INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY RESEARCH AND ANALYSIS

ISSN(print): 2643-9840, ISSN(online): 2643-9875

Volume 06 Issue 12 December 2023

DOI: 10.47191/ijmra/v6-i12-50, Impact Factor: 7.022

Page No. 5842- 5853

Effect of Petai Skin Extract Gel Administration on MITF Gene Expression and Melanin Count (in Vivo Experimental Study on Hyperpigmented Wistar Mice Exposed to UVB Exposed Hyperpigmentation)

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ABSTRACT: Prolonged exposure to high intensity UVB rays can induce the formation of Reactive Oxygen Species (ROS) which causes MITF activation thereby inducing melanin synthesis. Compounds contained in petai peel extract are known to play a role in inhibiting ROS production due to exposure to UVB rays. This study aims to determine the effect of administering petai peel extract gel on MITF gene expression and the amount of melanin in the skin tissue of mice exposed to high-intensity UVB. The research design was posttest only control group with a completely randomized design method. The samples studied were 24 mice exposed to UVB light with a wavelength of 302 nm and an energy of 390 mJ/cm2/day 3 times a week for 2 weeks. This research was carried out in four groups, namely the healthy group (K1), the negative control group (K2), treatment 1 (K3) with 10% petai peel extract gel and treatment 2 (K4) with 20% petai peel extract gel. MITF gene expression was analyzed using qRT-PCR and melanin was observed by specific staining with fontana-masson. qRT-PCR analysis showed that there was a significant decrease in MITF gene expression between groups K4 and K3 compared to group K2. Analysis of the amount of melanin also showed that there was a significant decrease in the mean in the K3 and K4 groups compared to K2 with a p value <0.05. Administration of petai peel extract gel can reduce the expression of the MITF gene and the amount of melanin in hyperpigmentation mice exposed to UVB light.

KEYWORDS: UVB exposure, petai peel extract, MITF, melanin

I. INTRODUCTION

Ultraviolet B (UVB) radiation is the main factor causing hyperpigmentation, cessation of collagen synthesis and inflammation of the skin.[1–3] Hyperpigmentation is a condition in which there is an increase in the amount of melanin in the skin which can be visually observed in the presence of brownish pigment then blackening.[4,5] Hyperpigmentation caused by exposure to UVB light is controlled by certain genes, proteins, and enzymes, such as tyrosinase, a transcription factor associated with *melanocyte inducing transcription factor (MITF),* tyrosinase-related *protein* 1 (TRP-1), and dopachrome *tautomerase* (TRP-2).[6] Activation of MITF leads to increased excessive melanin production in the skin.[7]

Increased exposure to UVB rays due to ozone layer damage in recent years and lifestyle changes have become important concerns as they can induce hyperpigmentation that increases cases of skin aging.[1–3] People with fair skin and older age have a higher risk of the effects of hyperpigmentation.[4,5] Although little research has been done on the specific incidence of hyperpigmentation, the incidence of melasma in men is 10%, while women prevalence is greater, especially at the age of 20-45 years. The ratio of melasma cases in women and men in Indonesia is 24:1.[5] Among the most common symptoms of aging, hyperpigmentation and facial wrinkles are the most common, this condition can affect a person's social interactions[6]. Until now, treatment with several chemical agents such as arbutin, azelaic acid, kojic acid, and hydroquinone was the main choice for preventing or treating skin hyperpigmentation and inducing collagen synthesis.[7,8] 9–11 [9–11].[12,13]

Traditionally, petai seeds have been used by local people as a treatment for various ailments, especially skin diseases.[14,15] Based on the explanation above, petai seeds have been widely used. However, petai skin by the community is just thrown away and has

not been widely used. Therefore, petai skin is used in the form of innovation into petai skin extract gel which acts as an antioxidant and anti-inflammatory. *Petai skin* also contains various types of polyphenols and flavonoids which are believed to be potential sources of bioactive compounds that are beneficial to health.[16] According to Isromi,[12] Petai bark (*Parkia speciosa*) contains flavonoids, phenols, and tannins that can be used as topical anti-inflammatories.[13] According to Rianti,[17] Petai skin contains flavonoids and phenols that can act as antioxidants. Other studies have also reported that petai has antioxidant properties[18–20] and anti-inflammatory.[16,21]

Previous studies have also reported that hyperpigmentation caused by exposure to UVB rays is regulated by various genes, proteins, and enzymes such as tyrosinase, and MITF.[22] In the melanin production pathway, tyrosinase plays a role in converting L-tyrosine *to* L-3,4-dihydroxyphenylalanine (L-DOPA).[23] L-DOPA is then oxidized to *L-DOPAquinone*, which then forms eumelanin and pheomelanin.[24] Other studies have also shown that chronic UVB exposure can cause oxidative stress, which activates phosphorylation of *mitogen-activated protein kinases* (MAPK) such as ERK, JNK, and p53 that accelerate melanin production.[26,29] Activation of these pathways increases transcription of MITF, which is a key factor in the activation of the enzymes tyrosinase, TRP-1, and TRP-2, which play a role in melanin production.[29,30] The flavonoid content in petai extract can inhibit ROS, which leads to suppression of MITF expression.[31,32] Other studies report that flavonoid compounds can increase the expression of antioxidants such as *superoxide dismuthase* (SOD) so that it can inhibit the MITF *pathway* causing melanin production to decrease.[33] Flavonoid compounds contained in petai skin extract are thought to have the ability to reduce melanin production through antioxidant mechanisms.[34,35] However, studies on the effect of topical gels of petai skin extract on melanin and MITF in hyperpigmentation models due to UVB exposure have not been conducted. Based on this background, the researchers wanted to analyze the effect of petai skin extract in the form of a topical gel on MITF gene expression and the amount of melanin in the skin of hyperpigmented mice exposed to UVB.

II. MATERIAL AND METHOD

Study Design and Experimental Animals

The study was a post test only control group with a randomized design method complete with five repetitions per treatment. The study subjects used male rats of the wistar strain, aged 2-3 months with a body weight of 200-250 grams that fit the inclusion and inclusion criteria, adapted for 5 days. This study used 4 treatment groups, a group of healthy mice without UVB irradiation (K1), a negative control group, UVB-induced mice with base gel treatment (K2), a UVB-induced rat treatment group with a 10% topical dose of petai skin extract gel treatment (K3), and a UVB-induced rat treatment group with a 20% topical dose of petai skin extract gel treatment (K4).

Research Materials

Research materials consist of materials for treatment such as water base gel, ketamine, xylazine, ethanol, aquades, rat feed, and chloroform.

Research Equipment

This research uses several equipment to make animal models including UV light (broadband with a peak emission of 302 nm) with an energy of 390 mJ/cm2, razors, exposure cages, maintenance cages, drinking water for rats and hair cutters. The tools used for data collection are vacutainer, hematocrit tube, 5 mL pot, 6 mm biopsy punch, centrifuge, micropipette, 1000 uL micropipette tip, and 1.5 mL vial tube. Tools used for data analysis include microplate readers, microscopes, staining jars, coated desk glass, cover glass, and laptops.

How to Make Petai Skin Extract Gel

±600 grams of petai peel is cut into small pieces, dried at 50-60°C and mashed into a dry powder. The process of making petai bark extract uses maceration techniques. Dried coolie petai powder is extracted using 70% ethanol then filtered and the filtrate is accommodated, the residue then macerated again by the same method. The choice of this solvent is because ethanol is able to filter active ingredients that are polar, semipolar and nonpolar. It is expected to produce an optimal amount of extract. The ethanol content is evaporated using a rotary evaporator to obtain a viscous extract. The results of evaporation are then thickened using a waterbath. The resulting viscous extract is used for the manufacture of gels. Gelling is done by mixing a hydrogel gel base (Katecho) of 200 mg with thick petai peel extract at P1 as much as 10% and P2 as much as 20%. Furthermore, to remove the aroma on pete added essential oil of lemon fruit. Stirring is carried out under aseptic conditions to form a homogeneous mixture of characteristics observed under a microscope.

Effect of Petai Skin Extract Gel Administration on MITF Gene Expression and Melanin Count (in Vivo Experimental Study on Hyperpigmented Wistar Mice Exposed to UVB Exposed Hyperpigmentation)

Table 1. Petai bark extract gel formula.

Composition	Dosage 10%	Dosage 20%
Hydrogel (Catecho) https://www.katecho.com/hydrogel/	180 mg	160 mg
The mapi leather eprint	20 mg	40 mg

Dosage Assignment

Before the study is carried out, the dosage to be used for research is first determined. Previous research using extract gels containing flavonoids at a dose of 20% for topical use can reduce melanin levels[91]. The use of petai skin extract gel was carried out every day as much as 200 mg / rat so that the extra dose of petai peel used was 20 mg of extract in 180mg of gel per rat for a dose of 10% and 40 mg of extract in 160 mg of gel per rat for a dose of 20% (Table 1) [92].

UV-B Display

(1) Mice that had been adapted for 1 week were anesthetized with a mixture of ketamine (60 mg / kgbb) and xylazine (20 mg / kgbb). **(2)** The hair on the dorsal part of the rat shaved clean with a size of 5x5 cm. **(3)** Rats' backs were exposed to UV light (broadband with peak emission 302 nm) with a minimum dose of erythema 390 mJ/cm2 15 minutes six times in two weeks. **(4)** Rat Behaviour 1 (10% dose skin extract gel) and Behavior 2 (20% dose skin extract gel) were then treated topically using a mapi skin extract gel given once a day for 14 days after UV-B irradiation.

Paraffin Block Making

(1) Dehydration: input pieces of tissue in alcohol stratified from 30%, 40%, 60%, 50%, 70%, 80%, 90%, 96% (stratified) to remove fluid from the tissue. Put the tissue into the alcohol-xylol solution for 1 hour then put the tissue in the pure xylol solution for 2 x 2 hours. (2) Paraffinization and Embedding: tissue input in liquid paraffin for 2 x 2 hours. Wait until the paraffin solidifies, cut the tissue in paraffin 4 microns thick with microtomes. The result of the tissue piece is attached to a glass object that has previously been smeared with polylysine as an adhesive. Put the tissue on the glass of the deparafination object in the incubator and heated to a temperature of 56-58°C until the paraffin melts.

Validation and analysis of post-UVB Melanin painting and treatment

(1) Melanin painting is done using the Masson Fontana painting method with the following stages: (1) The tissue slide is deparafinized. (2) The slide is incubated in Working Silver Solution for 2 minutes, then rinse with water. (3) The slide is incubated in a 0.1% solution of Gold chloride 10 minutes, then rinsed with water. (4) The slide is incubated in a 5% solution of Hypo for 5 minutes then rinsed with water. (5) The slide is incubated in Nuclear-fast red solution for 5 minutes, then rinsed with water. (6) The slide is carried out by dehydration process, then install the desk glass.

Analysis of the amount of melanin was carried out using a digital analysis method based on where the slides were photographed using an Olympus CX41 microscope (Olympus, Japan) at a magnification of 400 times using the Optilab Pro camera (Miconos, Indonesia). Each preparation was photographed as many as 3 fields of view using JPEG format. The photos were edited using Adobe Photoshop CS3 software version 10.0.1 (Adobe Systems Inc., San Jose, CA, U.S.A) to select the epidermis layer using the *Polygonal Lasso tool.* The calculation of epidermal area in *pixels* was done using ImageJ software version 1.47t (National Institutes of Health, Bethesda, MD) using the *red channel* in the RGB *stack* by setting the *threshold* to near the maximum. An area of the epidermis is necessary to normalize the amount of melanin. The calculation of the amount of melanin in *pixels* was done using ImageJ software version 1.47t using the *red channel* by setting the *threshold*. The normalized amount of melanin is calculated according to the following formula for per field of view:.[92].

Quantitative Analysis of MITF Gene Expression using RT-PCR

(1) RNA extraction and cDNA synthesis: RNA isolation of skin tissue was performed using TRIzol® reagent, (Invitrogen Life Technologies) and cDNA manufacturing using iScript cDNA Synthesis Kit (Bio-Rad iScript gDNA Clear cDNA synthesis Kit Catalog) using Reverse Transcriptase PCR (RT-PCR) thermal cycler C1000 (Bio-Rad). (2) MITF Gene Expression Determination: MITF was amplified using PCR-RFLP Technique, using PCR 2x PCR Master mix solution (iNtRON,® catalog number 25027) in a 0.2 mL vial tube with a total volume of 50 uL for 1 sample. PCR was performed using the thermal cycle of DNA: Applied Veriti Biosystems 96.

Table 2. PCR Mix CXCL8 Components.

Component	Kind
Primer	Forward MITF 5'-GCTGGAGACGGAACTCTGCT-3' Reverse MITF

	5'-GAGTGGGAGGGAGAGTGAGG-3'
Reagent	Trizol Reagen
Rna Transcribed	High Capacity cDNA Reverse Transcription
cDNA	SYBR Green

Network Sampling and Storage

Mice after 24 hours of the last treatment were turned off by cervical dislocation for tissue retrieval. Skin tissue is taken using a 6 mm punch biopsy in the induced part of the skin. Skin tissue samples are preserved in a later RNA solution to maintain RNA quality. Skin samples in RNA are stored at -20oC until the PCR analysis process is carried out.

III. RESULT

Petai Skin Extraction

Petai peel extract in this study was obtained by maceration method using ethanol solvent and produced an extract yield of 8.00%. The phytochemical screening results of petai bark extract show that petai skin is positive for phenol, phenolic, tannin, flavonoid, terpenoid, and saponin group compounds (Appendix. In this study, the total flavonoids and phenolics in petai bark extract were also determined using spectrophotometric methods. In 1 gram of petai bark extract contains flavonoids of 65.27mg ± 1.20 and phenolics of 44.70mg± 1.22. These results prove that most of the compounds contained in petai bark extract are flavonoids.

Hyperpigmentation Model Validation

In this study using a hyperpigmentation model. Animal models induced hyperpigmentation with 302 nm UVB irradiation with an energy intensity of 390mJ/cm2 for three times a week for two weeks. Validation of hyperpigmentation is observed on day 14. In coloring *Fontana masson* shows that there is a significant increase in melanin production which is characterized by brown pigment in the epidermis (melanocyte cells). In the group given UVB irradiation (negative control) the amount of melanin increased by 46.5% (Figure 1).

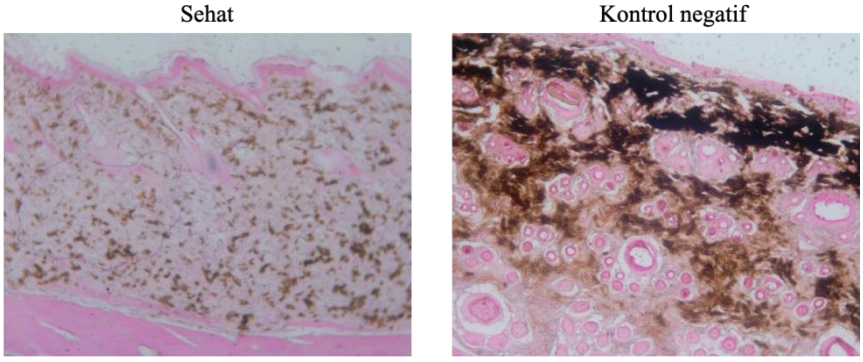

Figure 1. Validation of hyperpigmentation by masson fontane staining (A) Healthy mice and (B) UVB irradiated mice.

Effects of 10% and 20% Doses of Petai Skin Extract Gel on MITF Gene Expression

In this study, researchers found that petai peel extract gel was able to reduce MITF gene expression and melanin count in hyperpygemntation model mice significantly dose-dependent (Table 3; Figure 2).

Table 3. Data from MITF and Melanin Gene Expression Research

Variable	**Group**			
	Sehat=5 Mean±SD K1	Negative control n=5 Mean±SD K2	Gel Extract Kulit Petai Dosage 10% n=5 Mean±SD K3	Gel Extract Kulit Petai Dosage 20% n=5 Mean±SD K4
Expressive has MITF	1.03±0.01	3.52±0.84	0.84±0.05	0.80±0.15
Saphiro wilk	0,415*	0,723*	0,181	0,362
Levene test				0,001**

One way ANOVA				0,000***
Total Melanin	10.95±2.48	32.36±3.92	$27.98±2.58	28.34±2.24
Saphiro wilk	0,627	0,251	0,963	0,675
Levene test				0,687
Kruskal-Wallis Test				0,000***

Information:
*Saphiro Wilk test (p < 0.05 = abnormal)
** Levene's Test (p < 0.05 = not homogeneous)
Kruskal Wallis (p < 0.05 = different meanings)

Based on the results of the study shown in table 3. The mean expression of the MITF gene in the gel group of petai skin extract dose was the lowest dose of 20% (K4) (0.80±0.15), then followed by the average expression of the MITF gene in the gel group of petai skin extract dose of 10% (K3) (0.84±0.05). The highest ratio in the negative control treatment group (K2) was 3.52±0.84. The MITF gene expression data of all groups are normally distributed, shown *by Shapiro Wilk* results obtained a value of p>0.05 and also have inhomogeneous data variants indicated by the results *of Levene's Test* with a value of p = 0.001 (p < 0.05). The distribution and variant of MITF gene expression data are normal and inhomogeneous, so parametric statistical analysis with *One way-ANOVA* produces a value of p = 0.000 (p < 0.05) so that there is a significant difference in the average expression of MITF genes between the four groups. The significant *One way ANOVA* test results were followed by the *Tamhane Post Hoc* test to see which group was the most influential.

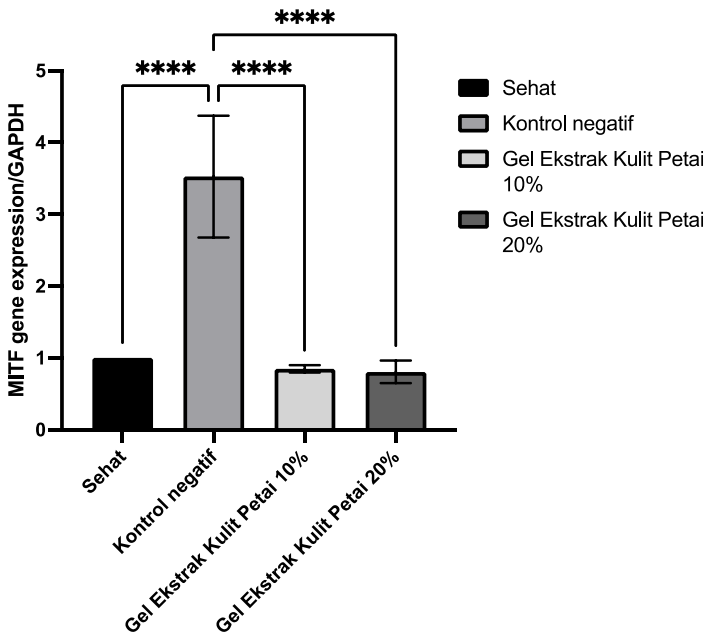

Figure 2. Graph of MITF gene expression across research groups

Table 4. Post Hoc Tamhane Test of MITF Gene Expression in Each Group

Group	Comparison Group	Say.	95% confidence interval	
			Lower Limit	Upper Limit
K1	K2	0,005*	-3,9532	-1,0502
	K3	0,002*	0,0891	0,2642
	K4	0,111	-0,0561	0,4883
K2	K3*	0,003*	1,2298	4,1269
	K4	0,003*	1,2936	4,1464
K3	K4*	0,993	-0,2182	0,3015

Sign *(p<0.05) indicates a significantly different group

Effect of Petai Skin Extract Gel Administration on MITF Gene Expression and Melanin Count (in Vivo Experimental Study on Hyperpigmented Wistar Mice Exposed to UVB Exposed Hyperpigmentation)

Based on the data above, the average comparison between the K2 group with K3 (0.003) and K2 with K4 (0.003) showed a significant difference, while the comparison between the K3 and K4 groups (0.993) there was no significant difference. In the comparison of K1 and K2 obtained a value of 0.005 (p <0.05) so that there is a significant difference between the two groups. The most significant decrease in MITF gene expression was shown in the administration of 20% petai peel extract with a lower limit value of 1.2936 and an upper limit value of 4.1464. The results *of the post hoc tamhane* test on MITF gene expression data showed that the administration of petai skin extract gel that can reduce MITF gene expression in male rats wistar strain hyperpigmentation model.

Effects of Petai Skin Extract Gel Doses of 10% and 20% on Melanin Expression

In this study, researchers found that petai skin extract gel was able to reduce the amount of melanin in hyperpygemntation mice significantly depending on dose (Table 3; Figure 3). Based on the results of the study shown in table 3. The average amount of melanin in the K3 group (27.98±2.58) was the lowest, then followed by the average amount of melanin in the K4 group (28.34±2.24). The highest amount of melanin data was in the negative control group of 32.36±3.92. Data on the amount of melanin of all normally distributed groups (p>0.05) based on *Shapiro Wilk's results*. Based on homogeneity analysis with Levene test shows homogeneous data (p > 0.05). The distribution of data on the amount of normal melanin and homgen so that parameteric statistical analysis with *one way ANOVA resulted in a value of p* = 0.000 (p < 0.05) so that it was stated that there was a significant difference in the average amount of melanin between the four groups. The results of a *significant one-way ANOVA* test were followed by *a post hoc LSD* test to see which group was the most influential.

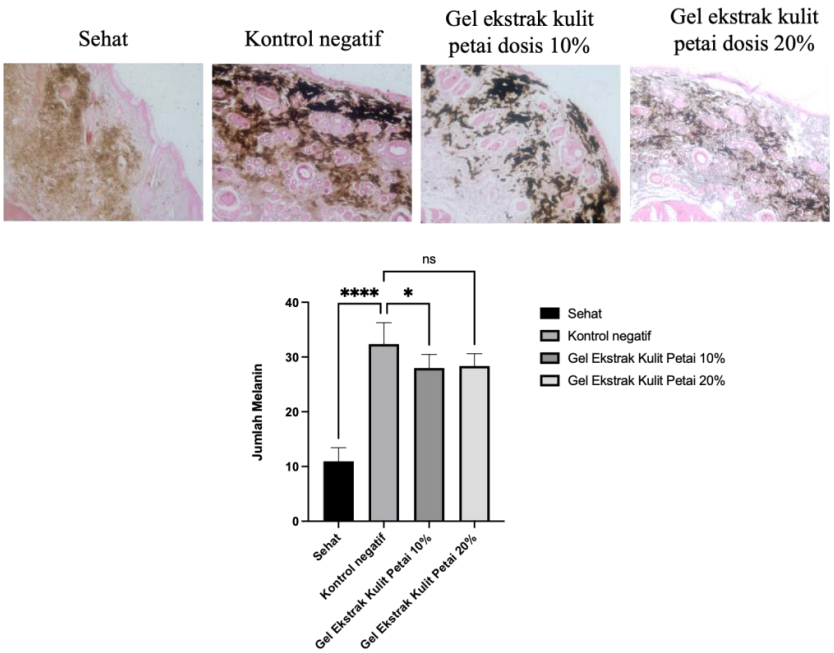

Figure 3. (A) Melanin staining picture in all treatment groups and (B) Graph of melanin count in all study groups

Table 5. *Post Hoc LSD* Test on Melanin Count in Each Group

Group	Comparison Group	Say.	95% confidence interval	
			Lower Limit	Upper Limit
K1	K2*	0,001*	-24,8654	17,9580
	K3	0,001*	-20,4904	-13,5830
	K4	0,001*	-20,8520	-13,9446
K2	K3*	0,016*	0,9213	7,8287
	K4*	0,025*	0,5596	7,4670
K3	K4	0,829	-3,8154	3,0920

Sign * indicates a significantly different group.

Based on the data above, an average comparison of K2 (negative control) with K3 (10% petai skin extract gel) (0.016) and K4 (20% petai skin extract gel) (0.025) was obtained, which means that there is a significant difference, while K3 and K4 (0.829) have no significant difference. In the comparison of groups K1 and K2 obtained a value of 0.001 ($p < 0.05$) so that there is a significant difference between the two groups. The highest value of melanin amount was most significantly shown in the administration of 10% petai skin extract gel with a lower limit value of 0.9213 and an upper limit value of 7.8187. The results *of the LSD post hoc test* on melanin count data showed that the administration of petai skin extract gel that can reduce the amount of melanin in male rats of the Wistar strain hyperpigmentation model.

IV. DISCUSSION

UVB exposure is a major risk factor for skin hyperpigmentation characterized by increased expression of melanin-forming proteins such as MITF.[93] UVB light irradiation induces DNA damage through the formation of oxidative ROS, resulting in activation of several melanogenesis pathways.[94] Recent research proves that petai bark extract which contains various secondary metabolite compounds such as flavonoids, alkaloids, tannins, and saponins can suppress ROS formation because it has antioxidant activity. The ability of extracts that can suppress ROS may prevent melanin production. [35,95] This study aimed to determine the effect of petai skin extract gel administration on MITF gene expression and melanin amount in hyperpigmented mouse models. This study used male rats of the wistar strain because it includes mammalian vetebrata with skin structures similar to human skin. Test animals were induced exposure to UVB light 302 nm with an energy intensity of 390mJ/cm2 three times a week for 2 weeks [93].

This study showed that MITF gene expression in the K3 (10% petai skin extract gel) and K4 (20% petai skin extract gel) groups decreased significantly compared to the hyperpigmented rat group that did not receive therapy (negative control). This suggests that decreased expression of the MITF gene can prevent hyperpigmentation. Flavonoid compounds derived from petai bark extract may inhibit TGF-β thereby inhibiting MITF activity through inhibition of the PI3K/Akt pathway.[96] TGF-β suppression has the potential to inhibit melanin biosynthesis in B16 melanoma cells. The ability of flavonoid compounds to inhibit TGF-β has also been reported to inhibit the cAMP/protein kinase A pathway and induce GLI2, which then suppresses MITF, the central transcription factor of melanogenesis.[97] Meanwhile, through the SMAD pathway, TGF-β suppression in inhibiting the process of melanogenesis by sending signals through ligand-specific heteromeric receptors, namely serine / threonine kinase receptors that function for phosphorylation and activation of (R)-Smad receptors, which leads to the formation of complexes with (Co)-Smad, Smad4, and transcriptional regulation of target genes so as to suppress MITF expression [98].

This study further demonstrated the inhibitory effect of petai skin extract gel on the amount of melanin. This is due to petai bark extract which inhibits MITF so that it blocks TRP-1 activity and TRP-2 correlates with activation of eumelanin and pheomelanin formation pathways. Suppression of excessive TRP-1 expression can inhibit melanin synthesis [99]. Previous research reported that increased TRP-1 expression correlated with increased amounts of melanin due to UVB irradiation. However, increased TRP-2 expression is associated with melanoma cell proliferation [100]. In this study, the administration of petai skin extract gel significantly and dose-dependent decreased the amount of melanin. This shows that the administration of petai skin extract gel through MITF downregulation thus prevents melanin formation in melanocyte cells.

V. CONCLUSION

1. Administration of petai bark extract at doses of 10% and 20% significantly decreased MITF gene expression in male rats of Wistar strain model UVB-induced hyperpigmentation.
2. Administration of petai bark extract at doses of 10% and 20% significantly decreased the amount of melanin in male rats of Wistar strain model of UVB-induced hyperpigmentation.

ACKNOWLEDGEMENT

The authors declare no conflict of interest and no involvement of sponsors or funding for this research.

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