J Prima Med Sains (2024) 6(2):169-176
doi.org/10.34012/jpms.v6i2.5743

ORIGINAL ARTICLE

Effect of gotu kola leaf extract cream on collagenization and histopathological picture of rat skin exposed to Ultraviolet B light

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Abstract

Changes due to genetic conditions (internal and chronological aging) overlap with aging symptoms triggered by environmental conditions (extrinsic aging). Until recently, exposure to the sun's ultraviolet (UV) radiation was considered the leading cause of extrinsic skin aging; both UVB (290-320 nm) and UVA (320-400 nm) caused photoaging, and due to their physical properties, UVB radiation was especially harmful to the skin condition. This study aims to analyze and test the effect of administering gotu kola leaf extract cream (*Ceentella asiatica*) on collagenization in Wistar rats (*Rattus norvegicus*) exposed to ultraviolet-B light. This study uses a posttest-only control group design, where only observations are made to the control group and treatment after the treatment is given. The research results show that gotu kola leaf extract contains phytochemicals that can be used as an antioxidant and anti-inflammatory ingredient because they contain flavonoids, saponins, tannins, and phenolic compounds. This research concludes that pegagan leaf extract contains phytochemicals that can be used as medicinal ingredients because it contains high antioxidant and anti-inflammatory compounds. The average percentage of collagen growth in the control group compared to treatment groups P1, P2, and P3 after exposure to UVB light was very high, and the group control of collagen growth was not significant and tended to be stable.

Keywords: gotu kola leaf, collagen, UVB light, rattus norvegicus

Introduction

Climate change affects people worldwide, manifesting as regional and global climate variations over extended periods. It primarily stems from human activities, notably the release of greenhouse gases like carbon dioxide from fossil fuel combustion. This phenomenon poses significant health risks, including skin-related issues and disease outbreaks. Directly, climate change can intensify skin problems through extreme weather events associated with global warming, leading to higher rates of skin infections, inflammatory diseases, and premature aging. Indirectly, disruptions to natural systems contribute to the spread of vector-borne and waterborne diseases, many of which adversely affect skin health.[1,2]

The skin is a barrier organ that separates the body from the external environment. Due to the many physical, chemical, and microbial disturbances that affect the skin, various types of immune cells reside in or are recruited to the skin to maintain skin homeostasis. The skin serves several functions, such as acting as a barrier against water loss and pathogens and protecting against various forms of trauma, including thermal, chemical, and ultraviolet radiation. The skin keeps us in contact with our environment through numerous nerve endings, regulates body temperature, enhances metabolic functions, and synthesizes vitamin D.[3]

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UV-induced photoaging is characterized by modifications to the dermal extracellular matrix (ECM), leading to wrinkles, fragility, weakness, roughness, impaired wound healing, and increased epidermal thickness. In addition, excessive ultraviolet B (UVB) radiation causes the formation of intracellular reactive oxygen species (ROS). This leads to oxidative stress and skin inflammation by activating mitogen-activated protein kinases and upregulating transcription factors, such as activator protein 1 (AP-1). Furthermore, UVB-induced ROS can increase the expression of matrix metalloproteinase-1 (MMP-1) in fibroblasts, triggering skin photoaging. MMP-1 degrades type 1 collagen, the main component of the ECM that provides structural support to the skin, resulting in dermal breakdown and skin aging.[4]

Long-term exposure to UVB and UVA radiation from sunlight alters the properties of collagen fibers in the dermis, reducing skin elasticity and causing wrinkles. UV radiation increases the risk of long-term damage, such as photoaging, photoimmunosuppression, and photocarcinogenesis. UVA radiation negatively affects epidermal keratinocytes and dermal fibroblasts, inducing long-term changes. The changes caused by UVB radiation are primarily visible in the epidermis but also penetrate the upper dermis. The harmful effects of ultraviolet exposure include side effects on the skin, such as sunburn, photodermatosis, hyperpigmentation, premature skin aging, precancerous lesions, and cancer.[5]

Human skin, an essential part of the innate immune system, has various molecular mechanisms that protect it from UV exposure. The first is the epidermal structure, which provides the first defense against harmful external agents. Additionally, immune cells such as Langerhans and T lymphocytes are in the skin. Another form of protection for the skin is melanocytes. Melanin, a pigment synthesized by these cells, inhibits the penetration of UV radiation into the living layers of the epidermis by absorbing it.[6]

A common feature of photoaging is the reduced expression of dermal fibers. Excessive exposure to UV radiation increases the formation of reactive oxygen species (ROS), which can damage the skin's main structural proteins, collagen and elastin. Skin affected by photoaging experiences increased inflammatory mediators, collagen degradation, reduced collagen synthesis, and increased elastotic tissue, resulting in wrinkles, reduced collagen synthesis, and decreased skin elasticity. Anti-aging agents can be used to address this condition. One of the most common anti-aging agents used to reduce the effects of free radicals is antioxidants, which can be obtained externally (exogenous antioxidants), one of which comes from gotu kola (*Centella asiatica*) plant.[7]

Gotu kola is a plant used to prevent or treat diseases in traditional medicine in Asian countries. Gotu kola contains various bioactive compounds, including phenolic compounds, triterpenes, minerals, and vitamins. In dermatology, gotu kola is used to treat minor wounds, hypertrophic wounds, burns, psoriasis, and scleroderma. It has been observed that gotu kola preparations can stimulate fibroblast proliferation, thus enhancing type I collagen production and reducing the formation of stretch marks and inflammatory reactions. Additionally, compounds isolated from gotu kola can improve blood microcirculation in the skin and prevent excessive fat accumulation in cells. Gotu kola is an active ingredient in skin care formulations in cosmetics due to its antioxidant, anti-inflammatory, anti-cellulite, and anti-aging activities.[8] Therefore, gotu kola extract, rich in triterpenes, is a valuable raw material with a broad spectrum of cosmetic actions. Based on the background presented above, the research question is: does the cream containing gotu kola leaf extract affect the histopathological characteristics of the skin in Wistar rats exposed to ultraviolet B radiation?".

Method

This study uses a posttest-only control group design, where only observations are made to the control group and treatment after the treatment is given. The research sample was made of white rats of the Wistar strain weighing 160-200 grams and 2-3 months old. The grouping of test animals was carried out randomly. The researchers used 6 Wistar strain mice for each experimental group, bringing the total number of test animals to 24. The grouping of test animals was carried out randomly into 4 test groups. The control group (P-0) rats were exposed to ultraviolet B light and applied a base cream for 14 days. In the Treatment-1 (P-1) group, the mice were exposed to ultraviolet B light and given a gotu kola leaf extract cream with a concentration of 2.5% daily for 21 days. In the Treatment-2 (P-2) group, the mice were exposed to ultraviolet B light and given gotu kola leaf extract cream with a concentration of 5% daily for 21

days. In the Treatment-3 (P-3) group, the mice were exposed to ultraviolet B light and given a gotu kola leaf extract cream with a concentration of 7% daily for 21 days.

The tools used include rat cages, drinking fountains, shavers, digital scales, surgical equipment, microscopes, rulers, cameras, maceration tools, filters, rotary evaporators, evaporation cups, and water baths. Tools for in vitro testing include measuring flasks 10 ml, 25 ml, 100 ml, test tubes, test tube racks, BioHit micropipettes 1000µL, measuring pipettes, spatulas, vials, incubators, pH meters, cuvettes, centrifuges, centrifuge tubes, UV-Vis spectrophotometers, beaker glass, Philips brand UVB lamp PL-S9W/01/2P, Materials used include, wistar strain white rat, aquadest, 96% ethanol, label paper, rat feed and drink, gotu kola leaf extract cream.

The manufacture of gotu kola leaf extract uses the maceration method. Fresh gotu leaves weighing 50 grams are finely cut and mixed with 96% ethanol. The mixture is then stirred in a shaker for 24 hours at a speed of 200 rpm. The results are filtered to separate the pulp and filtrate. The filtrate is then evaporated at 40°C with a pressure of 100 MBar to produce a gotu leaf extract. The viscous extract obtained is weighed and stored for further use. Gotu kola leaf extract cream is made with an oil-in-water (M/A) emulsion type. The cream is made in several concentrations, namely 0% (base), 2.5%, 5%, and 10%.

Exposure to light was carried out with a frequency of 3 times a week (Monday, Wednesday, and Friday), starting with 50 mJ/cm2 for 50 seconds in the first week, followed by 70 mJ/cm2 for 70 seconds in the second week and 80 mJ/cm2 for 80 seconds in the last week with a total UVB received of 840 mJ/cm2. Irradiation occurs daily at 10.00 WIB using Phillips UVB lamps PLS9W/01/2P. Gotu kola leaf extract cream is applied after the rats are exposed to UVB rays. At the time of exposure to UVB rays, the extract was given 2 times a day for 3 weeks, 20 minutes after exposure to UVB rays at 09.40 WIB, 10.00 WIB, and 4 hours later, the administration started again at 14.00 WIB. The application of gotu kola leaf extract cream is still given on days without irradiation. After the 21-day period ended, the mice were euthanized using an excess dose of ketamine (125mg/kg BB) intramuscularly in an anaerobic jar 48 hours after the last irradiation. The skin sampling process is carried out with a biopsy in the back area where the skin will be taken and cleaned of hair; the skin is cut with a thickness of approximately 2 mm to subcutaneous with a length of 2 cm and a width of 2 cm. After that, a histopathological preparation was made, and the amount of dermal collagen was calculated as *post-test data.* The rest of the unused rat organs will be buried.

The preparation of histopathology preparations is based on research conducted by Suwiti (2010), namely a sample in the form of skin that has been fixed with 10% formalin, dehydrated, and successively cleaned with one session of solution (3 times 10% formalin, 70% alcohol, 96% alcohol, 3 times absolute alcohol, 3 times xylol, and 2 times liquid paraffin) within 23 hours. The sample is then blocked with liquid paraffin; after cooling for 30 minutes, it is cut with a microtome. Before mounting, it is stained with the HE method by soaking in xylol I, II, and III for 5 minutes each. Finally, it is soaked in absolute alcohol I and II for 5 minutes. Before soaking in HE solution (15 minutes), immersion in aquadest is carried out for 1 minute. The sample is again soaked in aquadest (1 minute), then 5-7 minutes in 10% acid alcohol, twice in aquadest for 1 minute and 15 minutes. After that, it is stained with eosin. The prepared is then soaked 4 times with 96% alcohol for 3 minutes each. Next, it is cleaned in xylol I and xylol II for 5 minutes.

When all treatments have been completed, the researchers performed a biopsy on the skin of the rat's back (2 cm x 2 cm x 2 mm). Then, they assessed by immunohistochemical staining to calculate MMP-1 expression and Picro Sirius Red staining to observe dermal collagen density. The data from the results of histopathological observations collected and scored are then analyzed. The data from the research results are tabulated, then the changes found are analyzed and presented descriptively. The normality analysis of the data used in this study is the *Kolmogorov-Smirnov normality test.* The normal distributed data apanila $p > 0.05$. After conducting a data normality test, it was followed by a homogeneity test using *Levene's test.* The data were expressed as homogeneous when $p > 0.05$. The data that had gone through the normality and homogeneity tests were then processed again using *the t-test* to see the differences in comparison between groups.

Results

Skin/tissue sampling was carried out on the 16th day, where previously the rats were sacrificed by inhalation using chloroform, the back area to be taken was cleaned of hair that began to grow back, the

skin was cut with a thickness of ± 3 mm to the *subcutan* and 2.5 cm long (Nanda et al., 2017). Tissue samples were stained with *hematoxylin* staining for 5 minutes and rinsed using running water for 10 minutes. Then the sample is stained with *eosin* staining for 2 minutes and continued by inserting the sample into a stratified alcohol solution, clearing with silos, and ending with the closure of the tissue slide with a cover glass that has been coated with adhesive material.

Red Haematoxylin-Eosin staining with a light microscope connected to a digital camera and using OptiLab Viewer 2.2 software observed Collagen density. The preparation was followed with a magnification of 400 times on 5 fields of view. Image storage is done by pressing the camera icon on the taskbar *of OptiLab Viewer* 2.2 software. The images that have been obtained are then stored and processed using Image J software. The analysis particle menu is selected for the percentage of collagen density in one area. The parameters obtained are in the form of a percentage of area area (%). The description and data of the observation results can be stored in a file.

Table 1 shows that the mean percentage of collagen density in the group of control mice without any treatment (KN) is 36,490±0,816. The average percentage of collagen density in the treatment I (P1) group of rats exposed to UVB light and given a dose of 2.5% gotu kola leaf extract cream was 42±2621.359. The average percen-tage of collagen density in the group of rats treated II (P2), exposed to UVB light and given a 5% dose of gotu kola leaf extract cream, was 44±963.2,389. The average percentage of collagen density in the mice treated III (P3) group, exposed to UVB light and given a dose of 7% gotu kola leaf extract cream, was 51,662±2,529. Based on the observed data, it can be concluded that the average area percentage of collagen density is the best P3 group, namely the treatment group that is given gotu kola leaf extract cream at a dose of 7%. Then, the average area percentage of the worst collagen density was the control group, which was given UVB exposure treatment but not gotu leaf extract cream. The results of the ANOVA test show a significant difference in the percentage of wound healing between groups (p = 0.000). This shows a significant difference in the mean percentage of collagen density between the four groups.

Table 1. Collagen density in rat skin tissue

Repetition	P0	P1	P2	P3
1	37,426	42,707	48,928	51,032
2	35,469	39,968	42,416	54,965
3	37,265	43,198	46,273	49,305
4	36,339	43,814	42,936	48,365
5	35,656	41,560	44,259	50,325
6	36,785	42,325	44,965	54,765
Shoes	+2	+2	+2	+3
Mean	36,490	42,262	44,963	51,662
SD	0,816	1,359	2,389	2,529

Table 2. ANOVA test results

Comparison of wound healing percentages	Number of comparisons	df.	p
Between Groups	713.664	3	0,000
In a Group	70,070	20	
Total	786,733	23	0,000

Table 3. Results of the t-test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% CI Lower	95% CI Upper
Results	Equal variances assumed	.838	.382	-8.918	10	.000	-5.772000	.647214	-7.214083	-4.329917
	Equal variances are not assumed..			-8.918	8.185	.000	-5.772000	.647214	-7.258625	-4.285375

Based on the output above, the value of the Sig. Levene's Test for equality of Variance is 0.383 > 0.05, so it can be interpreted that the data variance between the groups is homogeneous. Then, in the equal variances assumed part, it was known that the value of Sig (2-tailed) was 0.000 < 0.05, so it was concluded that there was a significant (real) difference between the mean results in the control group, treatment group 1 with a dose of 2.5%, treatment group 2 with a dose of 5%, treatment group 3 with a dose of 7%.

From the histopathological observations in the image above, namely in the control group, it can be explained that the collagen density image in the normal control group is purplish-blue and looks solid and ordinary. The condition of collagen density looks denser, and the fibers are not scattered because this group has not been given any treatment.

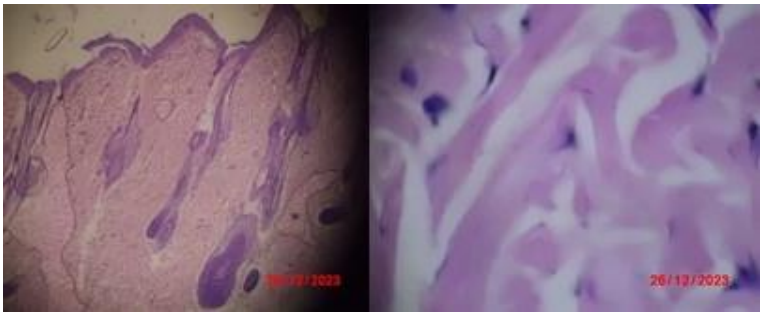

Figure 1. Collagen observation control group

From the histopathological observations in the image above, namely in treatment group I (P1) with the administration of gotu kola leaf extract cream at a dose of 2.5%, it can be explained that the collagen density image in the P1 group, the amount of collagen (blue color) has begun to exist, but it is still seen rarely. The condition of collagen density has already started to exist but is still rare in this group because, after exposure to UVB light, the group has creamed gotu leaf extract at a dose of 2.5%.

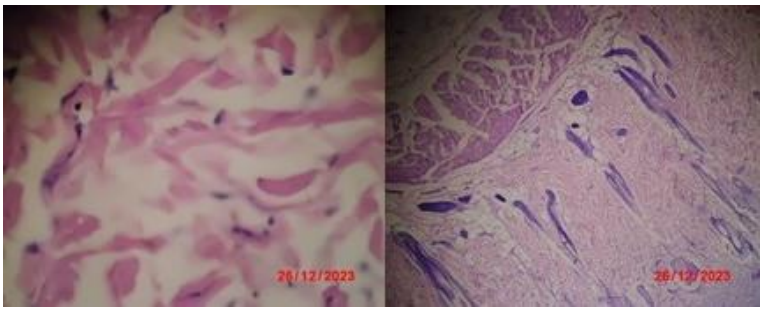

Figure 2. Observation of collagen group P1

From the histopathological observations in the image above, namely in treatment group II (P2) with the administration of gotu kola leaf extract cream at a dose of 5%, it can be explained that the collagen density image in the P2 group has more collagen than the P1 group and is tighter. The condition of collagen density has started to increase and begin to tighten because, in this group, after being exposed to UVB rays, the P2 group was given a 5% dose of gotu kola leaf extract cream.

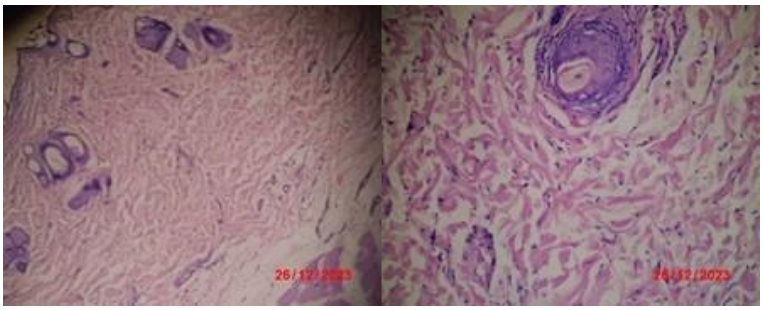

Figure 2. Observation of collagen group P2

The histopathological observations in the image above, particularly in treatment group III (P3), explain that the collagen density in group P3, which received 7% gotu kola leaf extract cream, was significantly higher and denser compared to groups P1 and P2. This group's high and dense collagen condition was observed after UVB exposure, followed by applying 7% gotu kola leaf extract gel.

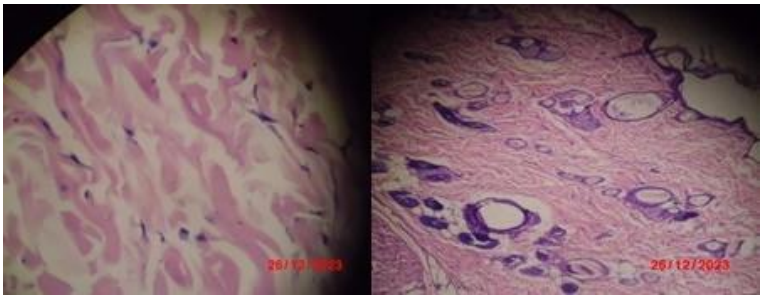

Figure 2. Observation of collagen group P3

From the histopathological observations in the previous images, starting from group K, P1, P2, and ending with group P3, the collagen density preparations were analyzed using Image J software with the area fraction method in five fields of view at 400x magnification. The control group showed the lowest percentage of collagen density compared to the other groups. The photo showing the collagen density in group P0 (marked by blue) shows almost no visible collagen. In the normal control group, compared to control groups P1, P2, and P3, the percentage of collagen density appeared more stable, as seen from the blue color in the collagen density photo in the table. This is because the normal control group was not

exposed to UV radiation like the other groups, resulting in more stable collagen formation than the different groups.

In the groups exposed to UVB radiation—P1, P2, and P3—the best collagen density was observed in treatment group P3, which was given 7% gotu kola leaf extract cream. This is due to the higher concentration of active compounds in the gotu kola extract gel, proportional to the increased dosage used in the cream formulation.

Discussion

Extrinsic skin aging, also known as photoaging, involves structural and functional changes that can be prevented and are primarily caused by unprotected exposure to ultraviolet radiation. UVB is the primary source of direct DNA damage and inflammation in photoaging. UVB rays can penetrate the epidermis and the upper dermis, contributing to general aging mechanisms and further collagen degradation and the production of elastotic material in the skin.[9] This study observed collagen growth based on the percentage of collagen growth area using Image J software. The extract used in this study was derived from gotu kola plant. Aloe vera leaves used were fresh, dark green aloe vera leaves. The aloe vera leaf extract was prepared through maceration.

Gotu kola leaf extract was made using the maceration method. 50 grams of fresh gotu kola leaves were finely chopped, then macerated in 96% ethanol, and shaken for 24 hours at 200 rpm. The extract was filtered to separate the residue from the filtrate, and the filtrate was evaporated at 40°C under 100 MBar pressure to obtain gotu kola leaf extract. The thick extract obtained was weighed and stored before use. Gotu kola extract was then processed into a cream formulation.

The compounds or chemicals in the gotu kola or aloe vera extracts, which tested positive in the phytochemical tests, include flavonoids, saponins, phenolics, and tannins. Flavonoids were detected through the reaction of the thick extract: 1 mL of aloe vera (gotu kola extract) was placed into a test tube, followed by adding 2 mg of magnesium powder and 3 drops of concentrated HCl. A color change to orange and yellow indicated the presence of flavonoids. The saponin content in the gotu kola extract was tested by shaking 10 mL of the thick extract vertically in a test tube for 10 seconds and then allowing it to stand for 10 seconds. A foam layer 3.8 cm high was observed. The tests for tannins and phenolics were performed by adding a few drops of 10% iron (III) chloride solution to 1 mL of the thick extract. The appearance of dark blue or blackish-green color indicated the presence of these compounds.

The experimental animal used in this study is a male white rat because it is easy to get and handle and has physiological and anatomical similarities with humans. The rats used were 25 heads, namely 5 rats in each group. Before treatment, the rats are acclimatized for 1 week first so that they can adjust. Each rat was grouped and marked, namely: regular control group (KN), negative control group (P0) without any treatment, and treatment group I (P1), which was given gotu kola leaf extract gel at a dose of 5% topically, treatment group II (P2) which was a group given gotu kola leaf extract gel at a dose of 10% topically, and treatment group III (P3) which was a group given gotu kola leaf extract gel with a dose of 15% topically.

Furthermore, skin/tissue sampling was carried out on the 16th day,, where previously the rats were sacrificed by inhalation using chloroform, the back area to be taken was cleaned of hair that began to grow back, the skin was cut with a thickness of ± 3 mm to the subcutan and 2.5 cm long. Tissue samples were stained with hematoxylin staining for 5 minutes and rinsed using running water for 10 minutes. Then the sample is stained with eosin staining for 2 minutes and continues by inserting the sample into a stratified alcohol solution, clearing with silos, and ending with the closure of the tissue slide with a cover glass that has been coated with adhesive material.

The results of the observation of collagen density preparation using J image software with the area fraction method using 5 fields of view with a magnification of 40x and from the results of the analysis of the above results that the normality and homogeneity tests showed the significance value in the Shapiro Wilk test, each group had a value of $p>0.05$. The statistical analysis test was continued with the ANOVA parametric test with a confidence level of $p<0.05$. In the ANOVA parametric test, a significance result of 0.000 was obtained. It can be stated that the significance value is ($p<0.05$), and there are significant differences between several groups. Statistical analysis continued using the Bonferroni test.

In the post-hoc Bonferroni difference test, the negative control group (P0) showed the lowest percentage of collagen density compared to the other group. There was a significant difference in the negative control group P0 with the P1 group, P2 group, and P3 group normal control group ($p<0.05$). The results of collagen density scoring in the negative control group P0 showed a score of +1 and an average collagen density of 28.037%, lower than that of the P1, P2, P3, and normal control groups of KN. This is because the negative control group was not given gel treatment, only exposed to direct UVB rays, so collagen formation in the negative control group was the lowest compared to other groups.

In the normal control group with the negative control group P0, P1 group, P2 group, and P3 group, there was a significant difference in collagen density ($p<0.05$). The collagen density scoring results in the normal group were higher than in the negative control group, with a score of +2 and an average collagen density of 36.333%. Because in the normal control treatment, they were not exposed to UVB rays like other groups, collagen formation was stable compared to different groups.

The average results of collagen density in the treatment group with gotu kola leaf extract gel were:group P1, group P2 and group P3 got significant results with average collagen density respectively of 41.969%, 44.302% and 50.798% or greater than the normal control group and negative control group. The results of the collagen density score in the P1 and P2 groups showed a score of +2, with the interpretation that the three formula groups had a moderate collagen fiber density, which was shown to be 10-50% per field of view. Meanwhile, in the P3 group, the collagen density score shows a score of +3, with the interpretation that the P3 group has a dense collagen density on average.[10]

The significant difference in results between the P1, P2, and P3 groups and the negative control group P0 showed a considerable effect on the increase in collagen in the skin of mice after exposure to UVB rays that damaged the skin tissue of the mice. This situation was affected by the rise in the dose of *gotu gotu* leaf extract in gel preparations, namely in the P1 group with an extract dose of 5%, in the P2 group with an extract dose of 10%, and in the P3 group with an extract dose of 15%. So the more significant the dose of *gotu gotu* leaf extract in the formulation of gel preparations, the more considerable collagen fibre density increases between P1, P2, and P3. This happens because gotu kola leaf extract gel contains flavonoid compounds and polyphenols with antioxidant and anti-inflammatory activities. The anti-inflammatory and anti-oxidative properties of various molecules, including flavonoids, polyphenols, carotenoids, vitamins C and E, and natural extracts, can prevent or stimulate collagen degeneration or synthesis.[11]

Gotu kola leaf gel's tannin and phenolic content acts as an astringent, which can reduce mucosal permeability and strengthen mucosal bonds, thereby preventing irritants. Indirectly, tannins affect the permeability of the mucosa and bacterial cell walls, causing the bacteria to shrink and die. The phenolic content in gotu kola leaves plays a role in preventing cell damage caused by free radicals, thus preventing inflammation and inflammatory processes.[12] Another factor that may have influenced the results of this study is the smaller sample size compared to previous studies, as this study only used 25 white rats or 5 rats per group. The sample size used in a study affects its outcomes, as a larger sample size reduces the chance of generalization errors.

Conclusion

In this study, the results showed that the collagen growth in the control group was not significant and tended to remain stable. This condition was because the control group was not exposed to UVB radiation, unlike the other groups, resulting in more stable collagen formation compared to the different groups. Meanwhile, the group treated with 7% gotu kola leaf extract gel had a more significant effect on collagen growth in the skin tissue of rats after UVB exposure compared to the groups treated with 2.5% and 5% gotu kola extract gel. This is because a higher dose of gotu kola extract contains more beneficial chemical compounds for collagen growth in the skin. The average percentage of collagen growth in the control group compared to treatment groups P1, P2, and P3 after UVB exposure was significantly different. This is because the control group did not receive the application of a cream containing active ingredients that could accelerate collagen growth in the skin tissue.

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