Prevention of Cytoadherence and Heart Cell Hypoxia of Balb/C Mices Infected with *Plasmodium Berghei* with Therapy of Pare (*Momordica charantia L*)

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Abstract. Background: Malaria is a disease caused by *Plasmodium* parasites (*P. falciparum, P. ovale, P. vivax, P. malariae, P. knowlesi*) infection often caused by the bite of female *Anopheles* mosquitoes which have *Plasmodium* parasites in their salivary glands. *Plasmodium* develops in the human liver and then invades red blood cells. This causes the symptoms of malaria. Cytoadherence is the adherence of erythrocytes infected by parasite on the endothelial surface of blood vessels due to mature parasites which causes adhesive molecules on the surface of erythrocytes to adhere with adhesive molecules on the endothelial surface of blood vessels. Causes of hypoxia in malaria include cytoadherence, sequestration, and anemia. Bitter melon (*Momordica charantia L.*), which is a traditional medicine, contains terpenoid and alkaloid substances which have anti-malarial properties. There hasn't been any study on the relationship between bitter melon and cytoadherence as well as hypoxia in malaria. ***Objective:*** To understand the effect of bitter melon therapy on decreasing cytoadherence and hypoxia in hepatocytes of Balb/c mice infected with *Plasmodium berghei.* ***Methods:*** This purely experimental research is conducted *in vivo* in a lab environment. There are 2 control groups, the positive control group which received anti-malarial therapy, and the negative control group which receives no therapeutic intervention. There are also 3 treatment groups, group 1 received a 4mg/gBW dose of bitter melon extract, group 2 received 8mg/gBW dose of bitter melon extract, and group 3 received

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12mg/gBW dose of bitter melon extract. Each group has 5 Balb/c mice infected with *P.berghei*. ***Results:*** There is a decrease in cytoadherence with a significant relationship (r = -0,917) and the most effective dose is 12mg/gBW. There is also a significant decrease in hypoxia with a significant relationship (r = -0,892) and the most effective dose is 12mg/gBW. ***Conclusion:*** Bitter melon therapy has a significant effect on decreasing cytoadherence and hypoxia in hepatocytes of Balb/c mice infected with *P.berghei* with the most significant dose of 12mg/gBW.

Keywords: Malaria · cytoadherence · hypoxia · *Momordica charantia L*

1 Introduction

Malaria is defined as a disease caused by the Plasmodium parasite, this disease can be transmitted by female Anopheles mosquitoes which contain the malaria parasite in their salivary glands. The five Plasmodium that cause malaria are Plasmodium falciparum, Plasmodium ovale, Plasmodium vivax, Plasmodium malariae, and Plasmodium knowlesi [1]. Based on a report from the World Malaria Report in 2015 there were 214 million positive cases of malaria, 88% were patients from Africa with 438,000 deaths [2]. According to the results of Riskesdas in 2010 and 2013 showed a decrease in malaria prevalence from 1.39% to 0.6% [3]. Data that has been confirmed using the API in Indonesia has decreased from 4.68 per thousand people in 1990 to 0.8 per thousand people in 2016. In numbers, malaria cases in 2016 were 218,450, while in 2017 there were 195,597 cases (Infodatin-Malaria., 2020). Even so, the eastern part of Indonesia still has a high API compared to the National API [3].

High morbidity and mortality in malaria caused by Plasmodium falciparum occurs due to infection with complications. One of the mechanisms that influence this event is sequestration and cytoadherence. Cytoadherence is the attachment of parasites in erythrocytes (EP) to the surface of the vascular endothelium at a mature stage, the attachment occurs when the adhesive molecule on the surface of the EP knob attaches to several other adhesive molecules on the surface of the vascular endothelium [4]. Meanwhile, sequestration occurs between the infected endothelium and the capillary vessels and post-capillary venules in several tissues such as the kidneys, bone marrow, lungs, heart, brain, liver, including the intervillous spaces of liver cells that form a mass of clots so that block the passage of blood in the capillaries of vital organs. The blockage causes leakage of plasma proteins and hypoxia followed by tissue edema. The event is exacerbated by the destruction of much of the hemoglobin which causes a decrease in oxygen delivery to the tissues. When oxygen in the tissues decreases it causes increased tissue damage, cell death and infarction. Blocked endothelium undergoes necrosis so that it ruptures and causes hemorrhage [5].

Hypoxia that occurs in the body will increase body responses such as VEGF, HIF-1α, and HIF-2α which will regulate the increase in blood supply to tissues experiencing hypoxia. No reactivity for HIF-1a was observed in any of the malaria cases despite several attempts using different protocols and antibodies, and despite excellent control staining. It therefore appears that widespread persistent stabilization of HIF-1a does

not occur in cases of severe malaria. HIF-2a was found with greater frequency in the vasculature in severe malaria cases compared to non-neurological controls [6].

Bitter gourd (*Mamordica charantia* L) is known to contain resin, saponins, alkaloids, and cucurbitacin-type glycosides [7]. In addition, the fruit also contains flavonoids, saponins, steroids, triterpenoids, carbohydrates, alkaloids, momordisin, vitamin A, vitamin B, vitamin C, and creatinine [8]. In the community, bitter melon is used as a medicine for various diseases such as cough, sore throat, fever, fever, malaria, diabetes, and others [9;10] The methanol content of bitter melon and bitter melon fruit water contains anti-plasmodal against Plasmodium falciparum [11].

2 Materials and Methods

2.1 Research Design

This research is a purely experimental type of research with a post-test control group design conducted in vivo in the laboratory. The study was conducted by comparing mice infected with malaria and then given bitter melon extract to the control group, namely mice infected with Plasmodium berghei but not given bitter melon extract.

The research was divided into five groups, namely:

1. Negative control group (n = 5), namely mice infected with *Plasmodium berghei* without being given bitter melon extract.
2. Positive control group, namely mice infected with Plasmodium berghei (n = 5), treated with DHP (dihydroartemisinin + piperaquine) with a therapeutic dose of 0.02496 mg/grBW every day.
3. Treatment group 1, namely mice infected with *Plasmodium berghei*
4. (n = 5), were given bitter melon extract at a dose of 4 mg/grBW.
5. Treatment group 2, namely mice infected with Plasmodium berghei
6. (n = 5), were given bitter melon extract at a dose of 8 mg/grBW.
7. Treatment group 3, namely mice infected with Plasmodium berghei *(n = 5), were given bitter melon extract at a dose of 12 mg/grBW.*

2.2 Infection with Plasmodium Berghei Strain ANKA

Inoculation of *P. berghei* strain ANKA (thawing result from liquid nitrogen) to donor mice was intraperitoneally at $1x10^6$/ml. Then on the 4th day after inoculation, parasitaemia was calculated by taking blood from the tail of the mice and then making a thin blood smear stained with Giemsa staining. The number of parasites was calculated per 1000 erythrocytes. Observations were made using a microscope with 1000x magnification. To count the number of erythrocytes, from the tail end of the donor mice, 10 L of blood was taken and diluted 103 with PBS solution, then the number of erythrocytes was calculated using the Naubauer counting chamber. The number of erythrocytes/ml of blood is known by the formula (N × 5 × 104 × dilution), where N is the number of erythrocytes. Furthermore, the number of donor mice parasites was calculated by multiplying the number of erythrocytes/ml of blood by the percentage of parasitaemia. The number of parasites to be given is 1×10^6/ml of blood, so the dilution to get the number

of parasites is the number of parasites/1 $\times$ 10^6. Mice were declared as infection donors for treated mice if the parasitaemia degree of donor mice reached more than 20–30%. Inoculation of experimental animals was carried out by means of diluted parasitaemia of donor mice injected into the peritoneal cavity of experimental animals. The inoculation procedure is to hold the nape of the mouse to Turning the body of the mouse so that it looks like the stomach of the mouse with the head lower than the body. The injection area was cleaned first with 70% ethanol. Next, a sterile needle was inserted at a 30-degree angle to the right or lower left quadrant of the mice's abdomen. Perform aspiration first to make sure the puncture is correct then the material is injected.

2.3 Isolation of the Liver of Mice

Mice were dissected for blood and liver collection on day 7 after administration of bitter melon extract to check variables. Surgery was carried out on the 7th day because from the research conducted by Roihatul, it was found that the degree of parasitemia in mice infected with *P. berghei* could be compared with mice that were in the positive or negative control group. The surgery was performed using a neck dislocation technique. Mice were placed on a board to be dissected. Surgery is performed by opening the skin of the abdomen and then the thoracic cavity. The liver was stored in an organ bottle with 10% formalin. The dissected mice were then buried in the ground.

2.4 Bitter Melon Extract

For the manufacture of bitter melon extract, the plant material of bitter melon (1.3 kg) was soaked in 6.5 L 80% methanol and stored in an orbital shaker (Orbital shaker) at 130 rpm for three days. After 72 h, the extract was filtered and the residue was macerated twice in the same way. Filtrate combined, concentrated with a rotary evaporator, and dried in oven at 40 °C. The crude extract was fractionated according to the Kupchan method by suspending 25 g of crude extract in ethanol (90%) and then defatted with petroleum ether and dried.

2.5 Inspection HIF-2α

The HIF2α examination was carried out using the immunohistochemical method. For immunohistochemical painting on slides, deparaffinization was carried out with xylol 2x10 minutes, absolute ethanol 1x5 minutes, ethanol 90% 1x5 minutes, ethanol 80% 1x5 minutes, ethanol 70% 1x5 minutes, sterile distilled water 3x5 minutes. The slides were then washed with sterile PBS for 3x5 minutes, dried and dripped with 3% H2O2 in methanol and incubated for 15–20 min. Next, it was washed with sterile PBS for 3x5 minutes. Then, antigen retrieval (AR) was performed using Heat induced epitope retrieval (HIER) by heating at 95oC in a water bath for 20 min in citrate buffer pH 0.6. The slide is cooled slowly. Unspecified protein blocking was performed by dripping 0.25% triton-x100 in BSA blocking buffer for 1 h at room temperature and washed with sterile PBS for 3x5 minutes. Next, the slides were dripped with primary antibody (primary antibody: FBS 5% = 1:100) in BSA blocking buffer, incubated overnight at

4oC. The next day the slides were washed with sterile PBS for 3x5 minutes. Incubated with rabbit anti-mouse anti-IgG secondary antibody for 60 min at room temperature, and washed with sterile PBS 3x5 minutes. After that, the slides were dripped with SAHRP (SAHRP in sterile PBS 1:500), incubated for 40 min at room temperature, washed with sterile PBS for 3x5 minutes, and washed with sterile distilled water for 3x5 minutes then added DAB chromogen (1:50), incubated for 30 min at temperature chamber, and washed with sterile PBS 3x5 minutes. Finally, the slides were counterstained with hematoxylin mayer, incubated for 5 -10 min at room temperature, and washed with sterile tap water 3x5 minutes. For the calculation of the percentage of HIF 2α is calculated based on the amount of HIF 2α expression in the cell nucleus and extracellular in the liver.

2.6 Inspection Cytoadherence

Slide cytoadherence with Hematoxylin and Eosin (HE) staining was carried out in stages. The liver cell tissue was washed with PBS 3–5 times to clean it from contaminants, then fixed in 10% formalin, after which it was dehydrated using graded alcohol (30%, 50%, 70%, 80%, 96% and absolute) each 60 min Clearing using xylol 2 times each 60 min, then infiltrated with soft paraffin for 60 min at 480C, then block in hard paraffin in the mold and allowed to stand for a day. The next day the liver cell tissue was attached to the holder and 4–6 m thick was cut with a rotary microtome. Conducted mounting on an object glass with 5% gelatin. Observed under a microscope to calculate the expression of cytoadherence in the liver of mice.

2.7 Data Analysis

The collected data were analyzed by statistical calculations from the Statistical Product and Service Solutions (SPSS) version 21 program. To include the statistical test, the data was previously tested for normality with the Shapiro Wilk test and homogeneity test with the Levene test. A data is said to be normally distributed if it has a P value $>$ 0.05. Homogeneity test was carried out to find the suitability of variance for each group of variables, homogeneous data was data with $p > 0.05$.

2.8 Research Ethics

Research Ethics was submitted to the Health Research Ethics Committee (KEPK) Faculty of Medicine and Health Sciences, State Islamic University of Maulana Malik Ibrahim Malang with ethical code number 52/EC/KEPK-FKIK/2021.

3 Research Result

3.1 Calculation Results of the Average Degree of Parasitemia of Experimental Animals

The degree of parasitemia was obtained from the blood smear of the tails of mice which were stained with Giemsa and then the infected erythrocytes were counted from 1000 erythrocytes under a microscope with 1000 times magnification and expressed in percent (%). Infected erythrocytes contain ringform, the walls are more elastic with larger than normal erythrocyte sizes.

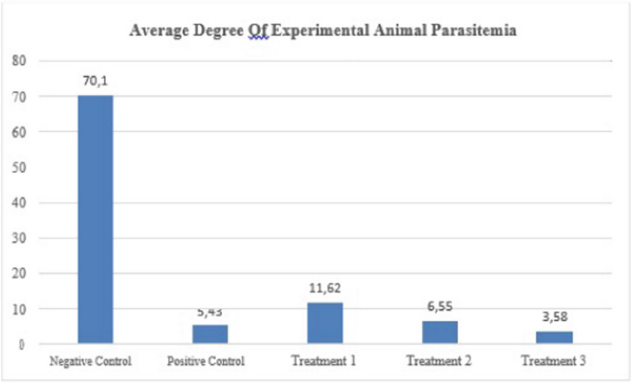

Fig. 1. Average degree of parasitemia in the treatment group

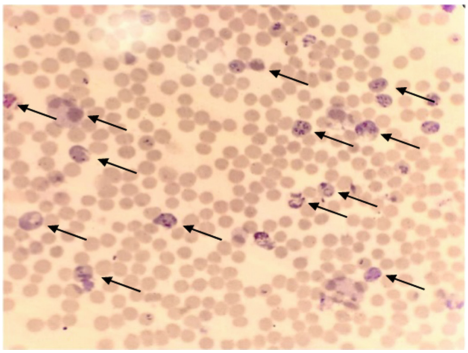

Fig. 2. Plasmodium berghei Infecting Erythrocytes

In the graph of the average degree of parasitemia, the highest incidence of parasitemia was found in the negative control group (70.1%), while the lowest incidence of parasitemia occurred in the treatment group 3 at 3.58% (Fig. 1).

Description: Erythrocytes infected with Plasmodium berghei are marked by arrows. The figure shows the various phases of Plasmodium, including the ring phase, mature schizont, and old schizont (Fig. 2).

3.2 Analysis of the Effect of Therapeutic Extract of Bitter Gourd (Momordica Charantia L.) on Cytoadherence Incidence in Balb/C Mice Infected with Plasmodium Berghei

After HE staining on the liver of mice. The calculation is done by counting the number of cytoadherence events from six fields of view in one preparation under a microscope with a magnification of 1000 times. From these observations, the average cytoadherence diagram is obtained as follows:

Figure 3 shows the mean cytoadherence and standard deviation, the negative control group has an average and standard deviation of 29.43 ± 19.09, for the positive control

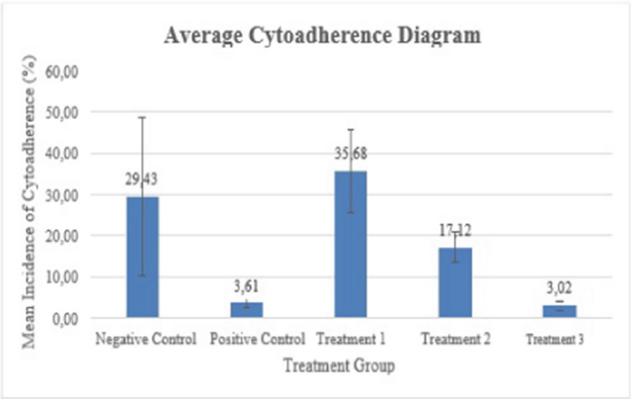

Fig. 3. Cytoadherence event diagram

group it is 3.61 ± 1.01, treatment group 1 is 35.68 ± 10.13, treatment group 2 was 17.12 ± 3.62, while treatment group 3 was 3.02 ± 1.03. By Therefore, the highest average was obtained in the treatment group 1, while the smallest average was obtained in the treatment group 3. Picture of cytoadherence as follows: (Fig. 4).

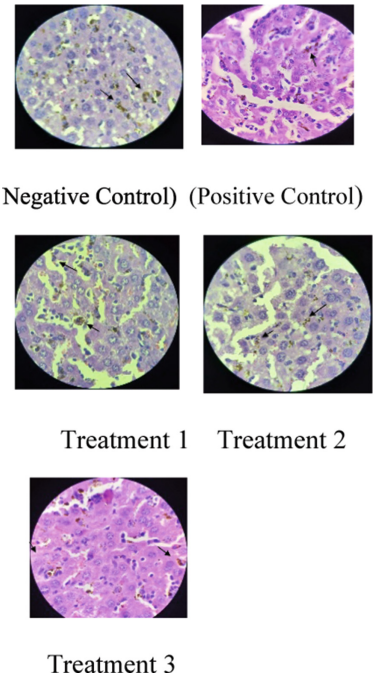

Fig. 4. Histology of cytoadherence events marked by arrows

Table 1. Pearson statistical correlative test between the administration of bitter melon extract therapy on cytoadherence in mice infected with *P.berghei*

	Cytoadherence
Treatment group	r = -0.917
	p = 0.000
	n = 18

The figure shows the incidence of cytoadherence in the control and treatment groups. The negative control group has a picture of many cytoadherence events, while in treatment 1 there are more cytoadherence events. The incidence of cytoadherence in the positive control group was less, followed by treatment group 3 which had a similar cytoadherence picture. This is in line with the diagram of the average incidence of cytoadherence. Then statistical analysis was carried out to prove whether there was a relationship between the administration of bitter melon extract therapy on cytoadherence in mice infected with *P.berghei*. To find a correlation or relationship, it is obtained through the Pearson statistical correlative test and the results are obtained in Table 1.

A correlation between the administration of bitter melon extract therapy (*Momordica charantia* L) and a decrease in the incidence of cytoadherence in the liver of experimental animals. The correlation relationship that is formed is a negative relationship with a very strong relationship (r = -0.917), which means that there is a relationship where when one variable is increased, the value of the other variable will decrease.

3.3 Analysis of the Therapeutic Effect of Bitter Gourd Extract (Momordica Charantia L.) on the Decrease in the Incidence of Hypoxia in Balb/C Mice Infected with Plasmodium Berghei

Hypoxia examination is calculated based on the percentage of HIF-2α. The incidence of hypoxia is checked and then presented. Histologically, there are differences in the incidence of hypoxia in experimental animals in the following Fig. 5:

As for the graph of the average incidence of hypoxia in the liver of mice infected with P.berghei as follows:

The picture above is a graph of the average data on the incidence of hypoxia in experimental animals that were treated. The negative control group had a mean and standard deviation of 26.60 ± 14.97, while the positive control group was 2.86 ± 0.47, in treatment group 1 (4mg bitter melon extract therapy) 33.09 ± 13.51, treatment group 2 (pare extract therapy as much as 8 mg) was 15.43 ± 6.37, while in treatment group 3 (12mg bitter melon extract therapy) it was 2.25 ± 0.91. The graph is followed by histological images of hypoxia, where the negative control group had the highest incidence of hypoxia and treatment group 1 was the group with the highest incidence of hypoxia. The low incidence of hypoxia occurred in group 5 (treatment group 3) which was almost the same as the incidence of hypoxia in the positive control.

Furthermore, to determine the relationship between the administration of bitter melon extract therapy with the incidence of liver cell hypoxia, the Spearman test obtained the

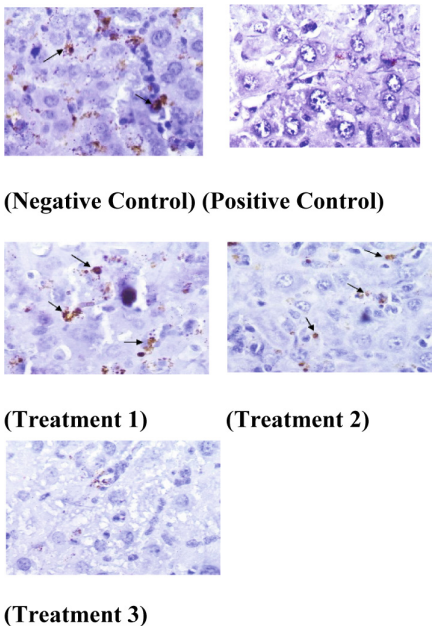

Fig. 5. Histological description of the incidence of hypoxia in experimental animals

Table 2. Spearman statistical correlative test between the administration of bitter melon extract therapy with the incidence of liver cell hypoxia

	Hypoxia
Treatment Group	r = -0.892
	p = 0.000
	n = 18

results in Table 2. The graph of the average incidence of hypoxia in the liver of mice infected with *P.berghei* shown in Fig. 6.

The results of the Spearman test above indicate that the significance value is below 0.01 so that there is a relationship between the administration of bitter melon extract therapy with the incidence of hypoxia in experimental animals. The correlation relationship formed is negative relationship of -0.0892 which is a strong negative relationship means that if one of the variables there is an increase in value, then the other variables experience a decrease in value.

3.4 Cytoadherence and Hypoxia Relationship Analysis

The relationship between Cytoadherence and hypoxia was examined by correlative test. Previously, the normality of the cytoadherence variable with hypoxia was tested and

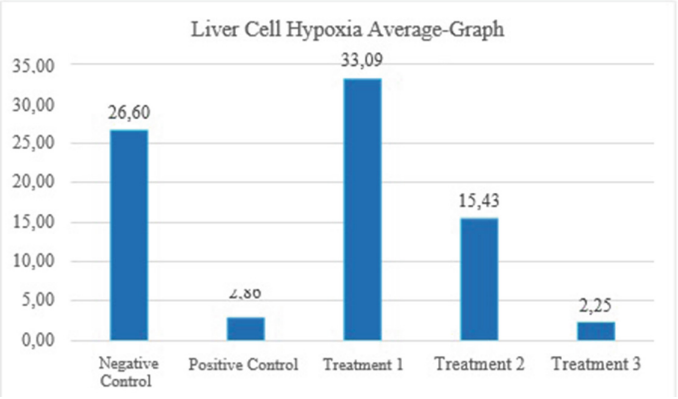

Fig. 6. Graph of the Average Incidence of Liver Hypoxia in Experimental Animals

Table 3. Pearson statistical correlative test between the cytoadherence and hypoxia

	Hypoxia
Cytoadherence	r = 0.890
	p = 0.000
	n = 30

the results were that both variables were data with a normal distribution. So that the statistical correlation test uses the Pearson test shown in Table 3.

4 Discussion

4.1 Effect of Pare Fruit Extract (Momordica Charantia L.) on Decreasing Cytoadherence Expression in Balb/c Mice Infected with Plasmodium Berghei

The calculation of cytoadherence in mice was calculated from observations under a microscope for five treatment groups, the calculations were then interpreted in a table count in terms of the percentage of cytoadherence incidence in experimental animals. This experiment used two types of control groups, namely the negative and positive control groups. The negative control group (KN) is the group infected with malaria and not given any therapy, while the positive control group (KP) is the experimental group of animals infected with malaria and given malaria therapy, namely DHP with a dose of 0.02496 mg/grBB mice. The next group is the treatment group, which is a group of mice infected with malaria and then treated with bitter melon fruit extract (Momordica charantia L.) with different doses, each of which is treatment group 1 (P1) of 4 mg/grBB, treatment group 2 (P2) of 8 mg/grBW, treatment group 3 (P3) was 12 mg/grBW. The control group was held to determine whether there was a relationship and comparison between the administration of bitter melon extract therapy with the incidence of cytoadherence in the liver of experimental animals infected with Plasmodium berghei. Positive

control was used to determine whether bitter melon therapy was as effective as DHP malaria therapy and to determine the dose of bitter melon therapy was almost the same as DHP therapy.

In the histogram data, the highest mean cytoaherence occurred in the negative control group, while the 3rd control group was the lowest average group. After cytoadherence data was obtained, statistical tests were performed to find the relationship between the therapy of bitter melon (*Momordica charantia* L.). The results of the correlation test are that there is a correlation between the administration of bitter melon extract therapy with the incidence of cytoadherence, the relationship that occurs is a negative significant correlation ($r = -0.917$) meaning that there is a very strong relationship, a significant negative correlation if the value of one variable is elevated then the other variables lower value [14].

Cytoadherence is the attachment of erythrocytes containing parasites (EP) to the surface of blood vessels after the surface of the erythrocytes forms a knob that has adhesive molecules so that they can stick to adhesive molecules from blood vessels [15]. As a result, there is blockage (obstruction) in the capillaries, resulting in tissue ischemia. In addition to obstruction, cytoadherence is also thought to cause immunological processes in the form of inflammatory mediators (TNF and Interleukins) which have an impact on impaired function in body tissues [16].

Bitter gourd contains alkaloids (momordisin), glycosides (momordisin, karantin), trichocyanic acid, resin, resinic acid, iron, calcium, phosphate salts, fatty oils consisting of oleic acid, linoleic acid, stearic acid, L-oleostearic [17]. Bitter gourd has chloroform extract which has moderate antimalarial activity (IC50 45.07 g/ml). The methanol extract (IC50 92.58 g/ml) and aqueous extract (IC50 454.23 g/ml) of bitter melon had weak antiplasmodium activity. It is suspected that the momordisin alkaloid content has an antiplasmodial effect [18]. In a study conducted by Shehab Ali et al. in 2014 in an in vitro study of the antiplasmodium activity of bitter melon, it was proven that the chloroform fraction of bitter melon was antiplasmodium (IC50 1.83 ± 0.0029 g/ml). In treatment group 1 (P1), treatment group 2 (P2), treatment group 3 (P3),

Decreased cytoadherence results were obtained. If it is continued with the comparative test, the significance in the treatment group 3 with a negative control is 0.000. This is related to bitter melon extract which has an antiplasmodial effect. If a comparative analysis was carried out between the dose of bitter gourd given to the decrease in the incidence of cytoadherence, a significant difference would be obtained, so that the null hypothesis was rejected, meaning that hypothesis 1 was accepted, namely that administration of bitter melon extract prevented cytoadherence of liver cells in Balb/C mice infected with *Plasmodium berghei.*

The inhibition process of Plasmodium which is inhibited by Alkaloids is by inhibiting the intracellular transfer of choline which is a compound for the biosynthesis of phospholipids in the formation of parasitic membranes that function to cover and protect the parasitophorus vacuole, subcellular compartment, and cytosol [19; 20]. Absorbed saponins in bitter melon will cause cell damage due to increased microbial permeability so that the materials needed by bacteria for life are lost, eventually the bacteria die [21].

Flavonoids have antimalarial effects, flavonoids are able to inhibit the development of the malaria parasite P. falciparum stage from the ring stage to the trophozoite stage and cause the schizont stage to grow with abnormal morphology [22].

4.2 Effect of Pare Fruit Extract (Momordica Charantia L.) on the Incidence of Hypoxia in Balb/c Mice Infected with Plasmodium Berghei.

Hypoxia is a decrease in the availability of O2 to levels below the threshold for the cell's ability to maintain its function. Tissues that are not well supplied with oxygen will not be able to sufficient oxygen needs so that the situation is hypoxic [22]. Cell response to hypoxic conditions is increased expression of the protein Hypoxia Inducible Factor (HIF), which is a transcription factor that plays an important role in maintain oxygen balance [23]. While HIF-1α and HIF-2α is often elevated in HCC, only HIF-2α correlates with high patient mortality [24]. This is in line with the results of the study, where there was an increase in HIF-2 in negative controls.

Bitter gourd has antioxidant, antitumor, neuroprotective, anti-inflammatory, and antimicrobial so that bitter melon is widely used as traditional medicine since ancient times. The flavonoids in bitter melon have antioxidant effects that play a role in inhibiting the clotting of blood platelets, increasing NO production which affects the dilation of blood vessels, as well as inhibit cancer growth. Another function of flavonoids is hepatoprotective, antithrombotic, anti-inflammatory, and antiviral [25].

Bitter gourd is widely used as a treatment for liver diseases because it has hepatoprotective properties, contains flavonoids, ascorbic acid, and Other components (tannins, saponins, triterpenes, and alkaloids) play a role in major in hepatoprotection. Hepatoprotective properties are related to the presence of antioxidants based on several previous studies [24].

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