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Moringa oleifera leaf extract ameliorates collagen degradation via the inhibition of MMP-3 expression in UVB-induced rats

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ABSTRACT Prolonged exposure to high-intensity UVB induces the formation of reactive oxygen species (ROS) in skin tissue, triggering an increase in matrix metalloproteinase-3 (MMP-3) enzyme production and leading to collagen degradation. *Moringa oleifera* (MO) contains bioactive compounds known for ROS-scavenging and anti-inflammatory properties. However, the precise molecular mechanism of action remains unclear, requiring the inhibition of MMP-3 activation and regulation of collagen deposition. This study aims to elucidate the potential effect of MO leaf extract-based gel in restoring collagen deposition by reducing MMP-3 activation in UVB irradiate-induced collagen loss in rats. This study employed a completely randomized design, comprising four groups: a healthy group without UVB radiation, a negative control group subjected to UVB radiation and receiving a placebo, and two treatment groups exposed to UVB radiation with 5% or 10% moringa leaf extract-based gel (MO-5% or MO-10%), respectively. Results showed that MO-5% and MO-10% significantly reduced MMP-3 gene expression and increased collagen density compared to the negative control group ($p < 0.05$). *Moringa oleifera* leaf extract ameliorates collagen degradation by inhibiting MMP-3 expression in UVB-induced rats, suggesting its potential as a pharmacological and cosmetic agent for UVB-induced skin damage.

KEYWORDS Collagen; MMP-3; *Moringa oleifera*; UVB exposure

1. Introduction

UVB-induced collagen loss may develop a stretch loss, reductions appearing in the epidermal layer thickness, collagen substance, elastic fiber deterioration, and increased wrinkling and dryness of the skin tissue (Gao et al. 2018; Lan 2019; You et al. 2019). Unrestrained collagen loss may also alter many dermatological diseases, including solar keratosis, chronic optic cheilitis, photo-elastic fibrosis, melanoma, basal cell carcinoma, sunspot-like mites, and squamous cell carcinoma (Bibbins-Domingo et al. 2016; Pittayapruek et al. 2016). Current clinical therapies for UVB-induced collagen loss, including dermabrasion, intense pulse, botulinum toxin injection, chemical peels, and laser resurfacing, still have some side effects (Kwon et al. 2016; She et al. 2019; Phansuk et al. 2022). Therefore, an effective strategy that can delay and prevent UVB-induced collagen loss with minimal side effects is of clinical importance. At the cellular level, continuous exposure to high-intensity UVB radiation may induce the formation of reactive oxygen species (ROS), which may activate the protein kinase pathway to upregulate the transcription of matrix metalloproteinase (MMP) enzymes leading to extracellular matrix (ECM) degradation (Wölfle et al. 2011; Ngo et al. 2017). Collagen and elastin are two of the structural proteins that make up the extracellular matrix (ECM), and they may be involved in maintaining skin elasticity retention (Shin et al. 2019). The primary building block of dermal ECM is type 1 collagen, which is produced from procollagen type 1 and transforming growth factor (TGF)-β1-stimulated synthesis of type 1 collagen can be impeded by matrix metalloproteinase (MMP)-3 (Hwang et al. 2012; Lin et al. 2017; Cole et al. 2018). Recent comprehensive studies have revealed that certain biometabolite compounds in *Moringa oleifera* (MO) extract, such as flavonoids, terpenoids, saponins, and tannins, have anti-aging potential effects in restoring collagen degradation (Meccariello and D'Angelo 2021; Wang et al. 2022). Nevertheless, the specific mechanism of MO in collagen restoration correlated to MMP-3 and collagen

production remains unclear.

Moringa oleifera (MO) leaves have several bioactive compounds, such as anthocyanins or flavonoids, which act as antioxidants and anti-inflammatories (Yuziana et al. 2021; Andy et al. 2022; Wang et al. 2022). Previous studies have shown that anthocyanins can inhibit the production of ROS in the skin due to UVB exposure to prevent collagen degradation (Yuziana et al. 2021; Andy et al. 2022). In addition, an inhibitor of ROS is also able to reduce the production of inflammatory molecules and trigger the M1-M2 polarization process and induce the TGF-β1 secretion (Masyithah Darlan et al. 2020; Putra et al. 2020a,b; Hamra et al. 2021). The secretion of this growth factor can trigger the SMAD2 and SMAD3 complexes activation, which plays a role in collagen production (Putra et al. 2018; Masyithah Darlan et al. 2020). Flavonoids are a group of phenolic compounds that act as antioxidants by binding metals or donating hydrogen atoms to another unstable molecule to prevent cell damage due to free radicals (Tanvir et al. 2017; Kim et al. 2019). Anthocyanins are water-soluble essential pigments that have a long conjugated double arrangement and can act as antioxidants with a radical scavenging mechanism (Jamil et al. 2018; Kim et al. 2019; Jeyaraj et al. 2021).

A prior work conducted both *in vitro* and *in vivo* showed that MO stem extract may enhance cellular antioxidant defense mechanisms and activate PPARα to shield skin keratinocytes from oxidative stress injury (Ezzat et al. 2020; Sunarto et al. 2020). Another previous study reported that MO leaf extract can reduce pro-inflammatory cytokines such as interleukin (IL)-1α, IL-8, NO, and PGE2 in human keratinocyte cells irradiated by UVB (Pandey et al. 2017). In addition, another report showed that MO leaf extract may inhibit the increase in ROS production and cell damage in UVB-irradiated HaCaT cells through the inhibition of MMP-1, MMP-3, and MMP-9 enzymes (Wang et al. 2022). Moreover, another study demonstrated that 3% MO leaf extract-based cream applied to the skin's surface may act as a ROS scavenger and regulate skin texture roughness, skin smoothness, and skin wrinkles (Yuziana et al. 2021). *Moringa oleifera* has strong antioxidant activity that has the potential for UVB skin protection due to its bioactive antioxidant compound (Andy et al. 2022; Wang et al. 2022). However, *in vivo* studies to provide scientific evidence regarding the benefits of 5% and 10% MO leaf extract in regulating MMP-3 expression and collagen synthesis in dermal tissue have not been studied. Therefore, this study investigated the effect of MO leaf extract-based gel on MMP-3 gene expression and collagen density in UVB irradiate-induced collagen loss in rat skin tissue.

2. Materials and Methods

2.1. Preparation of MO leaf extract and extract-based gel

The dried MO leaves were obtained from *Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional* (B2P2TOOT), Tawangmangu, Karanganyar, Central Java, Indonesia. The authentication of MO plant was also carried out by B2P2TOOT. The 600 g sifted MO leaf was macerated with 0.25 L 96% ethanol solvent for 72 h and the extract-containing ethanol was evaporated with a rotary evaporator (RV 10, IKA, Germany) until a concentrated substrate was obtained, based on a previous method (Andy et al. 2022) with slight modification. Qualitative phytochemical analysis of saponins, tannins, flavonoids, triterpenoids, alkaloids and steroids was performed using previous methods (Bagheri et al. 2020; Setyawan et al. 2021). Total flavonoid content was performed using spectrophotometry approach (Multiskan High, Thermo Scientific) (Septiani et al. 2021).

To produce 5% and 10% (w/w) ethanolic MO-leaf extract-based gel, 7.5% (w/v) acrylamide-based gel was prepared. Based on the Cosmetic Ingredient Review Expert Panel The acute dermal LD_{50} for a solution containing acrylamide (24% in water) was reported to be >5000 mg/kg in rats. Therefore, 7.5% acrylamide in this study is suggested to be safe for experimental animals. A 100 mL ddH_2O was added to 7.5 g acrylamide (Himosap-GAS100, Derypol, Barcelona, Spain) and stirred to dissolve at 40 °C to produce colloidal solution (Bergfeld et al. 2017). The 7.5% (w/v) acrylamide solution and the MO extract at a ratio of 20:1 and 10:1 to produce 5% and 10% (w/w) ethanolic MO-leaf extract-based gel, respectively. 7.5% (w/v) acrylamide-based gel was used for the placebo group.

2.2. UVB irradiate-induced collagen loss rats and MO leaf extract-based gel administration

A total of 24 healthy male rats (250 ± 25 g) were fed and watered ad libitum and raised at 28 °C temperature and a 12-h photoperiod. After a week of acclimation, rats were randomly distributed into four groups: UVB non-irradiation (healthy control), UVB irradiation (negative control), UVB irradiation and 5% of MO leaf extract in 200 mg water-based gel (MO-5%), and UVB irradiation and 10% of MO leaf extract in 200 mg water-based gel (MO-10%). Every group had six rats in it. UVB radiation, which has a peak emission at 302 nm (CL-100M, UVP, USA), was used in this work. An earlier method involved exposing rats to 160 mJ/cm^2 of UVB light for 30 min every day for five days in a row (Kim et al. 2019). The treating gels were topically applied on the hairless posterior rat skin daily for up to 14 days. The negative control group rats received placebo gel treatment, which was water-based gel without MO. On day 15, the rats were anesthetized intramuscularly with a solution of ketamine (60 mg/kgBW) and xylazine (20 mg/kgBW). The dorsal skin tissue was taken and cut into two parts: one was fixed in 10% for-

malin for pathological staining and the other was stored in RNA later for RNA detection. All procedures involving animal treatments were approved by the Ethics Committee of Faculty of Medicine, Sultan Agung Islamic University (no. 227/VIII/2022/Komisi Etik).

2.3. MMP-3 mRNA level by qRT-PCR

Total RNA from rat skin tissue was extracted with TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Briefly, first-stranded cDNA was synthesized from 0.5 µg total RNA of sample using Super-Script II (Invitrogen, Massachusetts, USA). SYBR No ROX Green I dye (SMO-BIO Technology Inc., Hsinchu, Taiwan) was used for reverse transcription in a real-time PCR instrument (PCR max Eco 48), and mRNA levels of the MMP-3 (forward, F: 5′-TACCTGAACCCGTGTTGCTCTC-3′; reverse, R:5′-GTTGCTGAGGTATCGCCAGGAA-3′) and GAPDH (F: 5′- TGACAACTTTGGCATCGTGG-3′; R: 5′-GGGCCATCCACAG TCTTCTG-3′) (Yao et al. 2015; Pratiwi et al. 2019) were run using the respective primers. The thermocycler was set up as follows: a 10-minute initial step at 95 °C, 40 cycles at 95 °C for 15 s, and a 1-minute rest period at 60 °C. The cycle quantification (Cq) was used to record the gene expression. Eco Software v5.0 was used to collect the data (Illumina Inc., San Diego, CA, USA). All data analyses used the $2^{-\Delta\Delta Ct}$ method (Livak method).

2.4. Collagen deposition histological analysis with Masson's trichrome staining

After 5 days of treatment, the paraffin-embedded skin tissues were sliced, deparaffinized, and stained with Masson's trichrome (Bio-Optica, Milan, Italy) manufactured protocols and previous method (Xianyuan et al. 2019). The collagen deposition was observed in the green-blue color area under an optical microscope observation (Olympus, Tokyo, Japan) and quantified by measuring the green-blue area in 5 different fields of view on each slide using ImageJ software (NIH, USA).

2.5. Statistical analysis

SPSS version 26.0 was used to complete the statistical analysis (SPSS Inc., Chicago, IL, USA). Every value was shown as mean ± standard deviation (SD). The collected and collated data were subjected to tests for homogeneity using the Levene test and normality using the Shapiro-Wilk test. Intergroup differences were performed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test using a p value < 0.05.

3. Results and Discussion

3.1. Phytochemical analysis of MO leaf extract

Phytochemical analysis of the MO leaf extract was carried out to screen the presence of certain secondary biometabolites contained-MO leaf extract. The screening results of the phytochemical analysis qualitatively showed that the MO leaf extract positively contained saponins, tannins, flavonoids, and triterpenoids and did not contain alkaloids and steroids (Table 1). An analysis of the total flavonoid content was then carried out to determine the precise content of flavonoid using the spectrophotometric method. The results of the flavonoid test showed that the MO leaf extract contained flavonoid 20.69 ± 1.06 mg/g MO extract.

TABLE 1 Phytochemical analysis compound of MO leaf extract.

Compound	Results	Methods
Alkaloid	-	Wagner
Saponin	+	Forth
Tannin	+	$FeCl_3$ 1%
Flavonoid	+	Willstatter
Steroid	-	Liebermann-Burchard
Triterpenoid	+	Liebermann-Burchard

Note: + = present, - = absent

3.2. MO downregulated mRNA level of MMP-3 in UVB irradiate-induced collagen loss rat skin

The critical factors for skin collagen loss were considered to be MMP activation and reduction in procollagen synthesis. To assess the certain mechanisms of MO in regulating MMPs and procollagen activity, the MMP-3 mRNA levels were ascertained by qRT-PCR. As shown in Figure 1, the mRNA levels of MMP-3 from the UVB-irradiated group were markedly elevated compared with the UVB non-irradiated group, whereas 5% and 10% MO-based gel significantly decreased UVB-induced MMP-3 expression by 1.61 and 1.93 times, respectively. Thus, dose-dependent MO may be potent as a collagen loss regulator by decreasing the MMP pathway, and the 10% MO-based gel may regulate MMP-3 gene expression nearly a natural condition.

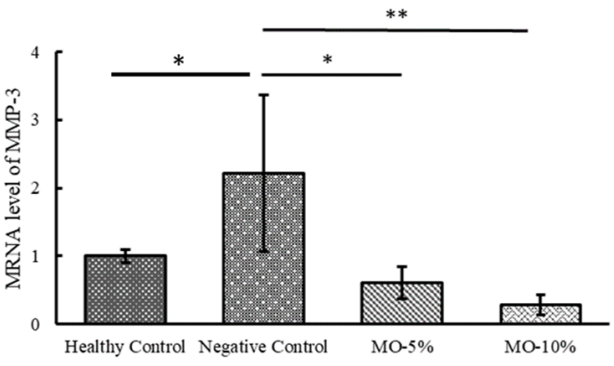


FIGURE 1 The effect of *Moringa oleifera* leaf extract-based gel on MMP-3 gene expression. The expression of MMP-3 is increased in the negative control group and significantly decreased in MO-5% and MO-10% groups. Healthy Control: without UVB irradiation; Negative Control: UVB irradiation plus placebo gel; MO-5% and MO-10%: UVB irradiation plus treatment gel containing 5% and 10% MO, respectively. Data are presented as mean ± SD (n = 6). $^{*}p < 0.05$; $^{**}p < 0.001$

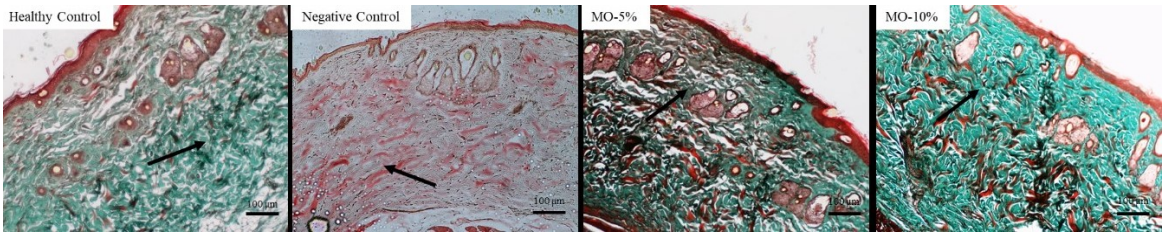
3.3. Moringa oleifera restored the amount of collagen deposition in UVB irradiate-induced collagen loss in rat skin

Masson's trichrome staining was carried out to verify the effect of MO leaf extract on collagen deposition in the UVB irradiate-induced collagen loss rat skin tissue. The percentage of collagen area is measured by calculating the area colored green-blue, which represents the number of collagen fibers (Hong et al. 2020). The comparative analysis resulted that dermal collagen deposition was decreased after UVB radiation (negative control group) (0.79 ± 0.1) and significantly increased after being treated with the MO leaf extract-based gel both in the MO-5% group (40.45 ± 0.7) and MO-10% group (51.52 ± 0.6) (Figure 2a and 2b). This result suggested that the antioxidant-biometabolite compound in MO leaf extract-based gel may restore collagen deposition. Interestingly, collagen deposition in MO-5% and MO-10% groups were higher than in the healthy control group (36.97 ± 0.7).

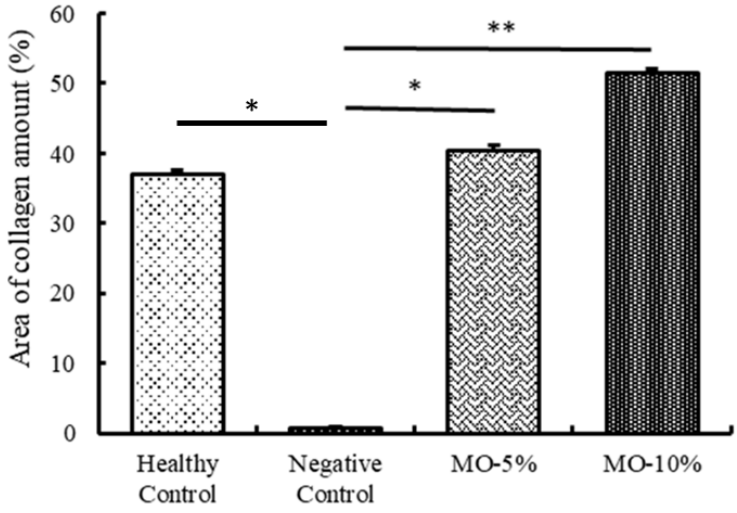
3.4. Discussion

The results of this study showed that MO leaf extract-based gel might induce MMP-3 gene downregulation and increase collagen deposition in UVB irradiate-induced collagen loss in rat skin, resulting in dermal collagen restoration. These results suggest that the protective effect of MO leaf extracts might be able to restore collagen loss by inducing the MMP-3 gene downregulation and increasing collagen amounts. External influences such as smoking, UV radiation, and mechanical stress combine with intrinsic factors like genetics and hormone fluctuations to trigger collagen disintegration (Park et al. 2018). UVB-exposed skin contains large amounts of ROS, which react with DNA, RNA, proteins, and fatty acids, resulting in oxidative damage (Wölfle et al. 2011). Oxidative stress initiates a cascade of signaling pathways, leading to the induction of activator protein 1 (AP-1) and downregulation of TGF-β, followed by MMP expression and inhibition of collagen synthesis (Kwon et al. 2019). On the other hand, *Moringa oleifera* has active compounds, one of which is anthocyanin that acts as an antioxidant and anti-inflammatory and might reduce MMP-3 levels and increase collagen density (Yuziana et al. 2021; Andy et al. 2022). This study aims to determine the effect of MO leaf extract-based gel on MMP-3 gene expression and collagen density in collagen loss Wistar rats due to UVB exposure.

This study found that 5% and 10% MO leaf extract-based gel extract significantly downregulated MMP-3 gene expression in UVB irradiate-induced collagen loss



(a)



(b)

FIGURE 2 Collagen amount histological analysis with Masson's trichrome staining. (A) Microscopic appearance of the skin tissue with MT staining under 10× observation magnification. (B) Area of collagen amount (green-blue area) were analyzed with ImageJ. Data are presented as mean ± SD (n = 6). The negative control group showed a lower amount of collagen than the MO leaf extract-based gel in either the 5% or 10% group. The arrows in the green-blue color area indicate collagen deposition. Healthy Control: without UVB irradiation; Negative Control: UVB irradiation plus placebo gel; MO-5% and MO-10%: UVB irradiation plus treatment gel containing 5% and 10% MO, respectively. $^{*}p < 0.05$; $^{**}p < 0.001$. Scale bar = 100 µM.

rats. These results are in line with previous studies that revealed that MO has antioxidant abilities in reducing ROS levels. The increase in ROS augmentation may activate the transcription factor nuclear factor (NF)-κB, which regulates the transcription of various pro-inflammatory molecules, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-6 (She et al. 2019; Lee et al. 2020). High levels of TNF-α and IL-6 synergistically activate various signaling pathways, such as JAK/STAT and mTOR, leading to an increase in MMP-3 gene expression, the major degradation factor for collagen (Nosenko et al. 2019; Lee et al. 2020). In this study, the flavonoid level showed that 20.69 ± 1.06 mg/g MO extract might significantly play a role in inhibiting ROS production. Previous research showed that high-level flavonoids in MO play a key role in regulating ROS production through direct and indirect mechanisms (Wang et al. 2022). A previous study reported that flavonoids have a direct mechanism in inactivating ROS through electron transfer to form stable covalent bonds (Andy et al. 2022). On the other way, several previous studies have also reported that anthocyanins induce the catalyzation of superoxide dismutase (SOD), a ROS inhibitor (Wang et al. 2022).

This study also found that 5% and 10% MO leaf extract-based gel significantly increased collagen density in UVB irradiate-induced collagen loss rats which is indicated in turquoise green on the slide. These results are consistent with previous studies that stated that MO might increase collagen density in a rat with *Pseudomonas aeruginosa* infection. Previous studies also reported that the decrease in pro-inflammatory factors due to antioxidants negatively inhibits the expression of anti-inflammatory cytokines, including TGF-β (Gao et al. 2018; Chen et al. 2021). TGF-β is a prototypical fibrogenic cytokine, which may increase the production of ECM genes in activated skin fibroblasts and inhibit matrix-degrading enzymes through the SMAD pathway, mainly associated with collagen synthesis (Restimulia et al. 2022, 2021). The activated SMAD 2/3 binding to Smad 4 leads to an entry of the protein complex into the cell nucleus, resulting in activation of the procollagen transcription (Lin et al. 2017; Gao et al. 2018).

In addition, the toxicity test showed that 24% acrylamide in water is the maximum level of safety (Bergfeld et al. 2017). So, it implied that 7.5% acrylamide in water (as a based hydrogel) was safe in cosmetics formulation. Taken together, MO has the potential and safe as a pharmacological and cosmetic agent for UVB-induced skin damage. However, this study has several limitations, first, we did not properly examine MMP-3 protein expression, wrinkle formation, epidermal thickness and collagen deposition to strengthen this evidence (Mariati et al. 2021). In addition, we have not measure aging-associated molecules such as NF-κB, IL-6, TNF-α, IFN-γ, TGF-β, and SOD after administration of MO leaf extract-based gel in UVB irradiate-induced collagen loss rats. The investigation on the expression level of molecules involved in the signaling pathway, i.e. TGF-β/Smad as conducted by Nuryana et al. (2023), will provide a better understanding on molecular mechanism. Furthermore, we did not perfectly represent this effect in human skin. Besides, we have not yet determined the optimal dose and duration of MO leaf extract-based gel treatment. Finally, further research related to MMP-3 protein expression, wrinkle formation, epidermal thickness and collagen deposition, molecular interactions, and dose determination, especially on human skin, needs to be carried out to strengthen the evidence for MO treatment in UVB protection.

4. Conclusions

These findings demonstrate that *Moringa oleifera* leaf extract-based gel may downregulate MMP-3 gene expression and increase collagen deposition in the UVB irradiate-induced collagen loss in rat skin.

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Authors' contributions

RR: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript; TS: conception and design, supervising, data analysis and interpretation, manuscript writing, final approval of the manuscript; AP and AIM : supervising, data analysis and interpretation, critical reviewing, final approval of the manuscript. NH and SYA : manuscript writing, collection and/or assembly of data. IA and RS : data analysis and interpretation, and critical reviewing. SAH : collection and/or assembly of data.

Competing interests

The authors declare no conflict of interest.

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