IJSCIA

International Journal of Scientific Advances
ISSN: 2708-7972
Volume: 4 | Issue: 3 | May – Jun 2023 Available Online: www.ijscia.com
DOI: 10.51542/ijscia.v4i3.12

The Administration of 15% Sensitive Plant (Mimosa pudica) Leaf Extract Cream Inhibited the Increasing of Tyrosinase Enzyme and the Amount of Melanin in Male Guinea Pig (Cavia porcellus) Skin Exposed to Ultraviolet B

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ABSTRACT

Background: The leaf of sensitive plant (*Mimosa pudica Linn*) has a fairly high content of flavonoids, which work in inhibiting the tyrosinase enzyme. Changes in the formation of this enzyme will cause the melanin pigment to be disrupted, resulting in the appearance of skin pigmentation disorders (hyperpigmentation). The purpose of this study was to prove the effectiveness of 15% sensitive plant leaf extract cream on guinea pig skin exposed to ultraviolet B (UVB). ***Methods****:* This is true experimental research using randomized post-test only control group design. The subjects were 30 male guinea pigs randomly divided into 3 groups, namely the control group (K), treatment group 1 (P1), and treatment group 2 (P2). Control group was not given any treatment, P1 was smeared with basic cream and exposed to UVB and P2 was smeared with 15% sensitive plant leaf extract cream and exposed to UVB for 2 weeks. After 2 weeks, the tyrosinase enzyme level were examined using ELISA and melanin amount were examined using Masson Fontana staining. The result was analyzed using comparative test between groups. ***Results****:* The tyrosinase enzyme in P2 group was 5,44±0,63 ng/ml, which is lower than K group (8,60±0,16 ng/ml (p<0,001)) and P1 (12,96±2,21 ng/ml (p<0,001)). The mean melanin amount in P2 was 0,09±0,07 %, which was also lower than K (0,31±0,24 % (p=0,049)) and P1 (0,53±0,47 % (p=0,043)). ***Conclusion:*** Administration of sensitive plant leaf extract cream inhibited the increase in tyrosinase enzyme and melanin amount in guinea pig exposed to UVB and normal subjects.

Keywords: sensitive plant; tyrosinase; melanin; UVB

INTRODUCTION

One of the skin problems caused by UVB is hyperpigmentation which appears due to an increase in skin pigment substances. Hyperpigmentation is a condition in which dark patches appear on the outer skin due to excessive melanin production. One of the reasons for local or spot pigmentation is due to an increase in the uneven distribution of melanin.[1] There are several mechanisms that can affect melanin formation, including inhibition of melanin migration, inhibition as an antioxidant agent and direct inhibition of tyrosinase.[2]

Tyrosinase is the main key in the process of melanin synthesis. The mechanism starts from the hydroxylation of the amino acid tyrosine by the enzyme tyrosinase to L-DOPA. Then L-DOPA is oxidized to DOPAquinone, and DOPAquinone is converted to DOPAchrome. DOPAchrome will be converted by the enzyme tyrosinase to DHI (5,6-dihydroxyindole) or catalyzed by the enzyme DOPAchrome taumerase (TRP2) to DHICA (5,6-dihydroxy-indole-2-carboxylic acid).[3,4]

Melanin is produced in melanosomes which are present in the basal layer of the epidermis. Despite having a light-shielding function in human skin, hyperpigmentation can cause aesthetic problems. Misuse of hazardous materials such as mercury, as a skin whitening agent, will cause the skin to turn black and trigger skin cancer. Currently there are many cosmetic developments from natural ingredients that are focused on inhibiting the activity of the tyrosinase enzyme as a melanin inhibitor. These materials can be compounds derived from plants with the potential to cause side effects relatively smaller than chemicals.[5]

One of the natural ingredients is the sensitive plant (Mimosa pudica), which is commonly found in environments that have a tropical climate in Indonesia.[6] This plant has a fairly high flavonoid content, especially in the leaf.[7] Apart from flavonoids, there are many other active ingredients such as mimosine, terpenoids, glycosides, alkaloids, quinines, phenols, tannins, saponins and komarins.[8]

Flavonoid has the ability to depigment the skin. In the process of melanogenesis, flavonoids directly inhibit tyrosinase activity.[9]

The results of phytochemical analysis data from sensitive plant leaf that were carried out at Biochemistry Laboratory of the Faculty of Agricultural Technology, Udayana University showed that the leaf extract contained 30,280 mg/100 mg of flavonoids, which is higher than the flavonoid content in red pomegranate extract of 238.63 mg/100mg. The results of the study by Siahaan et al., proved that the content of flavonoids in pomegranate extract can prevent an increase in the amount of melanin [10]. This indicates that the sensitive plant leaf extract can inhibit the levels of the tyrosinase enzyme and the amount of melanin. The purpose of this study was to find out and analyze the benefits of the sensitive plant leaf extract on the tyrosinase enzyme and the amount of melanin.

METHODS

Study Design and Experimental Animals

This research is a descriptive quantitative using the true experiment method with a randomized posttest only control group design. The experiment was carried out at the Integrated Biomedical Laboratory of Drug and Experimental Animal Development of Udayana University. The sample needed in this experiment was 27 male Wistar rats (n=9), 3-4 months old, weighing 300-350 grams. To anticipate drop out, 10% of total sample were added, with the total amount to 30 rats divided into 3 groups: control and treatment group 1 and 2 (n=10). This research has been approved by the ethics commission of Udayana University, Bali, (B/80/UN14.2.9/PT.01.04/2022).

Sensitive plant (Mimosa pudica) Leaf Extract Production Preparation of the extract and examination of the phytochemical ingredients of sensitive plant leaf were carried out at the Faculty of Agricultural Technology, Integrated Services Laboratory, Udayana University, while the cream extract was processed at PT. Nekhawa Ubud, Bali. 1 kg sensitive plant were obtained from a hydroponic medicine garden village in Surabaya. The obtained sensitive plant leaf was cleaned and made into powder. 1000 grams of the powder was then added with 96% ethanol and incubated for 24 hours. After 24 hours, it was filtered with Whatman filter paper, resulting in dregs of filtrate. At 45 degrees, the ethanol dregs were combined and evaporated to obtain 200 grams of ethanol extract in the form of a paste. The process of making the sensitive plant leaf extract into a base cream by providing a cream-based composition was as followed: Add Sepigel 305 as an emulsifier with a concentration of 10%, then add 2% lanol, 2% dimethicone and 0.5% phenoxyethanol and continue mixing until the ingredients become cream.

Experimental Animal Treatment

Guinea pigs used as experimental animals were adapted for 7 days before treatment and then randomly divided into 3 groups. All guinea pigs were shaved on their backs. In the control (K) group, the guinea pigs were not given any treatment. In treatment group 1 (P1), the guinea pigs were smeared with basic cream and exposed to UVB. In treatment group 2 (P2), the guinea pigs were smeared with 15% sensitive plant leaf cream extract and exposed to UVB. The UVB was given at a dose of 65mJ/cm2 for 65 seconds for 2 weeks. The topical ingredient was applied 20 minutes before UVB exposure and repeated after 4 hours. The cream was still applied twice a day to the guinea pigs even without UVB irradiation.[11] After 48 hours of last irradiation, the guinea pigs skin samples were taken to prevent the effects of acute irradiation. Anesthesia used was the combination of 12mg/kg BW ketamine and xylacine 1.5.[12] The tissue was taken using punch biopsy method to examine the tyrosinase enzyme level using the ELISA method and to examine the amount of epidermal melanin. The guinea pigs were then given back to the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Udayana University.

Tyrosinase Enzyme Level Examination

The tyrosinase enzyme level was examined using BT Lab brand ELISA kit, Cat No E0098Gp with following steps: (1) Crush approximately 2 mg of skin tissue, homogenized in 1 ml of PBS and place the tube in ice. (2) The supernatant was obtained by centrifugation at 12000 rpm for 10 minutes. (3) Preparation of standard solutions (4) Add 50µl of standard solution to each well plate. (5) Add 40µl of sample to the well plate and add 10µl of anti-TYR antibody then add 50µl of streptavidin-HRP to the well plate and standard well (not in the control well). (6) Incubate for 60 minutes at 37°C (well covered with sealer). (7) The sealer was opened and the well was washed 5 times with wash buffer, leaving it for 1-2 minutes each washing. (8) Add 50µl Substrate Solution A to each well then add 50µl Substrate Solution B. (9) Incubate for 10 minutes at 37°C, cover with a plastic seal. (10) Add 50µl Stop Solution to each well. The blue color will change to yellow. (11) Determined by optical density (OD) and read by ELISA reader.

Melanin Amount Examination

A. Preparation of histological preparations:

(1) Immobilization stage. Soak the back skin tissue in 10% formalin phosphate buffer for 24 hours then cut the tissue and soak it in graded alcohol. (2) Dehydration stage. Soak the skin tissue in 30% or 40% alcohol, 50%, 70%, 80%, 90%, 96%, 100% 3 times for 25 minutes each. (3) Washing stage, using wipes to clear and compare alcohol:xylene (1:1) time range of 2 x 120 minutes. (4) Embedding step begins with a 60-minute infiltration process with pure paraffin, the tissue was embedded in melted paraffin and formed into blocks for 1 day to facilitate microtome sectioning. (5) Cutting stage, using a 5 mm thick Leica 820 microtome, then apply the tissue to an adhesive-coated glass object, dry, and finally stain it with Masson-Fontana.xt

B. Masson-Fontana staining:

(1) Deparaffinization stage by immersing the slide in xylane twice for 5 minutes each. (2) Rehydration stage: Soak tissue slides in 100% alcohol, twice for 5 minutes. Then soak in 95% and 70% alcohol, twice for 3 minutes respectively. Lastly, soak tissue slides in water twice for 3 minutes. (3) Use silver solution 56C with heating technique for 1 hour. Then examined through a microscope until the tissue looks black. (4) Use distilled water (dH2O) to wash 4 times for 1 minute each, and drip 0.2% Gold Chloride solution, leave for 5 minutes. (5) Use distilled water to wash then add 5% Sodium thiosulfate, leave for 5 minutes. (6) Wash using distilled water and paint using 0.1% Nuclear Fast Red for 5 minutes. (7) Wash using distilled water 4 x 1 minute. (8) Place the tissue slide in 95% alcohol for 1 minute. (9) Place the tissue slide in absolute alcohol for 5 minutes. (10.) Insert tissue slides in xylane for 2 x 5 minutes. (11) Mounting with entellan. (12) Cover using a cover glass.

C. Observation and Calculating Melanin:

Digital fast analysis can calculate the amount of melanin, prepare histologic preparations on objects photographed using the optilab Pro camera and Olympus Cx40 Microscope and magnify 40x focus on the object. Photos taken 3x (right side, center side, and left side of the preparation).

Taking the melanin dominant field of view and indicating the use of black areas. The result will be saved in JPEG format. With Adobe Photoshop CS3 version 9.0 software, using the magic wand feature of the Adobe Photoshop CS3 program, turn the melanin black. Then use the inverse function to select non-black pixels and the delete function to delete them so that the remaining pixels become black. The amount of melanin was calculated using Image J software as a percentage of the black melanin pixel area by comparing the pixel areas across the grid. Total melanin= ∑pixel area melanin/ ∑pixel total area epidermal x 100%.

Statistical Analysis

Statistical analysis was performed with SPSS Version 22. Normality test was assessed using Shapiro-Wilk test and homogeneity test was assessed with Levene's test. Comparability test was assessed using Anova T-test when the data is normal and homogeny, and Mann-Whitney test when it is abnormally distributed.

RESULTS

Normality test on tyrosinase enzyme and melanin expression for each group was done using Shapiro-wilk test, presented on Table 1. Based on Table 1, it was concluded that the data was normally distributed (p> 0.05).

TABLE 1: Normality Test.

Group	n	*p*	Desc
Tyrosinase Enzyme			
Control	10	0.065	Normal
P1	10	0.608	Normal
P2	10	0.801	Normal
Melanin Amount			
Control	10	0.200	Normal
P1	10	0.416	Normal
P2	10	0.295	Normal

p = significance >0.05

Levene homogeneity test is presented in Table 2. Based on Table 2, it was concluded that the data was not homogeny (p < 0.05) and a Post Hoc test was carried out with Dunnett T3 test.

TABLE 2: Homogeneity Test.

Variables	F	p	Desc
Tyrosinase Enzyme	8.56	0.001	Not homogeny
Melanin Amount	11.15	<0,001	Not homogeny

p = significance >0.05

Comparability test on average level of tyrosinase enzyme between groups using One Way Anova test and presented in Table 3.

TABLE 3: Comparability Test of Tyrosinase Enzyme.

Subject Group	n	Mean Tyrosinase Enzyme	SD	F	p
Control	10	8.60	0.16		
P1	10	12.96	2.21	80.79	<0.001
P2	10	5.44	0.63		

Analysis of comparative test in Table 3 showed the average tyrosinase enzyme level in control group was 8.60±0.16 ng/ml, P1 was 12.96±2.21 ng/ml and P2 was 5.44±0.63 ng/ml. Analysis of significance with One Way Anova test showed the value of F=80.79 and p<0.001, meaning that the mean levels of tyrosinase enzyme in the three groups were significantly different (p<0.05).

To find out which groups were different from the control group, it was necessary to carry out Post Hoc test with the Dunnett T3 test. The test results are presented in Table 4.

TABLE 4: Post Hoc Test.

Groups	Mean Difference	p	Desc
Control and P1	-4.36	<0.001	Significantly different
Control and P2	3.16	<0.001	Significantly different
P1 and P2	7.52	<0.001	Significantly different

Table 4 showed that the mean tyrosinase level in P1 was higher than control group, the mean level in P2 was lower than the control group and the mean level in P2 was lower than P1.

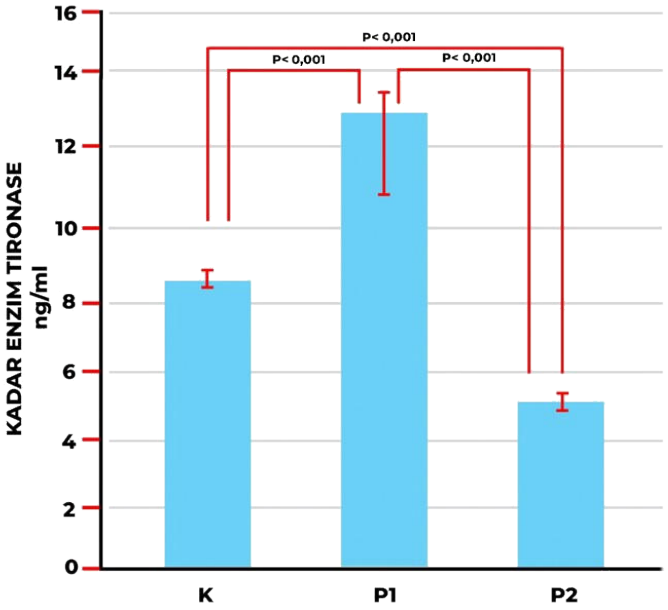


FIGURE 1: Comparison of Tyrosinase Enzyme between Groups.

Comparability test on average level of mean amount of melanin between groups using One Way Anova test and presented in Table 5.

TABLE 5: Comparability Test of Melanin Amount.

Subject Group	n	Mean Melanin	SD	F	p
Control	10	0.31	0.24		
P1	10	0.53	0.47	5.20	0.012
P2	10	0.09	0.07		

Analysis of comparative test in Table 5 showed the average melanin amount in control group was 0.31±0.24 %, P1 was 0.53±0.47 % and P2 was 0.09±0.07 %. Analysis of significance with One Way Anova test showed the value of F=5.20 and p=0.012, meaning that the mean melanin amount in the three groups were significantly different (p<0.05).

To find out which groups were different from the control group, it was necessary to carry out Post Hoc test with the Dunnett T3 test. The test results are presented in Table 6.

TABLE 6: Post Hoc Test.

Groups	Mean Difference	p	Desc
Control and P1	-0.22	0.491	Insignificantly different
Control and P2	0.23	0.049	Significantly different
P1 and P2	0.45	0.043	Significantly different

Table 6 showed that the mean melanin amount in P1 was higher than control group, the mean amount in P2 was lower than the control group and the mean amount in P2 was lower than P1.

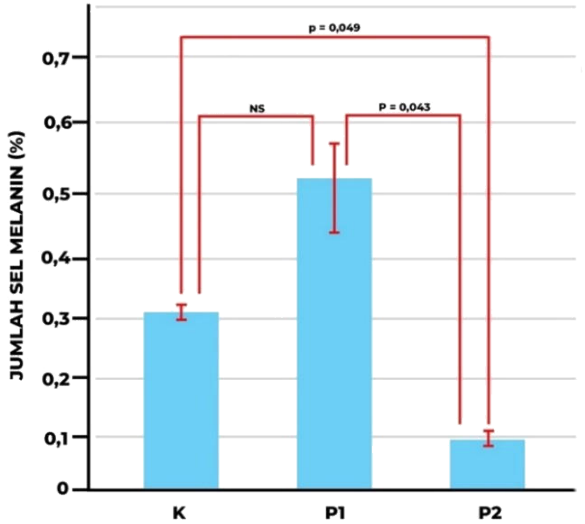
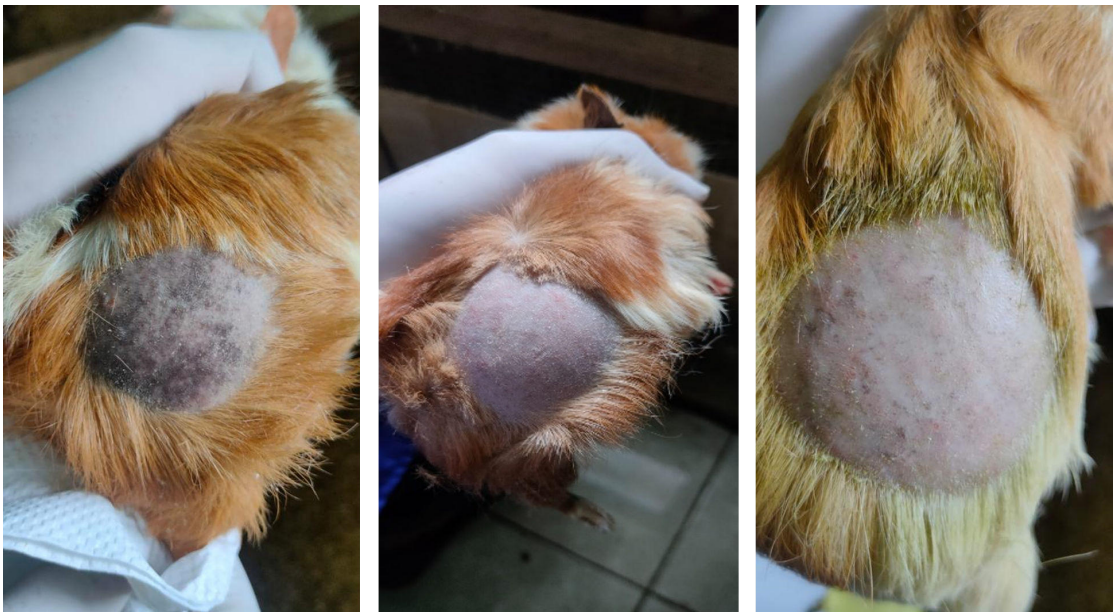


FIGURE 2: Comparison of Melanin Amount between GroupsMacroscopic and Microscopic Features of Guinea Pig Skin Melanin Expression.

Changes in the macroscopic appearance of the guinea pig skin were seen after 2 weeks of treatment. The skin on the back of the guinea pig was shaved before the punch biopsy was performed. It was seen that the skin of the guinea pig in group K was reddish white and in group P1 there were black spots on the skin.

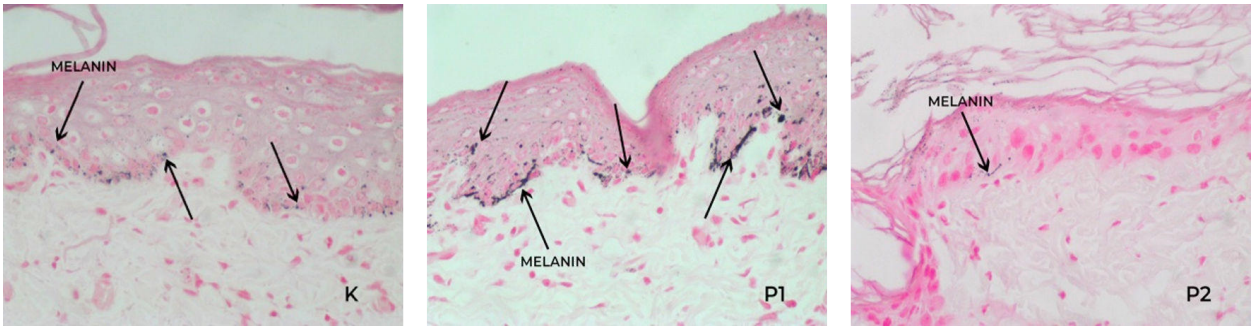
Whereas in the P2 group, the guinea pig skin color was reddish white with a few black spots. Comparison of guinea pig skin between the control group, group P1 and group P2 is presented in Figure 3.



Control (K) Treat 1 (P1) Treat 2 (P2)

FIGURE 3: Macroscopic Comparison of Guinea Pigs Skin between Groups.

Changes in the histopathological microscopic picture were also seen in the 40x magnification microscopic image focused on the object. Melanin that had been stained with Masson-Fontana stain would appear black. In the K group picture, black spots appeared on the basal layer and in the P1 group there were more black spots along the basal layer. Whereas in the P2 group, there were no black spots in the basal layer. Comparison of microscopic images between the control group, P1 group and P2 group is presented in Figure 4.



Control (K) Treat 1 (P1) Treat 2 (P2)

FIGURE 4: Macroscopic Comparison of Melanin Amount with Masson Fontana Staining.

DISCUSSION

Scientifically, sensitive plants are known as plants that contain lots of flavonoids. This study proved that the leaf of the sensitive plant can be used to inhibit the levels of the tyrosinase enzyme and the amount of melanin in the skin. The research showed that the administration of 15% sensitive plant leaf extract cream can inhibit the increase in tyrosinase enzyme levels, with an average of 5.44 ng/ml after exposure to UVB, which was lower from the treatment group 1, with an average tyrosinase enzyme level of 12.96 ng/ml. In addition, the average tyrosinase enzyme level in the treatment group 2 was lower than that in the control group (K), which was 8.6 ng/ml ($p< 0.001$), meaning there are significant differences in the levels of the tyrosinase enzyme in the various treatments (K, P1, and P2).

Inhibition of tyrosinase activity (monophenolase and diphenolase) reduces melanin synthesis.[13] This is also supported by the research which showed that after the administration of 15% sensitive plant leaf extract cream and exposure to UVB, an average amount of 0.09% melanin was obtained, which was lower than the treatment group 2 (0.53%). In addition, the average amount of melanin in the treatment group 2 was lower than that of the control group, which was 0.31% ($p\leq 0,05$).

The phytochemical content in sensitive plant leaf used in this study were flavonoids 30280.75 mg/100g, phenols 6076.07 mg/100g and tannins 401.27 mg/100g. When compared with the flavonoid content in red pomegranate extract of 238.63 mg/100 mg, this study gave results that

significantly inhibited the increase levels of the enzyme tyrosinase and the amount of melanin in the skin.

However, in this study, the average levels of the tyrosinase enzyme and the amount of melanin in group P2 were lower than in group K. If the tyrosinase enzyme and the amount of melanin were large, the skin color would be darker; conversely, if the body had little melanin, the skin color would appear paler. This can lead to the risk of hypopigmentation, which is a condition where there is a skin tone that is lighter than the overall skin color. Hypopigmentation has a higher risk of developing skin cancer because the skin will be more susceptible to damage from ultraviolet light exposure. In addition, psychological problems can also be a risk for hypopigmentation, such as experiencing insecurity and social anxiety due to uneven skin appearance.

The positive side effect of giving the sensitive plant (Mimosa pudica) Leaf Extract Cream is as a skin barrier which is proven because it has components in the form of tannins, lactones, flavonoids, saponins, and many oligo elements which have anti-inflammatory, analgesic, and antiseptic effects). The negative side effect of giving sensitive plant leaf extract in the form of cream on the skin is the occurrence of an allergic response. Forms of allergies that can arise immediately in the form of acute spontaneous urticaria and irritant contact dermatitis immediately up to < 48 hours of use.[14]

The study's results by Rivera-Arce et al. (2007) did not find any risk of giving the Sensitive Plant (Mimosa pudica) Leaf Extract Cream to normal people; this is because none of the study respondents had a history of allergies or sensitive skin.[15] The same thing was also done by research by Villarreal et al. (1991).[16] The toxicity of Sensitive Plant (Mimosa pudica) Leaf Extract was not found in a Sci-Finder, PubMed, Science Direct, or Google Scholar data search for the period 1990 to 2020 by Kumar (2021).[17]

Currently hydroquinone is the gold standard in the treatment of skin hyperpigmentation. However, hydroquinone is often misused as an additional bleach in cosmetic products to attract consumer attention. Long lasting effects that occur when hydroquinone is administered without close supervision for medical purposes are skin cancer, impaired kidney and liver function due to the presence of hydroquinone which accumulates in the body.

The results of this study showed that there was a significant difference in the levels of the tyrosinase enzyme and melanin expression caused by the administration of 15% sensitive plant leaf extract. Topical use of 15% sensitive plant leaf extract is expected to be able to inhibit the negative effects due to continuous exposure to UVB on the skin.

CONCLUSION

Administration of 15% sensitive plant (Mimosa pudica) leaf extract inhibit the increase of tyrosinase enzyme and melanin amount in guinea pigs exposed to UVB.

Conflict Of Interest

All researchers declare that there is no conflict of interest related to this article.

Funding

This research is self-funded.

Author's Contribution

All authors contribute equally in compiling this research article.

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