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In Vivo Antioxidant and UV-Photoprotective of Extract Pasak Bumi (Eurycoma Longifolia Jack.)

Edyson[1], Angga Maulana Edward Pardede[2], Hardiandy Gilbert Nugraha[2], Mashuri[3], Eko Suhartono[1,a)]

[1]*Departement of Medical Chemistry/Biochemistry, Faculty of Medicine, Universitas Lambung Mangkurat*
[2]*Medical Study Program, Faculty of Medicine, Universitas Lambung Mangkurat*
[3]*Departement of Radiology, Faculty of Medicine, Universitas Lambung Mangkurat*

[a)]Corresponding author: ekoantioxidant@gmail.com

Abstract. UV radiation on skin acutely caused oxidative damage and skin lesion, erythema. Oxidative damage formed because of the increase of reactive oxygen compound (ROS) and decreasing of endogen antioxidant activity that caused the damage on the skin. Pasak bumi is a plant that has antioxidant compound such as flavonoid and alkaloid that can reduce the forming of ROS due to acute UV radiation. This research was pure experimental research with rats (*Rattus norvegicus*) as its research subject. The used sample was 24 samples and divided into four treatment groups, that were selected using simple random sampling method. Pasak bum root was extracted using ethanol by maceration method. Superoxide dismutase enzyme activity and superoxide anion level were measured using Misra and Fridovich method; hydrogen peroxide level was measured using the colorimetric method, and erythema index was measured using chromameter based on L*a*b* coloring system. The data were statistically analyzed using *post-hoc Tukey* test and *Mann-Whitney* test with 95% of the reliable level; which resulted in significantly different results between groups that radiated by UV light and groups that were radiated by UV light and given the pasak bumi extract. Based on those results, it could be concluded that pasak bumi root extract could affect: superoxide anion level, superoxide dismutase enzyme activity, hydrogen peroxide level, and erythema index on skin rat radiated by UV light.

Keywords: oxidative damage, acute UV, pasak bumi extract.

INTRODUCTION

Oxidative damage is a condition when there is an imbalance between the production of reactive oxygen compound (ROS) and an antioxidant defense that causes the damage of some cells such as oxidation protein, oxidation DNA, and peroxidation lipid. The emergence of oxidative damage is one of them can be caused by UV radiation [1].

Ultraviolet (UV) is an electromagnetic wave that has a wavelength (λ) between 100 nm – 400 nm. UV light spectrum can be classified in three groups: UV-A (λ = 315 – 400 nm), UV-B (λ = 280 – 315 nm) dan UV-C (λ = 100 – 280 nm). The energy distributions of UV light that occurred in a media is named UV radiation [2]. Each decade (since 1970) ozone layer has decreased by 3% and increase UV radiation by 12% on earth surface. The increasing of UV radiation intensity have a serious impact on living things on earth. Excessive UV radiation causes erythema and sunburns and chronically causes the risk of skin cancer through the free radical formation [3]. When the skin is radiated by UV light, the skin will perform several mechanisms as self-protection through swelling of the epidermal layer and

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increased melanin synthesis. However, the photobiological effects of UV light produce free radicals and cause oxidative damage resulting in cell damage. UV light exposure containing photon energy will induce superoxide ($\bullet O_2^-$) anion radical formation and activates O_2 triplet into singlet O_2. Next, $\bullet O_2^-$ which affects the skin cell membrane will form hydrogen peroxide (H_2O_2). If H_2O_2 levels are too much in the cell and reach toxicity level (10µM-100µM), it will cause oxidative damage and cell damage [4]. Matsumura et al .'s study states that changes in the skin begin within the first hour after UV radiation, and the skin changes will peak at 24 hours later [5].

One of the ways to reduce or prevent oxidative damage is a compound that has antioxidant properties. These antioxidants can be obtained from natural ingredients derived from plants. Pasak bumi (*Eurycoma longifolia* Jack) is one of these plants. Pasak bumi is a plant that can be easily found in South Kalimantan and is one of the 13 superior plants that have been determined by the Indonesian government because it contains active substances in all parts. Bioactive substances in the pasak bumi, flavonoids, and alkaloids, are known as direct scavengers on free radicals, inhibit enzymes that play a role in the formation of free radicals, and stimulate antioxidant enzymes [6]. Budianto R et al .'s research states that the pasak bumi root can be an antioxidant, which is a barrier to protein damage due to free radicals [7].

The pasak bumi extract which is an antioxidant is suspected to be able to protect the skin radiated by UV light acutely. However, research on the role of pasak bumi as an antioxidant through inhibition of UV-induced oxidative damage has never been done. Therefore, this research will analyze the role of pasak bumi as an antioxidant in inhibiting oxidative damage to the skin due to UV radiation by measuring the activity of SOD enzymes, superoxide anion levels, hydrogen peroxide levels, and erythema index.

MATERIALS AND METHODS

Sample Preparation

The subjects used in this study were 24 wistar male rats (*Rattus norvegicus*) with an average weight of 200-250 grams and aged 8-10 weeks. The rats were acclimatized for 1 week before being treated to provide the same physical and psychological conditions. Rats were divided into 4 groups with each group had 6 rats, they were P0: rats without being given the extract of the pasak bumi root and UV exposure; P1: rats were exposure with UV light without the addition of pasak bumi root extract; P2: rats were given extract of pasak bumi root without radiation; P3: rats were given extract of pasak bumi root and radiated.

Determination of Plants

The root of the pasak bumi plant was obtained from Martapura City, South Kalimantan; determined at the Laboratory of Mathematics and Sciences Faculty, Lambung Mangkurat University, Banjarbaru, South Kalimantan with a certificate: 135a/LB.LABDASAR/X/2018.

Ethanol Extract of Pasak Bumi Root

Extraction was done using the maceration method. A total of 20 grams of powder samples were included in the maceration tool and poured 1 liter of solution (ethanol) into the maceration tool contained with the sample, then stirred for 3 x 24 hours, and every 1 x 24 hours the filtrate was filtered with filter paper and the solvent was replaced with new solvent while stirring for 3 x 24 hours. The extract was collected and evaporated by using a rotatory evaporator at low pressure with a temperature of 50°C until the liquid extraction was reduced up to 1/10 part, then evaporated in the water bath to obtain a thick extract with fixed weight. The extract was placed in a sterile bottle that was tightly closed and stored in the refrigerator.

UV Light Exposure

The rats (*Rattus norvegicus*) shaved its hair 2 cm x 2 cm on the back, then in some groups smeared with pasak bumi extract at a dose of 1 mg / cm^2. The radiation was carried out for 24 hours by using a radiation box made of wood or a board measuring 106 cm long, 34 cm wide, and 53 cm high using the PHILIPS 30 watt UV-C lamp, with the distance between the lamp and the experimental animal was 40 cm.

Erythema Index Measurement

Erythema index measurement would use the method carried out in the research of Takiwaki *et al*. Rats that have been radiated for 24 hours were put into the chromameter tool and then photographed on the back skin to get the results of the erythema index based on the L* a* b* coloring system (L*: brightness, a*: red-green coordinate scale, b*: yellow-blue coordinate scale). After taking the data, the results of the quantitative data on erythema index would be displayed on the computer [8].

Skin Homogenate

After being treated, rats were terminated by injecting using ketamine anesthesia. The dorsal rats with a length of 2 cm and a width of 2 cm were taken, cleaned, then smoothed with 20% TCA (*Trichloroacetic acid*) for 1 ml and phosphate buffer (pH 7.4) for 3 ml. Then centrifuged at a speed of 2000 rpm for 20 minutes, then the supernatant was taken.

SOD Activity Analysis

The measurement of SOD activity in the supernatant was carried out using the Misra and Fridovich method of 500 µl of the supernatant added to 0.8 ml of carbonate buffer and 100 µl of epinephrine. The change in absorbance of each sample was then recorded at a wavelength of 480 nm spectrophotometer for 2 minutes at an interval of 15 seconds as well as for blank and standard solutions. One unit of SOD was defined as the amount needed to inhibit 50% of the auto-oxidation of epinephrine. The mixture was diluted 1/10 then read absorbance using a spectrophotometer [9].

Radical Superoxide Analysis

Superoxide levels could be measured indirectly by measuring the SOD activity in the supernatant with the Misra and Fridovich method for 500 µl of the supernatant added to 0.8 ml of carbonate buffer and 100 µl of epinephrine. The change in absorbance of each sample was recorded at a wavelength of 480 nm [9].

H_2O_2 Level Analysis

Measurements used the colorimetric method by using color as the indicator. The reagents needed to have consisted of a mixture of 50 ml of potassium dichromate 5% with 150 ml of glacial acetate, H_2O_2 0.2 M, and 250 ml buffer phosphate pH 7 with a concentration of 0.01 M. the changes in absorbance of each sample were then recorded at a wavelength of 570 nm.

Statistical Analysis

The results of the SOD enzyme activity data and superoxide anion levels were normally distributed and homogeneous, then a parametric test analysis was performed using the One-way ANOVA test at a reliable level of

95% (α = 0.05); it was found that the results were significantly different then a further test was carried out using the Tukey Post-hoc test, so that one treatment was different in meaning could be known among other treatments.

The results of erythema index data and hydrogen peroxide levels were not normally distributed and homogeneous, then a parametric test analysis was performed using the Kruskal Wallis test at a confidence level of 95% (α = 0.05); it was found that the results were significantly different, then further tests were carried out using the Mann Whitney test, so that one treatment could be known to have differed in meaning between the other treatments.

RESULTS AND DISCUSSION

SOD Activity and Superoxide Radical Levels

SOD is an enzyme that catalyzes superoxide radicals into peroxide acid. This enzyme contains Cu/Zn as a cofactor. UV exposure causes a decrease in SOD activity, but the administration of pasak bumi extract can increase SOD activity on UV-exposed skin (figure 1). UV exposure causes an increase in skin superoxide levels (figure 2), so many SOD is used to catalyze them. So that SOD activity decreases.

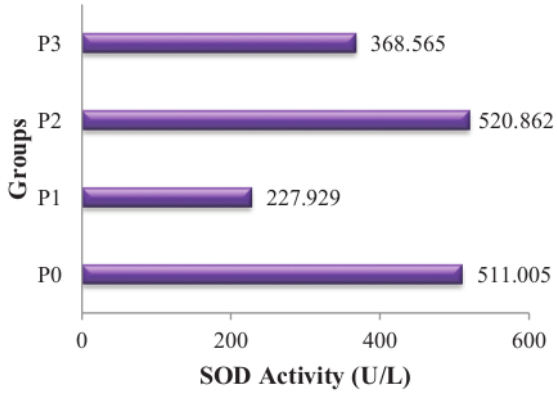

FIGURE 1. The Average of SOD Enzyme Activity

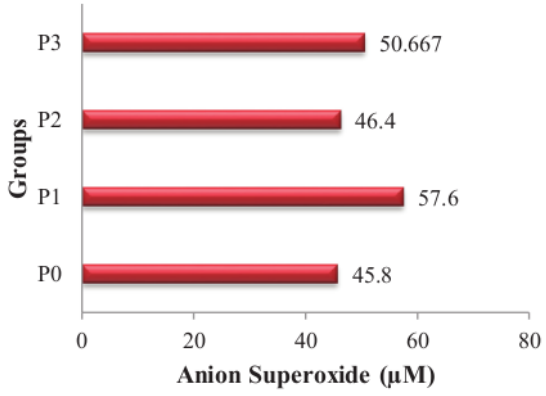

FIGURE 2. The Average of Superoxide Anion Level

The ANOVA test results concluded that SOD activity ($p = 0.04$; $p < 0.05$) and superoxide radical levels ($p = 0.03$; $p < 0.05$) had significant differences. The P0 and P2 groups showed SOD enzyme activity and superoxide anion levels. This was caused by the formation of superoxide anions physiologically in the process of cell respiration (mitochondrial oxidation phosphorylation). About 2% of the process underwent imperfect reduction and produced free radicals such as superoxide anions. According to Mann Hayyan, in physiological processes, the human body produces ROS around 5 grams per day and the body's biological system will produce SOD enzymes that specifically maintain superoxide anion levels in order not to cause oxidative stress [4,10].

The P1 group found SOD enzyme activity, but the activity value decreased when compared with P0 and P2 groups. This was caused by the formation of ROS, especially superoxide anions through activation of NADPH oxidase and the respiratory chain reaction process caused by exposure to UV light. ROS would be produced continuously by keratinocytes and fibroblasts so that there was an imbalance between ROS and antioxidants which caused depletion of endogenous antioxidants, that wathe SOD enzyme [6,11,12].

The P3 group showed that the value of SOD enzyme activity decreased and superoxide anion levels increased when compared to P0 and P2 groups, but the activity value increased and superoxide anion levels decreased when compared to group P1. This was due to the content of bioactive substances in the roots of pasak bumi, flavonoids, and alkaloids. Flavonoids could role as a scavenger directly on free radicals, inhibiting enzymes that played a role in the formation of free radicals and stimulating antioxidant enzymes. Alkaloids could also the role as a superoxide radical scavenger. So that the activity of endogenous antioxidant enzymes, the SOD enzyme, would increase and the superoxide anion level decreased [6,13,14,15].

The P0 group showed differences in SOD enzyme activity which were not significant with the P2 group. This showed that the administration of ethanol extract of pasak bumi roots on rat skin did not affect the activity of SOD enzymes if it was not exposed to UV light. However, there were some significant differences in the activity of SOD enzymes in P1 and P3 groups. This showed that the administration of ethanol extract of pasak bumi roots on rat skin had an influence in increasing the activity of SOD enzymes when exposed to UV light. The increase in SOD enzyme activity was suspected due to the bioactive substance ethanol extract of pasak bumi root which had the potential as an antioxidant capable of binding superoxide anions formed due to exposure to UV light and was able to increase the activity of endogenous antioxidant enzymes. This showed that the bioactive substance in the ethanol extract of the pasak bumi root was photosensitizer [16].

Hydrogen Peroxide Level

The results of the Kruskal-Wallis test concluded that peroxide levels ($p = 0.03$; $p < 0.05$) had significant differences (figure 3). Group P0 and P2 showed almost the equivalent amount of hydrogen peroxide. Hydrogen peroxide was part of non-radical ROS. The presence of hydrogen peroxide in normal conditions was the result of production in the mitochondria during the cellular respiration process. During the process, some O_2 would play a role in the formation of ATP and others would be reduced to form superoxide which was reactive. Furthermore, superoxide oxygen would be reduced back to hydrogen peroxide. In addition, hydrogen peroxide was also produced from oxidase enzymes including xanthine oxidase, urate oxidase and D-amino acid oxidase [4].

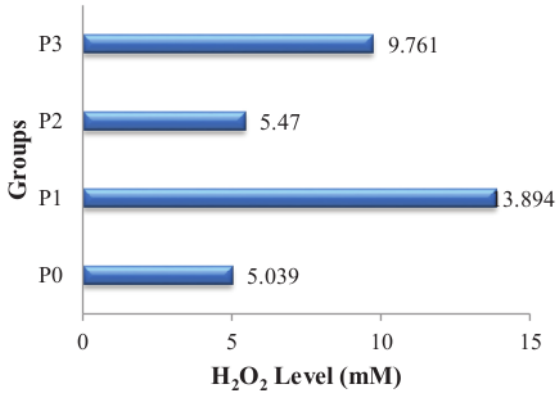

FIGURE 3. Average Hydrogen Peroxide Level

P1 group showed a significant increase in hydrogen peroxide levels compared to P0. This occurred because there was an increase in O_2 metabolism into superoxide radicals and the oxidation process in living cells formed an unstable O_2 derivative compound, SOR and one part of the SOR was hydrogen peroxide [4].

The P3 group showed a decrease in hydrogen peroxide levels when compared with P1 which was also radiated but smeared with pasak bumi extract. This was due to the bioactive content of ethanol extract of pasak bumi that were flavonoids and alkaloids. Alkaloids played a role in capturing superoxide radicals so that the formation of hydrogen peroxide from superoxide radical derivatives became inhibited [13]. The P0 group showed differences in hydrogen peroxide levels which were not significant with the P2 treatment group. This showed that the use of ethanol extract of pasak bumi had no effect if it was not exposed to UV light. But in the P1 and P3 groups, there were significant differences. This showed that the administration of ethanol extract from pasak bumi could reduce hydrogen peroxide levels when exposed to UV light. This reduction in hydrogen peroxide was suspected to be due to the presence of bioactive substances contained in ethanol extract of pasak bumi which played as an antioxidant capable of binding free radicals to prevent oxidative stress due to UV exposure. The protective effects of flavonoids in biological systems came from the ability to transferred radical free electrons, activated antioxidant enzymes, reduced radical alpha-tocopherol, and inhibited oxidation. Flavonoids protected from exposure to UV radiation and protected against the formation of ROS (Reactive Oxygen Species) [18]. In the treatment groups P2 and P3 had a significant difference because the treatment group P2 was not exposed to UV light but was smeared with ethanol extract of the pasak bumi root, so the levels of superoxide radicals received by rat skin were less than those in the P3 treatment group exposed to UV light and smeared with extracted ethanol root of pasak bumi. This showed that the bioactive substance in ethanol extract of pasak bumi was photosensitic [16]. Therefore the results of P2 were the same as the results of the P0 treatment group. The P3 treatment group obtained higher free radicals than the P2 treatment group. However, the hydrogen peroxide content produced could be reduced due to the help of antioxidants in the pasak bumi extract. This reduction in hydrogen peroxide levels could reach below the rate of toxicity of hydrogen peroxide (10μM-100μM) [4].

Erythema Index

Erythema index is an index that describes the redness of the skin due to UV radiation. This redness can be seen in figure 4. The results of the Kruskal-Wallis test concluded that erythema index ($p = 0.00$; $p < 0.05$) had significant differences (figure 5). P1 group showed a significant increase in erythema index because UV light induced erythema formation through a photochemical production mechanism. UV light-induced erythema formation through a

photochemical production mechanism. UV rays would break down oxygen into superoxide radicals which would damage cells. But there was a body defense mechanism that could reduce this.

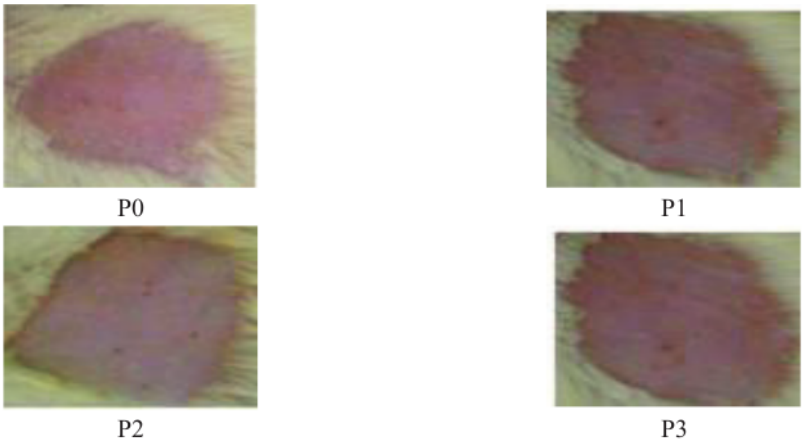

FIGURE 4. Description of White Rat Skin using the Chromameter Tool

UV light would make the skin became erythema when the cell's capacity to reduce superoxide radicals had exceeded its ability [17].

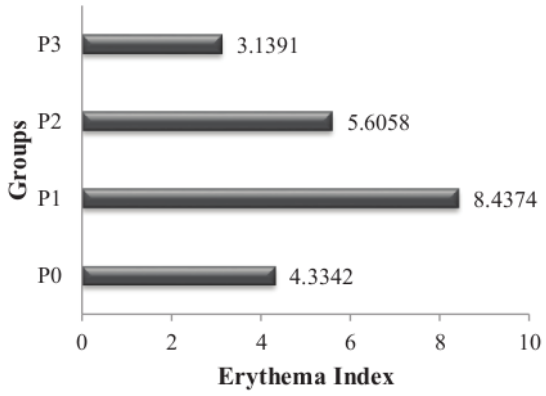

FIGURE 5. Average of Erythema Index

The P3 group experienced a reduction in erythema index compared to group P1 due to the bioactive content in ethanol extract of pasak bumi, they were flavonoids and alkaloids. These bioactive substances played a role in helping body cells to reduced superoxide radicals that entered the cell. The mechanism of bioactive substances reduced free radicals by binding to free radicals directly, by blocking enzymes that played a role in the formation of free radicals and then stimulating activation of the body's antioxidant enzymes. This mechanism could reduce the occurrence of erythema due to inflammation caused by superoxide radicals [13].

The P0 group showed no significant difference in erythema index with the P3 group. This was because the pasak bumi extract containing bioactive substances was able to bind existing free radicals so that it could reduce the erythema

index to almost the equivalent of the erythema index produced by the P0 treatment group [13]. Groups of P0 and P2 had significant differences. This was due to the ethanol content in the ethanol extract of the pasak bumi root which caused irritation and induced an increase in the erythema index in the skin of white rats.

CONCLUSION

It can be concluded that pasak bumi extract that had been smeared on rat skin which radiated by UV light acutely can increase SOD enzyme activity, and decrease superoxide anion level, hydrogen peroxide level, and erythema index significantly. In other words, pasak bumi extract has a role as a photoprotective agent.

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