Original Article

The Effect of Artesunate and Brotowali (*Tinospora Crispa*) Combination on Histopathological, and Expression of Nuclear Factor Kappa B (NF- κ B) in Renal Tubules of Mice Infected With *Plasmodium Berghei*

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ABSTRACT

Brotowali (BR) extract (*Tinospora crispa*) can be used as an antimalarial. **Aim:** to determine the effect of BR extract in histopathological and expression of NF κ B in mice tubules infected by *Plasmodium berghei* treated by artesunate (AR). **Method:** we used 42 C57BL / 6J strain mice as experimental animals, which were randomly divided into 7 groups : negative control (NC), positive control (PC), treatment group consist of AR 32 mg/kg (group 1); BR 70 mg/kg (group 2), combination of AR+BR 50 mg/kg (group 3), AR+BR 60 mg/kg (group 4), and AR+BR 70 mg/kg (group 5). Histological examination (hematoxylin-eosin (HE) staining) and expression of NF κ B (immunohistochemical staining) in the kidneys were performed on 7th and 14th. **Result:** compared to PC group, BR with doses of 70 mg until 14th day, improved the degree of tubular necrosis, interstitial fibrosis, tubular degeneration, and inflammatory cell infiltration ($p < 0.001$) but did not reach NC group ($p < 0.05$). The combination of AR+BR until the 14th day with dose of 50, 60, 70 mg all of dose improves significantly in-term of degree of tubular cell necrosis and inflammatory cell infiltration. The degree of interstitial fibrosis on 14th day only improved in group 4 and 5 ($p < 0.001$ and $p = 0.003$). The level of NF- κ B expression on day 7 and day 14 was reduced in group 2, group 4, and group 5 compared to PC group. There was positive correlation on 7th and 14th between NF- κ B expression and tubular degeneration, tubular cell necrosis, inflammatory cell infiltration, and interstitial fibrosis. **Conclusion:** the combination of AR+BR extract can improve histopathological features and reduce NF- κ B expression in mice tubules infected by *Plasmodium berghei* with an optimal dose was 60 mg/day for 7-14 days or 70 mg for 7 days.

Keywords: brotowali extract, *Plasmodium berghei*, renal tubule histopathology, NF- κ B expression

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INTRODUCTION

Malaria is a global health problem in more than 104 countries^[1], and it is estimated that there are currently 300-500 million cases each year, with more than one million people dying each year. Death due to malaria is mostly in a group of children and pregnant women.^[2]

The process of malaria pathogenesis is very complex. Thus, the process of site adhesion and sequestration of malaria pathogenesis is also associated with increased production of pro-inflammatory cytokines. Some studies indicate the expression of adhesion molecules in vascular endothelial cells and the production of proinflammatory cytokines is closely related to the activation of the kappa B nuclear factor (NF- κ B) transcription protein.^[3,4,5,6] Also reported transcription of Toll-like receptor signaling

(TLR) on the NF- κ B pathway is significantly increased in peripheral blood mononuclear cells in natural or experimental malaria infections. Nuclear factor-kappa B (NF- κ B) is a transcription factor that plays an important role in inducing the regulation of various genes in the inflammatory response and cell proliferation. Activated nuclear factor kappa B (NF- κ B) will induce the formation of immune system proteins and molecules such as Intercellular Adhesion Molecule-1 (ICAM-1), Vascular cell Adhesion Molecule-1 (VCAM-1), and cytokines such as Nuclear Tumors Factor-alpha (TNF- α), Interleukin-1 (IL-1) via gene transcription.^[3,5]

One of the clinical manifestations of severe malaria is through complications of malaria in the kidney with manifestations of acute kidney failure. Some hypotheses about the pathogenesis of renal failure in *Plasmodium falciparum* are mechanical obstruction of infected erythrocyte cells, mediation of the pathological glomerular immune system, loss of fluid through various mechanisms, and renal microcirculation changes. Erythrocytes that are infected by *P. falciparum* will experience cytoadherence, resetting/autoagglutination, and sequestration of small blood vessels (capillaries or venules) in various vital organs including the brain, liver, spleen, intestine, and kidney. In malaria patients with acute renal failure (ARF), there are fewer infected erythrocytes in the peritubular and

glomerular capillaries than in patients without ARF, but the extent of sequestration of infected erythrocyte cells in glomerular and tubulointerstitial capillaries is less than in cerebral blood vessels. So the process of cytoadherence and blockage by infected erythrocyte cells seems to be the only one of the pathogenetic mechanisms for malaria with ARF^[7]. Somsak (2013) study explained it was said that plasma creatinine urea could be used as monitoring of kidney damage.^[8]

The problem of antimalarial drug resistance raises various efforts. Some of them are developing new treatments to become an effective antimalarial drug or increasing the effectiveness of existing antimalarial drugs.^[9] Various types of efforts are currently being developed, one of which is a modulation of the immune response. It is based on the factors that mark the pathogenesis of severe malaria, such as hyperactivation of the immune system with the production of excess pro-inflammatory cytokines. All antimalarial drugs indirectly affect the immune response through its ability to damage parasites, thereby reducing the amount of antigen capable of activating the immune system. Besides these effects, some anti-plasmodial drugs also have a direct effect on the immune system, namely by reducing the production of proinflammatory cytokines, which play an important role in the early stages of severe malaria.^[10]

Brotowali (*Tinospora crispa* (L) Miers) is a traditional plant that has been known since 1900 and is said to be a medicine that can overcome various diseases such as rheumatic fever, malaria fever and general weakness. Rough extract of this plant contains tinocrisposid bitter substances and some alkaloids such as aporphine, berberine, and palmatine. They are efficient as antimalarial, antipyretic, antidiabetic, anti-inflammatory, and analgesic.^[11,12,13,14]

We investigated whether brotowali extract can affect the histopathological features and NF- κ B expression on the renal tubules in mice infected by *Plasmodium Berghei* treated by artesunate. It was based on information about the role of NF- κ B in inflammatory reactions as pathogenesis of malaria in the kidneys and brotowali extract profile as described above.

METHODS

Laboratory experimental research design, post-test only control group, using C57BL / 6J strain mice, which were divided into 7 groups, namely: negative control, positive control, treatment with intraperitoneal artesunate with a dose of 32 mg/kg/day (group 1), treatment with brotowali extract with a dose of 70 mg / kg/day (group 2), and a combination of artesunate-brotowali with several doses brotowali per day: 50 mg (group 3), 60 mg (group 4), and 70 mg (group 5). Each group consisting of 2 sub-groups dissected on the 7th day and the 14th day with a total sample of 42.

This research was conducted in the Parasitology and Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang, and Anatomical Pathology Laboratory of dr. Soetomo, General Hospital, Surabaya. The research animal was C57BL / 6J strain mice, female, weighing 20-25 grams, age 12-16 weeks, randomization was obtained from the Eijkman Molecular Biology Institute Jakarta. The ethical committee of the Faculty of Medicine, Universitas Brawijaya, Malang approved this study. The assessment of renal tubular structure was used to see the level of tubular histopathological damage by hematoxylin-eosin (HE) staining of NF- κ B expression. It was detected by using monoclonal antibodies against NF- κ B from NF- κ B/ p65 (R&D), Ab-1 staining of Thermo Scientific plant with catalog number RB-1638-R7.

Making Brotowali extract (*Tinospora crispa*)

The brotowali extraction was carried out at the Chemical Research Center-LIPI (Indonesian Institute of Sciences), Bandung. The method is 500 grams of dried brotowali herbs in the form of powder soaked by using 5L 70% ethanol v / v. The filtrate was filtered with a 200 micron mesh wire. The residue was soaked again using 5 L 70% v/v ethanol (repeated 3 times). Then, the filtrate obtained was decanted overnight, and the decantation results were filtered. The filtrate was evaporated using a Heidolph evaporator at a temperature of 50°C until it was concentrated (but can still be poured) then dried using a 50°C blower oven. Extraction results were then performed thin layer chromatogram (TLC) tests to determine the total content of phenols and flavonoids.

Plasmodium berghei infection

We inoculated mice with 1×10^6 erythrocytes infected by *Plasmodium berghei* intraperitoneally.

The degree of parasitemia was assessed in each group from day 1 to day 14.

Providing brotowali extract

The oral supplementation of brotowali stem extract of 50 mg, 60 mg, and 70 mg/day dissolved in 0.2 cc PBS solution is administered with sonde orally. It is starting on the 4th day after infection for 3 days for the mice group that were dissected on the 7th day. The mice group that were dissected on the 14th day administer.

Giving intraperitoneal artesunate

Giving artesunate is done by intraperitoneal injection of a dose of 32 mg/kg bb/day on days 4, 5, and 6 posts infection.^[16]

Kidney sampling

Mice were sacrificed with inhaled chloroform on the 7th and 14th days of post-infection. Mice in the supine position and opened abdominal incision along with the peritoneum sheath, then kidney tissue is taken and fixed in 10% formalin in a tube that has been labeled. Resection was performed with a 4 mm microtome, making histopathological preparations at the PA Laboratory dr. Soetomo Hospital Surabaya, and painting of immunohistochemistry at the Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang.

Hematoxylin-eosin (HE) staining

The slides were washed with PBS pH 7.4 for 5 minutes, stained with haematoxylin for 10 minutes. Soak in tap water for 10 minutes, then rinse with H₂O. Dehydrated with 30% and 50% serial alcohol, respectively 5 minutes. Stained with Eosin solution for 3 minutes, then rinsed with 30% alcohol. Wash with H₂O for 5 minutes and dry, then using an entellan for mounting and cover with a cover glass.

NF- κ B immunohistochemistry

The initial stage is defatting before the slide is heated to a temperature of 60°C for 60 minutes. Then added with the following solutions in sequence Xylol (2x10 minutes), Absolute Ethanol (2x10 minutes), Ethanol 90% (1x 5 minutes), Ethanol 80% (1 x 5 minutes), Ethanol 70% (1 x 5 minutes), Aquadest sterile (3x5 minutes). Then the Antigen Retrieval process is done with citrate buffer. The slide was immersed in a Chamber containing citrate buffer pH 6.0. then heated in a water temperature of 95°C for 20

minutes. Slides are removed from the water bath, then wait until it is at room temperature (± 20 minutes) then washed with PBS (3x2 minutes). Then the immunohistochemical staining process is carried out as follows; The first day, the slides were dropped with 3% H_2O_2 in methanol and incubated for 15 minutes, washed with PBS for 2 minutes 3 times. After that, Unspecific Protein

Blocking is done with Background Sniper drops, incubated 15 minutes at room temperature, then washed with PBS for 2 minutes 3 times. NF- κ B primary antibodies were dissolved in PBS buffer and 2% BSA overnight at 4°C. The second day the slides were incubated for 30 minutes secondary antibodies at room temperature then washed with PBS for two minutes three times. After incubating the enzyme Streptavidin Horseradish Peroxidase (SA-HRP) for 20 minutes at room temperature, then washed with PBS for 2 minutes 3 times and rinsed with Aquadest. Dripped Chromagen Diaminobenzidine (DAB) and DAB buffer with a ratio of 1:50 and incubated 3-10 minutes at room temperature, then washed with PBS for 2 minutes 3 times and washed with Aquades for 2 minutes 3 times. Furthermore, Mayer was dropped and tap water in a ratio of 1: 10 and incubated 5-10 minutes at room temperature, then rinsed with Tap water, dried and observed under a microscope at 1000 times magnification.

Histopathological Evaluation

Histopathological damage features in infected kidney tubules include tubular degeneration, tubular cell necrosis, tubular infiltration/interstitial, and interstitial fibrosis. The method of scoring histopathological changes in the kidneys is determined according to the method of Klopfeisch (2013).^[17] Histopathological Measurement of tubular degeneration scores are score 0: no degenerative changes in tubular cells, score 1: degenerative cells <25% of the field of view, score 2: if the degenerative cell counts 26 - 50% of the visual field, score 3: if the number of degenerative cells is between 51 - 75% of the visual field, and score 4; if the number of degenerative cells >76% from the field of view).

However, Measurement of the histopathological score of tubular epithelial cell necrosis are score 0: no necrotic changes, score 2: if the necrotic cells <25% of the visual field, score 4: if the number of necrotic cells is between 26-50%,

score 6: if the necrotic cells between 51-75% of the visual field, and score 8: if necrotic cells is >76% of the visual field.

Besides, Measurement of infiltration interstitial/ infiltration score of inflammatory cells are score 0: no inflammation cells in interstitial space, score 1: inflammation cells found <25% in interstitial space, score 2: inflammation cells found 26-50% in interstitial spaces, score 3: inflammation cells are found in 51-75% in the interstitial space, and score 4: inflammation cells are found in >76% in the interstitial space.

Measurement of interstitial fibrosis scores are score 0: no fibrosis, score 3: if fibrosis tissue <10% of the entire visual field, score 5: if fibrosis tissue 11 - 30% visual field, score 10: if fibrosis tissue > 30% from the whole field of view. The scoring value of the damage degree in each sample is the average number of all types of lesions that occur in 10 different fields of view on the tubules. The entire examination used a Nikon Eclipse Ci light microscope equipped with a calibrated 12 Megapixel Digital Camera Opti lab Plus and was equipped with Image Raster 3 image processing software.

Immunohistochemical methods examined the expression of NF- κ B in the tubular kidney structure of kidneys' mice of each group of mice. Data for each sample are assessed semi-quantitatively according to the modified Remmele method (Nowak *et al.*, 2007), that the Remmele scale index (Immuno Reactive Score / IRS) is the result of multiplying the percentage score of immunoreactive cells with the color intensity score on immunoreactive cells (**Table 1**).^[18] The IRS semiquantitative scale is the result of the multiplication between the positive cell percentage score (A) and the color reaction intensity score (B), so $IRS = (A \times B)$. The data for each sample is the average value of the IRS observed in 10 (ten) Field View (LP) different at a magnification of 400x and 1000x.

Statistical analysis

The data obtained in this study will be statistically analyzed using a nonparametric comparative test with the Kruskal Wallis test, the Mann Whitney Post Hoc Test, to compare the scores of more than two groups. All technical data

processing results were analyzed by computerization using Statistical Product and Service Solution software, IBM SPSS Statistics 20, with a significance level of 0.05 ($\alpha=0.05$) and a confidence level of 95% ($\alpha=0.05$).

Table 1. IRS Semiquantitative Scale

A	B
Score 0: no positive cells Score 0: no color reaction	Score 0: no positive cells Score 0: no color reaction
Score 1: positive cell less than 10% Score 1: Weak color intensity	Score 1: positive cell less than 10% Score 1: Weak color intensity
Score 2: positive cells between 11% - 50% Score 2: Medium color intensity	Score 2: positive cells between 11% - 50% Score 2: Medium color intensity
Score 3: Positive cells between from 51% - 80% Score 3: Strong color intensity	Score 3: Positive cells between from 51% - 80% Score 3: Strong color intensity
Score 4: Positive cells more than 80%	Score 4: Positive cells more than 80%

IRS, Immuno Reactive Score

The Spearman correlation test was used to determine the correlation between the level of histopathological damage to the renal tubules and the level of NF- κ B expression with the brotowali dose used and for the correlation between the degree of tubule damage to the level of NF- κ B expression. To find out how much influence the dose variation in the combination group of artesunate and Brotowali on NF- κ B expression and the degree of tubular damage using a linear regression test.

RESULTS

Test the total content of phenols and Brotowali flavonoids (*Tinospora crispa*).

The total content of phenols and flavonoids was measured by phytochemical tests and thin layer chromatograms (TLC). The total phenol content obtained from the ethanol extract of *Tinospora crispa* stem was $43.34 \pm 1.92\%$ per dry weight. The resulting total flavonoid content was 74.26 ± 1.32 per dry weight.

Histopathological changes in tubules

There were morphological changes in tubules in infected mice in the form of tubular degeneration ($p < 0.001$), tubular cell necrosis ($p < 0.001$), infiltration interstitial ($p < 0.001$) and interstitial fibrosis ($p < 0.001$) that were seen on the 7th day post-infection.

Table 2. Day 7 evaluation of renal tubules based on the treatment group compared to the positive control group

	Tubular degeneration	Tubular necrosis	Interstitial infiltration	Interstitial Fibrosis
Group 1 vs PC	0.048 *	0.001 *	0.115	0.086
Group 2 vs	<0.001 *	<0.001 *	<0.001 *	<0.001 *

Group 3 vs	0.077	0.026 *	0.019 *	0.016 *
Group 4 vs	<0.001 *	<0.001 *	<0.001 *	<0.001 *
Group 5 vs	<0.001 *	<0.001 *	<0.001 *	<0.001 *

Mann Whitney U test; * indicated significant statistically.

PC, positive control group, group 1 (artesunate); group 2 (brotowali), group 3 (artesunate+brotowali 50 mg); group 4 (artesunate+brotowali 60 mg); group 5 (artesunate+brotowali 70 mg).

Table 3. Day 14 evaluation of renal tubules based on the treatment group compared to the positive control group

Group	Tubular degeneration	Tubular necrosis	Interstitial infiltration	Interstitial Fibrosis
Group 1 vs PC	0.048 *	0.359	0.007 *	0.657
Group 2 vs PC	<0.001 *	<0.001 *	<0.001 *	<0.001 *
Group 3 vs PC	0.243	<0.001 *	<0.001 *	0.412
Group 4 vs PC	<0.001 *	<0.001 *	<0.001 *	<0.001 *
Group 5 vs PC	<0.001 *	<0.001 *	<0.001 *	0.003 *

Mann Whitney U test; * indicated significant statistically.

PC, positive control group, group 1 (artesunate); group 2 (brotowali), group 3 (artesunate+brotowali 50 mg); group 4 (artesunate+brotowali 60 mg); group 5 (artesunate+brotowali 70 mg).

Figure 1. Histopathology Renal Tubules of mice infected with *Plasmodium Berghei*.

Light blue arrows indicate tubular degeneration. Dark blue arrows indicate hemozoin in blood vessels. Hemozoin red arrow in the intertubular. Green arrows indicate interstitial infiltration of inflammatory cells. White arrows indicate interstitial fibrosis. Brown arrows indicate tubular cell necrosis. (HE staining, 1000x magnification).

Spearman correlation test showed a negative correlation between the brotowali dose in combination dose with the degree of tubular degeneration on the 7th day which showed the higher the brotowali dose the lower the degree of damage to tubular degeneration ($r = -0.491$; $p < 0.001$) with the regression equation $Y = 5.475 - 0.292 X$, $R^2 = 29.3\%$ while on the 14th day showed a positive correlation indicating the higher the brotowali dose the greater the degree of tubular degeneration ($r=0.196$; $p=0.062$) with a regression equation $Y = 0.933 + 0.150X$, $R^2 = 4.40\%$. In tubular cell necrosis the Spearman correlation shows a negative correlation on the 7th and 14th day ($r=-0.569$; $p < 0.001$; $r = -0.289$).

$p=0.006$) with the regression equation of days 7 and 14 is $Y = 6,056 - 0.333 X$, $R^2 = 37.4\%$; $Y = 4.667 - 0.167 X$, $R^2 = 8.30\%$. Inflammation cell infiltration on the 7th day there was no significant correlation ($p > 0.05$), while on the 14th day the Spearman correlation showed a positive correlation which means that the higher the brotowali dose the greater the degree of inflammation cell infiltration ($r = 0.310$; $p = 0.003$) the regression equation is $Y = 0.556 + 0.083 X$, $R^2 = 5.20\%$. In the degree of interstitial fibrosis, the Spearman correlation shows a negative correlation on day 7 which shows the lower the brotowali dose the greater the degree of interstitial fibrosis ($r = -0.538$; $p < 0.001$) with a regression equation $Y = 6.606 - 0.283X$, $R^2 = 29.0\%$ whereas on the 14th day there was no significant correlation ($p > 0.05$)

Changes in NF- κ B expression levels

NF- κ B expression in tubules showed a significant increase on the 7th-day post-infection ($p < 0.001$) and did not differ significantly on the 14th day ($p = 0.264$)

Figure 2. Comparison picture of NF- κ B expression in tubules. In **figure A** above, the control group shows the absence of NF- κ B expression and in **figure B**, the treatment group shows the presence of NF κ B expression marked by a white arrow on the cytoplasm of tubular cells, green in the interstitial tubular blood vessels. (IHC, 1000x magnification).

Table 4. Day 7 evaluation of Expression NF- κ B based on the treatment group compared to the positive control group

Treatment group	P-value
Group 1 vs PC	0.063
Group 2 vs PC	<0.001*
Group 3 vs PC	0.014*
Group 4 vs PC	<0.001*
Group 5 vs PC	<0.001*

Mann Whitney U test. * indicated significant statistically.

NF- κ B, nuclear factor kappa B; PC, positive control group, group 1 (artesanate); group 2 (brotowali), group 3 (artesanate+brotowali 50 mg); group 4 (artesanate+brotowali

60 mg); group 5 (artesanate+brotowali 70 mg).

Table 5. Day 14 evaluation of Expression NF- κ B based on the treatment group compared to the positive control group

Treatment group	P-value
Group 1 vs PC	0.943
Group 2 vs PC	<0.001*
Group 3 vs PC	0.152
Group 4 vs PC	<0.001*
Group 5 vs PC	<0.001*

Mann Whitney U test. * indicated significant statistically.

NF- κ B, nuclear factor kappa B; PC, positive control group, group 1 (artesanate); group 2 (brotowali), group 3 (artesanate+brotowali 50 mg); group 4 (artesanate+brotowali 60 mg); group 5 (artesanate+brotowali 70 mg).

Spearman correlation tests showed a negative correlation between brotowali doses in combination doses with NF- κ B expression levels in tubules on day 7th ($r = -0.774$ $p = 0.000$) which showed that the higher brotowali doses at dose combinations the lower the NF- κ B expression levels with a regression equation $Y = 12,556 - 0,933 X$; $R^2 = 63.30\%$. Whereas on the 14th day there was no correlation between the brotowali dose at the combined dose with the level of NF- κ B expression ($p = 0.268$).

Correlation of NF- κ B Expression and Histopathological Damage.

Tests with spearman correlation show a positive correlation between NF- κ B expression and the degree of tubular damage on day 7th of both tubular degeneration ($p < 0.001$; $r = 0.542$), tubular cell necrosis ($p < 0.001$; $r = 0.607$), interstitial infiltration ($p < 0.001$; $r =$

0.542), tubular cell necrosis ($p < 0.001$; $r = 0.607$), interstitial infiltration ($p < 0.001$; $r = 0.542$), tubular cell necrosis ($p < 0.001$; $r = 0.607$), interstitial infiltration ($p < 0.001$; $r = 0.542$); $p < 0.001$; $r = 0.642$) or inflammatory cell infiltration and interstitial fibrosis ($p < 0.001$; $r = 0.580$) with the tubular degeneration regression equation $Y = 1.417 + 0.156X$; $R^2 = 20.90\%$, tubular cell necrosis $Y = 1.517 + 0.246X$; $R^2 = 30.60\%$, and interstitial infiltration $Y = 0.926 + 0.142X$; $R^2 = 31.10\%$. On the 14th day also showed a positive correlation on tubular degeneration ($p < 0.001$; $r = 0.510$), tubular cell necrosis ($p < 0.001$; $r = 0.523$), interstitial infiltration ($p < 0.001$; $r = 0.565$), infiltration of fibrosis ($p < 0.001$; $r = 0.523$); $p < 0.001$; $r = 0.561$) with the tubular degeneration regression equation $Y = 1.741 + 0.127X$; $R^2 = 18.10\%$, tubular cell necrosis $Y =$

$2,023 + 0.234X$; $R^2 = 29.70\%$, interstitial infiltration $Y = 1.131 + 0.116X$; $R^2 = 30.20\%$ and interstitial fibrosis $Y = 2.772 + 0.257X$; $R^2 = 22.50\%$.

Figure 3. The relationship between the increase in brotowali dose in the combination of artesunate-brotowali (A) to increase the degree of tubular degeneration on the 7th (B) to increase the degree of tubular cell necrosis (C) to increase in interstitial infiltration (D) to increase in the degree of interstitial fibrosis (E) to increased NF- κ B expression on the 7th

Figure 4. The relationship between the increase in brotowali dose in the combination of artesunate-brotowali (A) to increase the degree of tubular degeneration on day 14 (B) to increase the degree of tubular cell necrosis (C) to increase interstitial infiltration (D) to increase the degree of interstitial fibrosis (E) to increased expression of NF-k β on day 14

DISCUSSION

Histopathological changes in Renal Tubules

In *Plasmodium berghei* infection on days 7 and 14, it was found that the morphological changes of tubules on the histopathological picture of rats in the form of tubular degeneration, tubular cell necrosis, interstitial infiltration / interstitial inflammation cells, interstitial fibrosis and hemozoin deposition. It is adjusted to the pathophysiology of *P. berghei* infection, which depends on 3 main factors, namely parasitic factors, host, and socio-geographic factors. The parasitic factors are cytoadherence, sequestration, drug resistance, speed of multiplication, antigenic variation, and malaria toxin. Host factors are immunity, proinflammatory cytokines, genetic, and age of mice.^[19] Histopathological changes are classified as acute tubular necrosis in the presence of erythrocytes paralleling the peritubular and glomerular capillaries.^[20] The process of changing erythrocytes causes sequestration of parasites, which will block capillary blood flow and cause interference with the microcirculation. The result is in tissue hypoxia and ends with kidney cell damage, subsequently causing acute tubular necrosis.^[19,21,22] It is similar to Punsaward (2013) that the modulation of intracellular signal transduction and NF- κ B signals in blood mononuclear cells is induced by hemozoin and Glycophosphatidylinositol (GPI) *Plasmodium falciparum*. Besides that, NF- κ B activation will cause the formation of pro-inflammatory cytokines such as TNF α and IL-8, etc. as well as Nitric Oxide and adhesion molecules such as ICAM-1. *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP-1) and proinflammatory cytokines and adhesion molecules play a role in the formation of severe malaria pathogenesis.^[6,23] Immunological mechanisms that occur are sequestration that causes rupture of schizonts and release of parasitic toxins, and from several studies, it is said to increase activation of severe malaria

pathogenesis. enzyme poly (ADP-ribose) polymerase-1 (PARP-1), all of which activate NF- κ B, which further increases proinflammatory cytokines. Nuclear factor-kappa Beta (NF- κ B) also increases the secretion of adhesion molecules such as ICAM-1.^[4,19,24] Loss of NF- κ B function causes various levels of immunodeficiency, thus causing various immune and inflammatory responses and deficiency of several antibodies.^[5,23]

Monotherapy injection of artesunate provides an improvement in tubular degeneration on the 7th and 14th day of post-infection, this is caused by the mechanism of antiparasitic *in vitro* through cutting the endoperoxide chain followed by position changes which results in carbon-centered radicals that alkalize and damage macromolecules in parasites.^[25] The degree of tubular cell necrosis in late 14th day infection does not show improvement. It might be due to the pharmacokinetic instability of the artesunate against parasitic protein^[25] so that the required dose is higher, but there are concerns about drug side effects.

The degree of interstitial fibrosis and interstitial infiltration/infiltration of inflammatory cells showed no improvement until the 14th day. It is possible because the pathological changes that occur by parasitic infections which release inflammatory cytokines by host immune cells and the artesunate mechanism in killing parasites by releasing free radicals.

Brotowali administration until 14th shows improvement in morphological changes in tubular degeneration, tubular cell necrosis, interstitial infiltration / inflammatory cell infiltration, and interstitial fibrosis significantly. It is due to the content of tinocrisposid compounds, flavonoids, such as quercetin, aporphine, berberine, and palmatine as anti-inflammatory and antimalarial compounds.^[13] It inhibits tubular damage caused by immune responses during malaria infection, either by host immune cells or parasites. According to

Rathe *et al.*, (2009), the compounds compound flavonoids inhibit the production of Nitric oxide (NO), Inducible Nitrite oxide (INOS) and NF- κ B activation in macrophage cells stimulated by lipopolysaccharides.^[26] Same as Sulaiman *et al.* (2008) and Ibahim (2011) explained flavonoids which inhibit the production of pro-inflammatory cytokines^[27,28] that owned by Brotowali is higher, but the antiparasitic effect is not too significant and shown in this study in the form of an increased of the parasitemia degree until the end of the study period. Besides, 2 mice spasms and death on the 12th day is possible because cerebral malaria increases by about 10% of all malaria cases infection in an experimental study with mice infected by *Plasmodium berghei*.^[29]

The result of this study showed by giving a combination of artesunate and brotowali doses of 50, 60, and 70 mg obtained optimal results on the combination of artesunate and brotowali at a dose of 60 mg/day in the form of improvement in the necrosis degree until the 14th day. The result is due to antiparasitic and anti-inflammatory effects.^[12, 13, 15, 30,31,32]

Changes in NF- κ B expression levels

In the positive control group, there was an increase in NF- κ B expression on the 7th day post-infection, and it was not significantly different from the 14th day. NF- κ B expression in vitro research shows that hemozoin (HZ) and GPI can stimulate monocytes and macrophages to synthesize proinflammatory cytokines through the NF- κ B activation pathway.^[4] It also cause an increased in various genes that mediate immune responses including inflammatory cytokines such as TNF α , IL-1, IL-6, adhesion molecules (ICAM-1, VICAM-1) which is responsible for pathogenesis of renal impairment in malaria. It happened where cytoadherence and rosetting which form parasite sequestration, in microcirculation cause occlusion of renal arteries resulting in anoxia of tubular cells.^[33]

In addition, other studies supported the theory that during malaria infection

modulation of intracellular signal transduction and NF- κ B signaling in blood mononuclear cells induced by hemozoin and *Plasmodium falciparum* GPI, and NF- κ B activation will increase the production of proinflammatory cytokines, chemokines, and nitric oxide. It is also influenced by the activation of the toll road like receptors (TLRs), which are important mediators in regulating innate non-specific immune responses that respond to infection, although TLRs respond to malaria infection, the pathogenesis still needs further research.^[23] Histopathological features of malaria in acute renal impairment vary between acute tubular necrosis (NTA), interstitial nephritis, and glomerulonephritis. glomerulonephritis is almost always histological findings.^[19,34] Despite, this is the most important and most frequent changes in tubules are found.^[34]

The administration of monotherapy artesunate does not reduce NF- κ B expression on days 7 and 14 post-infection. In contrast, the administration of brotowali monotherapy significantly reduces NF- κ B expression on the 7th to 14th day. Artesunate with active metabolites is dihydroartemisinin (DHA), which has antiparasitic activity. The antiparasitic activity happened by cutting the endoperoxide chain which damages macromolecules in the parasite, ^[25] but artesunate does not have the antioxidant and anti-inflammatory effects as in brotowali.

The administration of a combination of artesunate and brotowali doses of 50 mg/day only significantly reduced NF- κ B expression on the 7th day. In combination with brotowali 60 mg/day significantly decreased NF- κ B expression until brotowali administration until the 14th day, but did not achieve negative control as in the administration of a single brotowali dose of 70 mg for 14 days. It is probably due to the interaction of artesunate drug with brotowali crude extract. This interaction between drugs and herbs occurs through several mechanisms, including the metabolism of the cytochrome P450 enzyme by

herbal components that can change the absorption and metabolism of the drug by changing the transporter activity of the protein enzyme of the drug.^[38,39]

Brotowali contains berberine and palmatine compounds which are useful as anti-inflammatory,^[12,13,35] also contain total polyphenols which function as anti-oxidant, anti-nitrite oxidant, antiproliferative.^[36,28] The administration of brotowali until the 14th day can reduce the expression of NF- κ B greater than until the antioxidant, anti-nitrite oxidant, antiproliferative. 7th day. Brotowali contains berberine, palmatine, polyphenol, flavonoid compounds which are useful as anti-inflammatory agents.^[12,28] Rathee *et al.* (2009) stated flavonoids reduce the activation of NF- κ B^[26]. Hipol *et al.* (2012) added extract *Tinospora crispa* containing alkaloids, diterpenoid lactones, phenolics, inhibits the release of inflammatory mediators by membrane stabilization.^[37]

From the statistical evaluation, it can be seen that there is a biphasic effect on the decrease in NF- κ B expression at a combination of 60 mg doses on the 7th and 14th day. The dose of 70 mg on the 7th day decreased the expression of NF- κ B is greater than the combination of lower doses of 50 mg or at a high dose of 70 mg for 14 days. It is due to the effect of activation of the cytochrome P450 metabolic enzyme or the transport process (including fexofenadine and P-glycoprotein) by brotowali crude extract.^[38,39]

Correlation of NF κ B Expression and Histopathological Damage

In the statistical evaluation of this study, a positive correlation was obtained between NF- κ B expression and the degree of increase in the degree of tubular degeneration, tubular cell necrosis, interstitial infiltration, and fibrosis infiltration until the 14th day of observation. NF- κ B activation plays an important role in the pathogenesis of severe malaria through important mediators, such as Toll-like receptors (TLR) will cause activation of the

inflammatory response either through cellular immunity (TH1) which will release TNF α , IL-1, IL-6, IL-12, IL-18, INF- γ and cellular immunity (TH2) which will release IL-4 and IL-10.^[23,24]

Meanwhile, nitric oxide and adhesion molecules such as ICAM-1, CD-36, all of which cause cytoadherence and parasite sequestration which will clog blood flow capillaries and cause interference with the microcirculation that cause anoxia in tubular cells resulting in acute tubular necrosis and kidney disorders.^[6,19,21,22]

CONCLUSION

In conclusion, the results of the study found that the administration of brotowali extract (*Tinospora crispa*) can improve the histopathological picture and reduce the expression of NF- κ B in mice tubules infected by *Plasmodium berghei* with an optimal dose of 60 mg for 7-14 days or 70 mg for 7 days. There is an association between Histopathological damage to the tubules with increased NF- κ B expression.

The legitimacy of this study is that brotowali crude extracts cannot identify active substances that can repair histopathological damage. Also, brotowali crude extracts are possible to interact between ingredients and drugs in this study with artesunate, which can induce or inhibit the activity of other active substances.

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