

Cempedak (*Artocarpus integer*) Leaves Ethanol Extract Cream 2.5% Prevented the Increase of Tyrosinase Enzyme and Melanin in the Ultraviolet B-Exposed Male Guinea Pig (*Cavia porcellus*) Skin

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Abstract: **Background:** Excessive exposure of ultraviolet B (UV-B) to skin will stimulate an increase of tyrosinase enzyme and melanin formation and will cause skin hyperpigmentation. Cempedak leaves (*Artocarpus integer*) contain flavonoids, phenols, and tannins which have antioxidant and anti-tyrosinase activity that can inhibit the melanogenesis process. This study aimed to prove that giving a cream containing ethanol extract of cempedak leaves with a concentration of 2.5% could prevent the increase of tyrosinase enzyme levels and the amount of melanin in the UV-B-exposed guinea pig male skin. **Methods:** This study used a randomized post-test only controlled group design with 36 guinea pigs subject. The control group was given ultraviolet B exposure and basic cream, meanwhile the treatment group was given ultraviolet B exposure and 2.5% cempedak leave ethanol extract cream. Skin biopsy was done, followed by the examination of the tyrosinase enzyme by ELISA and examination of melanin by the histology with Masson-Fontana staining. **Results:** It showed that the mean of tyrosinase enzyme level in the treatment group was significantly lower than control group ($3.83 \pm 0.45 \text{ ng/L}$ vs. $74.71 \pm 0.37 \text{ ng/L}$; $p=0.01$). The mean of tyrosinase enzyme levels in the treatment group was also lower than control group (0.66 ± 0.85 pixel percentage vs. 3.06 ± 1.70 pixel percentage; $p=0.00$). **Conclusion:** The administration of 2.5% cempedak leave ethanol extract cream prevented the increase of tyrosinase enzyme levels and the amount of melanin in guinea pig skin that was exposed to UV-B.

Keywords: Cempedak leave, tyrosinase enzyme, melanin, ultraviolet B.

1. Introduction

Aging is a natural process that results in deterioration or changes in physical, psychological, and social conditions. The main factor from extrinsic that can accelerate the skin aging process is ultraviolet light. Ultraviolet B can cause direct damage to DNA, meanwhile UV-A has a longer wave length that can penetrate deeper into the dermis causing indirect oxidative damage through photosensitization reactions in the presence of reactive oxygen species (ROS). This will cause damage to DNA, proteins, lipids, cells, and tissues and cause acceleration of the aging process and skin problems such as dry skin, pigmentation spots, wrinkles, and sagging².

The increase of the ultraviolet exposure is inducing the escalation of melanogenesis stimulation as well as transfer from melanocytes to keratinocytes. This will lead to tanning as a response to skin color changing by the improvement of the cells' ability to absorb the lights and protecting the nucleus from damages caused by ultraviolet radiation²⁹.

Pigmentation disorders frequently found such as lentigo or lentigo solaris, melasma, post-inflammatory hyperpigmentation (PIH), melanoma, ochronosis, and freckles, or ephelides^{5,23}. Melasma, characterized by hyperpigmentation patches with a defined and symmetrical line on the face, neck, and arm. It is commonly found in women living in the area with high UV exposure due to the increase of the melanin amount.

Other contributing factors are genetics and hormones¹³. In other research has been confirmed the association between glutathione levels in the plasma with the severity of melasma, in mild melasma the plasma glutathione is higher than moderate and severe melasma³⁰.

Hydroquinone is a gold raw material for hyperpigmentation, but if used long-term and high concentrations can cause irritation and ochronosis side effects⁴. The use of natural substances like flavonoid compounds in plants as an anti-hyperpigmentation needs to be considered because of their low toxicity¹⁵.

Cempedak leaves are rich in phenolic content and contain terpenoid compounds, steroids, tannins, phenols, and flavonoids, also rich in phenolic compounds^{3,25}. The presence of phenolic compounds and flavonoids in cempedak leaves as antioxidants can ward off free radicals to inhibit oxidation, thereby slowing photooxidation from exposure to ultraviolet light³¹. The cempedak plant has been shown to have strong antioxidant activity in the leaves, stems, wood which is stronger than other component of *Artocarpus* plant³¹.

Several studies of cempedak leave extract as an antioxidant have been carried out^{1,12,17}. However, research on the benefits of cempedak extract as an anti-hyperpigmentation has not been carried out, therefore research is needed to provide scientific evidence regarding the benefits of giving cempedak leave ethanol extract in

inhibiting the increase in tyrosinase enzyme levels and the amount of melanin due to exposure to UV-B rays.

This study aimed to prove that giving a cream with ethanol extract of cempedak leave with a concentration of 2.5% could inhibit the increase of tyrosinase enzyme levels and the amount of melanin in the UV-B-exposed male guinea pigs' skin.

2. Material and Methods

This study used a randomized post-test only controlled group design. This research is conducted in the Advanced Biomedical Animal Unit Laboratory, Histology Laboratory of Medical Faculty of Udayana University Bali, Food Analysis Laboratory and Biochemistry Laboratory of Udayana University Bali, and Sentra Pathology Laboratory Bali. The 2.5% cempedak leave ethanol extract cream and basic cream were formulated in PT. Nekhawa, Ubud, Bali.

Preparation of 96% ethanol extract cempedak leaves

3 kg fresh cempedak leaves washed with water and then oven-dried at the temperature of 50°C for 12 hours, finely blended, and filtered with 20 mesh tea filter. Furthermore, macerated using 96% ethanol for 24 hours and stirred every 2 hours, then the resulting solution filtered with Whatmann paper, and the filtrate evaporated 20 minutes to purify the extract with the solvent used. The process completed when there is no more solvent left evaporated. The evaporation gave thick extracts to further filtered, labelled, and kept in dark bottles at the temperature of 40°C and obtained 74 gram of extracts.

Cream preparation

The formulation for 2.5% cempedak leave ethanol extract cream materials are cempedak leave ethanol extract with the concentration of 2.5%, 3% Sepigel 305, mixed into the water for 5 minutes, 2% lanolin, 0.5% phenoxyethanol, 2% dimethicone, blended into a cream form. To obtain cempedak leave ethanol extract with a concentration of 2.5%, it is required 2.5 grams cempedak leave ethanol extract in the total mixture of 100 grams of cream. The 2.5% cempedak leave ethanol extract cream was conducted in the PT. Nekhawa, Ubud Bali. Whereas the formulation for the basic cream materials is 3% Sepigel 305 as the emulsifier mixed in the water for 5 minutes, 2% lanolin, 0.5% phenoxyethanol, and 2% dimethicone was added and blended into a cream form. The basic cream was made in the PT. Nekhawa, Ubud Bali.

Experimental Animal

This study used 36 guinea pigs (*Cavia porcellus*) available from animal unit of Medical Faculty, Udayana University. The sample inclusion criteria in this research are healthy, 3 months old, male guinea pigs with 300-350 grams of weight. The guinea pigs were divided randomly into 2 groups, with 18 guinea pigs in each of the groups, the control group is treated with basic cream and ultraviolet B exposure, while the treatment group is treated with 2.5%

cempedak leave ethanol extract cream and UV-B exposure. A day before the treatment, guinea pigs from all of the groups being shaved on the back in 2x2 cm size. Hereafter, the guinea pigs in the treatment group are treated with 2.5% cempedak leave ethanol extract cream and the guinea pigs in the control group are treated with basic cream. The cream was applied 20 minutes before the UV-B light exposure. The application of the topical ingredients was repeated 4 hours after UV-B light exposure. On the day where the UV-B light exposure was not conducted, the topical ingredients application is still administered twice a day, in 09.40 am WITA and 2.00 pm WITA. Guinea pigs either from the control or treatment group are treated by UV-B light exposure using *Philips PL-S 9W/01/2P (Poland)* lamps 3 times a week (Monday, Wednesday, and Friday) for 2 weeks every 10.00 am WITA by the dose of 65 mJ/cm² (total dose of UV-B light 390 mJ/cm²) for 65 seconds. Distance between UV-B exposed to the guinea pigs was measured by using a *UV light meter*.

Biopsy was done after 48 hours of the final UV-B light exposure to avoid acute effects caused by the exposure. The tissues were divided into two part, one part was used for histological preparation and stained with Masson-Fontana, while from the other part, 2 mg of the tissue was taken for tyrosinase enzyme assay with ELISA.

Examination of melanin

The analysis melanin in the epidermis was conducted using *Optilab Pro Viewer 2.2 (Miconos, Indonesia)* connected to the microscope *Olympus CX21 (Olympus, Japan)*. Whole epidermis area was captured with 4x magnification and the images was saved in the form of JPEG. Analysis carried out by *digital image* using *Software Image J v.1.44i* manually. Then the color is adjusted by choosing the *image* function to choose the *color* function and choose the *split channels* function for every image file. There were 3 color options, which are the RGB (*Red-Green-Blue*) system, this research used blue color so that the black area (melanin area) is well-defined. Furthermore, blocking is conducted by using a yellow mark on the images, to block manually only the epidermis to be evaluated so that the dermis part is excluded. Hereafter, the *threshold* function is chosen to convert the images into black and white, where the black part showed melanin. Then *analyze particles* is conducted by pixel which is *size 120-infinity* to obtain *pixel area non-melanin*. The *pixel area melanin* percentage in the epidermis layer is calculated by deducting the total pixel area epidermis (100%) with the percentage of *pixel area non-melanin*¹⁸.

Tyrosinase enzyme level examination

Tyrosinase enzyme levels are examined by using Tyrosinase Assay Kit with ELISA method under the trademark of *Bioassay Technology Laboratory (No. catalog E2488Ra)* with the detection range of 3.75 ng/L-240 ng/L.

3. Results

Table 5.1: Descriptive Data of Tyrosinase Enzyme Levels and Total Melanin

Variable	Subject group	N	Average ±SD	Median	Min	Max
Level of Tyrosinase enzymes (ng/L)	Control	18	74.71±0.73	74.65	72.62	75.99
	Treatment	18	3.83±0.45	3.78	3.21	4.70
Number of Melanin (pixel percentage)	Control	18	3.06±1.70	2.97	1.01	7.98
	Treatment	18	0.66±0.85	0.22	0.00	3.02

n = number of samples

SD = standard deviation

The descriptive results of the table show that the mean levels of the tyrosinase enzyme and the amount of melanin in the treatment group with 2.5% cempedak leave ethanol extract cream were lower than the control group with the base cream that had been exposed to ultraviolet B rays. The results of the normality and homogeneity tests show that the data is homogeneous and normally distributed.

Analysis of significance using the independent t-test showed p value <0.001, which means that there were significant differences between two groups. These results indicate that the mean levels of the enzyme tyrosinase (3.83±0.45ng/L) and amount of melanin (0.66±0.85pixel percentage) in the treatment group were significantly lower than the control group (74,71±0,73ng/L; 3,06±1,70pixel percentage). The difference in the mean of the control group and treatment group levels of the tyrosinase enzyme can be observed in Figure 1 and 2.

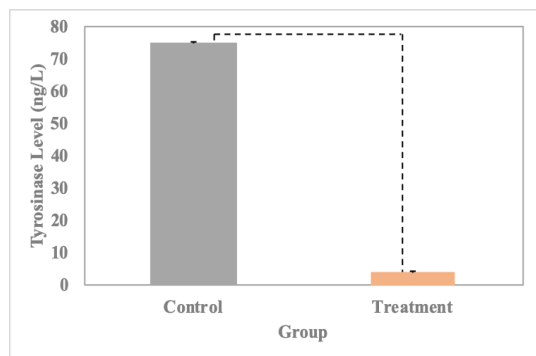


Figure 1: Tyrosinase level in treatment group was significantly lower than controlled group.

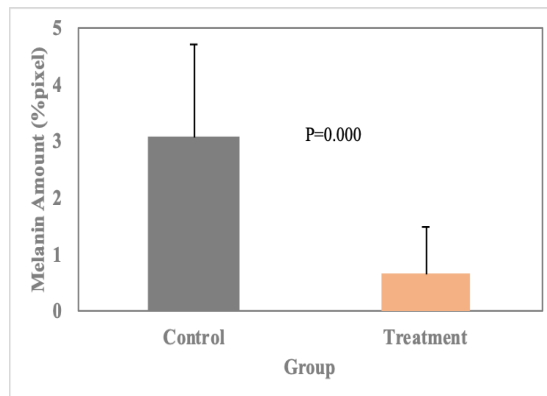
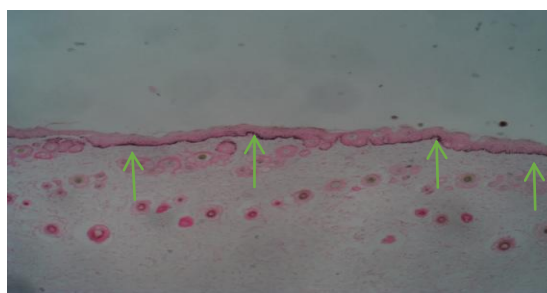
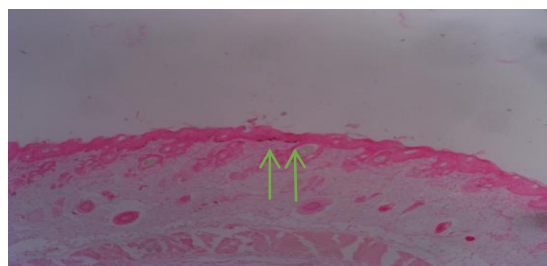


Figure 2: Melanin amount in treatment group was significantly lower than controlled group.

The standard deviation of the tyrosinase enzyme level (small standard deviation: 74.71±0.73ng/L; 3.83±0.45ng/L) had not overlap with each other, which indicating a data distribution that tends to accumulate around the mean thus indicating a normal distribution with a consistent data. On the other hand, the standard deviation of the melanin amount showed in the graph is overlapping, which indicating a wider data distribution according, particularly from the treatment group that is beyond the mean (0.66±0.85pixel percentage) thus indicating a less consistent data distribution.



A. Control group



B. Treatment group

Figure 3: Histopathologic of melanin in Guinea Pig skin (Masson-Fontana staining: 4x). The arrow point the area containing melanin pigment. The control group (A) showed more melanin than the treatment group (B).

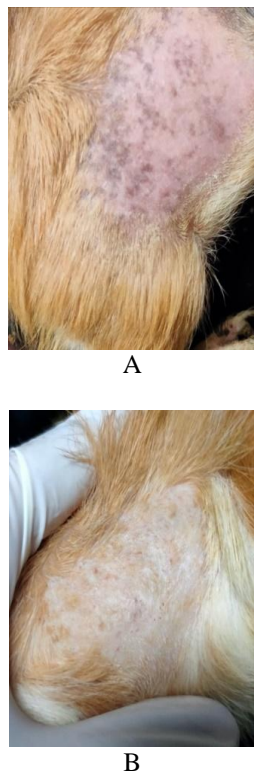


Figure 4: Skin appearance of guinea pig after treatment. The control group (A) showed more black spots than the treatment group (B).

4. Discussion

The results showed that the mean levels of the tyrosinase enzyme and the amount of melanin were higher in the control group that was given basic cream with repeated exposure to UV-B rays. Increased and repeated exposure to UV-B rays can lead to increased stimulation of melanogenesis by increasing melanin distribution, increasing the tyrosinase enzyme, and increasing the number of melanocyte cells as well as the transfer of melanocytes from melanosomes to keratinocytes²².

Exposure to UV-B rays can cause direct DNA damage by cross-linking adjacent pyrimidine bases and then generating free radicals. Meanwhile, ultraviolet B indirectly triggers superoxide anions which cause damage to DNA, stimulation of α Melanocyte Stimulating Hormone (α MSH), and protein kinase, as well as activation of the tyrosinase enzyme which causes melanogenesis²⁷.

Ultraviolet rays induce melanogenesis through melanocortin peptide bonds, namely α -Melanocyte Stimulating Hormone (α -MSH). Melanocortin1 Receptor (MC1R) binds to α -Melanocyte Stimulating Hormone (α -MSH) to produce adenylyl cyclase/protein G converts Cyclic Adenosine Triphosphate (cATP) to Cyclic Adenosine Monophosphate (cAMP) which activates Element Binding Protein (CREB). Protein Kinase-A (PKA) which stimulates Element Binding Protein (CREB) activates the Microphthalmia-Associated Transcription Factor (Mitf) to produce an important enzyme for melanogenesis, namely the tyrosinase enzyme⁸.

The mean levels of the tyrosinase enzyme and the amount of melanin in this study were significantly lower in the treatment group than in the control group with repeated exposure to ultraviolet B ($p < 0.001$). This shows that the ethanol extract of 2.5% cempedak leaves can effectively prevented the increase in tyrosinase enzyme levels and the amount of melanin.

It has been found that the average tyrosinase enzyme levels in the treatment group were lower than the control group, respectively $3.83 \pm 0.45 \text{ ng/L}$ and $74.71 \pm 0.73 \text{ ng/L}$. The existence of inhibition in increasing levels of the tyrosinase enzyme in this study will also inhibit the increase in the amount of melanin because melanin synthesis requires a key enzyme, namely the tyrosinase enzyme. This is indicated by the mean amount of melanin in the treatment group which was significantly lower, namely 0.66 ± 0.85 pixel percentage compared to the control group, namely 3.06 ± 1.70 pixel percentage.

The levels of the amount of melanin in the administration of 2.5% cempedak leaves ethanol extract cream on male guinea pig skin exposed to UV-B obtained in this study (0,699pixel percentage) were lower when compared to the the amount of melanin in the skin of male guinea pigs without treatment (0,894pixel percentage) and treating with basic cream to male guinea pig skins with exposure to ultraviolet UV-B (3,062pixel percentage). This shows that the administration of 2.5% cempedak leave ethanol extract cream can effectively prevented the increase in tyrosinase enzyme levels and the amount of melanin in male guinea pig skin exposed to UV-B.

The effectiveness of 2.5% cempedak leaves ethanol extract cream in preventing the increase in tyrosinase enzyme levels and the amount of melanin is due to the two mechanisms from the active compounds contained therein are mechanism is flavonoids, phenols, and tannins which function as antioxidants, as anti-tyrosinase and as inhibitor competitive tyrosinase. Flavonoids as metal ion chelators with adjacent hydroxyl and/or ketone and phenol sides as scavenger antioxidants release one hydrogen atom from the hydroxyl group so that ROS is not formed⁶. This prevent the formation of ROS due to exposure to UV-B to the skin so that the occurrence of melanogenesis is prevented, namely there is no increase in melanocyte activity to produce melanin, inhibits the transfer of melanocytes to keratinocytes, and inhibits the increase in tyrosinase enzyme activity²². Flavonoids can also inhibit α MSH and Mitf. This causes an prevention of the tyrosinase enzyme activity that can prevent the tyrosine conversion stage to 3,4-dihydroxyphenylalanine (DOPA) and DOPAquinone in the melanogenesis process¹⁴.

A number of compounds contained in several sources of natural ingredients have been researched which can inhibit the activity of the tyrosinase enzyme, consequently will inhibit melanin synthesis in the epidermis layer of the skin. However, from several sources of natural ingredients, they are still limited to being used as ingredients in cosmetics. This is due to its low activity as a tyrosinase inhibitor and considerations in terms of safety for its use¹¹.

Cempedak leaves have been widely used by people traditionally, one of which is to remove dark spots on the face and also to treat skin diseases²⁸. However, no previous research has proven that the cempedak leaves extract can have anti-hyperpigmentation activity. Therefore, it is expected that this research can provide information by proving that the ethanol extract of 2.5% cempedak leaf which is given topically in the form of a cream can prevent the increase in tyrosinase enzyme levels and the amount of melanin. It is also expected that there will be benefits from research on the ethanol extract of 2.5% cempedak leaf, which is an alternative treatment in anti-aging, especially anti-hyperpigmentation therapy which can help improve one's quality of life and self-confidence.

5. Conclusion

It can be concluded that the ethanol extract of 2.5% cempedak leaf prevented the increase in tyrosinase enzyme and melanin levels in male guinea pigs' skin exposed to UV B. It is necessary to know the potential side effect of ethanol extract of cempedak leaves on the skin at certain concentrations. Also, further research needs to be done to compare its effectiveness against the gold standard ingredient in anti-hyperpigmentation therapy, and further research as an anti-hyperpigmentation therapy in humans is needed before it can be widely used.

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