











The Mangosteen Peel Ethyl Acetate Extract-based Cream Inhibits Ultraviolet-B Radiation-induced Hyperpigmentation in Guinea Pig Skin

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Abstract

BACKGROUND: Ultraviolet B (UVB) radiation is the main factor causing the aberrant melanin pigments leading to skin hyperpigmentation. Retinoic acid and hydroquinone are the primary preference for the skin whitening agents in preventing hyperpigmentation. However, these treatments could induce slight-to-severe irritation leading to skin cancer. Mangosteen peel possesses α -mangostin, the primary constituent of xanthenes in mangosteen peel that has potency as an anti-tyrosinase for treating issues of skin hyperpigmentation.

AIM: This study aims to demonstrate the capacity of mangosteen peel ethyl acetate extract-based cream in inhibiting the UVB radiation-induced skin hyperpigmentation in guinea pig.

MATERIALS AND METHODS: A total of 25 female guinea pigs were used to produce UVB-irradiated skin hyperpigmentation model. Guinea pig skins were treated with 12% mangosteen ethyl acetate extract-based cream. Mushroom tyrosinase inhibitor activity was used to evaluate the capacity of mangosteen extract in inhibiting tyrosinase activity *in vitro*. The melanin index in guinea pig skin after treatments was analyzed using a mexameter. The percentage of epidermal melanin-contained keratinocytes of skin tissues were analyzed using masson fontana. Pmel17 expression in cell surface was determined using immunohistochemistry. The level of tyrosinase in tissue homogenates was analyzed using Enzyme-linked immunosorbent assays.

RESULTS: Mangosteen peel ethyl acetate extract showed potent inhibitory activity against the mushroom tyrosinase. Its-based cream decreased melanin index, epidermal melanin, Pmel17 expression, and tyrosinase level in hyperpigmentation skin tissues.

CONCLUSION: Overall, our study demonstrates the capacity of mangosteen peel ethyl acetate extract-based cream in inhibiting the UVB radiation-induced skin hyperpigmentation in guinea pig.

Edited by: Sinisa Stojanowski
Citation: Harlisa P, Kariosentono H, Purwanto B, Dirgahayu P, Soetrisno S, Wasita B, Aliif I, Putra A. The Mangosteen Peel Ethyl Acetate Extract-based Cream Inhibits Ultraviolet-B Radiation-Induced Hyperpigmentation in Guinea Pig Skin. Open Access Maced J Med Sci. 2022 Nov 26; 10(A):1640-1648. https://doi.org/10.3889/oamjms.2022.10838
Keywords: Ultraviolet B; Hyperpigmentation; Mangosteen peel; Tyrosinase; Pmel17
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Received: 24-Aug-2022
Revised: 24-Oct-2022
Accepted: 16-Nov-2022
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Funding: This study was supported by Universitas Islam Sultan Agung and Universitas Sebelas Maret
Competing Interests: The authors have declared that no competing interests exist
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Introduction

Ultraviolet B (UVB) radiation is the main factor causing the high expression of melanin pigments leading to skin hyperpigmentation [1]. UVB exposure promotes an increase of reactive oxygen species (ROS) levels thereby increasing melanin synthesis, melanocyte proliferation, and accelerating the skin aging process [2]. ROS overexpression induces pro-inflammatory mediators and melanocyte mitogens expression by keratinocytes, such as α -melanocyte-stimulating hormones [3]. These mechanisms promote the activation of microphthalmia-associated transcription

factor (MITF) leading to the production of tyrosinase enzymes, tyrosinase protein-1 (TRP-1), and tyrosinase protein-2 (TRP-2) [4]. Tyrosinase plays an important role in excessive melanin pigment synthesis [5].

Repeated exposure to UVB is known to cause the aberrant accumulation of melanin leading to aesthetic problems, pigmentation disorders, and even cancer [6]. In 2015, after being exposed to 3 times the minimum erythema dose of UVB, 4.2% of 142 positive patients developed hyperpigmentation [7]. The previous study reported that UVB exposure also causes 8% incidence of squamous carcinoma known as melanoma skin cancer with high metastatic potential [8]. At present, chemical compounds such

as retinoic acid and hydroquinone are the primary preference for the skin whitening agents for preventing hyperpigmentation [9]. However, these treatments could induce slight-to-severe irritation leading to skin cancer [10]. Therefore, the strategy to inhibit melanin synthesis using non-irritating material for managing these problems is needed.

Utilizing antioxidants may be beneficial for slowing down the skin aging process because oxidative stress and skin aging are closely associated. In particular, mangosteen peel (*Garcinia mangostana* Linn.) possesses antioxidant, anticancer, anti-inflammatory, and antibacterial activity [11]. α -mangostin, the primary constituent of xanthenes in mangosteen peel has potency as an anti-tyrosinase for treating issues of skin hyperpigmentation [12]. The previous studies reported that the mangosteen peel contains phenolic and flavonoid polyphenols, both of which include phenol rings with one or more hydroxyl substituents that may scavenge ROS as a result of exposure to UVB radiation [13], [14]. Other studies also revealed that -keto group in mangosteen peel have a structure such as dihydroxyphenylalanine (DOPA) or tyrosine that could prevent melanogenesis in skin-induced UVB radiation [15], [16]. Physical and chemical characterization of mangosteen peel has been already reported by previous studies [11], [12], [13], [14], [15], [16]; however, the potency of the mangosteen peel extract-based cream and its mechanism in skin hyperpigmentation is still unknown.

In this study, we aimed to determine the effect of mangosteen peel ethyl acetate extract-based cream in inhibiting UV-B radiation-induced hyperpigmentation in guinea pig skin. We determined the chemical contents in ethyl acetate extract of mangosteen peel and employed the mangosteen peel extract-based cream in guinea pig with skin hyperpigmentation. We analyzed the tyrosinase level, melanin expression, epidermal melanin number, and melanin index in guinea pig skin hyperpigmentation model.

Materials and Methods

Chemicals and reagents

α -Mangostin, mushroom tyrosinase 4187 IU/mg, L-Tyrosine (dihydroxy phenyl alanine), was purchased from Sigma-Aldrich (USA). Ethyl acetate was purchased from Bratachem (Indonesia). Dimethyl sulfoxide extra pure was purchased from Acros® organic (Belgium). Ketamine 10% was obtained from Kepro B.V (Netherlands). Tyrosinase Elisa kit were purchased from MyBioSource (USA). Masson Fontana reagents were purchased from Bio Optica (Italy). HMB45 monoclonal

antibody was purchased from Diagnostic Biosystems (USA).

Mangosteen peel extract-based cream preparation and characterization

The Mangosteen was collected in from Bogor botanical Indonesia. Its botanical identity was determined and authenticated by a taxonomist. The extraction of Mangosteen peel was employed in Phytochemical Pharmacognosy Laboratory, Faculty of Pharmacy, University of Gadjah Mada, Indonesia. Collected mangosteen peel was cleaned through using water, cut into smaller pieces and dried. The mangosteen was mashed and extracted with ethyl acetate p.a. The mangosteen ethyl acetate extract was collected and used for the following experiments. The α -mangosteen contained in mangosteen peel ethyl acetate extract was then analyzed by high performance thin layer chromatography (HPLC) as previously described. Formulation of cream contains 12% mangosteen peel ethyl acetate extract was made in accordance to our previous study [17].

Measurement of inhibitory activity for mushroom tyrosinase

Various concentrations of mangosteen peel ethyl acetate extract and kojic acid as reference were mixed with distilled water to make a volume of 1 mL. Three milliliters of L-tyrosine were combined and incubated at 37°C for 15 min after 5 min incubation at room temperature. After that, 0.1 mL of the mushroom tyrosinase solution for mushrooms was added and incubated at 37°C for 25 min. Absorbance was measured at 470 nm and compared with blank without tyrosinase. The following equation was used to determine the percentage of enzyme activity inhibition and the IC₅₀ values:

$$\text{Inhibition (\%)} = (A_0 - A_1 + A_2)/A_0 \times 100 \quad (1)$$

where A_0 , A_1 , and A_2 are absorbance values of control, samples, and blank, respectively.

Animals

Eight-week-old female guinea pigs (*Cavia porcellus*) (weight, 250–300 g; n = 25) were obtained from Wates Veterinary Center, Yogyakarta, Indonesia. The experimental protocol for this study was approved by the Institutional Animal Care and Use Committee of Universitas Sebelas Maret Surakarta (Number 011/UN27.06/KEPK/EC/2020). The caged guinea pigs were kept in a room with regulated humidity and temperature (22 ± 1°C, 60 ± 5% humidity) with 12 h light/dark cycles. The guinea pigs were acclimated to individual cages after a 1-week quarantine. Food and

drink were provided ad-libitum during the trial period. The animals were divided into five treatment groups, healthy guinea pigs (P1), UVB-exposed guinea pig (P2), UVB-exposed guinea pig treated with base cream (P3), UVB-exposed guinea pig treated with 12% ethyl acetate mangosteen extract-based cream (P4), and UVB-exposed guinea pig treated with 5% hydroquinone-based cream (P5).

UVB exposure-induced hyperpigmentation

The UV source was supplied by a closely spaced array of Exo Terra Reptile UVB150-25 W Desert Terrarium Bulb (Canada). Bulbs were positioned 15 cm above the guinea pigs. The irradiation was measured using UV meter. After hair removal, the guinea pigs dorsal skin, except P1, was exposed to 65 mJ/cm² UVB radiation 3 times a week for 4 weeks. After 28 days induction, skin tissues and blood serum from guinea pig models were collected for following analysis.

Measurement of melanin index

The change in melanin pigmentation before and after treatments was assessed by a non-invasive approach after 28 days with three measurements each using a Mexameter (MX18, Germany Three distinct light wavelengths are emitted by the Mexameter probe (green, 568 nm; red, 660 nm; and infrared, 880 nm); a receiver then detects the light reflected by the skin. The average of the measured values was used to determine the level of pigmentation.

Histological study

After 28 days treatments, the guinea pigs were sacrificed and the dorsal skin tissues were taken. These samples were then fixed in 4% paraformaldehyde for 24 h and then embedded in paraffin. Sections (4 μ m thick) were stained with the Masson Fontana staining solution, according to manufacturer protocol (Bio Optica, Italy). Under a light microscope, the stained slides were observed. Epidermal melanin production was determined in a 435 \times 325 μ m area of section.

Immunohistochemistry

On 4-mm-thick, formalin-fixed, and paraffin-embedded skin tissues placed on glass slides, immunohistochemistry was carried out. Slides containing paraffin-embedded skin tissue were deparaffinized using xylene and alcohol. After rehydration, slides were incubated with anti-HMB45 monoclonal antibody (1: 100, Abcam, Cambridge, MA, United States) for targeting Pme17, followed by biotinylated secondary antibody. The detection was analyzed using streptavidin peroxidase, and the

expression intensity of Pme17 was semi-quantified using ImageJ software.

Measurement of tyrosinase concentration

Tyrosinase protein concentrations in guinea pig skin tissue homogenates were quantified using an enzyme-linked immunosorbent assays (ELISA) in accordance with the instructions provided with the ELISA kit (MyBioSource, San Diego, USA).

Statistical analysis

Statistical analyses were performed by SPSS v26. Data from at least three independent experiments were reported as mean \pm SD. All data were measured using Kruskal-Wallis test, and only significance levels of 0.05 or lower were reported. The following symbols represent statistically significant from the P2: *p < 0.05, **p < 0.01, and ***p < 0.001.

Results

Analysis of α -mangosteen in mangosteen peel extract

Using an HPLC, we determined the α -mangosteen content of Mangosteen Peel Extract. The α -mangosteen retention time is shown in Figure 1a. By using a standard calibration curve, the amount of α -mangosteen was calculated. The amount of α -mangosteen in mangosteen peel ethyl acetate extract was 86.753 \pm 0.321% (Table 1).

Table 1: α -mangosteen content in Mangosteen Peel Extract

Samples	Retention time	Area Percent
Mangosteen peel ethyl acetate extract	5.694 \pm 0.003	86.753 \pm 0.321

Mushroom tyrosinase inhibitor activity

As shown in Figure 1, mangosteen peel ethyl acetate extract showed potent inhibitory activity against the mushroom tyrosinase. The estimated IC₅₀ value was 77.147 μ g/mL (Figure 2a). On the other hand, kojic acid as reference standard showed more potent inhibitory activity in mushroom tyrosinase, with IC₅₀ of 1.99 μ g/mL (Figure 2b).

Mangosteen ethyl acetate extract-based cream alleviates melanin index in UVB radiation-induced hyperpigmentation skin

After several treatments, we then analyzed the melanin index in guinea pig skin using a Mexameter. As

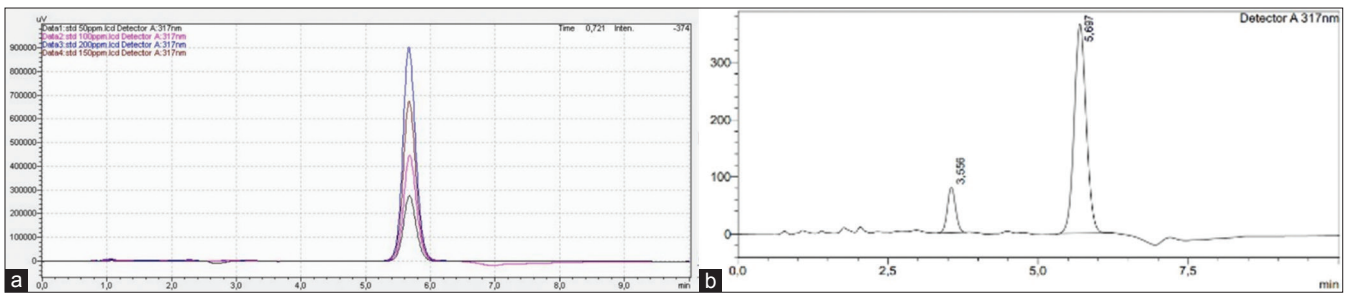


Figure 1: HPLC chromatogram for α -mangosteen standard (a) and α -mangosteen in mangosteen peel ethyl acetate extract (b)

shown in Figure 3, UVB exposure increased melanin index measurement in P2 by 360.40 ± 165.10 . P4 and P5 showed a significant decrease of melanin index in UVB radiation-induced hyperpigmentation compared to P2 ($p < 0.05$). P4 attenuated the melanin index by 55.64 ± 69.00 . On the other side, the P5 had optimum decrease of melanin index by 46.08 ± 33.33 .

keratinocytes percentage by $1.90 \pm 0.04\%$ and P5 showed optimum inhibition by $0.00 \pm 0.00\%$ (Figure 4b).

Mangosteen ethyl acetate extract-based cream attenuates pmel17 expression in UVB radiation-induced hyperpigmentation skin

Despite the evidence carried out that the majority of pigment-forming gene products are found in intracellular vesicles, the previous studies reported that the type I transmembrane protein Pmel17 is momentarily present at the cell surface. We then evaluated the expression of Pmel17 in guinea pig skin tissues after 28 days treatments using immunohistochemistry. Anti-HMB45 monoclonal antibody was used to detect Pmel17 in cell surface of guinea pig skin. As shown in Figure 5a and b, UVB exposure upregulated Pmel17 expression in cell surface of guinea pig skin by $37.56 \pm 0.21\%$. Both P4 and P5 significantly downregulated Pmel17 expression compared to P2 by $0.00 \pm 0.00\%$ ($p < 0.01$).

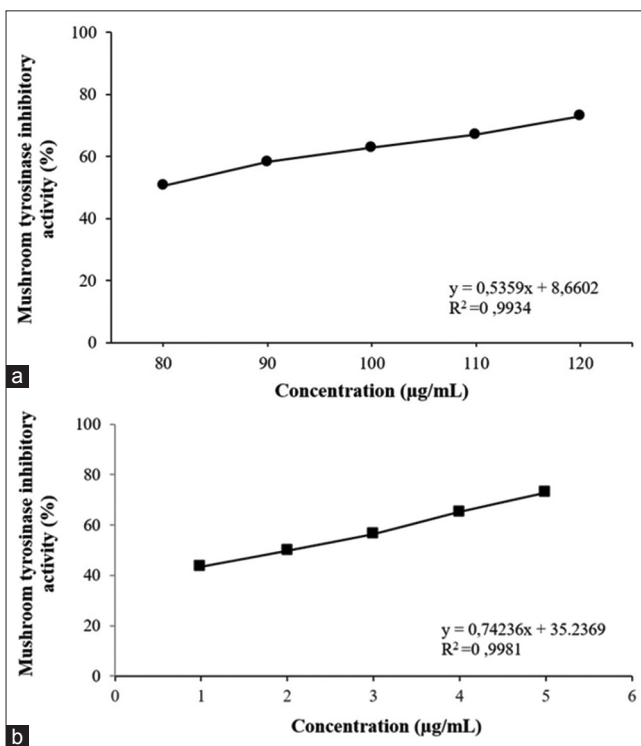


Figure 2: Effect of mangosteen ethyl acetate extract (a) and kojic acid (b) on mushroom tyrosinase inhibitory activity

Mangosteen ethyl acetate extract-based cream inhibits epidermal melanin in UVB radiation-induced hyperpigmentation skin

We measured the percentage of epidermal melanin-contained keratinocytes after 28 days treatments using Masson Fontana staining (Figure 4a). The Masson Fontana staining analysis showed that UVB exposure enhanced the percentage of epidermal melanin-contained keratinocytes in P2 by $66.12 \pm 0.34\%$. There was a significant decrease of epidermal melanin area percentage in P4 and P5 compared to P2 ($p < 0.05$). P4 decreased the epidermal melanin-contained

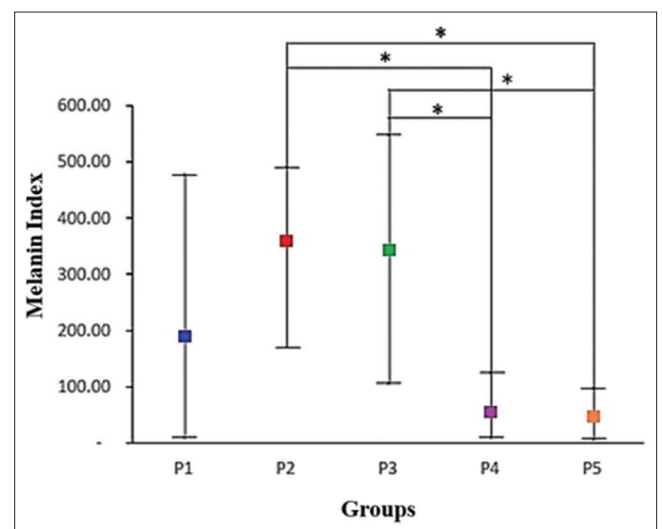


Figure 3: Effect of mangosteen ethyl acetate extract-based cream on melanin index ($n = 5$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Mangosteen ethyl acetate extract-based cream decreases tyrosinase level in uvb radiation-induced hyperpigmentation skin

We measured the level of tyrosinase in guinea pig skin tissue homogenates after 28 days treatments using ELISA. UVB exposure increased the level of tyrosinase in P2 by 9.04 ± 0.54 ng/mL. There was a significant decrease of tyrosinase level

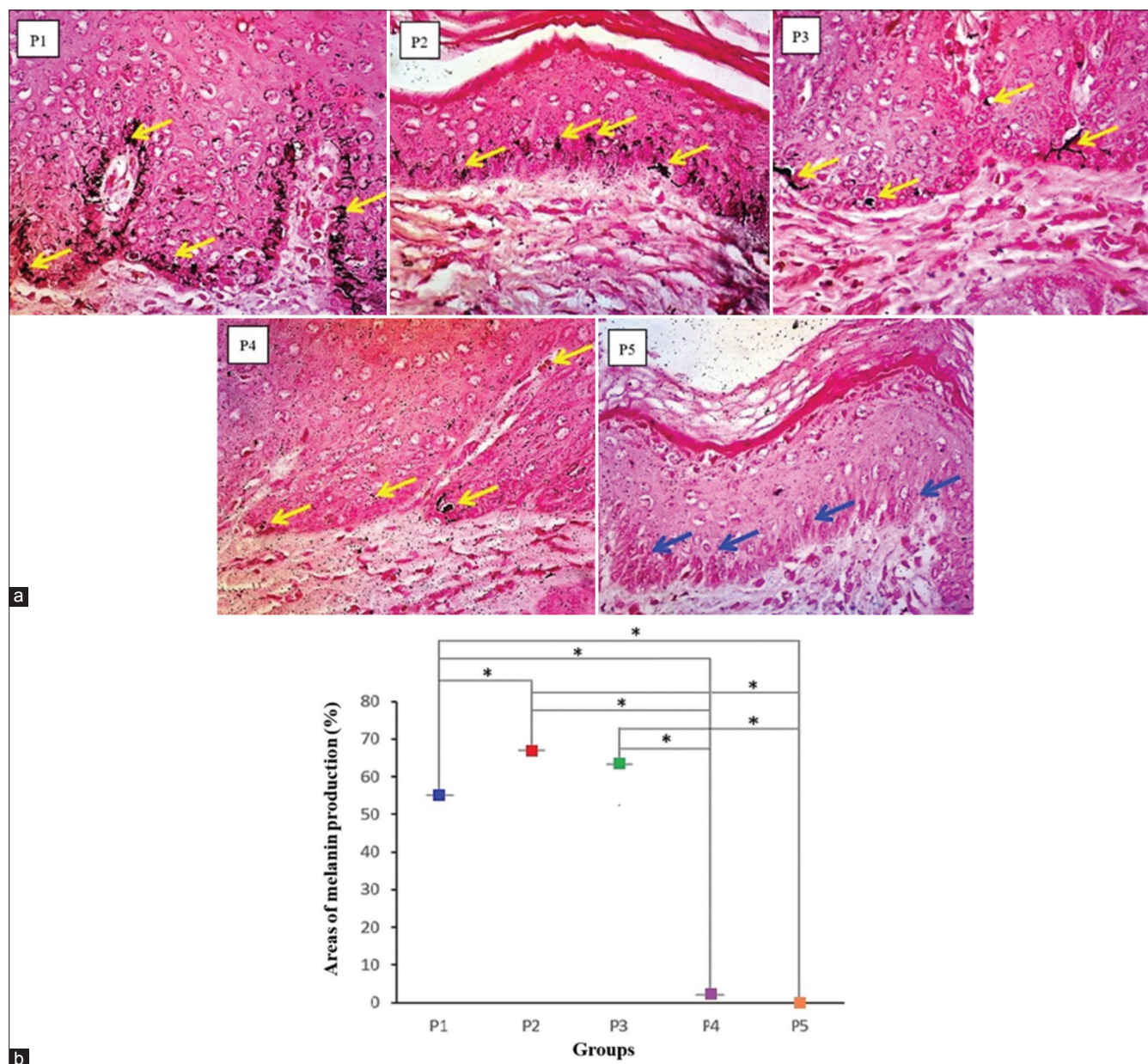


Figure 4: Epidermal melanin production was analyzed by Masson Fontana Staining. Arrows indicate melanin (a) Graphical representation of epidermal melanin areas (b) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

in P4 and P5 compared to P2 ($p < 0.01$). P4 inhibited the tyrosinase level by 5.11 ± 0.96 ng/mL. P5 showed optimum inhibition of tyrosinase level by 2.80 ± 0.36 ng/mL (Figure 6).

Discussion

Exposure to UVB radiation is a significant risk factor for skin photoaging, which is accompanied by inflammatory conditions, melanin accumulation, and impaired collagen production [18]. UVB exposure causes DNA damage by producing reactive oxygen species (ROS), which in turn triggers the melanin production pathway, an inflammatory response, and

the inactivation of collagen synthesis, which results in the development of skin cancer [19], [20]. Arbutin, kojic acid, hydroquinone, and sulfite are currently used as first-line therapies for hyperpigmentation issues [9]. However, there are a number of negative side effects associated with these agents, such as skin irritation, genotoxicity, and cell toxicity [21]. Since skin aging and oxidative stress are closely related, using antioxidants could be beneficial for reducing skin aging. Particularly, mangosteen peel contains α -mangostin, which is the main component of xanthenes and is effective as an anti-tyrosinase for treating skin hyperpigmentation [11]. In this study, we aimed to explore the capability of mangosteen peel ethyl acetated extract-based cream in inhibiting UVB radiation-induced skin hyperpigmentation in guinea pig animal model.

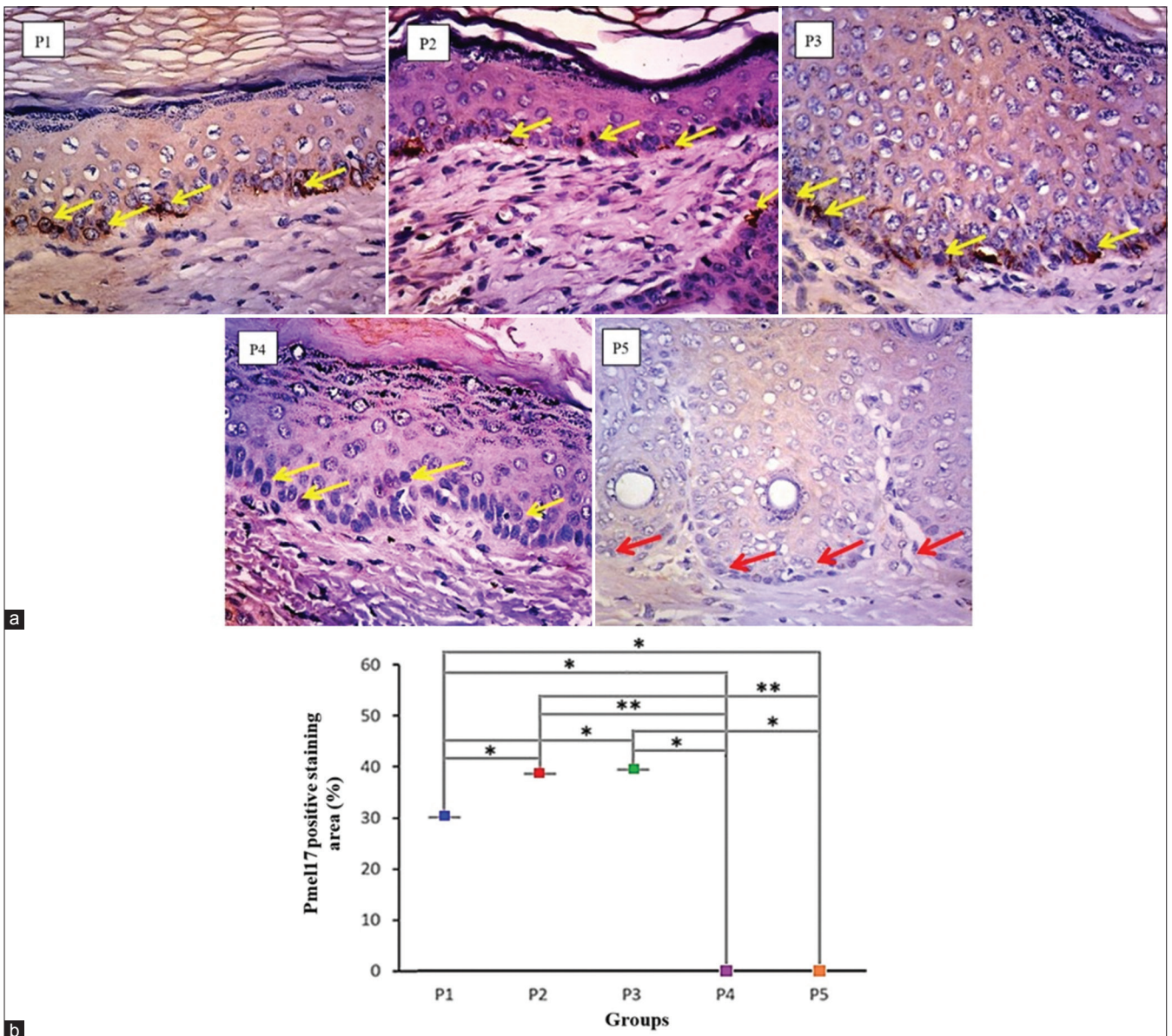


Figure 5: Pmel17 expression was analyzed by immunohistochemistry. Arrows indicate positive staining of Pmel17 (a) Graphical representation of Pmel17 expression (b) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Although mangosteen peel ethyl acetate extract is as crude extract, it contains a significant compounds and demonstrated pharmacological effects. Using HPLC, metabolite profile showed that mangosteen peel ethyl acetate extract contains a high amount of α -mangosteen (Figure 1). The previous studies reported that there are 40 xanthenes in the pericarp of the mangosteen fruit, with alpha-, beta-, and gamma-mangostin being the most prevalent ones [22], [23]. They contain a distinctive chemical composition that includes a tricyclic aromatic system with isoprene, hydroxyl, and methoxyl groups [24]. Garcinone, mangostin, isomangostin, and garcimangosone are phytochemicals that may be found in mangosteen peel [25]. Several studies conducted *in vitro* and *in vivo* have revealed that these substances have anti-oxidant, anti-proliferative, pro-apoptotic, anti-inflammatory, and anti-carcinogenic properties [12], [13], [14], [26]. In particular, α -mangostin could

play a major role as anti-tyrosinase in restoring skin hyperpigmentation [12].

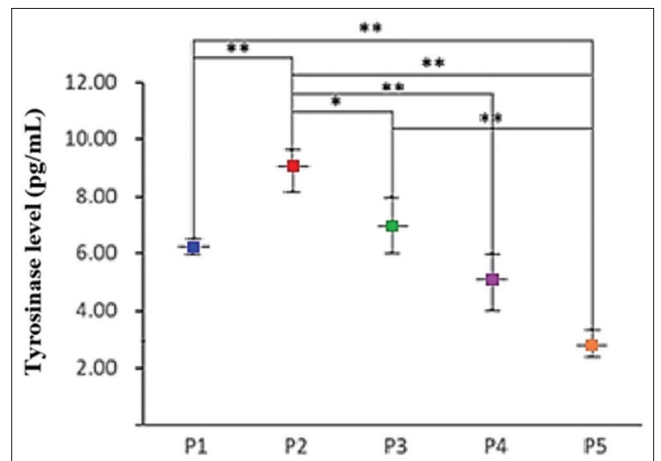


Figure 6: Tyrosinase level was analyzed using ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Tyrosinase, an enzyme involved in the production of melanin, can be measured as one way to ascertain the potential mechanism by which mangosteen peel ethyl acetate extract functions [27]. Tyrosine, the initial substrate for the formation of melanin, is transformed into L-DOPA by tyrosinase. Tyrosinase also participates in the subsequent phase in the production of melanin, which is the transformation of DOPA into DOPAquinone. As a result, controlling tyrosinase is a potent method for reducing melanin production [28]. In this study, *in vitro* mushroom tyrosinase activity analysis revealed that mangosteen peel ethyl acetate extract directly inhibited tyrosinase activity. Our analysis was in line with a previous investigation revealing the mechanism involved in the melanogenesis inhibitory by some constituents in mangosteen peel ethyl acetate extract that could reduce the expression of tyrosinase [11], [12], [13], [14]. To confirm our observation, we evaluated the *in vivo* analysis of tyrosinase levels in skin tissues that will be revealed in following explanation.

In vivo analysis was performed on UVB-irradiated guinea pigs, a popular animal model for hyperpigmentation study [29], [30], [31]. To generate hyperpigmentation model, animals exposed by a total of 780 mJ/cm² UVB radiation dosage in 4 weeks. Then, guinea pigs dorsal skin was treated using samples. In our melanin index measurement, the UVB-irradiated control group increased melanin index after 4 weeks of UVB exposure. The 12% mangosteen ethyl acetate extract-based cream was significantly downregulated melanin index, although the hydroquinone-based cream administration performed more optimum decrease (Figure 3). Moreover, Masson Fontana staining was used to analyze a change in the histology caused by the mangosteen ethyl acetate extract-based cream depigmenting action. Melanin is generated by melanocytes in the epidermis and activated by the expression of cytokines and growth factors released by UV-irradiated keratinocytes [19]. Our study reported that there was significant increase of melanin in the UV-irradiated guinea pig epidermal skin. However, group that received mangosteen ethyl acetate extract-based cream treatment provided a significant inhibitory effect of UV-induced melanin formation (Figure 4).

To improve our understanding regarding the process behind depigmenting effects of mangosteen ethyl acetate extract-based cream, we analyzed the level of tyrosinase and Pmel17 expression in UV-irradiated guinea pig skin tissue, which are involved in melanin formation. Our results showed that mangosteen ethyl acetate extract-based cream significantly inhibited tyrosinase expression in the dorsal skin of guinea pigs. Previous studies revealed that after the formation of DOPAquinone, the DOPAchrome is generated. TRP-2 participates in the conversion of DOPAchrome to 5,6-dihydroxyindole-2- carboxylic acid, which is an intermediate in the synthesis of eumelanin [32]. The

melanin pigment is produced by melanosomes, a type of lysosome-related organelle [33]. The structural organization of melanosomes is composed by several proteins, including Pmel17. This protein plays an important role in stage II melanosomes, which is generated from the elongation of those vesicles and the appearance within of unique fibrillar structures [34]. Our study reported that mangosteen ethyl acetate extract-based cream significantly reduced the expression of Pmel17 in the UV-irradiated dorsal skin of guinea pigs. These analysis confirmed our suggestion that mangosteen ethyl acetate extract-based cream effectively inhibits the production of melanin pigment in UVB irradiation-induced hyperpigmentation. In this study, we did not analyze the ROS concentration in the guinea pig skin tissues. We also did not analyze expression of MITF as the main transcription factor of tyrosinase, TRP-1 and TRP2 as the tyrosinase regulator in melanin formation.

Conclusion

Overall, our study demonstrates the capacity of mangosteen peel ethyl acetate extract-based cream in inhibiting the UVB radiation-induced skin hyperpigmentation in guinea pig. This investigation has thus provided more evidence regarding the potency of mangosteen peel ethyl acetate extract-based cream as possible anti-melanogenesis agents.

Acknowledgment

The author greatly appreciated to The Dean of Faculty of Medicine and The Rector of Universitas Islam Sultan Agung and Universitas Sebelas Maret for support during the study. We acknowledge Dono Indarto, Agung Putra, and Iffan Alif for their assistance in conducting this work. The study was supported by the Stem Cell and Cancer Research, Faculty of Medicine, Universitas Islam Sultan Agung. This study was also funded by Universitas Islam Sultan Agung and Universitas Sebelas Maret.

Author Contribution

PH, BW, and HKS: Data gathering and study conception. PH, BP, and PD: Study design. PH, IA, and AP: Writing and submission of manuscript. PD, S, and BW: Critical editing and approval of final draft.

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