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Effect of Petai Bark Extract Gel on *Tyrosinase* and *TRP1* Gene Expression (In *Vivo* Experimental Study on Hyperpigmented Wistar Mice Exposed to UVB Model)

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ABSTRACT: UVB irradiation can induce the formation of Reactive Oxygen Species (ROS) which causes activation of melanin synthesis through activation of tyrosinase and tyrosinase related protein-1 (TRP1). Secondary metabolites 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 the expression of the tyrosinase and TRp1 genes in mouse skin tissue exposed to 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 390mJ/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. Tyrosinase and TRP1 gene expression was analyzed using qRT-PCR. qRT-PCR analysis showed that there was a significant decrease in tyrosinase and TRP1 gene expression between groups K3 (tyrosinase 3,19±2,12 and TRP1 4,96±3,42) and K4 (tyrosinase 0,65±0,44 and TRP1 2,22±1,18) compared to group K2 (tyrosinase 17,92±3,77 and TRP1 35,91±4,52). Administration of petai peel extract gel can reduce the expression of tyrosinase and TRP1 genes in hyperpigmentation mice exposed to UVB light.

KEYWORDS: UVB exposure, petai peel extract, tyrosinase, TRP1

I. INTRODUCTION

Hyperpigmentation is the formation of black pigment on the skin due to an increase in the amount of melanin caused by exposure to ultraviolet B (UVB) rays.[1] UVB radiation can stimulate melanin production in skin cells, especially melalocytes, which are responsible for causing black color to the skin. [2] *Tyrosinase* and *tryrosinase-related protein 1* (*TRP-1*) are enzymes involved in melanin biosynthesis.[3] *Tyrosinase* plays a role in the early stages of melanin synthesis, while *TRP1* is involved in the maturation and spread stages of melanin.[4] Excessive expression of these two enzymes can lead to excessive melanin production, thus contributing to hyperpigmentation.[5] Some standard hyperpigmentation therapies such as retinol, hydroquinone, and tranexamic acid cause cancer in long-term use.[6] Therefore, a safe and effective therapeutic approach is needed to prevent hyperpigmentation due to UVB exposure, one of which is using natural antioxidant compounds.

The use of some chemical agents such as arbutin, azelaic acid, kojic acid, and hydroquinone in the prevention of hyperpigmentation is reported to have adverse side effects, including genotoxicity, skin irritation, contact dermatitis, and an increased risk of skin cancer.[7] In 2015, approximately 4.2% of 142 subjects exposed to three times the minimum erythema dose (MED) UVB 390mj/cm2 experienced hyperpigmentation. [8] In Indonesia, cases of hyperpigmentation account for about 0.25-4% of all cases of skin diseases. [9,10] Standard treatment of skin hyperpigmentation focuses on physical and chemical protection against UVB exposure without affecting melanin production pathways such as *tyrosinase* and *TRP1*.[7]

The use of natural antioxidants is proven to inhibit melanin synthesis and prevent hyperpigmentation.[11] One of the natural antioxidants is *Parkia speciosa* extract known as petai or *bitter bean*. *Parkia speciosa bark* contains bioactive compounds such as flavonoids and tannins, which have been shown to have antioxidant and anti-inflammatory activity.[12,13] Petai skin polyphenolic compounds such as *cinnamic acid, isoferulic acid, caffeic acid, and ferulic acid* have antioxidant activity by inhibiting melanocyte degradation so that hypermelanogenesis does not occur.[13,14] Previous research reported that antioxidant compounds can inhibit

the expression of *tyrosinase* and *TRP1* in melanocyte cells so that excessive melanin production does not occur.[15] However, no studies have examined the effect of petai peel extract on *tyrosinase* and *TRP1* gene expression in models of hyperpigmentation due to UVB exposure.

Previous research has shown that administering extracts containing flavonoids and polyphenols either through topical application or oral administration can reduce free radical levels[16], which has the potential to prove the effect of petai peel extract on *tyrosinase* and *TRP1* gene expression in the skin of hyperpigmented mice due to UVB exposure. Based on this information, the purpose of this study was to evaluate the effect of petai peel extract on *tyrosinase* and *TRP1* gene expression in wistar strain rats that experienced UVB-induced hyperpigmentation.

II. MATERIAL AND METHOD

Study Design and Experimental Animals

This research is an *in vivo* experimental research with *Post Test Only Control Group Design*. The study subjects used 24 male rats of wistar strains aged 2-3 months with a body weight of 200-250 grams according to the criteria of inclusion and inclusion, adapted for 1 week. This study used 4 with the following details: 2 treatment and intervention groups, 1 treatment group that did not get intervention (control) and 1 group of healthy mice. Data measurement was carried out after the intervention. On the 15th day, female mice BALB/C were taken skin tissue samples to check MMP1 levels and IL-6 levels.

Research Materials

The materials used in this study consisted 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

The peel of ±500 grams petai is cut into small pieces, dried at 50 – 60 ° C and mashed into a dry powder. Then the dry powder is extracted through a maceration process using 70% ethanol for 72 hours then filtered and the filtrate is accommodated, the residue then macerated again by the same method. The ethanol content is evaporated using a *rotary evaporator* to obtain a viscous extract. The extract content was validated by qualitatively measuring secondary metabolite compounds by drip reactions, namely the measurement of flavonoids, alkaloids, terpenoids, tannins, saponins, and steroids. The viscous extract obtained is then stored at 2-8°C.

Alkaloid Content Check

Examination of alkaloid content using Wagner examination. Here is the procedure for Wagner's examination: **(1)** An extra 3 mL of petai skin is placed in a porcelain dish. **(2)** Added 5 mL HCl 2 M. **(3)** Stirred and cooled at room temperature. **(4)** Added 0.5 g NaCl then stirred and filtered. **(5)** The filtrate obtained is added HCl 2 M as much as 3 drops. **(6)** Filtrate plus Wagner reagent. **(7)** The formation of precipitate indicates the presence of alkaloid content.

Standard Curve Making of Quercetin

Method for determining the total content of flavonoids by creating a standard calibration curve of quercetin. Procedure for making quercetin solution: **(1)** Variation of quercetin solution concentration of 5, 10, 15, 20 and 25 mg/L. **(2**) Each solution is taken as much as 2 mL **(3)** Added 0.1 mL AlCl3 10% and 0.1 mL CH3COONa 1 M. **(4)** The mixture is allowed to stand at room temperature for 30 minutes. **(5)** A maximum wavelength measurement is made at one of the concentrations. **(6)** The maximum wavelength measurement result is used to measure the absorbance of each fraction.

Determination of Total Flavonoids of Petai Skin Extract

The determination of total flavonoids in petai bark extract was: **(1)** Each fraction weighed 0.06 g and dissolved in 10 mL of methanol. **(2)** The solution is taken as much as 2 mL then added 0.1 mL AlCl3 10% and 0.1 mL CH3COONa 1 M. **(3)** The mixture is allowed to stand for 30 minutes at room temperature. **(4)** Absorbance was measured at a wavelength of 433 nm using a UV-Vis spectrophotometer. **(5)** The results of this wavelength measurement are then compared with the quercetin standard curve.

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Quantitative Analysis of *Tyrosinase* and *TRP1* gene expression using RT-PCR

(1) RNA extraction and cDNA synthesis[58] RNA isolation of skin tissue is carried out using the reagent TRIzol®, (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) Determination of tyrosinase *and* TRP1 *amplified gene expression* 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.

Table 1. PCR Mix Components

Component	Kind	Sequences
Primer	Forward *Tyrosinase*	5'- AATCGCTTAGGTAAGAA-3
	Reverse *Tyrosinase*	5'- GTTGCTGAGGTATCGCCAGGAA-3'
Primer	Forward *TRP1*	5'-GCTTAAATTGCCAATTGAATA-3'
	Reverse *TRP1*	5'-AGGGAGGGGACTTATCTG-3'
Reagent	Trizol Reagen	
RNA transcribed	High Capacity cDNA Reverse Transcription	
cDNA	SYBR Green	

(3) The calculation of tyrosinase and TRP1 gene expression is calculated in a ratio value compared to the house keeping expression of the GAPDH gene so that the unit of calculation is the ratio of mRNA gene expression level to house keeping gene expression.

III. RESULT

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 was observed at day 14 in one healthy mouse and one negative control mouse (K2). In *Fontana masson* staining shows that there is a significant increase in melanin production which is indicated by brown pigment in the epidermis (melanocyte cells). In the group given UVB irradiation (negative control) the amount of melanin increased to 46.5% (Figure 1).

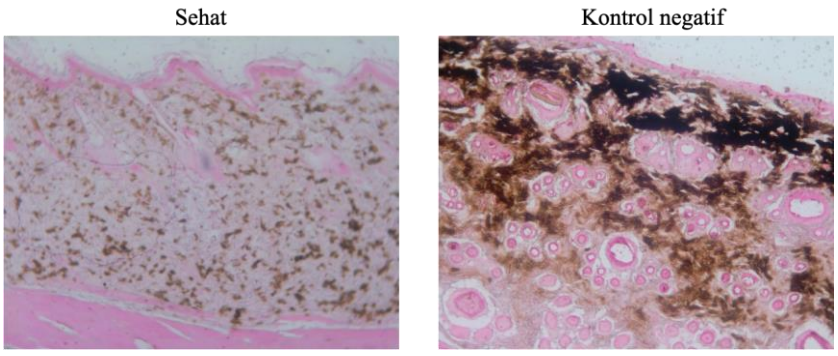

Figure 1. Validation of hyperpigmentation by masson fontana staining (A) Healthy mice and (B) UVB irradiated mice. 100x magnification

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

In this study, researchers found that petai bark extract gel was able to reduce tyrosinase and TRP1 gene expression in hyperpygemntation mice significantly dose-dependent models (Table 1; Figure 2).

Effect of Petai Bark Extract Gel on Tyrosinase and TRP1 Gene Expression (In *Vivo* Experimental Study on Hyperpigmented Wistar Mice Exposed to UVB Model)

Table 1. Tyrosinase and TRP1 Gene Expression Research Data

Variable	Group			pvalue
	Sehat=5 Mean±SD (K1)	Control negative n=5 Mean±SD (K2)	Petai bark extract gel dosage 10% n=5 Mean±SD (K3)	Petai bark extract gel dosage 20% n=5 Mean±SD (K4)
Express there Tyrosinase	1.00±0.01	17.92±3.77	3.19±2.12	0.65±0.44
Saphiro wilk	0,421	0,150	0,137	0,215
Levene test				0,001
One way ANOVA				0,001***
Expressive of having TRP1	1.01±0.01	35.91±4.52	4.96±3.42	2.22±1.18
Saphiro wilk	0,421	0,448	0,017*	0,158
Levene test				0,001
Kruskal-Wallis Test				0,001***

Information:

*Saphiro Wilk test (p < 0.05 = abnormal)

** Levene's Test (p < 0.05 = tidak homogeneous)

Krsuskal Wallis/ one way ANOVA (p < 0.05 = there is a difference in meaning)

Based on the results of the research shown in table 1. The average expression of the tyrosinase gene in the K4 group was the lowest (0.65±0.44), then followed by the average expression of the tyrosinase gene in the K3 group (3.19±2.12). The highest ratio in the negative control treatment group (K2) was 17.92±3.77. The mean value of the healthy group expression is used as the *base line* value of the ratio, so that all treatment groups are compared to the healthy group that is worth the ratio of one. Tyroisnase 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 shown by the results of *Levene's Test* with a value of p = 0.001 (p < 0.05). The distribution and variant of tyrosinase gene expression data are normal and inhomogeneous, then parametric statistical analysis with *the one way ANOVA* test produces a value of p = 0.001 (p < 0.05) so that it is stated that there is a significant difference in the average expression of the tyrosinase gene between the four groups. The significant *results of the ANOVA one-way* test were followed by *a post hoc Tamhane* test to see which group was the most influential.

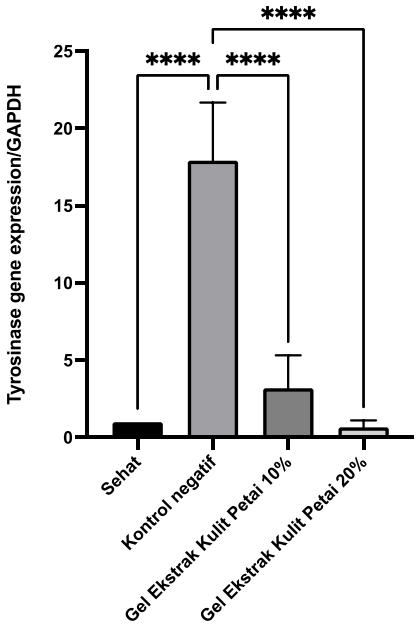

Figure 2. Tyrosinase gene expression graph in the entire study group

Effect of Petai Bark Extract Gel on Tyrosinase and TRP1 Gene Expression (In *Vivo* Experimental Study on Hyperpigmented Wistar Mice Exposed to UVB Model)

Table 2. *Post hoc Tamhane* test of tyrosinase gene expression in each group

Group	Comparison Group	Sig.	95% confidence interval	
			Lower Limit	Upper Limit
K1	K2	0,001*	-43,285	-29,827
	K3	0,203	-9,807	1,927
	K4	0,288	-3,243	0,827
K2	K3*	0,001*	25,642	39,590
	K4	0,001*	28,864	41,839
K3	K4*	0,513	-2,884	8,348

The * sign (p<0.05) indicates a significantly different group.

Based on the data above, the average comparison between the K2 group with K3 (0.001) and K2 with K4 (0.001) showed a significant difference, while the comparison between the K3 and K4 groups (0.513) there was no significant difference. In the comparison of K1 and K2, a value of 0.001 (p <0.05) was obtained so that there was a significant difference between the two groups. The most significant decrease in tyrosinase gene expression was shown in the administration of 20% petai skin extract with a lower limit value of -2.884 and an upper limit value of 8.348. The results *of Tamhane's post hoc* test on tyrosinase gene expression data showed that the administration of petai skin extract gel that can reduce tyrosinase gene expression in male rats of wistar strain hyperpigmentation model

In this study, researchers found that petai skin extract gel was able to reduce TRP1 gene expression in hyperpigmented mice significantly dose-dependent model (Table 1) Based on the results of the study shown in Table 1. The average expression of the TRP1 gene in the K4 group (2.22±1.18) was the lowest, then followed by the average expression of the TRP1 gene in the K3 group (4.96±3.42). The highest TRP1 gene expression data was in the negative control group of 35.91±4.52. The TRP1 gene expression data of groups K1, K2, and K4 are normally distributed, but the K3 group data (p<0.005) are not normally distributed based on *Shapiro Wilk*'s results. The distribution of TRP1 gene expression data is abnormal, so non-parametric statistical analysis with *the Kruskal-Wallis* test produces a value of p = 0.001 (p < 0.05) so that there is a significant difference in the average expression of the TRP1 gene between the four groups. The results of *the significant Kruskall-Wallis* test were followed by the *Mann-Whitney* test to see which group was the most influential.

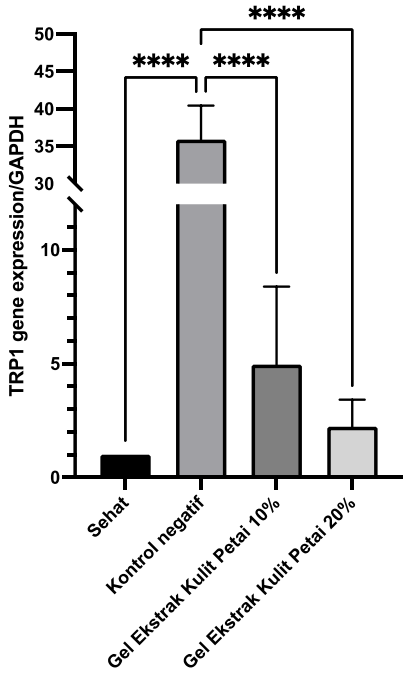

Figure 3. Graph of TRP1 gene expression across research groups

Effect of Petai Bark Extract Gel on Tyrosinase and TRP1 Gene Expression (In *Vivo* Experimental Study on Hyperpigmented Wistar Mice Exposed to UVB Model)

Table 3. *Mann-Whitney* TRP1 Test in Each Group

Group	Comparison Group	Sig.	95% confidence interval	
			Lower Limit	Upper Limit
K1	K2*	0,001	0,225	0,771
	K3	0,677	-2,538	0,135
	K4	0,272	-2,528	0,315
K2	K3*	0,000	-3,002	-2,887
	K4*	0,000	-2,992	-2,215
K3	K4	0,090	-1,373	1,563

The * 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) (0.000) and K4 (20% petai skin extract gel) (0.000) was obtained, which means that there is a significant difference, while K3 and K4 (0.090) 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 TRP1 gene expression value was most significantly shown in the administration of 20% petai skin extract gel with a lower limit value of -2.992 and an upper limit value of -2.215. The results *of the Mann-whitney* test on TRP1 gene expression data showed that the administration of petai skin extract gel that can reduce TRP1 gene expression in male rats of Wistar strain hyperpigmentation model.

IV. DISCUSSION

Exposure to UVB radiation is a major risk factor for skin hyperpigmentation, characterized by increased expression of melanin-forming enzymes such as tyrosinase, TRP1, and TRP1.[23,25] UVB radiation has been shown to increase oxidative stress due to DNA damage so that it can activate melanin formation pathways such as the *nuclear factor kappa beta (*NF-kB) pathway and *the melanocyte inducing transcription factor* (MITF) pathway.[19,26] In the process of melanogenesis, tyrosinase plays a role in converting L-tyrosine *into* L-3,4-dihydroxyphenylalanine *(L-DOPA)*.[27] L-DOPA is then oxidized to *L-DOPAquinone*, which then forms eumelanin and pheomelanin[28] Thus causing a blackish color on the skin. Recent research confirms that petai peel extract containing various secondary metabolites such as flavonoids, tannins, and saponins can suppress the formation of ROS due to its antioxidant activity.[17,21,29] The extract's ability to suppress ROS could potentially prevent melanin production.[18]

This study aimed to determine the effect of petai skin extract gel administration on tyrosinase and TRP1 gene expression in mouse hyperpigmentation models. Test animals were exposed to UVB radiation at 302 nm with an energy intensity of 390mJ/cm2 three times a week for two weeks. The results showed that in the negative control group there was an increase in the amount of melanin in mice up to 46.5%. In the administration of petai peel extract gel, it was shown to reduce the expression of tyrosinase and TRP1 genes in the K3 (petai peel extract gel 10%) and K4 (petai fruit peel extract gel 20%) groups significantly compared to the hyperpigmentation control group (negative control). This suggests that petai bark extract gel can prevent hyperpigmentation through the mechanism of lowering the expression of tyrosinase and TRP1 genes.

Secondary metabolite compounds derived from petai fruit peel extract such as flavonoids, phenolics, saponins, tannins and terpenoids are thought to inhibit MITF activity through inhibition of the PI3K/Akt pathway.[4,19,20] Previous research has also reported that flavonoid compounds can inhibit TGF-β via the cAMP/protein kinase A pathway and induce GLI2, then suppress MITF, a central transcription factor in melanogenesis.[30] Inhibitory MITF will prevent the synthesis of tyrosinase enzyme, so L-tyrosine cannot be converted into L-DOPA. Decreased L-DOPA levels were also reported to decrease TRP1 and TRP2 expression.[24] Secondary metabolite compounds in extracts such as flavonoids and phenolic compounds are reported to inhibit TGF-β thereby suppressing melanogenesis by signaling through specific ligand heteromeric receptors, namely serine/threonine kinase receptors that phosphorylate and activate (R)-SMAD receptors. This leads to the formation of complexes with (Co)-Smad, Smad4, and transcriptional regulation of target genes, ultimately suppressing the expression of melanogenesis-regulating enzymes such as tyrosinase, TRP1, and TRP2.[4,23]

Petai bark extract inhibits tyrosinase, consequently inhibiting the activity of TRP1 and TRP2 which correlate with the pathways of eumelanin and pheomelanin formation. Excessive suppression of TRP1 expression can inhibit melanin synthesis. Previous research reported that increased TRP1 expression correlated with increased melanin due to UVB irradiation.[23,31] However, increased TRP2 expression is associated with melanoma cell proliferation. In this study, significant and dose-dependent application of petai peel

extract gel prevented hyperpigmentation. This showed that gel application of petai bark extract decreased the expression of tyrosinase and TRP1 genes, thereby preventing melanin formation in melanocyte cells.

The limitation of this study is the lack of examination of ROS and MITF levels after application of petai fruit peel extract gel, so that the direct molecular mechanism of the extract regarding the prevention of melanin production is still unknown.

V. CONCLUSION

1. Pemberian ekstrak kulit petai pada dosis 10% dan 20% secara bermakna berpengaruh terhadap penurunan ekspresi gen tyrosinase pada tikus jantan galur Wistar model hiperpigmentasi yang diinduksi sinar UVB.
2. Pemberian ekstrak kulit petai pada dosis 10% dan 20% secara bermakna berpengaruh terhadap penurunan ekspresi gen TRP1 pada tikus jantan galur Wistar model hiperpigmentasi yang diinduksi sinar UVB.

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