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The Use of Rosella (*Hibiscus sabdariffa* L.) Extract Cream to Prevent Decreasing of Total Collagen in the Skin of Wistar Rats Exposed to Ultraviolet-B Light

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study aimed to prove that the cream of Rosella flower extract (*Hibiscus Sabdariffa* L.) could prevent a decrease in the amount of collagen in the skin of Wistar rats exposed to ultraviolet-B light.
Study Design: This type of research is experimental, using a post-test-only control group design by giving 20% Rosella flower extract cream.
Place and Duration of Study: This research was conducted at the Laboratory of the Faculty of Pharmacy, University of North Sumatra. The research was conducted in 2020.

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Methodology: The study consisted of two groups with a total sample of 12 male Wistar rats in each group. The P0 group, namely the control group, was exposed to UVB rays and smeared with cream with the essential ingredients. The P1 group was exposed to UVB rays and 20% Rosella flower extract cream. UVB exposure is given for 5 weeks with exposure capacities, namely: 40 mJ/cm2 (the week I), 60 mJ/cm2 (week II), 80 mJ/cm2 (week III), 100 mJ/cm2 (week IV) and 120 mJ/ cm2 (week V), then a biopsy was performed to examine the amount of collagen calculated through histopathological examination of skin tissue after staining with Hematoxylin and Eosin (H&E).
Results: The results showed that the average amount of collagen in the P0 group was 49,899±5,89%. The mean of the P1 group was higher by 56.358±6.81%. The comparison test results with the independent t-test obtained a t-value of 2.485 and a p-value of 0.021 ($p < 0.05$). The findings explained a significant collagen amount difference between the P0 group and the P1 group.
Conclusion: The conclusion was that administration of 20% Rosella (*Hibiscus Sabdariffa* L.) flower extract cream prevented a decrease in the amount of collagen in the skin of Wistar rats exposed to ultraviolet-B light. It is recommended that further research is needed that examines the same topic as the study but with a combination of Rosella flower extract>20%.

Keywords: Rosella flower extract cream; collagen; ultraviolet B.

1. INTRODUCTION

Aging is a process that occurs in all organs but is most visible in the body's skin. Skin aging is a continuous and multifactorial phenomenon that is the reduction of size, the number of cells, and the deceleration of organic function that is on cellular levels, even molecular levels [1]. The skin aging process in every individual depends on multifactor that affect skin aging. There are 2 causative factors of premature aging that is internal factors like stress, the weakness of durability, hormonal change, and body health, also for an external factor like free radicals, ultraviolet radiation, and pollution [2].

Free radicals are one of the causative aging factors. Free radical is not stable relative molecule, and they have unpaired electrons on the outside of the orbit and making them reactive for finding the pair of electrons. Electrons of unpaired free radicals easy to pull electrons on other electrons, which makes free radicals very reactive [3]. Free radical can also break the collagen and elastin, a protein that protects the skin to stay moist, soft, flexible, and elastic. That tissue will be break it causes for free radical, especially in the facial, it makes indentation and wrinkles in the skin due to long exposure of free radical [4].

Collagen is the most one of protein in the human body. The function of collagen is as a tissue that stretched by and protect the body skin for any outside factor. The type of collagen that found in the body skin are type I, type II, type III, type IV, type V, type VII and type XVII [1]. For prevent damage occurs collagen it causes of ultraviolet light exposure, it has one attempt to reduce the effect with consumption of antioxidant, it causes antioxidant known to have ability to inhibit establishment of ROS [5].

The one of flower plant type that have effect as antioxidant is rosella Flower (*Hibiscus Sabdariffa* L.). The flower petals of rosella that have flavonoid like flavanol and anthocyanins pigment [6]. Anthocyanins can prevent activated of multifactorial transcription that causes the degradation of mature collagen and inhibit the establishment of new collagen. Anthocyanin is a compound that has a conjugated double-bond system and functions to prevent cell damage caused by prolonged exposure to ultraviolet light [7].

Some previous research that has relevant with this experiment is nasifa and husni (2018), they reviewed article about the potential of antioxidant in flower petals (*Hibiscus sabdariffa* l.) as anti-aging. The other research was found that flower plant of rosella (*Hibiscus sabdariffa* L.) has potential as anti-aging for some part of that flower, that are leaf, fruit, and petals of rosella (*Hibiscus sabdariffa* L.) has proven effective as antioxidant [8].

There is an antioxidant potential in the flower of rosella (*Hibiscus Sabdariffa* L.) especially flavonoid that can make the rosella (*Hibiscus Sabdariffa* L.) extract cream to prevent the decrease of total collagen.

2. MATERIALS AND METHODS

2.1 Extraction for Rosella Extract

The first step of the extraction is by making the simplicity after collecting and preparing fresh Rosella Petals, about 100 grams. The simplicity has to be washed, dried, and put in the oven at 50oC temperature for 1-2 days. And then, the powdered flower has to be extracted by maceration technique using 70% ethanol solvent for 24 hours and covered by aluminum foil to keep it from evaporation; after that, the powdered Rosella will be saved in a place away from direct sunlight. After it is cooled, the powder is filtered for better extraction quality. Furthermore, the wastes will be extracted again with new ethanol, the same amount as before. Finally, the extract will be used as a raw material for the cream.

2.2 Formulation Cream with Rosella Extract (*Hibiscus sabdariffa* L.)

Formulation Cream with Extract Rosella (*Hibiscus Sabdariffa* L.) has some steps, that is:

The oil Phase: That made by melting some materials: alcohol, adeps lanae, paraffin liquid, and stearate acid; add on paraphil paraben and keep the temperature about 700C.

The water phase: That made by melting paraben methyl in hot water at about 900C temperature adding glycerin triethanolamine, and keeping the temperature at about 700C. The cream is made by mixing the oil phase with the water phase and stirring for 3 minutes. Allowed stand for 20 seconds; after that, stir until homogeny cream has been made, add ethanol extract of Rosella (*Hibiscus Sabdariffa* L.) and homogenize [3].

2.3 Preparation of Wistar Rats

The total of 24 rats as a trial animal experiment adapted by 1 week. The rats have 2 groups, with 12 rats in every group and have exposure to UVB and given basic material cream in the first group (P0) and the second group (P1) given with extract Rosella Rosella (*Hibiscus Sabdariffa* L.) cream.

Every rat from the two groups (P0 and P1) was shaved in the back hair and then applicated basic material cream for the first group (P0) and the second group (P1) be given Extract Rosella cream 20%. The absorption time for topical material in the skin is 20 minutes; after that, applicated material for two groups and exposure to UVB and applicated material can be repeated for the next 4 hours to give time for ROS. UVB is about 280 to 320 nm wavelength [9].

The Exposure to UVB was given 3 times every week from the third week until the seventh week, along with giving the food for an animal trial period. The intensity of exposure is 40 mJ/cm2 (First week), 60 mJ/cm2 (Second week), 80 mJ/cm2 (Third week), 100 mJ/cm2 (Fourth week) dan 120 mJ/cm2 (Fifth week), [10].

The rats euthanized by injecting a high dose of ketamine (125 mg/kg BB) using the intramuscular technic in anaerobic jar [11] 48 hours after the last radiation, to avoid the effect of acute radiation. The process of collecting the skin samples form back of the body after all the hair was shaved, and cut it with around 2mm until subcutaneous with 2 cm long and 2 cm wide. After that, the histopathology preparation and total counted collagen on dermis will be used as a post test data. And then, the remains of the rat's organ will be buried.

2.4 Histopathological Preparations

Fixation step: The skin rats' tissue was soaked in phosphate buffer formalin liquid 10% for 1 day. And then do trimming for the tissue to be taken.

Dehydration step: the tissue of skin rats soaked by graded concentrate alcohol, there are 30%, 40%, 50%, 70%, 80%, 90%, 96%, each one does 3 times over 25 minutes.

Clearing step: the tissue was added to the clearing agent (alcohol: toluene 1:1) for 30 minutes and dipped into pure toluol until it was clear.

Embedding step: after infiltration 4 times with pure paraffin, then the tissues were embedded in liquid paraffin, allowed to form blocks (+/- 1 day) so they could be easily sliced with a microtome.

Cutting stage: tissue cutting process using a macro tome, 6µ thick, serially, the 5th, 10th, 15th slices were taken to make slides with H&E stain, affixed to an object glass that had been smeared with adhesive, dripped with aquadest and dried.

2.5 Stain with Hematoxylin and Eosin (H&E)

Tissues that still contain paraffin are deparaffinized and hydrated. Stain the cell nucleus with Haematoxylin Weigert's for 8 minutes and wash the preparation for 10 minutes with running water. Then stain with H&E for 1 hour to clear the stain to a near balance wherein additional time does not improve results, and shorter times are not recommended even if the color looks good. Wash with acid water two times, then remove excess water physically by gently shaking. Dehydrate it with 100% ethanol three times, clean it in a Xylene solution, and then mount it in an acidic medium.

2.6 Observation Result

The amount of collagen was calculated using the fast digital analysis method. Each preparation has to photograph using an LC evolution camera and an Olympus Bx51 microscope with an objective magnification of 400 X. The field of view taken is the field of view that has the most collagen which is marked with a bright red area, and then the preparation is photographed three times, namely the left, middle and right sides of the preparation. Photos saved in JPEG format [12].

3. RESULTS AND DISCUSSION

3.1 Results

This research is an experimental study using a post-test-only control group design. Experiments were carried out to determine the potency of rosella flower extract cream (*Hibiscus Sabdariffa* L.) in preventing a decrease in the amount of collagen in the skin of Wistar rats exposed to ultraviolet-B light. Researchers used 24 male Wistar rats (Rattus norvegicus) aged 2 months. There were 12 rats each for the treatment group (P0), which was exposed to UVB and given the basic ingredient cream, and 12 rats for the treatment group (P1) which was exposed to UVB and given Rosella flower extract cream (*Hibiscus Sabdariffa* L.) The rats from group P0 were applied with base material cream and in the group; P1 was applied 20% rosella flower extract cream. After applying the material in both groups, UVB exposure was given 3 times per week from week 3 to week 7. The exposure intensities were 40 mJ/cm2 (week I), 60 mJ/cm2 (week II), 80 mJ/cm2 (week III), 100 mJ/cm2 (week IV), and 120 mJ/cm2 (week V).

The rats were euthanized by using an excess dose of ketamine (125 mg/kg BW) intramuscularly in an anaerobic jar 48 hours after the last irradiation to rule out the effects of acute irradiation. After cleaning the hair, the process of taking skin samples from the back area, cut with a thickness of approximately 2 mm to the subcutaneous with a length of 2 cm and a width of 2 cm. Furthermore, histopathological preparations were made, and the amount of dermal collagen was calculated as post-test data. After that, the remaining rat organs that were not used were buried.

3.1.1 Histopathology images of wistar rat skins after treatment

After treatment, the back skin tissue of Wistar rats was biopsied for histopathological examination. Collagen will stain bright red on Hematoxylin and Eosin (or H&E stain). An overview of collagen in the dermis of Wistar rats is presented in the following Figs 1 to 3.

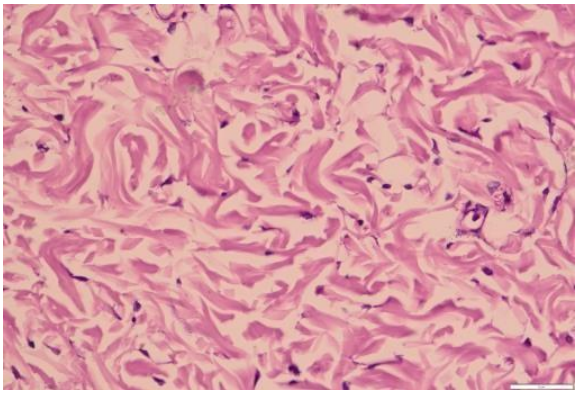

Fig. 1. Image of Wistar Rat Skin's Collagen with Stain Hematoxyllin and Eosin (or H&E Stain)

Wistar rat dermis histopathological tissue with 400x magnification. Collagen is indicated by black arrows. In the P0 group (treated group exposed to UVB and given a cream base) visible collagen is bright red, not intact and in small amounts marked with white areas indicated by blue arrows between bright red areas; in group P1 (Rosella flower extract cream 20%) visible collagen is bright red, abundant, thick, intact and fills almost the entire field of view of the dermis tissue.

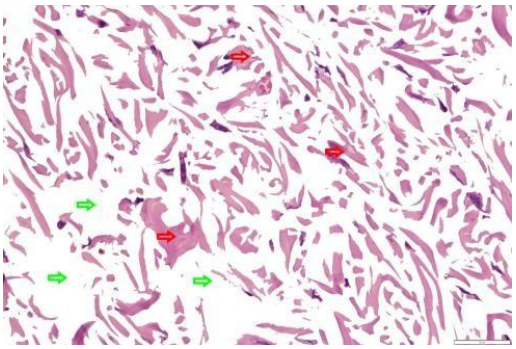

Fig. 2. Wistar Rat Skin Collagen with Hematoxylin and Eosin Stain 40X Magnification in Control

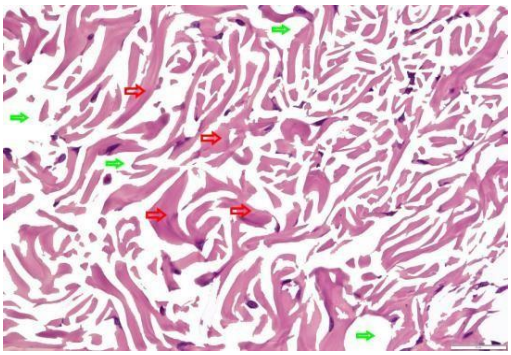

Fig. 3. Wistar Rat Skin Collagen with Hematoxylin and Eosin Staining 40X Magnification on Treatment

Explanation: Wistar rat dermis histopathological tissue with 40x magnification. Collagen is indicated by the red arrows. In group 1 (control or cream extract base) collagen is visible in red, not intact and in small amounts marked with a white area indicated by a green arrow between the red areas; In group 2 (Rosella flower extract cream 20%), collagen was seen to be red, abundant, thick, intact and filled the dermis tissue field of view.

3.1.2 Average amount of collagen

The results of the average amount of collagen in the P0 group (the treatment group exposed to UVB and given the base cream) with a mean of 49,899 and a standard deviation of 5.895 and group P1 (Rosella flower extract cream 20%) with a mean of 56.358 and a standard deviation of 6.805. It can be seen that the mean of P1 is greater than P0, as presented in Table 1.

The highest percentage of collagen in the P1 group was 67.52% and the lowest was 40.44%. The highest P0 group was 56.61% and the lowest was 40.82%, which is presented in the following Fig. 4.

3.1.3 Analysis result of normality and homogenity test

Normality test: The normality test of the research data used the Shapiro-Wilk test. Collagen data after treatment, the results showed that the data were normally distributed with values of P0 0.097>0.05 and P1 2.00>0.05 are presented in Table 2.

Homogenity test: Test the homogeneity of the data using Levene's test. Collagen data after treatment showed that the data were homogeneous with a p-value of 0.849 > 0.05, presented in Table 3.

Table 1. Average amount of collagen on P0 dan P1 group

No	Treatment Group	Number of ample (n)	Mean (%)	Deviation Standart
1.	P0 Group	12	49.899	5.895
2.	P1 Group	12	56.358	6.805

Table 2. Collagen data noarmality test result groups P0 and P1 after threatment

No	Treatment Group	Number of Sample (n)	Significance (%)	Information
1.	P0 Group	12	0.097	Normal
2.	P1 Group	12	0.200	Normal

Table 3. Collagen data homogenity test results between P0 and P1 groups after treatment

Variable	Number of Sample (n)	Significance (%)	Information
Collagen	24	0.849	Homogen

Fig. 4. Average Collagen of Base Cream (P0) and Rosella Flower (P1)

Table 4. Differences in the Mean Amount of Collagen between Groups After Being Given Rosella Flower Extract Cream

Subject Group	Number of Sample	Mean (%)	Deviation Standart	t-Value	Significance (P)
P0	12	49.899	5.895	2.485	0.021
P1	12	56.358	6.805		

Comperetic analysis of exposure effect: Comparative analysis was carried out to analyze the difference in mean between groups after being given treatment in the form of cream of base ingredients and cream of Rosella flower extract 20%. The results of the analysis of significance with an independent t-test are presented in Table 4.

3.2 Discussion

After being given treatment, the analysis showed that the average amount of collagen in the P0 group was 49,899±5,895, and the P1 group was 56,358±6,805. The results of the comparison test show that the t-value of 2.485 has a p-value of 0.021, which is smaller than the alpha ($p < 0.05$). These data indicate a significant difference in the amount of collagen between the two groups being compared. This study is relevant to the findings from [13], explaining that the IC50 value of rosella maceration extract was 11,940 g/mL based on the antioxidant test. In comparison, rosella reflux extract was 25,942 g/mL. These two extracts are very active as antioxidants, and the results of the lipstick formulation test are homogeneous, pH 4, melting point 50-700C, strength test 600-900 grams, and do not cause irritation. Another study revealed that rosella (*Hibiscus sabdariffa* L.) flowers have the potential as an anti-aging part of the leaves, fruit, and petals of the rosella (*Hibiscus sabdariffa* L.) plant, which have been shown to contain antioxidants.

In this study, the amount of collagen was seen by histopathological preparations of skin tissue with Sirius red staining. Sirius red shows a bright red color in intact collagen. The P0 group had a lower percentage of collagen, so the collagen damage that occurred in the P0 group was more significant, with an average difference of 0.910. The amount of dermal collagen was less in the P0 group due to repeated exposure to UVB rays. Repeated exposure to UVB rays produces ROS. Approximately 50% of skin damage due to photoaging is caused by ROS generation. The formation of ROS occurs in the skin when the skin is exposed to UV light. ROS damages skin through direct chemical modification reactions to mitochondrial DNA (mtDNA), cell lipids, deoxyribonucleic acid (DNA), and dermal matrix proteins, including collagen [14].

ROS produced by UV radiation activates cellular pathways, namely epidermal growth factor (EGF) cell receptors, interleukin (IL)-1, keratinocyte growth factor, and tumor necrosis factor (TNF)-α. Receptor activation is mediated by the protein-tyrosine phosphatase-K enzyme, which inactivates the EGF receptor. Receptor activation activates MAP kinase and C-Jun amino-terminal

kinase (JNK). Activation of the kinase activates the transcriptional complex of activator protein-1 (AP-1), forming C-Jun and C- Fos [15].

In addition to degrading mature collagen, UVB rays also inhibit collagen synthesis, mainly by downregulating the number of procollagen types I and III genes, resulting in acute loss of collagen in the skin [16]. Degradation of collagen by UVB rays occurs incompletely; collagen degradation leads to the accumulation of collagen fragmentation, which will reduce the structural integrity of the dermis. The accumulation of collagen fragmentation will inhibit the growth of new collagen and have a negative regulatory effect on the synthesis of new collagen [17].

In the P1 group, namely the group that was given Rosella flower extract cream 20%, it was found that the percentage of collagen was more significant than the P0 group. This shows that the increase in collagen can be caused by antioxidants found in 20% Rosella flower extract. By the opinion of [18]. an increase in the consumption of natural antioxidants found in fruits, vegetables, flowers, and other parts of plants can prevent diseases caused by oxidative stress and slow down the aging process of the skin because these ingredients contain colleagues.

Rosella flowers contain glycosides, flavonoids, saponins, triterpenoids, steroids, tannins, carotenoids, polyphenols, anthraquinone, and anthraquinone glycosides [19]. Rosella flower contains many vitamins, minerals, and essential bioactive compounds, such as organic acids, phytosterols, and polyphenols, which are antioxidants [20]. Antioxidants are needed to delay or inhibit oxidation reactions by free radicals or neutralize and destroy free radicals that can cause damage to cells and biomolecules such as DNA, proteins, and lipoproteins in the body, which can eventually trigger diseases, including degenerative diseases [21]. In their function as collagen protectors, phenolic compounds work as chain-breaking antioxidants or scavenger antioxidants by releasing one hydrogen atom from the hydroxyl group [22] so that the effect of reducing the amount of collagen due to free radicals can be prevented.

According to research by Bei et al. [23], anthocyanins act as collagen protectors through 3 mechanisms, namely inhibition of tyrosine kinase phosphorylation which prevents the activation of MAP kinase, JNK, and transcription of the AP-1 complex, so that collagen degradation does not occur and protects against TGF-β and procollagen, so that collagen synthesis occurs new is not hampered. The following mechanism inhibits NFḰB transcription so that MMP-8 activation is inhibited. Inhibition of MMP activation causes collagen not to be degraded. The last mechanism is cAMP inhibition. cAMP activates protein kinase A, one of the activators of MMP.

Rosella flowers (*Hibiscus sabdariffa* L.) are also known to have many active compounds that function as antioxidants such as flavonoids, polyphenols, anthocyanins, and vitamin C [24]. Antioxidant substances in rosella can capture reactive oxygen species (ROS) and free radicals, reduce reactive O2, metabolize fat peroxidation into non-radical products, and prevent the generation of free radicals [25].

Vitamin C works as a collagen protector in the skin by donating two electrons from the double bond between the second and third carbons so that ROS are not formed. In this process, vitamin C will be oxidized to produce dehydroascorbic acid, which can then be reduced back to ascorbic acid with the help of the enzyme 4-hydroxyphenylpyruvate dioxygenase [7]. In the biosynthesis of collagen, vitamin C is a cofactor for the enzyme's prolyl hydroxylase and lysyl hydroxylase. Proline and lysine are found in collagen, and hydroxylation of both will stimulate the formation of new collagen [26].

4. CONCLUSION

Based on the results of the study, it can be concluded that administration of 20% Rosella (*Hibiscus Sabdariffa* L.) flower extract cream can prevent a decrease in the amount of collagen in the skin of Wistar rats exposed to ultraviolet-B light.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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