

The Application of 5.4% Lime Peel Etanol Extract Cream (*Citrus aurantifolia*) inhibited the Increase of Matrix Metalloproteinase-1 Expression and the Decreased Amount of Collagen in Guinea Pigs (*Cavia Porcellus*) that were exposed to Ultraviolet B Rays

Dwi Ariyanti

¹Student of Anti-Aging Medicine Concentration, Biomedical Science, Faculty of Medicine Udayana University, Bali, Indonesia

Abstract: 80% of skin aging is caused by UV light exposure, which will increase the ROS production, one of which is characterized by an increase in Matrix Metalloproteinase-1 expression (MMP-1) and a decrease in the amount of collagen in the skin. Botanic antioxidants have been shown to reduce the incidence of photo aging. Lime peel contains vitamin C, flavonoids, saponins, and tannins which prevent the formation of free radicals that inhibit the formation of excessive enzymes of Matrix Metalloproteinase. The purpose of this study is to prove that giving ethanol extract 5.4% lime peel cream can inhibit the increase of Matrix metalloproteinase enzyme expression and decrease the amount of collagen in guinea pigs exposed to ultraviolet B. This study used a randomized posttest only control group design. The research subjects were guinea pigs (*Cavia porcellus*), male, healthy, local strain, age 3 months, weight 300-350 grams, one hybrid, totaling 36 guinea pigs which were divided into two groups, each with 18 individuals. The control group was given exposure to UV B rays and basic creams and the treatment group was given exposure to UVB rays and ethanol extract cream of lime skin 5.4%. Total UVB radiation for 4 weeks was 840mJ / cm², the treatment of base cream and lime peel extract cream of 5.4% in each group was given every day, 20 minutes before and 4 hours after UVB radiation. MMP-1 enzyme expression was examined using the MMP-1 examination kit with immunohistochemical methods, while the amount of collagen was examined using histopathological methods with Pico Sirius Red staining. Then the data analysis was done based on the results obtained in the form of T independent test. The results showed the mean expression of MMP-1 in the treatment group was significantly lower than the control group (9.26±2.29% vs 26.83±5.37%; p<0.001). The mean collagen amount in the treatment group was higher than the control group (82.32±2.50% vs 59.29±3.38%; p<0.001). Based on the results of this study, it was concluded that application of 5.4% lime peel extract cream inhibited the increase in MMP-1 expression and decreased the amount of collagen in guinea pigs that were exposed to Ultraviolet B rays.

Keywords: Lime Peel Etanol Extract, Matrix Metalloproteinase, Collagen, Ultraviolet B

1. Introduction

In general, people surrender to the condition that growing old must experience all kinds of illnesses, setbacks, shortages and helplessness. Currently, Anti-Aging is increasingly developing with various breakthroughs to solve the problem of aging. Human life expectancy to be able to live longer with better health quality. This is the basis for which anti-aging medicine is increasingly being developed and is now a new concept in medical science. The aging process can actually be considered the same as disease, so it can and must be prevented or treated. The concept of anti-aging treatment is how to prevent premature aging, slow down the rate of aging and improve the signs of aging [1-3].

Indonesia is a tropical country with exposure to ultraviolet rays of the sun all year round, so that Indonesia's population is very vulnerable against skin aging. The adverse effects of sunlight on the skin include sunburn, wrinkles, pigmentation, reduced skin volume and sagging skin. The increase in MMP-1 expression and the degradation of collagen due to UV rays was basically mediated by the two most responsible mechanisms, namely the induction of AP-1

and down regulation of TGF-β type II. Where the activation of AP-1 and TGF-β type II was preceded by the formation of ROS. ROS formation occurs less than 30 minutes after UV exposure and peroxide levels are doubled in human skin [3-4].

The method of using natural antioxidants is believed to provide more natural benefits to the skin, is safer and relatively cheap. Botanical antioxidants have also been shown to decrease the incidence of photo carcinogenesis and photo aging caused by increased ROS [3]. The body naturally makes antioxidants, including superoxide dismutase (SOD), catalase, and glutathione. Sometimes this natural protection is not sufficient; therefore additional antioxidant protection from the outside of the body is required. One source of natural antioxidants is lime. Lime (*Citrus aurantifolia*) is a plant originating from Asia and thrives in tropical climates. Oral administration of lemon peel extract (*Citrus limon*) by Ahmad (2018) proved that there was a decrease in MMP-1 expression and an increase in the amount of collagen in male white rats Wistar strain (*Rattus norvegicus*) exposed to UVB rays. Hindun et al.

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(2017) examined the potential of lime peel as a tyrosinase inhibitor and found that the total flavonoid content of lime peel was 0.667% w / w and inhibition concentration (IC) 50 42.11 mg / mL. Based on research conducted by Auliasari et al. (2019) regarding the use of lime peel waste in a gel preparation formula as an anti-wrinkle, the results obtained with an extract concentration of 1.8% which is the formula that has the best antioxidant activity with an IC50 value of 68.85 ppm [6-7].

Although there has been research on topical ingredients containing lime peel extract as anti-aging skin, there are many things that are not certain about the mechanism of action of these ingredients and the effects they have on MMP-1 and collagen, therefore it is necessary to conduct a study on its ability. Lime peel ethanol extract cream which contains natural antioxidants to protect the skin from damage caused by exposure to ultraviolet B rays.

2. Material and Methods

2.1 Lime Peel Extract

As many as 2 kg of lime peel washed, drained and sliced. Then dried in an oven at a temperature of 50-55°C for 24 hours. Then the dried orange peel is roughly powdered using a pollinator machine and is passed a sieve with mesh number 40. The lime peel powder was put into a maceration vessel, added with 100 ml of 96% ethanol in a ratio of 1: 1 until completely immersed and mixed homogeneously. The mixture was macerated at room temperature for 3 days. The filtrate was obtained by filtration. The solvent evaporated the entire filtrate using a rotary evaporator vacuum to obtain a thick ethanol extract of lime peel.

2.2 Lime Peel Extract Cream

Cream making is done in the cosmetics industry of PT. Nekhawa Ubud, Gianyar, Bali. Formulation of cream base ingredients: Sepigel 305 as emulsifier with a concentration of 3% mixed into water for 5 minutes, then add 1.8% lanol, 1.8% dimethicon and 0.5% phenoxy ethanol, continue mixing until the ingredients form a cream, to get 5.4% concentration of lime peel extract cream needed 5.4 grams of extract in a total mixture of 100 grams of cream.

2.3 Animals

The experimental animals in this study were male guinea pigs (*Cavia porcellus*), 3 months old, brown in color with a weight of 300-350 grams with standard dietary feed using HI-GRO 552 and vegetables. Food composition of guinea pigs consists of 17-20% protein, 3-4% fat, 35-40% carbohydrate. For drinking water is used ad libitum. Drinking water is put into bottles that are hung on the walls of the cage.

Experimental research was posttest only control group design, using 36 male guinea pigs (*Cavia porcellus*) which were divided into 2 (two) groups, namely the control group was given UVB exposure and placebo cream and the treatment group were given exposure to UVB rays and lime

peel extract cream. The control group was given exposure to UVB light and base cream while the treatment group was given exposure to UVB light and lime peel extract cream 5.4%. Total UVB radiation for 4 weeks was 840 mJ / cm², treatment of basic cream and lime peel extract cream 5.4% in each group was given every day, 20 minutes before and 4 hours after UVB radiation.

2.4 Picro Sirius Red Staining of Collagen and Quantification

Briefly, after euthanized, the biopsy of skin was immersed into formalin buffer for a night. The skin dehydrated by using grading ethanol, clearing and embedding into paraffin at 60°C. The thickness of cutting was 5 µm and then placed on object glass with poly-lysine. Slides then deparafinized in a series xylene, rehydrated in grading ethanol, stain with Picro Sirius Red.

2.5 Immunohistochemistry of MMP1

Antigen retrieval was using Citrate buffer pH 6 boiled with 800-watt microwave for 5 minutes, then cooled in room temperature. Next, the skin washed 2 times in PBS, then peroxidase blocking for 30 minutes, FBS 5% blocking for 2 hours and incubation with rabbit anti guinea pig MMP1 antibody (1:250) for 1 night in closed humidified box followed with labeled polymer-HRP (Dako Envision) for 30 minutes, and then DAB mixture for 10 minutes. Then counterstain with Gill Hematoxylin then mounting with Entellan. Photomicrograph was using 400x magnification by microscope CX-41 (Olympus, Japan) and camera OptilabPro (Miconos, Indonesia). Picture of skin was obtained and analyzed to count the percentage of epidermal cell which expressed tyrosinase. Each sample was captured 3 fields for 400x magnification.

2.6 Statistic

The data obtained then analyzed with independent T-Test by using SPSS 16.0 software.

3. Results

Between Control and Treatment Group, Independent T-test analysis found significant difference in amount of collagen ($p < 0.05$) (Figure 1). The control group had collagen for 59.29% and the treatment group for 82.32%. This study showed that Lime Peel Extract Cream prevent reduced amount of collagen significantly. The mean collagen amount in the treatment group was higher than the control group ($82.32 \pm 2.50\%$ vs $59.29 \pm 3.38\%$; $p < 0.001$). Figure 3 and 4 showed Picro Sirius Red Staining for collagen in Control and Treatment group respectively.

Independent T-test analysis found significant difference in percentage of fibroblasts that expressed MMP1 ($p < 0.05$) (Figure 2). The control group expressed MMP1 for 26.83% and the treatment group for 9.26% ($9.26 \pm 2.29\%$ vs $26.83 \pm 5.37\%$; $p < 0.001$). This study showed that Lime Peel Extract Cream reduced expression of MMP1 significantly. Figure 5 and 6 showed immunohistochemistry of MMP1 in Control and Treatment group respectively.

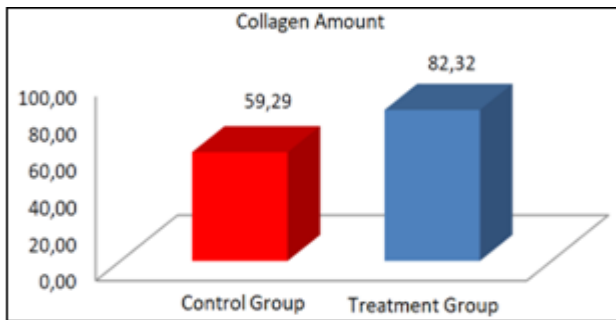


Figure 1: Collagen Quantification between Control and Treatment Group

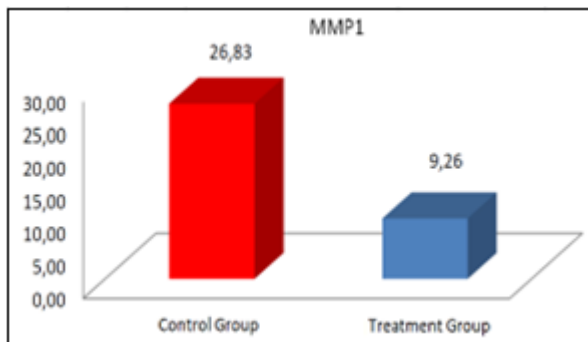


Figure 2: Expression of MMP1 between Control and Treatment Group

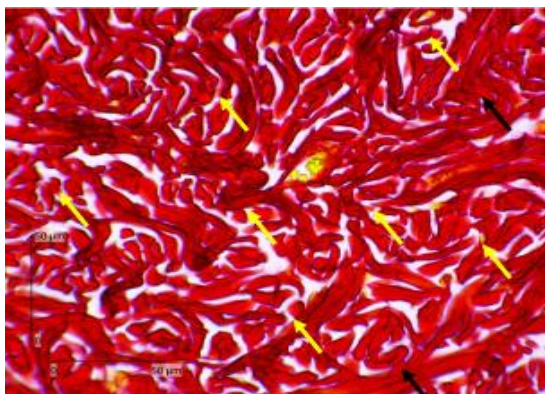


Figure 3: Picro Sirius Red Staining for Collagen in Control Group (400 X magnification). There were damage to the structure and structure of collagen with red collagen fibers that appear thin. Black arrows indicate intact collagen fibers. Yellow arrows indicate collagen fibers that broken.

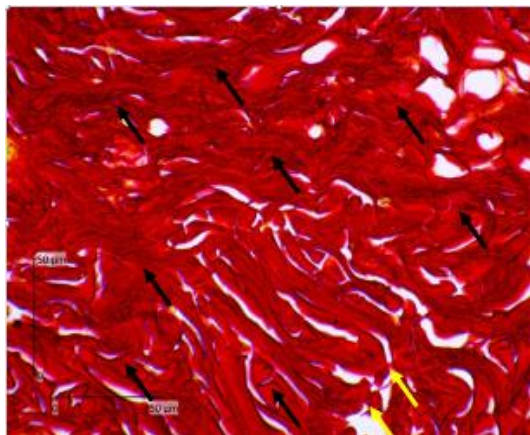


Figure 4: Picro Sirius Red Staining for Collagen in Treatment Group (400 X magnification). Collagen expression with red collagen fibers appears wider and

thicker. Black arrows indicate intact collagen fibers. Yellow arrows indicate collagen fibers that broken.

4. Discussion

The MMPs generally MMP1 is the main mediator of collagen degradation in photo aged skin. The MMP enzymes degrade collagen and elastin fibrils, which are important for skin strength and elasticity. The activity of MMP1 in the skin will increase even with a brief UV radiation, which will cause wrinkles on the skin, which is a sign of photo aging. Thus, inhibition of MMP is one way to prevent skin damage from UV exposure. The administration of 5.4% lime peel ethanol extract cream containing flavonoids, vitamin C, tannins and saponins can neutralize free radicals, so there is no oxidative stress which triggers a series of chain molecular reactions that can increase the formation of AP-1 then stimulates the transcription process MMP enzyme which plays a role in the collagen degradation process. The collagen-breaking MMP enzymes are most affected by exposure UV rays are of the MMP-1 type. ROS together with AP-1 also has a role in inhibiting collagen synthesis by inhibiting the type 2 receptor of TGF-β [8-16]. Flavonoids and saponins contained in lime peel extract act as inhibitor compounds for metalloproteinase enzymes. Flavonoids also play a role in accelerating the process of converting procollagen to collagen, thereby accelerating collagen biosynthesis. The stimulation of collagen biosynthesis is thought to be due to inhibition of the metalloproteinase enzyme by flavonoids.

In this study, the administration of 5.4% lime peel ethanol extract cream was applied to the skin of male guinea pigs exposed to UVB light and the results of the calculation of the mean expression of MMP-1 in the treatment group were 9.26 lower than the control group of 26, 83. While the inhibition of the decrease in the amount of collagen, the results of the calculation of the mean amount of collagen in the treatment group that were given 5.4% lime peel ethanol extract cream were 82.32 higher than the control group of 59.29. So that there is a significant difference in the mean expression of MMP-1 and the amount of collagen between the treatment group and the control group given 5.4% lime peel ethanol extract cream.

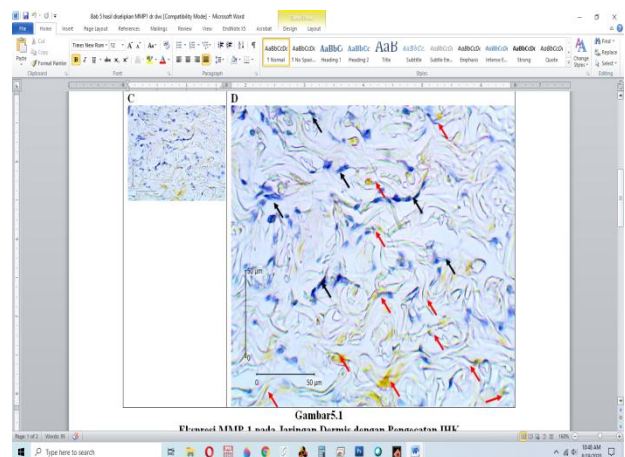


Figure 5: Immunohistochemistry of MMP1 in Placebo Group (400X magnification). It appeared that MMP-1

expression (brown color) was increased compared to Figure 6. Red arrows showed fibroblast expressing MMP-1. Black arrows indicate fibroblast that not expressed MMP-1.

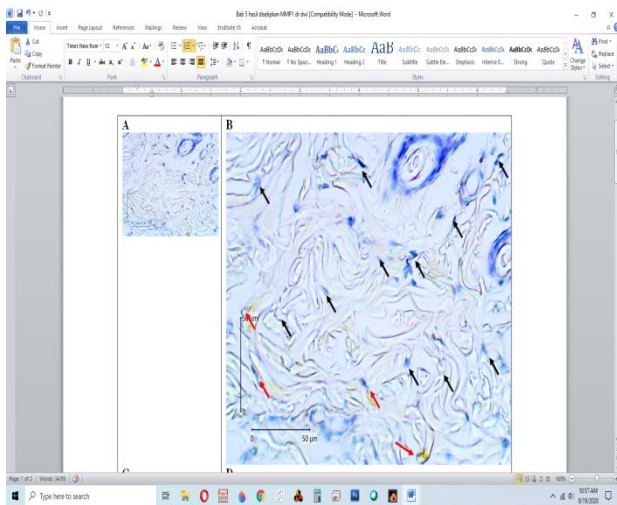


Figure 6: Immunohistochemistry of MMP1 in Treatment Group (400X magnification). There was less expression of MMP-1 (brown color) than in Control Group

5. Conclusion

The application of 5.4% lime peel extract cream inhibited the increase in MMP-1 expression and decreased the amount of collagen in guinea pigs that were exposed to Ultraviolet B rays.

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Author Profile



Dwi Ariyanti Student of Biomedical Magister Program (Antiaging Medicine), Faculty of Medicine, Udayana University