal fibroblast which incubated with 12-o-tetradecanoylphorbol-13-acetate showed p38 inhibition compared to control without quercetin group [13]. Previous study of quercetin on human dermal fibroblast showed no effect on p38 activation [14]. There has been no previous study on the effects of quercetin on the inhibition of phosphorylated p38 in keloid fibroblasts. This study showed that p-p38 expression was inhibited by 10 µg/mL quercetin, although its expression increased in the 20 µg/mL quercetin group. Other studies have shown the opposite effects of quercetin on p38 phosphorylation. A study of 20 µM quercetin on a human keratinocyte cell line (HaCaT) induced with ultraviolet B showed inhibition of p38 phosphorylation compared to the control group [15]. A previous similar study of 50 µM quercetin on a human keratinocyte cell line (HaCaT), which was induced with ultraviolet B, showed an increase in p38 phosphorylation compared to the control group [16]. These studies were consistent with our findings that a lower concentration of quercetin inhibited phosphorylated p38, whereas a higher quercetin concentration increased its expression. The inhibitory effect of 10 µg/mL quercetin on p38 phosphorylation had potential as a TGF-β/Smad signaling pathway inhibitor in keloid pathogenesis. Other studies have been conducted to determine the effects of new drugs on p38 phosphorylation inhibition in keloid

fibroblasts. A study of madecassoside in primary earlobe keloid fibroblast culture showed p38 phosphorylation inhibition [17]. Inhibition of the p38/MAPK signaling pathway in rabbits that underwent cleft surgery showed scar tissue proliferation through the Smad signaling pathway [18]. This is the first study to demonstrate the effect of quercetin on Smad7 expression in keloid fibroblast cultures. Quercetin inhibited Smad7 expression in keloid fibroblast cultures at concentrations of 10 and 20 µg/mL. These results contrasted with the inhibitory effect of quercetin on p38 phosphorylation, which increased Smad7 expression. The effect of quercetin on Smad7 expression did not appear to be mediated by p38 inhibition. The inhibitory effect of quercetin on Smad7 expression may be due to an increase in methyl-CpG-binding protein 2 (MeCP2). MeCP2 can inhibit promoter methylation of some genes, such as the Smad7 gene promoter [19]. The expression of MeCP2 is increased in human keloid fibroblasts [20]. There has been no publication of the effect of quercetin on MeCP2 expression in keloid fibroblasts. A study on quercetin in neuronal and non-neuronal cells showed an increase in MeCP2 expression [21]. Previous studies have shown that quercetin inhibits Smad7 expression in non-dermal fibroblasts. Quercetin increased Smad7 mRNA expression on human corneal fibroblast compared to control without quercetin [22]. The other study showed that quercetin increased Smad7 expression of mouse mesangial kidney cell line (SV40 MES 13 cells) [23]. The other study showed the quercetin effect on Smad7 upregulation on myocardium of myocardial infarct mice model [24]. One study showed Smad7's important roles in wound healing process which could decrease inflammation, fibrosis formation, and epidermal hyperplasia induced by TGF-β1 [25]. The previous study showed contrary results that Smad7 inhibition could repair reepithelization in wound healing of mice [26]. Increasing of Smad7 expression was one factor which was involved in skin aging process. A study showed UV radiation exposure of dermal fibroblasts to UV radiation induced Smad7 expression by increasing the Activator protein–1 (AP-1) transcription factor [27]. Smad7 inhibition was used as a therapeutic target in the aging process. A study on hydrogen peroxide (H_2O_2) in the human fibroblast cell line Hs68 showed the increased Smad7 expression, which demonstrated that *Poria cocos* could be used in skin aging therapy by inhibiting Smad7 expression [28].

This study demonstrated the inhibitory effect of quercetin on nuclear Smad2/3 but not on cytoplasmic Smad2/3 expression. This result supported the hypothesis that quercetin inhibits nuclear translocation of Smad2/3. Inhibition of Smad2/3 nuclear translocation by quercetin may be facilitated by inhibition of p38 phosphorylation. Nuclear translocation of Smad2/3 increased in keloid fibroblast and induced by TGF-β1. Inhibition of p38 could inhibit Smad2/3 nuclear translocation [11]. A previous study showed an inhibitory effect of quercetin on Smad2, Smad3, and Smad4 expression using western blotting, but did not explain whether the western blot procedures were performed by cytoplasmic or nuclear extraction [29]. The nuclear translocation of Smad2/3 inhibition as keloid target therapy has been reported in some studies. Inhibition of tumor necrosis factor [TNF]-stimulated gene-6 (TSG-6) on phosphorylated Smad2/3 nuclear translocation in human keloid fibroblasts inhibited cell proliferation, invasion, and collagen synthesis [30]. The other study showed the role of interleukin-10 (IL-10) in the nuclear translocation of phosphorylated Smad2/3 in human keloid fibroblasts, which also inhibited fibroblast proliferation and collagen synthesis [31]. The study of silibinin, a flavonoid, showed that silibinin inhibited the phosphorylation of Smad2 and Smad3, and their nuclear translocation in keloid fibroblasts. This inhibition caused a decrease in collagen type I expression [32]. The study of quercetin on the nuclear extraction of Smad2/3 expression in keloid fibroblasts has not been reported. The inhibitory effect of quercetin on Smad2/3 phosphorylation and Smad4 nuclear translocation has been studied in a human retinal

pigment epithelial cell line (ARPE-19) as a proliferative vitreoretinopathy treatment [33]. However, this study did not demonstrate the effects of quercetin on collagen type I in keloid fibroblasts. The quercetin concentration used in this study was not more than 20 µg/mL or equivalent to 66.17 µM. The previous study showed the similar result that 40 µM quercetin did not influence collagen type I expression on human fibroblast culture [14]. The previous study showed quercetin inhibition on collagen type I expression of keloid fibroblast. The inhibitory effect was observed at 25 µg/mL quercetin, and the effect appeared faster at a concentration of 50 µg/mL [8]. The discrepancy between the two studies last mentioned was probably caused by the different quercetin concentrations used. The inhibitory effect of quercetin on collagen type I in keloid fibroblasts may be observed after incubation with higher concentrations of quercetin. Our previous study showed that by using sub-cytotoxic concentration of *Physalis angulata* leaf extract at 0.63 µg/mL, it showed inhibition effects on collagen type I and tissue inhibitor of metalloproteinase I (TIMP-1) in keloid fibroblast [34]. The extract of *Physalis angulata* leaf has been used as a medicinal herb through active compounds such as quercetin [35].

Conclusion

This study demonstrated an inhibitory effect of 10 µg/mL quercetin on phosphorylated p38 expression in keloid fibroblasts and showed quercetin inhibition effect on Smad2/3 nuclear translocation. In contrast, 10 and 20 µg/mL quercetin inhibited Smad7 expression, theoretically increasing Smad2/3 expression. This result indicated that the inhibitory effect of quercetin on Smad2/3 nuclear translocation was probably not mediated by Smad7. This study showed that quercetin could regulate keloid fibroblast TGF-β/Smad pathway by inhibited p-p38 and Smad2/3 nuclear translocation, which could be used as biomolecular basis for quercetin as keloid prevention in wound healing process.

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