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RESEARCH ARTICLE

Collagen Thickness and Density in BALB/c Mice Exposed to UVB Light after using Siam Weeds Cream (*Chromolaena odorata* L.)

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ABSTRACT:

Introduction: Ultraviolet B (UVB) radiation can cause damage to the skin's dermis layer, which can lead to collagen fragmentation. Damage to collagen generates wrinkles, which are a sign of aging. **Objective:** The purpose of this study was to determine how Siam Weeds cream (*Chromolaena odorata* L.) altered the thickness and density of collagen in the skin of BALB/c mice after UVB exposure. **Methods:** There were four groups of 24 BALB/c mice altogether: normal control, negative control (Cream Base with UVB), positive control (UVB), and treatment (*C. odorata* cream and UVB). Just before and after the mice were exposed to UVB at 500 mJ/cm^2, the Siam Weeds cream formulation was applied at a concentration of 1%. Mice were slaughtered, and a back skin excisional biopsy was conducted to analyze collagen. **Results:** the treatment group differed significantly both in thickness and density of collagen against the positive control group and the negative control group ($p<0.05$) and There was no statistically significant difference between the treatment and normal control groups ($p<0.05$). **Conclusion:** A 1% dosage of *C. odorata* cream can improve the thickness and density of collagen in UVB-exposed mice's skin.

KEYWORDS: *Chromolaena odorata* L., Cream, Collagen, UVB.

INTRODUCTION:

The skin is the body's biggest organ, accounting for roughly 15% of an adult's total body weight. This organ serves a variety of critical activities, including providing protection from the external environment, whether physical, chemical, or biological. In addition, the skin plays a role in thermoregulation and prevents excessive water loss from the body. The purpose of the skin is to protect the body's surface[1].

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The epidermis, dermis, and subcutaneous layers are the three primary layers of the skin. Collagen, a protein fiber with roughly 15 different hereditary variants in the human skin, is the major component of the dermis especially the extracellular dermal matrix[2].

Collagen is found in various places throughout the body, including tendons, ligaments, bone layers, and the dermis. Collagen is the most stress-resistant component of the skin. Collagen contains elastic strands that help to keep the skin supple and resist deformation and tearing. Collagen accounts for 70% of the skin's dry weight[3].

The skin is damaged by ultraviolet (UV) radiation from the oxidizing environment. The amount of hydrogen peroxide in the skin doubles after 10-20 minutes of UV exposure. Hydrogen peroxide can also quickly cause the creation of additional reactive oxygen species (ROS). Photooxidation, photo isomerization, and photooxidation are some of the photochemical dangers that solar UV radiation poses to living cells. The generation of reactive peroxides (H_2O_2), as well as the pathobiological effects of ultraviolet radiation (UVA and UVB), create free radicals and damage DNA. Free radicals are thought to be one of the key mechanisms that accelerate the premature aging process[4].

UV light causes oxidative damage to macromolecules by producing free radicals that collect in cells and the extracellular matrix. UV radiation mostly affects the skin. Type I collagen fibrils, which make up the majority of human connective tissue (skin, bones, and tendons), are constantly exposed to UV radiation. Skin wrinkling is aided by photo-degradation of collagen, which may also contribute to skin cancer. Several studies have shown that UV radiation can degrade collagen by cleaving polypeptide chains and aromatic amino acids without disrupting the triple helix structure of nitrogen-carbon bonds. UV exposure appears to increase the normal aging process of collagen degradation[5].

Ultraviolet-B radiation affects the development of procollagen I and III, as well as the production of pro-matrix metalloproteinase-1 and -3, which are both produced and secreted by fibroblast cells[6].

It is critical to maintain a balance between oxidants and antioxidants in order to prevent cellular damage caused by oxidative stress. This can be accomplished by consuming antioxidants or utilizing antioxidants externally as cosmetics. Because of its abundant phytochemical components, such as alkaloids, flavonoids, flavanones, essential oils, phenolics, saponins, tannins, and terpenoids, *Chromolaena odorata* (Family: Asteraceae) has been widely recognized to have an antioxidant profile. Eupoline, chromomorphic acid, quersetagetin, and quercetin are all major components of this plant. The antioxidant characteristics of medicine or plants enhance the creation of fibroblasts and the proliferation of keratinocytes in the wound, making wound healing more efficient[7]. Based on the literature review above, it is necessary to conduct a study to assess the effect of topically applied *C. odorata* leaves on the thickness and density of collagen in the skin of BALB/c mice after exposure to UVB.

MATERIALS AND METHODS:

Materials:

BALB/c mice were obtained from PT. Biomedical Technology Indonesia Bogor, and the following ingredients were used: water-saturated n-butanol fraction, ether, formalin buffer 10%, liquid paraffin, stearic acid, cetyl alcohol, α– Tocopherol, glycerin, DMDM hydantoin, phenoxyethanol, propylene glycol, viscolam®, flavoring, Trichrome Masson staining Light microscopy, UVB light lamp (Dermapal Daavlin® USA).

Methods:

This is a quantitative laboratory experimental study with a pure experimental research design (True Experimental): post test only control design[8]. This study was carried out at the Hasanuddin University Teaching Hospital's Anatomical Pathology Laboratory for histological examination. The Biomedical Research Ethics Commission on Experimental Animals, Faculty of Medicine, Hasanuddin University, approved this study with the number 1238/UN4.6.4.5.31/PP36/2019.

Handling of experimental animals:

The researchers employed male BALB/c mice that were 5-7 weeks old and weighed 15-25 grams. Mice were given free access to food and water and were acclimatized for one week before testing. Temperature (23±2^o C), humidity (50±5%), and light (12 hour light/dark cycle) were all controlled and divided into four groups. Each group had six experimental animals. Normal control (no treatment or healthy skin), negative control (exposed to UVB and given a placebo cream), positive control (exposed to UVB), and treatment were the groups (exposed to UVB and given *C. odorata* cream).

Mice were exposed to UVB rays three times a week for four weeks, at a dose of 500 mJ/cm^2 at a distance of 20 cm from the source. Following that, all experimental animals were anesthetized with ether and a dislocation in the cervical region excision biopsy was conducted on the skin of the middle back at a size of 2x2 cm, which was subsequently kept in 10% buffered formalin for histological examination.

Manufacturing and Formulation of Cream:
The following table shows the ingredients in *C. odorata* cream:

Table 1. *C. odorata* cream formulation (oil-in-water)

S. No	Ingredients	Concentration (%)
1	Water-saturated n-butanol fraction *C. odorata* leaf extract	1
2	Stearic acid	3
3	Liquid paraffin	10
4	Cetyl alcohol	3
5	α – Tocopherol	0.1
6	Glycerin	10
7	DMDM hydantoin	0.5
8	Phenoxyethanol	0.5
9	Viscolam®	3
10	Propilene glycol	10
11	Fragrance	0.005
12	Aquadest Ad	100

All ingredients were weighed according to the calculations in table 1. Stearic acid, cetyl alcohol, liquid paraffin, and phenoxyethanol were melted in a water bath at 70ºC (oil phase). Water, propylene glycol, glycerin, and DMDM hydantoin were heated to a temperature of 70ºC (aqueous phase). The oil phase was added to the water phase, then the viscolam emulsifier was added, and was stirred with an electric stirrer at 4000 rpm to form a stable emulsion. The water-saturated n-butanol fraction was pulverized in a mortar and gradually added to the cream base, stirring until homogeneous, before being transferred to a beaker holding the remaining base and swirled until homogeneous again. After the cream has been slightly warmed, the tocopherol and flavoring are added and stirred until smooth.

At each application, cream base and *C. odorata* cream were distributed uniformly on the backs of mice from each cream group (negative control group and treatment group), with as much as 0.1mg/cm^2 in the irradiation area. Before the mice were slaughtered, the mice were left for twenty-four hours after the irradiation finished to remove the effects of acute irradiation[9].

Histophatology of Collagen[10]
Excisional biopsy was used to obtain preparations for histological examination from the skin of the mice's backs (dorsal). After being fixed with a formalin buffer, each specimen was put on a level surface and divided into two halves. The tissue sections in the centre were cut perpendicularly to a thickness of 4µM and stained with the Trichrome Masson stain for collagen. Trichrome Masson staining was used to determine the average thickness of collagen from the lower edge of the basement membrane to the upper edge of the subcutaneous fat tissue. The results of three fields of vision with a magnification of 200x are printed at 100% on A4 paper, and the thickness of each field of view is measured with a ruler in centimeters (cm).

A light microscope with a magnification of 200x was used to measure collagen density. Three raters evaluated each sample. The following criteria were used to determine the density of collagen fibers:

Collagen fibers appear very thin or tiny. (+) or 1: collagen fibers are very thin or tiny. (++) or 2: collagen fibers are scattered very thinly or only slightly. (+++) or 3: scattered collagen fibers (++++) or 4: collagen fibers densely spread.

Data analysis:
The Shapiro-Wilk test was used to determine whether the primary data of collagen thickness and density was normal. The Levene's test was used to determine the data's homogeneity. To analyze the differences between the study groups, Oneway ANOVA and Kruskall-Wallis were used. The results of the analysis were declared significant or there was a difference if the p value was < 0.05. Then the statistical test was continued with a further difference test using the Tukey HSD and Mann-Whitney U tests.

RESULT:
There are differences in thickness between 4 groups: I (normal control), II (Cream-Based and UVB negative control), III (UVB positive control), and IV (Cream *C. odorata* and UVB treatment), which can be seen in the following figure:

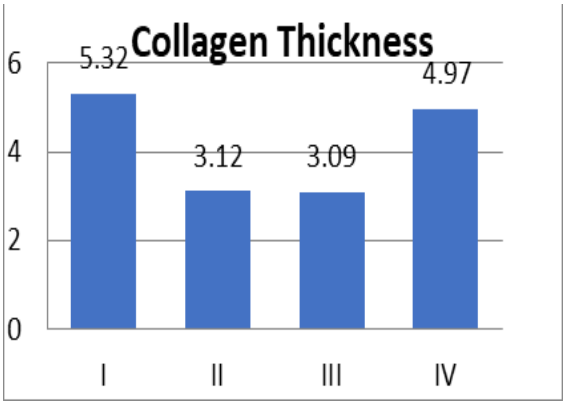

Figure 1. Graph of the average thickness of collagen on histopathological observations

There are descriptive differences between groups I, II, III, and IV, as shown in Figure 1, a graph showing the average thickness of collagen on histological findings. The values of groups I and IV are nearly identical, as are the values of groups II and III.

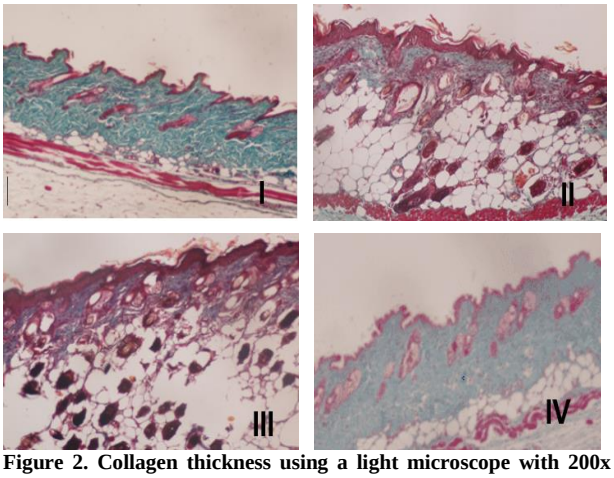

Figure 2. Collagen thickness using a light microscope with 200x magnification. I (Normal), II (Cream Base + UVB), III (UVB), IV (Cream *C. odorata* + UVB).

The average thickness analysis was obtained after the normality and homogeneity tests were performed with the Shapiro-Wilk and Levene's tests, which revealed that the data was homogeneous and normally distributed (symmetrically) with sig values or p > 0.05 where the homogeneity test result is p=0.357 and the normality test result is at p=0.794 (group I); p=0.390 (group II); p=0.540 (group III), and p=0.214.

Table 2. Average thickness of collagen by histopathological observation

Group	n	Min	Max	Mean	SD	F	P
I	6	4.82	5.94	5.32	0.433	23.230	0.000*
II	6	2.64	3.77	3.12	0.461		
III	6	232	4.31	3.08	0.740		
IV	6	4.25	5.86	4.96	0.711		

* Oneway ANOVA; p<0.05; significant

With F=23.230 and sig. or p=0.000 (p 0.05), the results of the Oneway ANOVA test (table 2) suggesting that the groups are significantly different. Follow-up testing was performed using the Post Hoc test, often known as Tukey HSD. average collagen thickness between groups Table 3 summarizes the test results.

Table 3. Tukey HSD Advanced Difference Test on average collagen thickness on histopathological observations.

Group	I	II	III	IV
I		0.000*	0.000*	0.735
II			1.000	0.000*
III				0.000*
IV				

** Post Hoc Tukey HSD; p<0.05; significant*

Table 3 demonstrates that the collagen thickness in Group I (Normal) differed considerably from Groups II (Cream Base and UVB) and III (UVB), but not from Group IV (Cream *C. odorata* and UVB), and there was no significant difference between Group II (Cream Base and UVB) and Group III (UVB) (UVB).

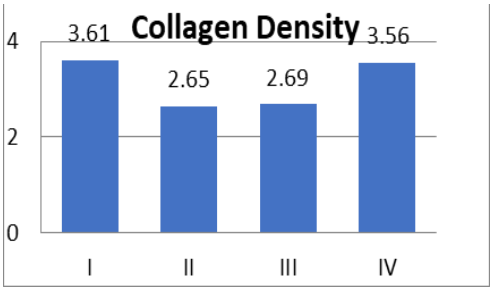

Figure 3. Graph of the average density of collagen on histopathological observations.

The graph of the average collagen density on histopathological observations in Figure 3 is descriptive, with differences between groups I, II, III, and IV. Groups I and IV have nearly identical values, and Groups II and III have nearly identical values.

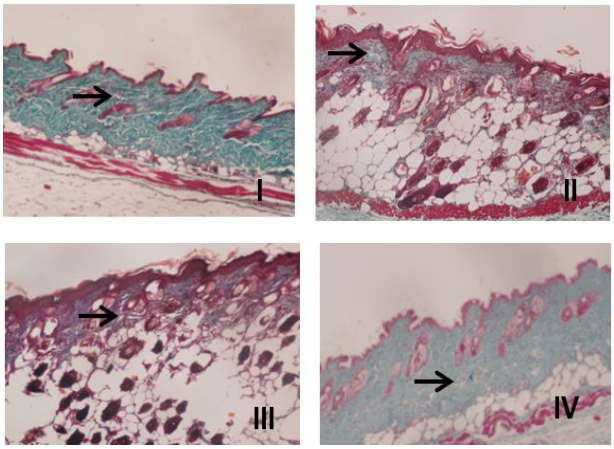

Figure 4. Collagen density using a light microscope with 200 x magnification. I (Normal), II (Cream Base + UVB), III (UVB), IV (Cream *C. odorata* + UVB).

After performing the normality and homogeneity tests with the Shapiro-Wilk and Levene's Tests, the average density analysis revealed that the data was homogeneous and normally distributed (symmetrically) with sig values or p > 0.05 where the homogeneity test result is p=0.131 and the normality test result is at p=0.333 (group I); p=0.101 (group II); p=0.263 (group III), and p=0.437.

Table 4. Average collagen density by histopathological observation

Group	N	Mean Rank	Chi-Square	P
I	6	18.67	15.561	0.001*
II	6	6.83		
III	6	7.00		
IV	6	17.50		

** Kruskall-Wallis; p<0.05; significant*

The Kruskall-Wallis test (table 4) has a sig. or p=0.001 (p<0.05), suggesting that the groups are significantly different. Follow-up tests were carried out using the Post Hoc test, namely Mann-Whitney U, the average thickness of collagen between groups. The test results are shown in a five-table format.

Table 5. Mann-Whitney U Follow-Up Test on the average collagen density on histopathological observations

Group	I	II	III	IV
I		0.007*	0.006*	0.562
II			0.868	0.007*
III				0.006*
IV				

** Post Hoc Mann-Whitney U; p<0.05; significant*

Table 5 demonstrates that the collagen thickness in Group I (Normal) differed considerably from Groups II (Cream Base and UVB) and III (UVB), but not from Group IV (Cream *C. odorata* and UVB), and there was no significant difference between Group II (Cream Base and UVB) and Group III (UVB) (UVB).

DISCUSSION:

Collagen is an extracellular protein that plays a key function in skin organ elasticity and mechanical protection. Collagen is significantly enhanced by the amino acids glycine, hydroxyproline, and hydroxylysine. In the papillary and adventitia dermis, type I collagen is the primary element, while the reticular dermis contains big, strong collagen bundles. Type IV collagen is found in the basement membrane zone, while type VII collagen, which is predominantly produced by keratinocytes, is the fibrils' main structural component[11].

UVB is a type of ultraviolet light with a wavelength of 290-320nm that can penetrate the earth's surface and damage skin. UVB penetrates the papillary dermis and is widely absorbed into the epidermis. Erythema, hyperplasia, hyperpigmentation, sunburn, dry skin, and diminished suppleness are among the side effects of UVB exposure[12].

BALB/c mice weighing 15-25grams were used in this investigation. In the area that would be irradiated, the mice's backs were shaved. At 08.40 am, which is 20 minutes before irradiation (allowing absorption time of topical materials on the skin), and at 13.00 pm, which is 4 h after irradiation, the mice's backs were uniformly treated with a cream base and the *C. odorata* cream. On days when there is no irradiation, topical materials are nevertheless applied. Before the mice were slaughtered, they were left for twenty-four hours after the irradiation was finished to eliminate the effects of acute irradiation. In this study, there were significant differences in collagen thickness and density depending on the sample group ($p<0.05$), with statistical test results indicating that the thickness and density of collagen in the treatment group (*C. odorata* cream) were significantly higher than the positive and negative control groups.

UV radiation can activate growth factor cytokine receptors (growth factors) on the surface of epidermal keratinocytes and dermal fibroblast cells. 15 minutes after UV exposure, epidermal growth factor receptors (IL-1) and tumor necrosis factor (TNF-) should be activated in keratinocytes and fibroblasts. When these receptors are active, the MAP kinase complex is activated, which then activates the nuclear transcription factor activator protein-one (AP-1). ROS are oxidants that decrease the protein tyrosine phosphatase enzyme through the oxidation process. Growth factor receptors are increased when this enzyme is blocked, which activates AP-1[13]. Overexpression of the c-jun component of AP-1 as a result of fibroblast culture can diminish collagen-1 expression. MMP collagenase (MMP-1), stromelysin-1 (MMP-3) and 92-kd gelatinase B (MMP-9) are all expressed by AP-1 in the dermis and epidermis, causing collagen and other proteins in the extracellular dermal matrix to be damaged. AP-1 can suppress the expression of procollagen-1, procollagen-3, and TGF dermal fibroblast genes, resulting in decreased collagen synthesis[14].

Furthermore, UVB-induced increased production of reactive oxygen species (ROS) in the skin can cause skin aging. Even when the skin already exists, the body has its own defense system against ROS. The continual production of ROS disrupts both the enzymatic and non-enzymatic antioxidant defense systems in the skin[15]. UVB also exacerbates the inflammatory response by activating and encouraging inflammatory cells that damage the skin. UVB-produced ROS can block MAPK signaling and activate nuclear factor-B (NF-KB) and activation protein 1 (AP1), resulting in the release of inflammatory cytokines such tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6. Furthermore, increased matrix metalloproteinase (MMP) activity in response to ROS production induces collagen breakdown, which disrupts ECM structure and function. UV light also promotes the creation of cyclooxygenase-2 (COX-2) in the skin, which exacerbates inflammation by catalyzing prostaglandin E2 synthesis and boosting the expression of an inducible nitrating factor[16]. Antioxidants are chemicals that can delay the oxidation of proteins, carbohydrates, lipids, and DNA in low quantities[17]. Antioxidant processes are classified into three types, each of which can occur naturally or artificially: Superoxide dismutase, glutathione reductase, catalase and certain micronutrients are among the first-line antioxidant defenses (e.g., Se, Cu, Zn); Glutathione, vitamins C and vitamin E, carotenoids, albumin and flavonoids are examples of second-line antioxidant defenses; and the third-line antioxidants include complex groups of repair enzymes for DNA and proteins, oxidized lipids, and peroxides (e.g. Proteases, DNA repair enzymes, etc.). Through a balance of free radicals and antioxidants, antioxidants mitigate the effects of physiologically produced reactive oxygen species in cellular metabolism[17]. When these two

components are out of balance, ROS production takes over and contributes to the harmful process of oxidative stress. Activation of signaling pathways such as gene transcription, cell cycle, proliferation, and apoptosis, as well as changes in proteins and lipids, inflammation, immunosuppression, DNA damage, and activation of signaling pathways such as gene transcription, cell cycle, proliferation, and apoptosis, are all part of this process[18].

As a result, maintaining normal skin homeostasis and oxidants requires regulating ROS levels, which can protect individuals from disease by neutralizing ROS production.

C. odorata leaves are formulated in the form of a cream (oil-in-water), with the aim of being used as a topical[19,20]. *C. odorata* leaves had an antioxidant effect in previous studies and were formulated in cream form to facilitate their use. The antioxidant effect of *C. odorata* leaves is tightly linked to flavonoids and polyphenols[21]. The ability of these chemicals to act as antioxidants is directly linked to their ability to donate electrons. The presence of electron donation activity is connected to antioxidant activity since it indicates the reducing power of bioactive substances. Cream preparations are common preparations that are popular due to their simplicity.

The findings of this study can be utilized as preliminary proof that *C. odorata* leaves can protect the skin from collagen damage. *C. odorata* leaves can be used to protect the skin, particularly from UVB rays.

CONCLUSION:

Applying a 1% concentration of *C. odorata* cream to the skin of BALB/c mice exposed to UVB radiation increased the thickness and density of collagen, according to this study.

CONFLICT OF INTEREST:

There are no conflicts of interest declared by the authors of this study.

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