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Thrombocyte Counts in Mice After the Administration of Chloroform Fraction of *Eleutherine palmifolia* L(Merr)

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Abstract. Dengue Hemorrhagic Fever (DHF) is a disease that can cause a decrease in the number of thrombocyte cells. Dayak onion bulbs (*Eleutherine palmifolia*) Is a Kalimantan plant that is thought to increase the number of thrombocyte cells in the blood. This study aims to determine the activity of chloroform fraction of *Eleutherine palmifolia* on the number of Thrombocyte cells. Experimental research using 25 animals divided into 5 groups, namely chloroform fraction extract dose of 50 mg/ KgBW, dose of 100 mg/ KgBW, dose of 200 mg/ KgBB, saline solution, and Psidii® syrup (positive control). Thrombocyte counts before and at 3 days after dosing revealed significantly higher mean counts after dosing with the as compared to the mean count at hour 0. There was only a non-significant rise of thrombocyte counts in the group having received saline solution in mice. The results showed the administration a dose of 100 mg/ KgBB was able to significantly increase Thrombocyte cells count in mice.

Keywords: Dengue Hemorrhagic Fever (DHF), *Eleutherine palmifolia*, Chloroform fraction, Thrombocyte

1. Introduction

Dengue Hemorrhagic Fever (DHF) is a disease caused by dengue virus which is transmitted through the bite of *Aedes aegyptii* mosquito. Reactions in the body due to the entry of the dengue virus are reduced Thrombocyte cells. Thrombocytes are part of several large cells in the bone marrow. Thrombocytes play an important role in the formation of blood clots [1], also function to prevent and treat bleeding in patients with thrombocytopenia [2]. The decrease in Thrombocyte count in the body (thrombocytopenia) causes the body to experience bleeding easily, so that more Thrombocytes are needed to repair damaged blood vessels [3].

Several studies of medicinal plants have been carried out as an effort to increase the number of Thrombocyte cells. One of them is Sundaryono (2011) research, stating that the administration of total flavonoid compounds from the stem of the plant *Jatropha multifida* L with a dose of 0.028g / KgBB orally in mice can increase the number of Thrombocyte cells by 543,000 / mm3. Other plants that contain flavonoid compounds are dayak onions (*Eleutherine bulbosa* Urb.). Dayak onion is one of the ornamental plants, the part of the plant that is commonly used is the part of the tuber and the leaves[4]. The content contained in dayak onion bulbs consists of flavonoids, saponins, polyphenols, alkaloids, glycosides, steroids, phenolics, tannins, triterpenoids and quinolones [5].

According to Noorrasidah (2016), the total flavonoid content of dayak onion bulbs calculated as quercetin in the ethyl acetate fraction had an average of 4.57 mg / 100 grams. Research by Muharni et al. (2013) states that flavonoids in the form of quercetin can inhibit the action of the reverse transcriptase enzyme which is a catalyst for viral replication and can increase the number of Thrombocyte cells in the blood. It is important to test the activity of active Dayak onion Bulb Isolate on increasing the number of Thrombocyte cells in male mice.

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2. Methodology

2.1 Materials and Animal Tests

2.1.1 Material

The material to be studied is dayak onion bulbs, the chemicals used are Na. 0.5% CMC, heparin, Psidii® syrup, EDTA, distilled water, 95% ethanol, methanol, ethyl acetate, n-hexane, chloroform, hydrochloric acid (HCl) 2N, amyl alcohol, Mg powder, iron (III) chloride (FeCl3) 1%, Acetic Acid Anhydrous, concentrated sulfuric acid (H2SO4), sodium citrate, NaOH, mayer reagents, dragendorf reagents, and bouchardat reagents. Supporting materials such as tissue, aluminum foil and filter paper.

2.1.2 Animal Test

The test animals used were white mice of male sex (*Mus musculus*). Test criteria were carried out by inclusion, male white mice aged 2-3 months and weighing between 20-40 g.

2.2 Research Procedure

2.2.1 Plant Determination

Plant determination is carried out to ensure that plant identity is used, so that errors in the collection of materials to be studied can be avoided. Determination of dayak onion plants that will be used in the study was conducted at the Laboratory of Physiology, Faculty of Mathematics and Natural Sciences, Mulawarman University, Samarinda.

2.2.2 Dayak Onion Extraction and Fractionation

Maseration extraction method was carried out using 95% ethanol. A total of 10 kg of simplicia powder of Dayak onion bulb (*Eleutherine bulbosa* Urb.) Which had been sieved with a mesh 40 sieve was macerated with a 95% ethanol solvent as much as 20 L (1:10) slowly stirred until the solvent soaked the entire dayak onion tuber powder, then macerated for 2 hours and soaked for 24 hours, then filtered using filter paper. Remaseration is done twice. The resulting maserate is then concentrated with a rotary evaporator at a temperature of 50^0C and then evaporated in a water bath until the total ethanol extract is obtained.

Dayak onion tuber extract obtained was then fractionated with a solid-liquid method using n-hexane, chloroform and ethyl acetate solvents in stages. Fractionation was carried out by adding n-hexane solvent to the extract with a ratio of 1:10 which was divided into 5 stirring times using the help of a magnetic stirrer for ± 10 minutes, then the filtrate was separated. The residue is fractionated again using chloroform and ethyl acetate solvent sequentially with the same work procedure. Furthermore, the filtrate concentrated on the water tangas obtained by the fraction of n-hexane, chloroform, ethyl acetate and residual fraction (ethanol fraction), then the yield was calculated.

2.2.3 Phytochemical Screening

1). Test Alkaloids
A total of 5 mg of sample was put into a test tube then added 1 ml of 2N HCl and 9 ml of distilled water, heated on a water bath for ± 2 minutes, cooled and filtered. Filtrate is used for experiments, namely Mayer, Bouchardat, and Dragendorf Reagents.
2). Flavonoid test
A total of 5 mg of sample is put into a test tube, plus 10 ml of distilled water, heated and filtered. Some of the filtrate was added with 1 ml of concentrated HCl, 50 mg of Mg powder and 2-3 drops of amyl alcohol. Then shake and let it separate, if it forms yellow, orange or red in the amyl alcohol layer it gives an indication of flavonoids.
3). Saponin test
A total of 5 mg of sample was put into a test tube, 10 ml of hot water was added and shaken for 10 seconds. Then 1 drop of 2N hydrochloric acid is added, if a permanent foam is formed, it gives an indication of saponin.
4). Test Tannin
A total of 5 mg of sample is put into a test tube, then added 10 ml of distilled water, bring to a boil and cool. The filtrate is diluted to almost no color, then 1-2 drops of 1% solution of iron (III) chloride (FeCl3). If it forms blackish blue or blackish green gives an indication of tannins (MOH, 1995).
5). Steroid / Triterpenoid test
A total of 5 mg of sample is put into a porcelain cup and added with 5 ml of n-hexane solvent, let it evaporate. The rest is added with 2 drops of anhydrous acetic acid reagent and 1 drop of concentrated HCl. If a purple or red color arises, then it turns into greenish blue indicating the presence of steroids / triterpenoids.
6). Quinone test
A total of 0.05 g of the sample is dissolved in 10 ml of water and placed on a water bath until a solution is formed. Then a few drops of NaOH 1 N are added to the solution. The formation of red filtrate shows the presence of quinone compounds.

2.2.4 Preparation of Test Animals

Male white mice that will be used in the study are first prepared and conditioned for 1 week before testing. Preparation of test animals is done so that test animals can adapt to new environments, control health and homogenize their food. Weighing mice every day for 1

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week before testing, with the aim of knowing the physical condition of the test animals seen from weight gain.

2.2.5 Preparation of Test Preparations

Test preparation is made by suspending the ethyl acetate fraction of dayak onion tuber with the addition of Na. CMC 0.5%. The sample is weighed based on the concentration of each dose, then suspended with Na. CMC 0.5%

2.2.6 Blood Sampling

Blood sampling is carried out through peripheral blood vessels. The selected blood vessels are blood vessels from the tail, by cleaning the location of taking blood using a tissue, then the tail is cut ± 0.5 cm from the tip of the tail, this is intended so that the injury is not too large and minimizes the effect of the infection. Dripping blood is stored in containers that have been given EDTA.

2.2.7 Treatment of animals test

To measure the initial Thrombocyte level, all the test animals were first taken blood and fasted for 6 hours then injected with heparin intraperitoneally 37.8UI / 20gBB to reduce the Thrombocyte count. After 24 hours, the blood of all animals that have experienced thrombocytopenia are taken. Then the test animals were given further treatment according to the dose level.

The study used 90 test animals divided into 3 dose groups for each test compound in the number of 5 individuals per group (total ethanol extract, n-hexane fraction, chloroform fraction, ethyl acetate fraction and ethanol fraction), and 2 test groups for 0.5% CMC (negative control), and Psidii® syrup (positive control). The test was carried out by measuring the number of Thrombocyte cells in normal mice, after induced heparin and after being given oral test preparation for 3 consecutive days.

2.2.8 Thrombocyte Amount Calculation

The calculation of Thrombocyte counts was carried out at the Chemistry Laboratory of the Mathematics and Natural Sciences University of Mulawarman Samarinda using an automatic device, namely hematology analyzer. The tool will run for ± 1 minute and the results will automatically exit.

3. Result and discussion

3.1 Phytochemical screening

Testing of secondary metabolites aims to determine the presence of secondary metabolites in natural material samples. Test results of phytochemical screening on total ethanolic extract (ET), n-hexane fraction (H), chloroform fraction (K), ethyl acetate fraction (EA) and ethanol fraction (E) Dayak onion bulbs in Table 1.

Table 1. Preliminary qualitative phytochemical analysis

NO	Plant Constituents	ET	H	K	EA	**E**
1	Alkaloids	+	-	+	+	+
		+	-	+	+	+
2	Flavonoids	+	+	+	+	+
3	Saponin	-	-	-	-	-
4	Tannin	+	-	-	+	-
5	Steroids	-	-	-	-	-
6	Quinone	+	-	+	-	+

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3.2 Test Thrombocyte Enhancement Activity in Mice

This study aims to determine the effect of dayak onion active fraction on increasing the number of Thrombocyte cells in mice that have been induced by heparin. Positive control used as a comparison in this study is Psidii® syrup. This drug is commonly used as a positive control in other studies, Psidii® syrup also contains guava leaves which, according to Soegijanto et al. (2010), secondary metabolite content in guava leaves can increase the number of megakaryocytes in the bone marrow so that it can increase the amount Thrombocyte cells.

The test animals used were male mice because the biological condition of male mice was more stable when compared to female mice whose biological conditions were influenced by the etrus cycle, male sex was also chosen so that the response to increased Thrombocytes was not affected by estrogen and progesterone hormones. In addition to gender uniformity, the test animals used also have weight-for-body uniformity (between 20-40 g) and age (2-3 months). This aims to reduce biological variability between the test animals used, so that it can provide a relatively more uniform response to the effect of Thrombocyte enhancement observed in this study. If the test animal has weight outside the range, it can affect the results obtained. This is because the greater the weight, the wider the circulatory system that the active substance must take to reach the peak concentration in the plasma, thus slowing its onset and vice versa. However, this is considered to have no major effect because it is overcome by dose adjustment.

Table 2. Activity of Increase Thrombocyte in mice

Group	Dose	Average% of Activity Increase Thrombocyte
Psidii® syrup	32.5 mg/ KgBB	84.13±3.99
Na CMC 0,5%	0,3 ml/20 g BB	-4.71±0.85
Total Ethanolic Extract	120 mg/ KgBB	-3.91±020
	240 mg/ KgBB	11.75±1.88
	480 mg/ KgBB*	16.27±1.44
n-Hexane Fraction	30 mg/ KgBB	36.19±3.70
	60 mg/ KgBB*	42.05±4.23
	120 mg/ KgBB	17.21±1.10
Chloroform Fraction	50 mg/ KgBB	29.20±1.24
	100 mg/ KgBB*	82.28±8.11
	200 mg/ KgBB	55.32±5.30
Ethyl Acetate Fraction	20 mg/ KgBB	1.82±0.56
	40 mg/ KgBB	8.24±1.02
	480 mg/ KgBB*	13.21±0.67
Ethanolic Fraction	25 mg/ KgBB*	31.62±0.20
	50 mg/ KgBB	15.47±1.88
	100 mg/ KgBB	8.73±1.44

* Best activity

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To measure the initial Thrombocyte level, all the test animals were first taken blood and fasted for 6 hours (only given drinking water) before being induced with heparin, this is so that the food contained in the gastrointestinal tract in the body of the mouse does not affect the effects of the preparations in test animals. The decrease in Thrombocyte count in this study was done by induction of heparin intraperitoneally 37,8UI / 20gBB, where heparin can prevent blood coagulation due to the incorporation of antithrombin cofactors with heparin, so this causes the joining of thrombin 1000 times faster than normal [6]. Generally, the total blood that can be taken is around 7.5% of the total blood volume of 1.8 ml. However, because of the sensitivity of the hematology analyzer Thrombocyte test instrument in analyzing a minimum of 0.1 ml of blood volume, the blood sample taken in the study was increased to 10% of the total blood volume. Keep in mind that taking too much blood in small animals will cause shock, stress and even cause death.

Blood samples that have been collected are then stored first in the refrigerator to be sent and analyzed collectively. According to Lindstrom et al. (2015), blood samples of rats and mice with EDTA are more stable and can be stored at room temperature or in the refrigerator for up to 48 hours. However, Stokol et al. (2014) suggested that if the sample is not directly examined, blood with EDTA should be stored in the refrigerator immediately after taking blood from the test animal.

Table 2 show the activity of increasing blood Thrombocytes in the highest white mice in the Chloroform fraction, One Way Anova test with LSD showed that Chloroform fraction ($p > 0.05$) on positive control so that it could be an alternative candidate for future dengue drugs. Research conducted by Supomo and Syamsul (2017) states that the administration of purified extract of dayak onion tuber with a dose of 400 mg / KgBB (optimum dose) orally in mice can increase the number of Thrombocyte cells by 135,000 / mm3. The results showed that the administration of dayak onion chloroform fraction was able to significantly increase Thrombocyte cell count in male white mice (*Mus musculus*) at a dose of 100mg / KgBB with a mean of 82.28 ± 8.11% (262.600 / mm3). The content in dayak onions is flavonoid flavonol [7] and quercetin [8]. Thrombocytes are formed in the bone marrow of megakaryocytes because of the stimulation of a humoral stimulator called trombopoetin. In increasing Thrombocytes in the blood, quercetin which is part of flavonoids acts as a thrombopoetin which can stimulate the proliferation and differentiation of megakaryocytes. Megacariocytes are cells from Thrombocytes, therefore if there are a large number of megakaryocytes produced, Thrombocytes are also formed. The mechanism of the increase in Thrombocyte count occurs through an increase in the number of cytokines, especially GM-CSF, IL-3 and stimulation of proliferation and differentiation of megakaryocytes, so as to increase the number of Thrombocyte cells in the blood. In addition, quercetin compounds can improve repairment of antibody formation both IgG and IgM and have been shown to reduce vascular permeability so as to prevent plasma leakage and prevent shock that causes death. The decrease in vascular permeability causes unused Thrombocytes to cover damaged vascular endothelium so that Thrombocyte counts will increase again [9].

4. Conclusion

The administration a dose of 100 mg/ KgBB of Dayak onion chloroform fraction was able to significantly increase the number of Thrombocyte cells in male white mice (*Mus musculus*)

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