Clitoria ternatea Flower Extract-Based Gel Elevates TGF-β1 Gene Expression and Collagen Density in UVB-Induced Collagen Loss Rat Skin

Ekstrak Bunga Clitoria ternatea Berbasis Gel Meningkatkan Ekspresi Gen TGF-β1 dan Kepadatan Kolagen pada Kulit Tikus yang Kehilangan Kolagen yang Diinduksi UVB

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ABSTRACT

Ultraviolet B (UVB) irradiation on the skin induces collagen loss by the ROS pathways and pro-collagen factors, mainly the TGF-β family. Many studies of antioxidant compounds in Clitoria ternatea have shown to induce decreasing ROS production, in the collagen loss process. However, the molecular mechanism of action is still not clearly studied, inducing the mechanism of TGF-β1 activation and regulation of collagen loss need to be explore. This study aimed to investigate the effect of Clitoria ternatea flower extract-based gel on TGF-β1 gene expression and collagen density in UVB-induced rat skin. A randomized control group post-test-only design was conducted on 20 male Wistar rats aged 8-10 weeks (150-250 grams). Rats were divided into four groups: Untreated group (Healthy Control), UVB+Based gel group (Negative Control), 5% Clitoria ternatea flower extract-based gel group (T-5%), and 10% Clitoria ternatea flower extract-based gel group (T-10%). Rats were exposed to UVB for 5 consecutive days and treated with gel every day for two weeks. At week 3, rat skins were isolated for TGF-β1 gene expression using qRT-PCR, and Masson Trichrome-collagen specific staining. Comparative analysis of qRT-PCR showed that the lowest mean TGF-β1 expression were seen in the Negative Control group (0.01±0.00), followed by T-5% (0.11±0.22), Healthy Control group (1.01±0.01) and T-10% (2.32±2.46). According the MT staining, the highest amount of collagen is in the Healthy Control group (43.83±5.3), followed by T-10% (38.39±3.1), T-5% (29.04±3.2) and Negative Control group. (13.87±2.7). Clitoria ternatea flower extract-based gel may increase TGF-β1 gene expression and collagen deposition in the UVB-induced collagen loss rat skin.

Keywords: Clitoria ternatea, collagen loss, TGF-β1, UVB irradiation

ABSTRAK

Radiasi ultraviolet B (UVB) pada kulit menginduksi hilangnya kolagen melalui jalur ROS dan faktor pro-kolagen, terutama keluarga TGF-β. Banyak penelitian mengenai senyawa antioksidan yang terkandung dalam Clitoria ternatea yang telah membuktikan tejdanya penurunan produksi ROS, dalam proses kehilangan kolagen. Namun, mekanisme aksi secara molekuler masih belum diketahui dengan jelas, selain itu, proses induksi aktivasi TGF-β1 dan regulasi hilangnya kolagen masih perlu dieksplorasi. Penelitian ini bertujuan untuk menyelidiki efek ekstrak bunga Clitoria ternatea berbasis gel terhadap ekspresi gen TGF-β1 dan densitas kolagen pada tikus yang kultinya diinduksi dengan UVB. Rancangan penelitian post-test-only dengan pengelompokan acak terkontrol dilakukan pada 20 tikus Wistar jantan berusia 8-10 minggu (150-250 gram). Tikus dibagi menjadi empat kelompok yaitu kelompok yang tidak diberi perlakuan (kontrol sehat), kelompok gel berbasis UVB+ (kontrol negatif), kelompok gel berbasis ekstrak bunga Clitoria ternatea 5% (T-5%), dan kelompok gel berbasis ekstrak bunga Clitoria ternatea 10% (T-10%). Tikus dipapar UVB selama 5 hari berturut-turut kemudian diberi gel setiap hari selama dua minggu. Pada minggu ke-3, kulit tikus diisolasi untuk mempelajari ekspresi gen TGF-β1 menggunakan qRT-PCR, dan pewarnaan spesifik Masson Trichrome-collagen. Komparatif analisis qRT-PCR menunjukkan rerata ekspresi TGF-β1 terendah terlihat pada kelompok Kontrol Negatif (0.01±0.00), diikuti T-5% (0.11±0.22), kelompok Kontrol Sehat (1.01±0.01) dan T-10% (2,32±2,46). Menurut pewarnaan MT, jumlah kolagen tertinggi pada kelompok Kontrol Sehat (43,83±5,3), diikuti T-10% (38,39±3,1), T-5% (29,04±3,2) dan kelompok Kontrol Negatif. (13,87±2,7). Ekstrak bunga Clitoria ternatea berbasis gel terbukti dapat meningkatkan ekspresi gen TGF-β1 dan berkurangnya deposisi kolagen pada kulit tikus yang kehilangan kolagen akiab UVB.

Kata Kunci: Collagen loss, Clitoria ternatea, iradiasi UVB, TGF-β1

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INTRODUCTION

Repeated exposure to UVB irradiation caused the loss of elasticity, reductions appearing in the epidermal thickness, collagen content, elastic fiber deterioration, and increased wrinkle and dryness of the skin tissue (1–3). Uncontrolled skin photoaging can develop into numerous dermatological diseases, including solar keratosis, chronic optic cheilitis, photo-elastic fibrosis, melanoma, basal cell carcinoma, sunspot-like mites, and squamous cell carcinoma (4–6). On the other side, UVB irradiation-induced skin damage is also related to inflammation characterized by the of forming reactive oxygen species (ROS) (7,8). Augmented levels of ROS directly induce DNA damage and trigger secretion of interleukin-6, ensuing in overexpression of matrix metalloproteinases (MMPs) resulting in collagen degradation (9–11). The extracellular matrix (ECM) is constructed of multiple structural proteins including collagen and elastin, all of which play a potential role in skin elasticity (12). Type-1 collagen is the main constructional protein of dermal ECM and is synthesized from type-1 procollagen which is synchronized by the activity of transforming growth factor beta-1 (TGF-β1) (13,14). Recent studies have revealed that various bioactive components in butterfly pea flower (Clitoria ternatea) such as flavonoids, saponins, terpenoids, and tannins have anti-aging potential effects (15–17). In spite of this, the certain mechanism of Clitoria ternatea L. in inhibiting skin aging mainly correlated to TGF-β1 and collagen production has not been clearly studied.

Antioxidant is extensively used to reduce and protect the skin from the dangers of ultraviolet radiation and have the potential to maximize skin aging therapy (18). The current therapies, such as microdermabrasion, lasers, fillers, chemical drugs and even surgery, have drawbacks, including being expensive, having only temporary effect and can cause skin irritation (19). Currently, natural antioxidants from plants and fruits are known to have fewer side effects than those produced (15,18). The butterfly pea (Clitoria ternatea) is one of the natural antioxidant sources which is plentiful in phenolic content and contains terpenoids, anthocyanins, tannins, phenols, and flavonoids (15,20,21). The presence of phenolic and flavonoid compounds in Clitoria ternatea can counteract with free radicals by repressing oxidation, subsequently slowing photooxidation from UV exposure (22,23). Flavonoids are phenolic compounds with antioxidant activity due to their ability to bind metals or donate hydrogen atoms and consequently preventing free radicals-induced cell damage (17,24). Phenol has been proven as a chain-breaking and free radicals scavenger that prevent cellular oxidative stress (16). Tannin and anthocyanin are secondary metabolites that have long conjugated double bonds and perform as biological antioxidants by means radical scavenging mechanism (18,20).

Clitoria ternatea has shown potential as a UVB skin protection due to their bioactive antioxidant compounds (15,16,25). However, studies regarding the benefits of Clitoria ternatea flower extract in regulating TGF-β1 expression and collagen synthesis in dermal tissue are not yet available. Therefore, this study aimed to investigate the effect of Clitoria ternatea flower extract-based gel on TGF-β1 gene expression and collagen density in high-intensity UVB-irradiated rat skin tissue.

METHODS

Maceration and Extraction of Clitoria ternatea Flower

Maceration and extraction methods of butterfly pea flower were carried out based on the previous study with slight modifications (26,27). The dried Clitoria ternatea flower was blended until smooth and sieved with a mesh size of -20 +30. The 50-gm dried Clitoria ternatea flower was macerated with 250ml96% ethanol solvent for 3 days and the pulp was macerated with 0.125 L 96% ethanol solvent. The extract obtained was evaporated with a rotary evaporator resulting in thick extract.

Animal Model and Clitoria ternatea Flower Extract-Based Gel Administration

Twenty-four healthy male Wistar rats (250±25 g) CV=10% were fed ad libitum at 28°C (room temperature) and exposed to UVB (photoperiod) for 12 hours. After a week of acclimatization, rats were randomly divided into the following four groups: Untreated (Healthy Control), UVB irradiation and based-gel (UVB), UVB irradiation and 5% of Clitoria ternatea flower extract-based gel (T-5%), and UVB irradiation and 10% of Clitoria ternatea flower extract-based gel (T-10%). The dosage used was based on previous research which showed that Clitoria ternatea L. extract cream 5% has proven to decrease the MMP-1 levels and decrease the amount of collagen in Wistar rat’s skin exposed to UV-B (28). A dose of 10% was used to determine the maximum concentration limit that can be applied and determine its efficacy and safety. Each group consisted of five rats. This study used UVB light (emission at 302nm CL-100M, UVP, USA). Rats were exposed to UVB light of 160mJ/cm2 for 30 minutes for 5 consecutive days as mentioned in previous study with a slight modification (9). The gels were administered topically on the back skin daily up to 14 days. Negative Control group rats did not receive any treatment. All rats were terminated on 15th day and skin tissue was isolated for the next processes.

TGF-β1 Gene Expression by qRT-PCR

Total RNA from rat skin tissue was extracted with TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer’s protocol. Briefly, first-stranded cDNA was synthesized with 1 g of total RNA using Super-Script II (Invitrogen, Massachusetts, USA). SYBR No ROX Green I dye (SMOBIO Technology Inc, Hsinchu, Taiwan) was used for reverse transcription in a real-time PCR instrument (PCR max Eco 48), and mRNA levels of the TGF-β1 (F:5’-GTCAACTGTGGAGCAACACG-3’; R:5’-CGTCAAAAGACAGCCACTCA-3’) and GAPDH (F:5’-TGACAACCTTGGCCATCTGGG-3’; R:5’-GGGCCATCAAAGTCTTCTG-3’) was detected using the respective primers. The thermocycler conditions used were as follows: initial step at 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds, and 60°C for 1 minute. The gene expression was recorded as the Cycles threshold (Ct). Data were obtained using Eco Software v5.0 (Illumina Inc, San Diego, CA, USA). All reactions were performed in triplicate, and the TGF-β1 data and GAPDH data were calculated using the 2−ΔΔCt method (Livak method) in calculating gene expression from quantitative PCR results, quantitative gene expression data is often normalized to control expression levels or so-called “housekeeping” genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most commonly used housekeeping genes in comparison to gene expression data (29).
Collagen Staining

The skin tissue paraffin block was cut using a microtome to a thickness of 5μm then stained with Masson Trichrome (Bio optica, catalog #04010802) and observed under the light microscope (Olympus CX21, Tokyo, Japan). The percentage of collagen density was calculated from the area of collagenous tissue formed on each slide using ImageJ.

Statistical Analysis

Statistical analyzes were performed using the software SPSS 26.0 (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard deviation (SD). The data obtained were collected, compiled, and tested for normality with the Shapiro-Wilk test and the homogeneity test with the Lavene test. Data analysis used one-way ANOVA and continued with the Least Significant Difference (LSD) post hoc test using p-value <0.05.

RESULTS

Phytochemical Screening of Clitorea ternatea Flower Extract

Phytochemical analysis of the Clitorea ternatea flower extract was carried out to detect the presence of various secondary metabolites in the extract. The results qualitatively showed that the Clitorea ternatea flower extract contained alkaloids, saponins, tannins, flavonoids, and triterpenoids, and does not contain steroids (Table 1).

Table 1. Phytochemical screening of Clitorea ternatea flower extract

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Content</th>
<th>Test applied</th>
</tr>
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<tbody>
<tr>
<td>Alkaloid</td>
<td>+</td>
<td>Wagner</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
<td>Forth</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
<td>FeCl3 1%</td>
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<tr>
<td>Flavonoid</td>
<td>+</td>
<td>Willstatter</td>
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<tr>
<td>Steroid</td>
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<td>Lieberman Burchard</td>
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<tr>
<td>Triterpenoid</td>
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Note: caption: + = present, - = absent

Clitorea ternatea Flower Extract-Based Gel Increase TGF-β1 Gene Expression in UVB-induced Collagen Loss Rat Skin

UVB exposure has been proven to induce collagen degradation by decreasing the pro-fibrotic factor called TGF-β1. TGF-β1 gene expression was carried out to examine the effect of Clitorea ternatea flower extract on TGF-β1 gene expression in the UVB-induced collagen loss rat skin tissue. The results of the comparative analysis showed that TGF-β1 expression was decreased after UVB radiation (Negative Control group) (0.01±0.00), and significantly increased after being treated with the Clitorea ternatea flower extract-based gel both in the T-5% group (29.04±3.2) and T-10% group (38.39±3.1) (Figure 1A and Figure 2B). This result suggested that the metabolite compound in Clitorea ternatea flower extract-based gel may restore collagen deposition. On the other hand, collagen deposition in the T-5% and T-10% group was lower than in the Healthy Control group (43.83±5.3).

Figure 1. Effect of Clitorea ternatea flower extract-based gel on TGF-β1 gene expression (n=6±SD). The expression of TGF-β1 is decreased in the Negative Control group and significantly increased in the T-10% group, *p<0.05

Clitorea ternatea Flower Extract-Based Gel Increase Collagen Deposition in UVB-induced Collagen Loss Rat Skin

Masson trichrome staining was carried out to verify the effect of Clitorea ternatea flower extract on collagen deposition in the UVB-induced collagen loss rat skin tissue. The comparative analysis resulted that dermal collagen deposition was decreased after UVB radiation (Negative Control group) (13.87±2.7), and significantly increased after being treated with the Clitorea ternatea flower extract-based gel both in the T-5% group (29.04±3.2) and T-10% group (38.39±3.1) (Figure 2A and Figure 2B). This result suggested that the metabolite compound in Clitorea ternatea flower extract-based gel may restore collagen deposition. On the other hand, collagen deposition in the T-5% and T-10% group was lower than in the Healthy Control group (43.83±5.3).

Figure 2. Collagen analysis with Masson Trichrome staining

Note: (A) Microscopic appearance of the skin tissue with MT staining under 10x observation magnification. (B) Percentage of collagen content analyzed with ImageJ (n=6±SD). The NC group showed a lower amount of collagen than Clitorea ternatea flower extract-based gel either 5% or 10% group. The arrows indicate collagen deposition. *p<0.05. HMT: Masson Trichrome NC: Negative Control. Scale bar: 100µM
DISCUSSION

The result of this study showed that Clitoria ternatea flower extract induces TGF-β gene up-regulation and increase the collagen deposition in UVB-induced collagen loss. This results in dermal collagen restoration. This suggests that the protective effect of Clitoria ternatea flower extracts might be able to restore the UVB-induced collagen loss by inducing the TGF-β gene up-regulation and increasing the collagen amounts. The Clitoria ternatea flower extract contained several bioactive compounds that act as antioxidants such as flavonoids and phenols (15-17,30). Based on previous studies, it is widely known that flavonoids and phenols have a role as metal ion chelators and stabilized hydrogen atoms from the hydroxyl group. This reduces reactive oxygen species (ROS) formation, especially hydroxyl radicals (OH) and inhibit the occurrence of photoaging (9,20,30,31). The phenolic compounds in Clitoria ternatea, primarily flavonoids, serve as antioxidants that may counteract UVB-induced free radicals. The antiaging properties of plants are attributed to contain antioxidant substances, such as polyphenols, flavonoids, and anthocyanins. Flavonoids, ROS scavengers, may inhibit ROS overproduction resulting in the suppression of MAPK signaling pathways including ERK, JNK, and p38, which negatively activates the nuclear-factor kappa-B (NF-KB) pathway (32-35). The blocked-NF-kB pathway may induce the activation of anti-inflammatory cytokines, including TGF-β. For dermal fibroblasts, TGF-β is an important regulator of collagen homeostasis by stimulating procollagens I and III and down-regulating MMP-1 transcription.

Repeated exposure to UVB stimulates the cytokine receptors by increasing the transcription of AP-1, which results in TGF-β1 inactivation and collagen degradation (3,9,33,36). UVB irradiation modulates the TGF-β pathway at several levels; it decreases type II TGF-β receptor in the dermal fibroblast-cell membrane in vivo, stimulates the intracellular inhibitor of TGF-β1 signaling Smad-7, and reduces levels of connective tissue growth factor (CCN2), an essential mediator of TGF-β1 effects on collagen synthesis (37-41). At the intracellular level, UVB can cause DNA damage by cross-linking adjacent pyrimidine bases and generating free radicals or reactive oxygen species (ROS), consequently, resulting in transforming growth factor (TGF-β) inhibition and AP-1 (activator protein) activation (23,31,42). AP-1 inhibits the TGF-β/SMAD pathway, the primary regulator for type I procollagen production, which leads to a decrease in collagen synthesis (3,9,33). In another hand, over-accumulation of ROS may also activate NF-kB which is then translocated into the nucleus for inducing transcriptional activation and transcriptional regulation of MMP-1, a major factor of collagen type 1 degradation (10,31,32). This is in line with the results of a current study which showed that UVB exposure can decrease TGF-β1 gene expression and collagen deposition.

This study provides information by proving that 10% topical Clitoria ternatea flower extract-based gel may act as an alternative anti-aging treatment, especially UVB-induced collagen loss therapy inducing TGF-β expression and collagen deposition. The increase in collagen deposition in the 5% dose group might be due to myofibroblasts being activated by TGF-β and other growth factors such as FGF and PDGF. In addition, the amount of collagen is also influenced by the levels of MMP enzymes. Fibroblasts and myofibroblasts are stimulated by several growth factors such as TGFβ, platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF) (43). The previous study also revealed that Clitoria ternatea L. extract 5% cream decreased the collagen amount by decreasing MMP-1 level (28). Further studies on the effect of 10% Clitoria ternatea flower extract-based gel on other dermal collagen loss markers such as MMP-1, AP-1, NF-kB, and ROS levels both in vivo and in clinical studies are needed to strengthen scientific evidence of the Clitoria ternatea capability in restoring UVB-induce collagen-loss.

These findings demonstrated that gel-based Clitoria ternatea flower extract may increase TGF-β1 gene expression and collagen deposition in the UVB-induced collagen loss rat skin.

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest with this article content.
Oxygen Species in L929 Fibroblasts is Attenuated by Extended Production of UV-Induced Reactive
Total Etanol Bunga Telang (Clitoria ternatea L) dari Daerah Sleman with Methode DPPH
Ekstrak Etanol 70% Bunga Telang (Clitoria ternatea L) dari Daerah Sleman dengan Metode DPPH


Reduction of Skin Scarring Fibroblasts and its Potential Therapeutics for the IL-1β-Induced Fibrosis Effects of IL-10 on Dermal
Lysate on Skin Rejuvenation Characteristics. Produced with Fetal Bovine Serum or Human Platelet
Mesenchymal Stem Cell Conditioned Media SU.


Mechanisms of Dermal Aging and Antiaging Approaches. International Journal of Molecular

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