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Levels of Parasitemia and Tnf-α expression in Mice (*Mus musculus*) liver cells infected with *Plasmodium berghei* after administration with the flesh fruit extract of *Phaleria marcocarpa*

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ABSTRACT

Malaria is a disease caused by the *Plasmodium* parasite and is transmitted to humans through the bite of female Anopheles mosquitoes. The purpose of this study was to determine the effect of the extract of the flesh of the God's crown fruit (*Phaleriamar cocarpa*) with different doses on the level of parasitemia and TNF-α expression in mice (*Mus musculus*) liver cells infected with *Plasmodium berghei*. The object of research is 25 mice aged 6-8 weeks, with body weight between 20-30 g. The five treatment groups were the negative control group (K-), i.e. mice that were infected with *P. berghei* without the extract of the flesh of the God's crown and the positive control group (K^+) i.e. mice that were infected with *P. berghei* and given anti-malaria suldox drugs. The treatment group (P1) were mice infected with *P.berghei* and were given extracts of the flesh of the God's crown dose of 100 mg/kg BW. The treatment group (P2) were mice infected with *P.berghei* and were given extracts of the flesh of the God's crown dose of 150 mg/kg BW. The treatment group (P3) were mice infected with *P. berghei* and were given an extract of the flesh of the God's crowndose of 200 mg/kg. The results of the study aimed at observing the expression of TNF-α by immonohisto chemical methods in mice liver cells marked with a brown color on hepatocytes. At a dose of 200 mg/kg BW, TNF-α expression was not seen on hepatocytes along with an increase in the dose of the extract of the flesh of the God's crown fruit (*P. macrocarpha*).

Key words: *Extracts of the flesh of the God's crown fruit, Plasmodium berghei, Parasitemia level, TNF-α expression*

Introduction

Malaria is an infectious disease caused by a parasite of the genus *Plasmodium* (Nagendrappa *et al.*, 2015). Malaria is also still the third deadliest disease in the world after diarrhea and upper respiratory tract infections (URTI) (Alvarez *et al.*, 2010). According to Talapko *et al.*, (2019) in 2017 globally, malaria af-

fected an estimated 219 people and causing 435.000 death case.

Indonesia is one of the countries in Asia with highest malaria case (Bhatia *et al.,* 2013; Lubis *et al.,* 2017; Zein *et al.,* 2017; Nixon *et al.,* 2014). According to the Indonesian Ministry of Health's report (2016), the number of Annual Parasite Incidence (API) or the number of positive malaria cases per 1,000 population per year has decreased from 1.75 in 2011 and 0.85 in 2015, which shows the success of malaria control programs. Nevertheless, in some areas of Eastern Indonesia such as Papua, West Papua, East Nusa Tenggara, Maluku and North Maluku this number is still high.

The high number of malaria cases, especially in eastern Indonesia, is caused by various factors such as vector resistance to insecticides, plasmodium resistance to anti-malaria drugs, low public knowledge of malaria prevention, low accessibility of people to the healthcare, lack of experienced medical staff expecialy in areas with endemic area, and relatively poor of healthcare situation (Elyazar *et al.,* 2011). Then, environmental factors such as physical, biological environment and social culture that is closely related to the bionomic or nature of Anopheles mosquito as a malaria vector by increasing population, poor drainage in urban areas, and erratic rainfall over a certain period of time can cause malaria cases to remain high and eradication of malaria to be increasingly difficult and even cause high mortality due to malaria (Kritsiriwuthinan, 2011).

According to Bartoloni and Zammarchi (2012) mortality in malaria is mainly caused by fatal complications, including liver damage. Liver damage due to plasmodium infection is caused by female Anophles mosquitoes that contain *Plasmodium* sporozoites bite humans, then the sporozoites will enter the blood tissue. The state of parasites in the blood is called parasitemia (Pujiastuti *et al.,* 2015; Wijayanti *et al.,* 2018). The degree of parasitemia can be determined by counting the number of parasites present in the blood (Jun *et al.,* 2012; de Mast *et al.,* 2015; Margono *et al.,* 2016)

Through this bloodstream, the sporozoites will enter the liver tissue. In the liver, sporozoid will form new merozoites. Merozoites that remain in the liver will develop continuously resulting in the liver releasing erythrocytes infected with parasites in large amounts, causing a reduction in hemoglobin in blood, platelets in blood vessels, and necrosis in the liver (Rowe *et al.,* 2009; Autino *et al.,* 2012). Viriyavejakul *et al.,* (2014) states that plasmodium infection in malaria sufferers causes liver damage especially on Kupffer cells.

Liver that is damaged due to Plasmodium infection, can induce the release of Tumor Necrosis Factor Alpha (TNF-α). TNF-α is a polypeptide hormone produced by monocytes or macrophages and is activated by T-lymphocyte cells, antigens, Natural Killer cells, and mast cells in various infectious conditions caused by viruses, bacteria and other pathogens (Wong *et al.,* 2011). According to Perera *et al.,* (2013) in malaria sufferers, TNF-α is produced and released by immune system cells at various stages in the Plasmodium life cycle. Schofield and Grau (2005) states that if the disease even severe, the TNF-α expression tend to be higher, because the secreted TNF-α aims to provide a protective effect on immunity against the infected organ. However, TNF-á in excessive level will cause very heavy and fatal tissue damage, so that it can be observed in the form of necrosis (Beeson *et al.,* 2008).

Necrosis can be interpreted as damaged or even death of cells from a tissue or organ due to disease due to infection (bacteria, viruses, fungi, parasites), lack of oxygen (hypoxia), and extreme environmental conditions such as heat, radiation, or exposure to ultraviolet radiation (Montagne *et al.,* 2015). Necrosis can be characterized morphologically, namely cytoplasmic vacuolation, and rupture of the plasma membrane that induces inflammation in cells undergoing necrosis (Soni *et al.,* 2013).

The treatment of malaria in addition to using synthetic drugs, can also use plant parts such as roots, stems, leaves, fruit, and even seeds (Hafid *et al.,* 2015; Ekasari *et al.,* 2016). The treatment of malaria in Indonesia traditionally using plants has been known for centuries and is still maintained today. One type of plant that can be used as a natural ingredient for the treatment of various types of diseases including malaria is the God's crownfruit (*Phaleria macrocarpa*). The use of the God's crown fruit for the treatment of malaria is possible because of the content of secondary metabolites such as saponins, flavonoids, polyphenols, and alkaloids contained in it (Faried *et al.,* 2016).

Although it has been used for the treatment of many traditional diseases, the use of the God's crown fruit for the treatment of malaria due to Plasmodium infection, especially at the level of parasitemia and TNF-α expression has not been widely

reported. Thus, the purpose of this study was to determine the effect of the administration of the extract of the flesh of the God's crown fruit (*P.macrocarpa*) on the level of parasitemia and TNF-α expression in mice (*M. musculus*) liver cells infected with *Plasmodium berghei*

Materials and Methods

Test animals used were male mice, aged ± 6-8 weeks with an average weight of 20-30 grams, as many as 25 animals. Before being used as an experimental animal, mice were acclimatized for 7 days. This acclimatization aimed to condition animals in laboratory conditions. Mice are given food according to the standard in the form of pellets and given to drink aquadest. During the acclimatization, the Mice's behaviors and the ability to consume food were observed, and weighing was done at the beginning and end of the acclimation period

This study used 5 treatment groups, each group consisting of 5 mice, the treatment groups were divided as follows:

K (-) : Negative control group (infected with *P.berghei*)

K (+) : Positive control group (infected with *P.berghei* 0.2 mL until the 8th day, then it was given the malaria drug suldox with a dose of 0.2 mL from the 9th day to the 15th day.

Treatment I : The treatment group that was administered *P.berghei* 0.2 mL until the 8th day was given the extract of the God's crown fruit flesh dose (100 mg/kg BW) from the 9th day to the 15th day.

Treatment II : The treatment group that was administered *P. berghei* 0.2 mL until the 8th day was given the extract of the God's crown fruit flesh dose (150 mg/kg BW) from the 9th day until the 15th day.

Treatment III : The treatment group that was administered *P. berghei* 0.2 mL until the 8th day was given the extract of the God's crown fruit dose (200 mg/kg BW) from the 9th day to the 15th day

The dried flesh of the God's crown were mashed, then weighed as much as 500g and used for extraction. The extraction process was done by maceration method. The flesh of the God's crown meat powder was marinated with 70% ethanol for 24 hours. Furthermore, filtering was done with the vaccum system. The filtering results were then evaporated with a vaccum rotary evaporator at a temperature of 50^{0}C until a thick extract was obtained.

P. berghei infected mice weighing 20-30 grams, aged ± 6-8 weeks intraperitonially with a dose of 0.2 ml per tail. Making blood smear was done by taking blood from the tail of mice and then dripped on the glass object, dried, and fixed with methanol 5 minutes. Then, it was dropped with Giemsa staining to cover the surface of blood smear for 24 minutes. Furthermore, it was washed by running water in an oblique position and dried by air. Blood smear preparations were then observed under a microscope and parasitemia was calculated by looking at the number of infected erythrocytes. Parasitemia was calculated on days 10, 12 and 14 on eight fields of view, then the average was taken. Calculation of the percentage of parasitemia was done by using the formula: n / m x 100%, where n = the number of infected erythrocytes and m = the number of erythrocytes counted (1000) (Harijanto, 2000).

Mice surgery was performed by cervical dislocation (neck dislocation). Mice were positioned on the surgical board using a straight needle then dissected starting from the abdomen by using bent scissors. Each liver was removed and separated using fine scissors. Furthermore, it was cleaned from the attached fat, washed using 0.9% NaCl repeatedly which functioned to keep the cells of the organ from changing shape, then put in a 4% formaldehyde tube (Muntiha, 2001). Observation of TNF-α expression was carried out by immunohistochemical staining methods according to Jammal *et al*. (2015).Data from the calculation of parasitemia values were analyzed descriptively. Data from immunohistochemical observations in the form of photos of liver organ preparations are displayed in the form of images and are described based on visible results.

Results

The measurement results of the parasitemia level of mice

The results of the calculation of the level of parasitemia showed that on the tenth day, the twelfth day and the fourteenth day the level of parasitemia on the negative control was higher than all groups, and decreased with increasing dosages of the extract of the God's crown flesh. On the twelfth day and the fourteenth day the level of parasitemia decreased consecutively from negative control, positive control (0, 2 mL Suldox), and also the dosage treatment of the extract of the God's crown fruit (Table 1).

Parasitemia is a state of parasites in the blood. In malaria sufferers, the parasite is in the form of sporozoites originating from *Plasmodium* infection. Measured parasitemia levels on the tenth, twelfth and fourteenth days were higher than negative controls because in negative control, there were no preventive measures that could inhibit Plasmodium infection, so the level of parasitemia remained high in the blood. In the treatment of the God's crown extract dose, the level of parasitemia decreased according to the increase in the extract dose. This shows that the God's crown has the ability to antiplasmodium, which is the ability to inhibit plasmodium infection that is shown by decreasing the level of parasitemia in the blood of mice.

Observation Results of TNF-α Expression with Immunohistochemical Staining Method

Observation of TNF-α expression by immonohistochemical staining method (Figure 1) shows that TNF-α expression decreased with increasing dosage of the God's crown flesh extract *(P. marcocarpa*) on mice liver cells. TNF-α expression was marked with brown color on hepatocytes.

Figure 1 shows that TNF-α expression is very high in negative control (A), which is shown by the color of hepatocytes that are very brown, and decreases successively from positive control (B), to the treatment of God's crown extract (C) and (D), and at E (dose 200 mg/kgBB). TNF-α expression can no longer be seen, which is shown by the color of the picture that is getting cleaner and brighter.

Discussion

According to Altaf *et al.,* (2013), the decrease in parasitemia in mice given the extract of the God's crown fruit was caused by the extract of the God's crown fruit that contained an active compound namely alkaloids. Quartener alkaloid compound is a compound containing nitrogen quaternary that has been known to inhibit the growth of Plasmodium by blocking intracellular transport of choline. Choline compounds are needed for biosynthesis of phospholipids in the formation of parasitic membranes to cover parasitophorous vacuole, cytosol and various subcellular compartments. This block of choline transport has been used as a malaria treatment strategy.

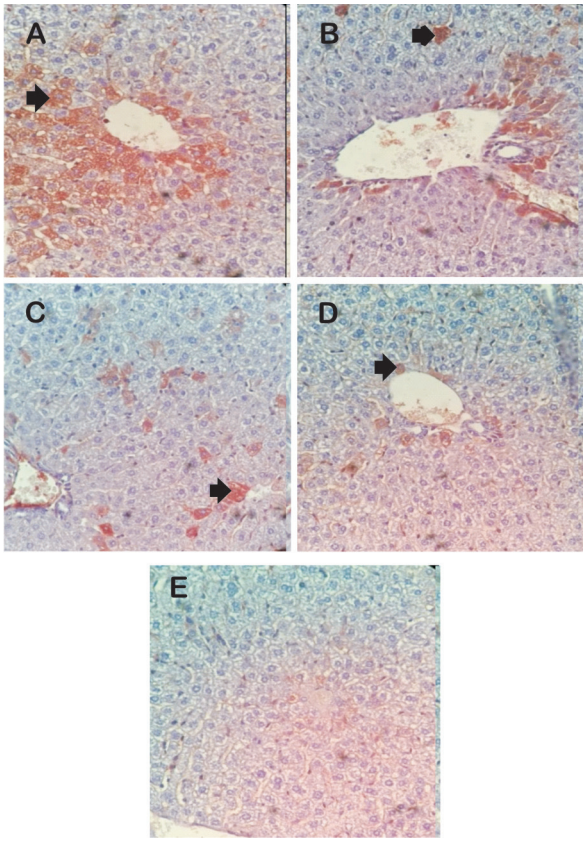

Fig. 1. Photomicrograph of TNF-α expression in mice liver cells (*M.musculus*) infected with *P. berghei* 1000x magnification. (A) Negative Control (B) Positive Control (malaria suldox drug) (C) given *P.macrocarpha* Flesh Fruit Extract 100 mg / kg BW, (D) 150 mg/kg BW, (E) 200 mg / kg BW. Arrows indicate TNF-α expression on hepatocytes.

Table 1. The Results of Parasitemia Calculation in Mice after *P. berghei* infection

Treatment	Average parasitemia(±) SD		
	day 10	day 12	day 10
Negative Control	24.00 ± 2.57	22.75 ± 0.95	22.00 ± 0.81
Positive Control(0.2 mL of Suldox)	23.25 ± 1.70	22.50 ± 1.29	21.50 ± 1.29
100 mg/kg	23.01 ± 1.43	22.05 ± 0.82	20.50 ± 0.57
150 mg/kg	23.00 ± 1.41	21.25 ± 0.95	19.75 ± 0.95
200 mg/kg	22.75 ± 0.95	20.50 ± 0.57	19.00 ± 0.40

If it is associated with the level of parasitemia as shown in Table 1, it can be seen that in negative control (A), severe plasmodium infection occurs (which is indicated by high levels of parasitemia and TNF-α expression). Then, in positive control and treatment of the God's crown fruit flesh extract, the level of parasitemia decreased, which was also followed by decreased expression of TNF-α (Figure B-E). These results are consistent with what was stated by Schofield and Grau (2005) that the more severe the disease, the TNF-α expression can be higher, whereas Cruz *et al.* (2016) stated that TNF-α will increase during Plasmodium infection.

The presence of TNF-α in infected cells aims to provide immune protection against these cells. In the B-E picture along with the decreasing level of parasitemia, the expression of TNF-α also decreases and even does not exist at all in cells that have recovered after administration of the God's crown extract. These results indicate that the administration of God's crown fruit extract can reduce the level of parasitemia and the expression of TNF-α in mice infected with *P. berghei* because it has secondary metabolite compounds such as phenolic, flavonoids, alkaloids, saponins, and tannins, which have antioxidant activity (Faried *et al.*, 2016).

Secondary metabolites found in the flesh of the God's crown have the ability to reduce the level of parasitemia because these compounds can bind to proteins contained in the cell membrane of parasites, which damage, interfere with infectious activity or even kill the parasite itself (Berthi *et al.*, 2018). The reduced parasites' ability to infect will be followed by a reduction in TNF-α expression, which leads to the repair of infected cells (de Gesso *et al.* 2015).

Percario *et al* (2012) states that the presence of *Plasmodium* infection can also induce the occurrence of oxidative stress, so reactive oxygen species (ROs) are formed, due to the presence of free radicals being released that are hydroxyl radicals in the liver (Guha *et al.,* 2006). Meanwhile, Bissinger *et al.* (2019) stated that hydroxyl radicals and hydrogene peroxide (H_2O_2) radicals released by erythrocytes infected with *Plasmodium* were about double compared to normal erythrocytes.

The formation of ROS will cause cell damage, but this can be inhibited by secondary metabolite compounds from the flesh of the God's crown, which is antioxidant. This inhibiting mechanism occurs because antioxidant compounds can contribute their hydrogen to free radicals so that the free radical ions turn stable. The stable state of ions causes a decrease in oxidative stress in the tissue and ultimately leads to the repair or regeneration of cells and tissues.

Conclusion

Based on the results of the study, it can be concluded that the level of parasitemia and expression of TNF-α in liver cells of mice (*Mus musculus*) infected with *Plasmodium berghei* decreased after administration of the God's crown flesh extract (*Phaleria marcocarpa*). The effective dose of the God's crown flesh extract (*P.marcocarpa*) is 200 mg/kg BW.

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