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Physical Stability and Antihyperpigmentation Activity of Berenuk (*Crescentia cujete* Linn) Leaves Ethanol Extract in Cream with Variations of Cremophor RH40 as Surfactant

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ABSTRACT

Indonesia has high exposure to sunlight which can cause skin problems such as hyperpigmentation. Treatment of hyperpigmentation with hydroquinone can cause side effects. Therefore, it is necessary to develop antihyperpigmentation products from natural ingredients to minimize these side effects. Berenuk leaves (*Crescentia cujete* Linn) have antioxidant activity with an IC50 value of 80.21 µg/ml in ethanol extract. This antioxidant compound can be used to treat hyperpigmentation problems. In its use, Berenuk Leaf Ethanol Extract (BLEE) is formulated in a cream dosage form. Cremophor RH40 is a type of nonionic surfactant that can affect the physical stability of cream. This study aims to determine the effect of Cremophor RH40 on the physical stability of cream preparations and their antihyperpigmentation activity. Cream preparations were made with varying concentrations of Cremophor RH40 5% (F1), 10% (F2), 15% (F3). Physical stability evaluation was tested using the cycling test method. Data were analyzed statistically with paired samples t-test. Analysis of antihyperpigmentation test data in the form of the amount of melanin in all test groups was analyzed for differences using Kruskal Wallis and continued with the Mann-Whitney test. BLEE cream's physical stability test result show that the most stable formula is formula 1 because it is stable up to the 5th cycle. in contrast, formula 2 only goes up to the 3rd cycle, and formula 3 in the first cycle has experienced 2-phase separation. BLEE cream (F1) has antihyperpigmentation activity because it has an average melanin intensity less than the negative and positive controls.

Keywords : Antihyperpigmentation, Berenuk Leaves, Cremophor RH40, Cycling test

INTRODUCTION

Indonesia is a tropical country where the possibility of skin exposure to sunlight is very high. One of the most dangerous components exposed to the sun is ultraviolet (UV) light. UV rays are classified into several types, namely photon lengths. UVA and UVB radiation UV-A, UV-B, and UV-C, which have different are considered the types of UV light that can cause pigmentation on the skin. UVB light will affect melanocyte proliferation through the mechanism of increasing the synthesis of alpha melanocyte stimulating hormone (α-MSH) (Saputra et al., 2021). This process will increase melanin synthesis by activating the tyrosinase enzyme, causing skin pigmentation disorders or hyperpigmentation (Vashi et al., 2017). One topical hyperpigmentation treatment often used is hydroquinone (Desai, 2014). Hydroquinon (1,4-dihydroxybenzene, HQ) has

been the gold standard for treating hyperpigmentation for over 50 years and has been successfully used to treat melanosis. The mechanism of action of hydroquinone as a skin lightener is by enzymatically inhibiting the oxidation of tyrosine to 3,4-dihydrophenylalanine (DOPA), inhibiting the activity of the enzyme tironase in melanocytes and reducing the amount of melanin directly (Lestari, 2017). However, long-term hydroquinone use can cause skin irritation, redness and ochronosis (Fabian et al., 2023). Therefore, it is necessary to develop products with natural ingredients to treat hyperpigmentation and minimize side effects.

Berenuk (*Crescentia cujete* Linn) is one of the natural ingredients that can be formulated as a preparation. Berenuk is often found on the roadside, but its utilization in the health sector is still very rare. Even though many parts of the berenuk plant contain compounds that can be useful (Atmodjo, 2019), one of the most utilized parts is the leaf. Berenuk leaves contain many secondary metabolites in the form of alkaloids, flavonoids, saponins, tannins, and steroids (Hasanah et al., 2017). Berenuk leaves in the form of ethanol extract have antioxidant activity, as evidenced by the IC50 value of 80.21 µg/ml (Das et al., 2014). Antioxidants can act as ROS scavenger so as to inhibit the hyperpigmentation process. Antioxidants can prevent oxidation reactions, by giving their electrons to free radical molecules. Hyperpigmentation can be prevented by antioxidants through the mechanism of inhibiting ROS (Reactive Oxygen Species) (Widyastuti et al., 2020). These antioxidant compounds can counteract free radicals and other problems, including antihyperpigmentation. Antioxidant compounds such as antihyperpigmentation work by inhibiting the oxidation reaction of the tyrosinase enzyme. Antioxidant compounds can also inhibit tyrosinase gene transcription and help reduce melanogenesis so as to reduce hyperpigmentation. (Maack and Pegard, 2016). Ethanol extract of berenuk leaves can be developed into dosage forms such as creams, because creams have good dispersibility on the skin, do not cause blockage of the skin and release active substances (Voight, 1994).

The cream is a preparation consisting of two phases, water and oil, so the manufacture of cream requires surfactants to maintain the physical stability of the cream. The physical stability of the preparation is an important parameter that must be tested to determine the quality of the stability of the preparation. One of them influences the quality of a good cream preparation, namely the use of surfactants (Arifin et al., 2022). Cremophor RH 40 is a non-ionic surfactant that can be used in making cream preparations because it can increase the solubility of a substance and has low toxicity compared to other surfactants so that the level of irritation is also low (Wu et al., 2017). Based on this description, this study aims to determine the physical stability of berenuk leaf ethanol extract cream during six storage cycles and its effectiveness in antihyperpigmentation.

METHODS

Research Material

The material used in the preparation of Berenuk Leaf Ethanol Extract (BLEE) cream are Berenuk Leaf Ethanol Extract (BLEE), PEG-40 Hydrogenated Castor Oil (pharmaceutical grade), Cetostearyl alcohol (pharmaceutical grade), Isopropyl myristate (pharmaceutical grade), Butyl hydroxy toluene (pharmaceutical grade), Glycerin (pharmaceutical grade), Nipagin (pharmaceutical grade), Nipasol (pharmaceutical grade), and aquadest (pharmaceutical grade). Materials used in testing antihyperpigmentation activity are hydroquinone, masson-fontana reagents, Neutral Buffered Formaldehyd 10%.

The equipment used in the study included analytical balance (Ohaous), porcelain cup, stirring rod, mixer (Maspion), pH meter (HANNA J0045115), Brookfield viscometer, dispersibility test kit, adhesion test kit, climatic chember (HWS-70BX), glassware (iwaki), narrowband UV B 311 nm lamp (Phillips), Olympus CX41 microscope and Optilab Pro camera, surgical equipment namely surgical scissors, scalpel, tweezers and surgical pad,

equipment for making histology consists of a microtome, object glass, and cover glass.

The animals used in the study were male guinea pigs, 3 months old with a body weight of 300-350 grams from a farm located in Kendal, Central Java.

Preparation of BLEE Cream

Preparation of the oil phase by weighing Cremophor RH40, Cetostearyl alcohol, Nipasol, Butyl hydroxy toluene (BHT), and Isopropyl myristate (IPM), then put into a cup and heat on a water bath while stirring until homogeneous. Preparation of the water phase by weighing nipagin and glycerin then put them into a cup coupled with distilled water and heating them using a water bath. The water phase is put into a mortar and added to the oil phase (mixing is done at the same temperature). Stir until a creamy mass is formed, then BLEE is added and stirred until homogenized. BLEE cream formula with variation of Cremopor RH40 concentration F1(5%), F2(10%), F3(15%) can be seen in Table 1.

Table 1. BLEE Cream Formula

No.	Materials	Function	F1(%)	F2(%)	F3(%)
1	BLEE	Active ingredient	1	1	1
2	Cremophor RH40	Surfactan	5	10	15
3	Cetostearyl alcohol	Thickener	5	5	5
4	Isopropyl myristate	Oil phase	5	5	5
5	BHT	Antioxidan	0.05	0.05	0.05
6	Glycerin	Emollient agent	15	15	15
7	Metyl Paraben	Preservative	0.18	0.18	0.18
8	Propyl Paraben	Preservative	0.02	0.02	0.02
9	Aquadest	Water phase	up to 100	up to100	up to100

Physical Stability Test of BLEE Cream Preparations

The physical stability test of BLEE cream preparation was carried out using the cycling test method. BLEE cream was put into a refrigerator at 4°C for 24 hours, then put into a climatic chamber at 40°C for 24 hours so that one cycle occurred. After experiencing six cycles of cycling test, BLEE cream was tested again for its physical characteristics, including organoleptics, homogeneity, pH, viscosity, dispersibility and adhesiveness.

Antihyperpigmentation Activity Test of BLEE Cream

1. Dutch Guinea Pig Treatment

Adaptation of Dutch guinea pigs was carried out for one week. The fur on the back of the hamster was shaved to a size of 3 x 3 cm, and warm water was applied. The guinea pigs were exposed to 311 nm UVB light for two weeks for five minutes daily. Dutch mules were randomly divided into four groups. Group I was treatment; group II positive control was given hydroquinone; group III was given BLEE cream preparation (F1); and group IV negative control was given cream preparation without active substance. All treatment groups were given the test material at night for two weeks. The guinea pigs were euthanized on day 15 with chloroform, and then tissue biopsies were taken at a depth of about 2 mm (to the subcutaneous) at the pigmentation lesion with a length of 3 cm. The skin tissue was placed in a 10% neutral buffer formaldehyde solution, and then histological preparations were made to examine the melanin area (Hastiningsih, 2015).

2. Preparation of Histology Preparations

The first stage is fixation, where guinea pig skin tissue is soaked in 10% phosphate-buffered formalin solution for one day. Then the

tissue parts to be taken are trimmed. Then, the dehydration stage is carried out where the guinea pig skin tissue is immersed in graded alcohol respectively 30%, 40%, 50%, 70%, 80%, 90%, and 96%, each three times for 25 minutes. The next stage, namely clearing, is done by putting the tissue into the clearing agent (alcohol: xylene 1: 1) for 30 minutes and dipping it in pure xylene until it is transparent. The embedding stage is carried out after infiltration four times with pure paraffin. Then, the tissue is embedded into liquid paraffin, left to form a block (± 1 day) for easy slicing with a microtome. The process of cutting the tissue was carried out using a Leica 820 microtome, 5 µ thick serially, the 5, 10, 15 slices were taken and then attached to a glass object that had been smeared with adhesive and finally painted with Masson-Fontana (Hastiningsih, 2015).

3. Staining with Masson-Fontana

Tissues that still contain paraffin, deparaffinized (slides soaked in xylene for 2 times for 5 minutes each), then rehydrated (slides soaked in ethanol 100%, 95%, 70%, dH2O each for 2 minutes) slides soaked with Silver Nitrate Fontana solution for 2 hours and incubated at 56ºC in the oven. Then, the slides were washed using dH2O three times, dripped with 1% Gold Chloride solution, and allowed to stand for 5 minutes. The slides were washed using dH2O, treated with 5% Sodium thiosulfate solution, and allowed to stand for 1 minute. Then the slide was washed using dH2O and painted using Nuclear Fast Red for 5 minutes. Then, the slides were washed twice using dH2O and dehydrated using 70%, 95%, and 100% ethanol for 20 seconds each. Then, it was cleared using xylene twice for 2 minutes each and mounted on an xylene-based medium. The painting result is a black melanin granule with a pink cell nucleus and a pale pink-pale cytoplasm (Hastiningsih, 2015).

4. Procedure Result Observation

The total of melanin was calculated by digital analysis method, each preparation was photographed using an Optilab Pro camera and Olympus CX41 microscope with 400 times magnification, each preparation was photographed three times and saved in JPEG format. The photos were edited using Adobe Photoshop CS3 software version 10.01 to select epidermal tissue using the Polygonal Lasso tool, namely the preparation's left, center, and right sides. The field of view taken is the field with the most melanin marked with a black area (Hastiningsih, 2015).

5. Procedure for Melanin Area Calculation

Calculating the total melanin in pixel units was performed with ImageJ version 1.47t software using channel red by setting the threshold. The normalized amount of melanin was calculated based on the following formula per field of view (McMullen et al., 2010).

$$\text{Melanin area} = \frac{\text{pixel melanin}}{\text{pixel epidermis}} \times 100\%$$

Data Analysis

Analysis of physical stability data of BLEE cream was carried out paired sample t-tests to compare the preparation before and after stability testing. It is stated that Cremophor RH40 influences physical stability if the significance value is < 0,05. The antihyperpigmentation test data in the form of the amount of melanin in all test groups was analyzed for differences using Kruskal Wallis and continued with the Mann-Whitney test. It is stated that there is a difference if the significance value is < 0,05.

RESULTS AND DISCUSSION

Physical Stability

Berenuk leaf ethanol extract cream preparation with variations of Cremophor RH40 as a surfactant was tested for physical stability using the cycling test method. This method is carried out by applying pressure in extreme temperatures, such as 4ºC and 40ºC during storage, causing the product to experience damage or degradation (ASEAN, 2013). Parameters for the stability test include organoleptic test, homogeneity, pH, viscosity, dispersibility and adhesiveness. The results of the organoleptical and homogeneity tests can be seen in Table 2.

Table 2. Organoleptical test, physical stability of BLEE cream

No.		Organoleptical			
		Color	Odor	Texture	Homogeneity
	Before storage				
1	F1 (5%)	Light green	Berenuk leaves	Semisolid	Homogeneous
2	F2 (10%)	Light green	Berenuk leaves	Semisolid	Homogeneous
3	F3 (15%)	Light green	Berenuk leaves	Semisolid	Homogeneous
4			After storage		
5	F1 (5%)	Green	Berenuk leaves	2-phase separation	Inhomogeneous
6	F2 (10%)	Green	Berenuk leaves	2-phase separation	Inhomogeneous
7	F3 (15%)	Green	Berenuk leaves	2-phase separation	Inhomogeneous

Organoleptic testing was carried out by observing the preparation's color, aroma and texture. The results of the physical stability test of BLEE cream formulas 1, 2 and 3 did not experience changes in aroma, but formulas 1, 2 and 3 experienced changes in color after storage which was initially light green to become more concentrated. Likewise, the texture of the preparation was observed where formula 1 experienced 2-phase separation after the 5th cycle storage, formula 2 experienced 2-phase separation after the 3rd cycle storage and formula 3 experienced 2-phase separation after the 1st cycle storage. It can be caused by variations in the concentration of cremophor RH40 as a surfactant in each formula. The higher the concentration of cremophor RH40 used makes the preparation more unstable because cremophor RH40 is unstable in high-temperature storage.

Homogeneity testing of BLEE cream is carried out to determine whether all ingredients and active substances have been mixed well (Tari & Indriani, 2023). The homogeneity test results changed to be inhomogeneous after storage in formulas 1, 2 and 3 (Table 2). It is because in the three cream formulas there is a separation of 2 phases after storage, so the mixture is not homogeneous.

The pH test results of BLEE cream are shown in Figure 1. This pH measurement aims to determine whether the cream made is safe and does not irritate the skin when used. The pH range requirement of a good topical preparation is in accordance with the natural pH of the skin, which is 4.5-6.5 (Purwatiningrum, 2014).

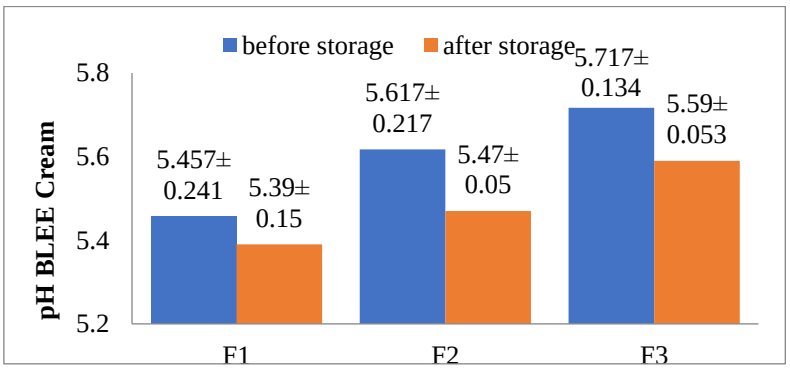

Figure 1. pH of BLEE cream

Increasing the concentration of Cremophor RH 40 in the formula did not give a significant change in pH value, because Cremophor RH 40 has a pH value that tends to

be neutral (Ataman-Chemicals, 2020). Data from the results (Figure 1) of the pH test of formulas 1, 2 and 3 before storage and after storage were analyzed using paired sample t-test statistics and showed a significant difference ($p < 0.05$). It shows that storage at extreme temperatures can affect the pH of the preparation (Suhery et al., 2016). Changes in the pH value of BLEE cream preparations during storage indicate a lack of stability (Putra et al., 2014). Storage at high temperatures results in hydrolysis, so the cream preparation has a decrease in pH (Magdalena et al., 2016). Based on the pH data (Figure 1), it still meets the criteria for the pH of the cream.

The results of viscosity testing (Figure 2). The viscosity test of BLEE cream aims to determine the amount of resistance produced by the cream. A good viscosity requirement for semi-solid preparations is 4000-40,000 cPs (Pratasik et al., 2019).

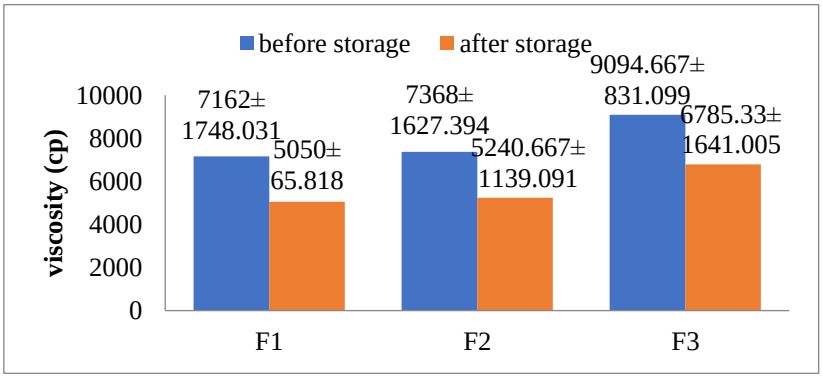

Figure 2. Viscosity of BLEE cream

The results of viscosity testing showed that the greater the concentration of cremophor RH 40 used, the greater the viscosity but did not increase significantly. The results of viscosity testing (Figure 2) of formulas 1, 2 and 3 before storage and after storage were analyzed using paired sample t-test statistics and showed a significant difference ($p<0.05$). After storage, both formulas 1, 2 and 3 experienced a decrease in viscosity. This happens because the high storage temperature causes melting of some ingredients in cream making, such as cremophor RH40 which has a melting point at 30ºC (Rowe et al., 2009). The high temperature obtained during storage will increase the distance between atoms so that the force between atoms will decrease, the distance becomes tenuous and causes the viscosity of the cream to decrease (Heroweti et al., 2023).

The results of the BLEE cream dispersibility test are shown in Figure 3. The dispersibility test aims to determine the ability of the cream base to spread so that it can be seen the ease of applying the preparation to the skin. Good dispersibility causes contact between the drug and the skin to be extensive, so that absorption into the skin takes place quickly (Pratasik et al., 2019).

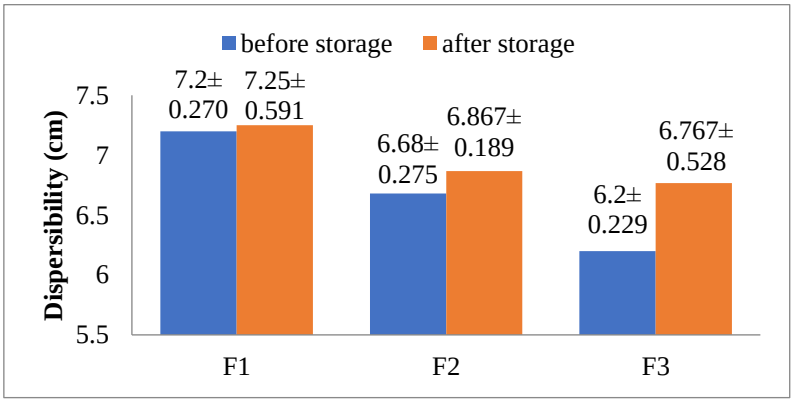

Figure 3. Dispersibility of BLEE cream

The results of testing the dispersibility of BLEE cream with variations in cremophor RH 40 concentration show that the higher the concentration of cremophor, the lower the ability to disperse. Dispersibility testing is carried out to see the ability of BLEE cream preparations to spread well on the skin during use. The greater the dispersibility of the preparation, the wider the active substance to diffuse into the skin. Dispersibility has an inversely proportional relationship with viscosity. The greater the dispersibility of the preparation, the smaller the viscosity (Rahmatullah et al., 2020). The results of the dispersibility test (Figure 3) analyzed using paired sample t-test statistics showed no significant difference ($p>0.05$) between the dispersibility of BLEE cream before storage and the dispersibility of BLEE cream after storage. BLEE cream preparations have increased dispersibility after storage and align with the theory that preparations that experience a decrease in viscosity have a high dispersion of the preparation.

Adhesion test results (Figure 4). Adhesion testing aims to determine the time required for the preparation to contact the skin surface and cause physiological effects and is related to the comfort of the preparation when used (Marviani & Rochman, 2021).

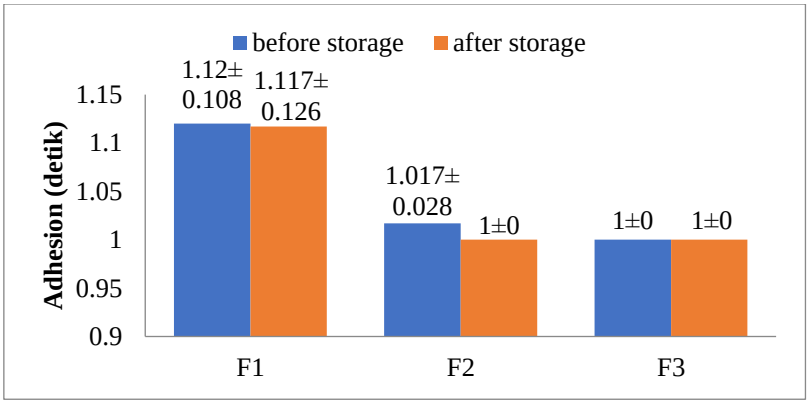

Figure 4. Adhesion of BLEE cream

The results of the BLEE cream adhesion test with variations in cremophor RH 40 concentration show that the greater the concentration, the smaller the adhesion. The test of adhesion aims to determine how long the contact time of the preparation is with the skin surface so that it causes a pharmacological effect. It is also related to comfort when the preparation is used (Marviani, T., & Rochman, 2021). The results of the adhesion test (Figure 4) of BLEE cream preparations analyzed non-parametric statistics with the Wilcoxon test showed no significant difference ($p>0.05$) in the adhesion of formulas 1, 2 and 3 of BLEE cream before storage and after storage. It shows that temperature does not affect the adhesion of the three formulas (Sari et al., 2021).

Antihyperpigmentation Test

The animals used for antihyperpigmentation testing in this study are guinea pigs because guinea pigs have biological similarities with humans and have several kinds of melanin. The test animals used were male guinea pigs to prevent the influence of the hormones Melanocyte Stimulating Hormone (MSH), estrogen and progesterone found in female guinea pigs (Fithria et al., 2017). The three hormones can stimulate melanin production, so it is feared that UV light stimulation can affect the process of melanin formation (Yamamoto et al., 2015).

Exposure to 311nm UVB light on the test animals was carried out on the skin of the guinea pig's back for two weeks so that the melanogenesis process occurs in the basal stratum so that melanin pigment is formed in the guinea pig's skin. The application of the preparation to the test animals was carried out for 2 weeks at night because the skin regeneration process naturally occurs at night so that the results obtained are maximized (Abdullah et al., 2022). The effectiveness of

antihyperpigmentation carried out in this study was seen from the integrated density of melanin cells in each treatment group in pixel units.

Based on histopathological preparations (Figure 5), which were analyzed in qualitative terms, it can be seen that there are differences in melanin intensity in the skin of test animals in each treatment group. The group without treatment has a high melanin intensity. It is not too much different from the group that is only given a cream base but the group given hydroquinone and the BLEE cream group (F1) have less melanin intensity then the group without treatment. Image j software analysis results in integrated density (Figure 6) values that express the number of melanin cell pixels from the skin of guinea pigs captured by masson fontana (Paquita et al., 2023).

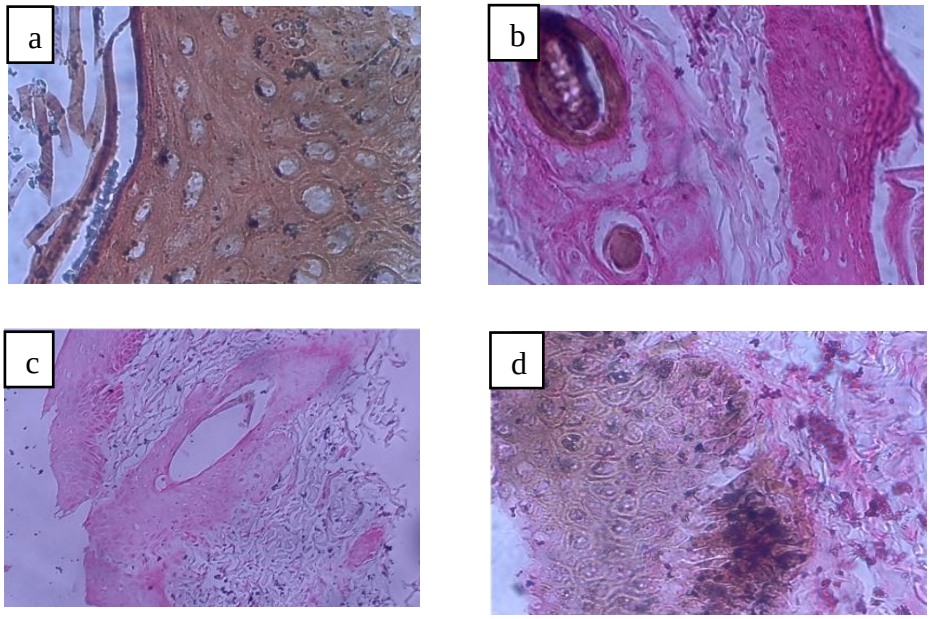

Figure 5. Histopathology Test Results. a (untreated), b (treated with hydroquinone cream), c (treated with BLEE F1 cream), d (treated with cream preparation without active substance)

The average integrated density of melanin analyzed by non-parametric statistics showed there was a significant difference between treatment groups ($p<0.05$), and the average intensity of melanin in the BLEE (F1) cream group was less than the negative control so that BLEE (F1) cream had effectiveness as antihyperpigmentation. Even the group given BLEE (F1) cream has better antihyperpigmentation activity than the positive control (hydroquinone cream).

According to previous research, flavonoid compounds are also known to inhibit the enzyme tyrosinase competitively, because they can bind to the active side of the enzyme so that it competes with its natural substrate, tyrosine (Nguyen et al., 2016). So, antioxidan compounds in berenuk leaf ethanol extract cream (*Crescentia cujete* Linn) can be used to treat antihyperpigmentation.

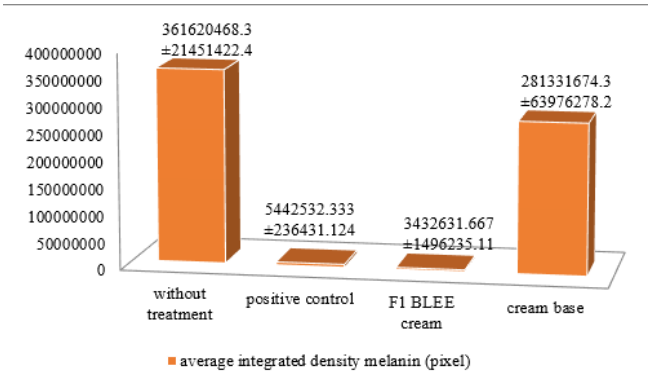

Figure 6. Average Integrated Density Melanin

CONCLUSION

The most physically stable berenuk leaf ethanol extract (BLEE) cream formula after testing with the cycling test method is F1 with a concentration of cremophor RH 40 as much as 5% which can be stable until the 5th cycle. BLEE cream is effective as an antihyperpigmentation with a concentration of berenuk leaf extract as an active substance of 1% and provides a better antihyperpigmentation effect than the positive control (hydroquinone cream).

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