

Antimalarial Effect of Doxorubicin on *Plasmodium Falciparum*: An in Vitro Study in FCR-3 Strain

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Plasmodium falciparum is the most common species of *Plasmodium* that causes malaria in Southeast Asia. Artemisinin, a drug with the mechanism of action by inducing oxidative stress in infected red blood cells (RBC) is currently used as the main therapy for malaria, after resistance to chloroquine has been found. However, evidence of artemisinin resistance was discovered in several regions in Southeast Asia. Therefore, a research is required to prove the existence of other drugs that have anti-malaria effects. A drug candidate, doxorubicin also can induce the formation of oxidative stress inside the cells. This study aims to determine the activity of doxorubicin to inhibit the development of *P. falciparum* in vitro. Red blood cell (RBC) infected with *P. falciparum* were treated with various concentrations of doxorubicin. Giemsa technique was applied to detect *P. falciparum* inside RBC. After 48 hours of incubation, the culture was observed to measure the number and the confluence of RBC and *P. falciparum* in the medium. This study revealed that doxorubicin reduced the number of RBC infected with *P. falciparum* lysis. The effective dose of doxorubicin-inhibit RBC cell lysis is 0.4 μ M, which only reduces 81% RBC cell lysis compared to the control group that reduces 95% RBC cell lysis. At this concentration also found a decrease in the number of *P. falciparum* cells in the medium. The results proved that doxorubicin has an inhibitory effect on the development of *P. falciparum* and can decrease the lysis of RBC due to *P. falciparum* infection. This findings provide an insight that doxorubicin is a potential candidate for antimalarial drugs.

Keywords: Artemisinin; Antimalaria; Oxidative Stress; Giemsa.

P. falciparum is the *Plasmodium* species that mainly causes malaria in Southeast Asia, including Indonesia (62.8%).¹ The Annual Parasite Incidence (API) rate in Indonesia, which states the number of positive cases of malaria per 1000 population is still relatively high (High category Cumulative Incidence (HCI) II (API = 50-100)),

especially in eastern Indonesia such as Papua, Nusa Tenggara Timur, and Maluku.²

Falciparum malaria caused by *P. falciparum* is the most severe type of malaria because it can generate high levels of parasitemia compared to other types of malaria. Falciparum malaria most often becomes severe malaria which can cause death.^{3,4}

Recently the recommended treatment for falciparum malaria is the use of artemisinin-based combination therapy (ACT). In Indonesia, dihydroartemisinin/piperaquine been applied as a combination with artemisinin since WHO recommended it as a first-line drug for malaria.⁵ Artemisinin works as an antimalarial drug by inducing oxidative stress causes the reduction of parasites in the body.^{6,7}

Resistance to both artemisinin and non-artemisinin components in artemisinin-based combination therapy emerging in Southeast Asia.⁸ This resistance can lead to treatment failure in malaria due to the reduced *P. falciparum* sensitivity toward artemisinin. This resistance condition can lead to the loss of the ability of artemisinin as an effective antimalarial drug, especially for the treatment of malaria with high severity. Hence, research and development of potential antimalarial drugs are needed to overcome the problem of *P. falciparum* resistance to various currently available antimalarial drug regimens. One of those is the use of doxorubicin as an example oxidative stress-inducing agent with a different mechanism of action from artemisinin.

Doxorubicin is an antibiotic derivative with similar effect with artemisinin by inducing oxidative stress. In carrying out its action, doxorubicin is involved in the inhibition of DNA and RNA synthesis.⁹⁻¹² In contrast to artemisinin which requires interaction with heme first to form free radicals, doxorubicin can be oxidized directly into a radical (semiquinone). Therefore, doxorubicin is a promising potential alternative drug candidate to replace artemisinin. The purpose of this study is to show the effect of doxorubicin administration on the inhibition of *P. falciparum* growth in vitro.

MATERIALS AND METHOD

Materials and Tools

In vitro study was conducted at the Parasitology Laboratory, Faculty of Medicine, Universitas Padjadjaran, Bandung. Stock culture of chloroquine-resistant *P. falciparum* strain FCR-3/Gambia with registration number ATCC30932 obtained from the Parasitology Laboratory, Faculty of Medicine, Universitas Padjadjaran was used. The materials used in this study included RPMI

medium (1640 R8578, Sigma Aldrich), RBC and serum from type O human blood, Phosphate Buffered Saline (PBS, Sigma Aldrich), doxorubicin (44583, Sigma Aldrich), and dye (Giemsa). Cell culture was carried out using a 25 cm² flask, while the treatment with doxorubicin was carried out using 6 wells plate. Cell culture incubation was carried out at 37° C using a CO₂ incubator.

Plasmodium falciparum cell culture preparation

The medium used to grow *P. falciparum* was complete medium and red blood. Complete medium was prepared by adding RPMI 1640 with serum from blood type O in a ratio of 9:1.13. Serum was obtained from venous blood taken using a vacutainer. Donor blood was centrifuged at 1,600 rpm for 10 minutes to extract the serum that presents in the supernatant. Serum was inactivated first at 56°C for 30 minutes before being added with RPMI 1640.

RBC was prepared by centrifuging the type O blood for 10 minutes at 1,600 rpm. The supernatant was discarded, and the precipitated pellet was washed with RPMI 1640 twice. Then the pellet was resuspended with RPMI 1640 in a ratio of 1:1. This suspension was stored at 4°C as a stock of RBC.

P. falciparum stock from -20°C was heated in a water bath at 37°C for 10 minutes. The stock was centrifuged for 10 minutes at 1,600 rpm. The pellet was washed with RPMI 1640 twice and then resuspended with 1 ml of complete medium. This suspension is ready to be used for further culture.

Plasmodium falciparum cell culture

RBC were counted using a hemocytometer so that it can reach 1 million blood cells. *P. falciparum* stock was added to complete medium and RBC in 50 ml Falcon tubes. This suspension was put into a 25 cm² flask with a volume of 5 ml. The flask containing cell culture was incubated in a CO₂ incubator. Medium is regularly replaced twice a week. After 1 week of maintenance, the cells were ready to be used for the next treatment.

Preparation of doxorubicin Solution

Doxorubicin was dissolved in distilled water to obtain an initial stock with a concentration of 8.6 mM. This stock was then diluted with RPMI 1640 to reach concentrations of 0.1µM, 0.2 µM, and 0.4µM. Fresh doxorubicin solution was used for cell treatment.

Doxorubicin treatment in *P. falciparum* culture

P. falciparum cells were cultured in a flask for 24 h, with a predetermined number of RBCs (1 million RBC per well in a 6-well plate). On day 1, the cells were harvested for further treatment with menadione. Parallel to this, the presence of parasites in RBC was detected using the Giemsa staining technique. Cell harvesting was done by transferring the culture medium into a falcon tube. Then the cells were washed twice with PBS, and the liquid was transferred to the same falcon. After that trypsin was added into the flask and incubate for 5-10 minutes in a CO² incubator to release the cells attached on the surface of the flask. The trypsinized cell suspension was used for the treatment of cells with doxorubicin. The treatment was carried out using a 6 wells plate.

The control used was a medium containing RBC, RBC and *P. falciparum*, as well as RBC and doxorubicin 0.4 M. Treatment of *P. falciparum* cells with doxorubicin was carried out with three concentration variants (0.1 μM, 0.2 μM, and 0.4 μM). Each treatment was repeated twice. After doxorubicin administration has been carried out, the culture is put in a CO² incubator for 2 x 24 hours.¹³

The controls used were wells containing medium, wells containing medium added with menadione with a concentration of 8μM, and wells containing medium and *P. falciparum*. Treatment wells consisted of medium added with doxorubicin with concentrations of 0.1μM, 0.2μM, and 0.4μM respectively. Each well was made with two

repetitions. The cells were incubated for 48 hours in a CO² incubator for further analysis.

Staining with Giemsa

Giemsa dissolved in PBS was used in this study (10% g/v). Giemsa staining was started by placing 10 μL of suspension on a slide. Then it was fixed with methanol and after that, add Giemsa dye for 10 minutes. The results were observed at 100x magnification to detect the presence of *P. falciparum* in RBC.

Cell culture observations were carried out twice (0 and 48 hours). The number of RBCs was calculated using a counting chamber or hemocytometer.¹⁴ The density of RBC and *P. falciparum* cells outside the RBC was determined by observation under a microscope.

RESULTS

This research consists of two stages, the addition of *P. falciparum* to RBC culture to ensure that *P. falciparum* infects RBC and the treatment stage with doxorubicin.

The first results showed the presence of a ring form of *P. falciparum* in the RBC, which also proved that *P. falciparum* had successfully infected the RBC (Figure 1). Cell culture treatment with doxorubicin was initiated by ensuring that doxorubicin administration did not cause RBC lysis. It can be observed in control wells containing RBC in complete medium without the addition of doxorubicin and control wells containing RBC with

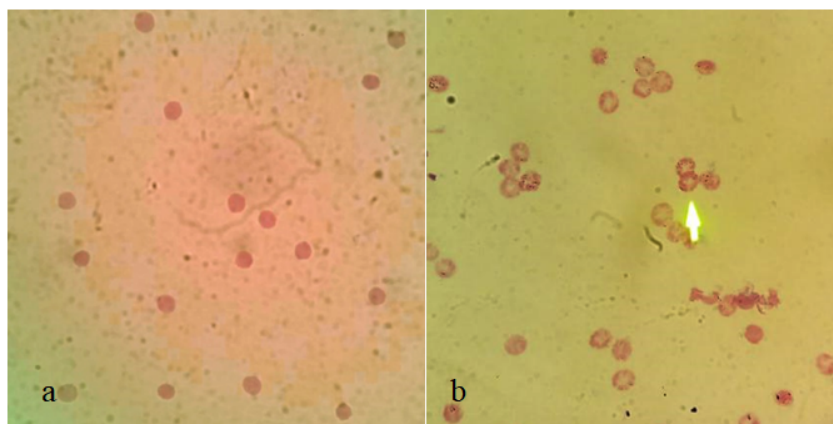


Fig. 1. Observations on the development of *P. falciparum* (a) RBC in complete medium without *P. falciparum* (control) (b) RBC infected by *P. falciparum* signed with the formation of ring (↑).

Table 1. Observation and Calculation of the Number of RBC and *P. falciparum*

	Observation time						
	Day 0			Day 2			
	Total number of RBC	Density of RBC (per mm ²)	Confluence of RBC (%)	Total number of RBC	Density of RBC (per mm ²)	Confluence of RBC (%)	Total number of <i>P. falciparum</i> observed outside red blood cell
Control	160000	305485	80	55000	90810	50	-
(A) RBC	160000	314767	80	6800	10810	30	+++
(B) RBC + <i>Plasmodium falciparum</i>	160000	318987	80	53000	89189	50	-
(C) RBC + doxorubicin	160000	303797	80	8100	2702	10	+++
Treatment							
(D) RBC + <i>Plasmodium falciparum</i> + doxorubicin 0.1 µM	160000	302953	80	17500	31891	20	++
(E) RBC + <i>Plasmodium falciparum</i> + doxorubicin 0.2 µM	160000	305485	80	30000	58378	40	+
(F) RBC + <i>Plasmodium falciparum</i> + doxorubicin 0.4 µM							

the addition of 0.4 μM doxorubicin. The results revealed that the two wells had almost the same percentage reduction in RBC number (65% and 66%, respectively), prove that the highest dose of doxorubicin in this study (0.4 μM) did not cause RBC lysis (Table 1, Figure 1).

Furthermore, the observations and calculations of the number of RBC infected with *P. falciparum* after adding of doxorubicin showed a decrease in the number of lysed RBC on the second day. The percentage reduction in RBC at 0.1 μM , 0.2 μM , and 0.4 μM concentrations of doxorubicin were 94%, 89%, and 81%, respectively. Percentage reduction in RBC in the three variants of doxorubicin concentration was smaller than the control wells containing RBC and *P. falciparum* without doxorubicin which decreased the number of RBC by 95%. The wells containing RBC and *P. falciparum* added with doxorubicin 0.4 μM had the lowest red blood cell lysis (81%)

compared to the other two variants of doxorubicin concentration, 0.1 μM and 0.2 μM with the decreasing number of RBC were 94% and 89%. These results suggested that doxorubicin can inhibit the development of *P. falciparum*, characterized by a reduced number of lysed RBC.

Reduction of lysed RBC was also confirmed by observing the RBC density per mm^2 and red blood cell confluency in the control and treatment groups. In general, the density and confluency of RBC on the second day in the treatment group was greater than that of the control wells containing RBC and *P. falciparum* without the addition of doxorubicin. Density and confluency of RBC were found to be the highest at a concentration of 0.4 μM doxorubicin compared to other variants of doxorubicin concentration. These observations are also in line with the lowest concentration of *P. falciparum* cells outside the RBC observed at a doxorubicin concentration of

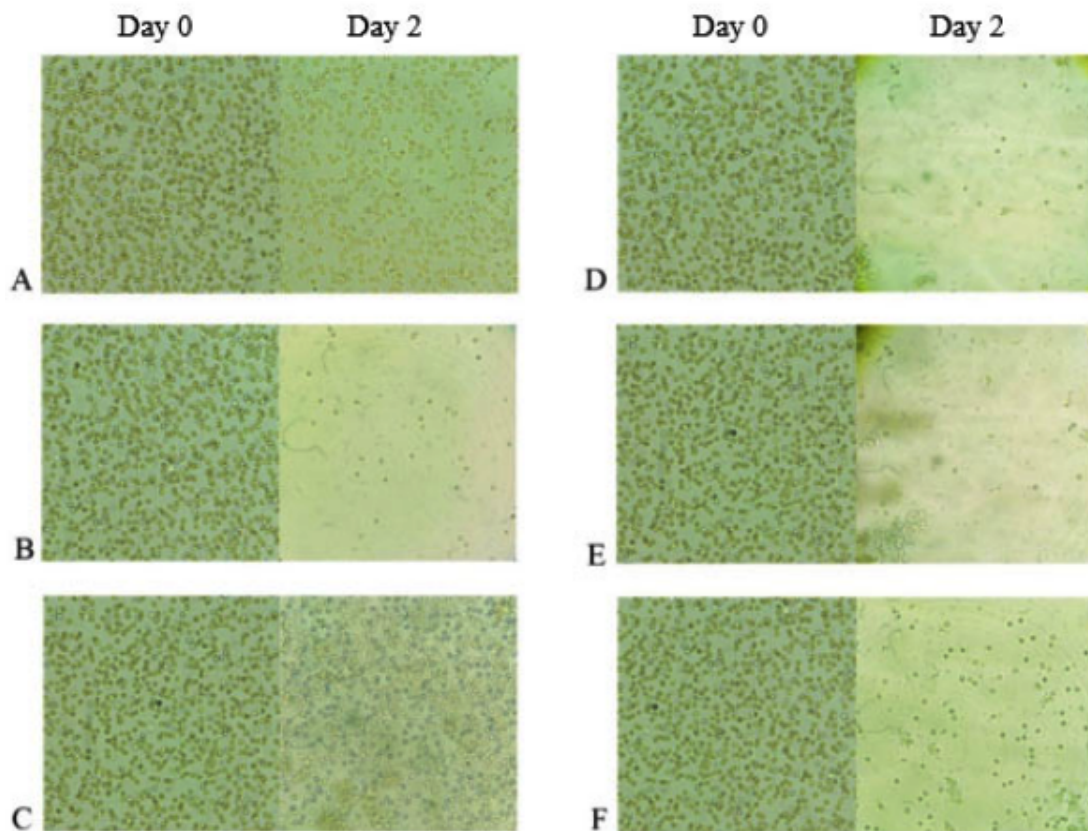


Fig. 2. Observation result of control and treatment (A) RBC (B) RBC + *P. falciparum* (C) RBC + doxorubicin 0.4 μM (D) RBC + *P. falciparum* + doxorubicin 0.1 μM

0.4 μM . Thus, the calculation of the number and density of RBC and *P. falciparum* revealed that doxorubicin could prevent infected RBC from lysis and inhibit the development of *P. falciparum* with the most significant effect found at the concentration of 0.4 μM doxorubicin.

DISCUSSION

Antimalarial drug discovery is crucial to overcome resistance problems. On the other hand, searching for potential antimalaria from existing drugs is preferable to simplify this endeavor. Some reports exhibited anticancer can also have antimalarial activity with different delivery ways: the combination of antimalaria with low-dose anticancer¹⁵ or repurposing anticancer as antimalarial drugs.^{16,17} Doxorubicin was established as a therapeutic agent.^{18,19,20} Hence this drug is one of the potential drug candidates to combat malaria disease. Several findings revealed that a compound can have multiple activities as anticancer and antilamaria. Anticancer targeting particular metabolism such as dihydrofolate reductase inhibitors (methotrexate, aminopterin, pemetrexate, edatrexate, pralatrexate or piritrexim), microtubulin assembly inhibitors (vinblastine, paclitaxel, tubulozole, docetaxel or dolastatin) and proteasome inhibitor (bortezimib) also active against *Plasmodium*.²¹ Anticancer drugs such as desatinib, oxaliplatin or irinotecan exhibit significant antiplasmodial activity.²² In addition, natural compounds such as vitamin C inhibit eukaryotic cell proliferation by causing oxidative stress in cancer cell²³ and blood-stage *Plasmodium*.²⁴

This investigation supports the antimalarial potential of doxorubicin in RBC infected with *P. falciparum* in vitro. Based on the data obtained, doxorubicin 0.4 μM had the greatest inhibitory effect on the development of *P. falciparum*, indicated by a decrease of lysed RBC. One of parameters that should be considered if doxorubicin will be tested in vivo is cytotoxicity of the drug against mammalian cell due to the compound can generate cardiomyopathy.^{25,26} The following are IC50 of doxorubicin on various cell lines when tested in vitro: 0.908 and 0.343 μM for prostate cancer cell line PC3 and DU145 respectively,²⁷ 1.1 and 0.72 μM for hepatocellular carcinoma cell line

Huh7 and HepG2, respectively,²⁸ 2.5 μM for human lymphoma Ramos cell line,²⁹ 0.8 μM for VX2 cell line,³⁰ 27.96 and 9.93 μM for osteosarcoma cell lines D17 and U2OS, respectively,³¹ 12.2009 μM for breast cancer MCF-7 and 10.339 μM for normal cell mouse fibroblast cell NIHT3T3.³² For those results we can concluded that the greatest concentration used in this study that cause greatest inhibitory of *Plasmodium* growth is still relatively low compared to most of IC50 of doxorubicin against various cell lines, hence increase of doxorubicin concentration is still possible. Moreover Lucas et al conducted a new doxorubicin delivery system to decrease cardiomyopathy: by loading the drug into RBC via electrophoretic method.³³ The finding not only give knowledge that delivery doxorubicin inside RBC will reduce cytotoxic effect on heart of animal model, but also prove that doxorubicin didn't destroy RBC if antimalarial effect of doxorubicin will be tested in vivo deliver via intravenous. Our research also found that the greatest concentration of doxorubicin (0.4 μM) did not generate RBC lysis in the absence of *P. falciparum*.

Doxorubicin is an antibiotic derivative drug that can work in living cells by increasing oxidative stress levels.³⁴ The same thing was also proven in a study conducted by Wang et.al. that doxorubicin can induce apoptosis in cells through various mechanisms.¹¹ Doxorubicin inhibit particular enzymes involve in DNA metabolism such as DNA topoisomerase II¹⁰ and DNA methyltransferase I (DNMTI)³⁵. Interestingly, doxorubicin also increases oxidative stress in cells by the oxidation of the molecule into less stable semiquinones.⁹ The release of Reactive Oxygen Species (ROS) in cells can induce cell membrane damage, lipid peroxidation, increased oxidative stress, and DNA damage that can lead to cell apoptosis. This mechanism is different from the increased oxidative stress by artemisinin which requires interaction with heme first to form free radicals. Therefore doxorubicin is promising potential drug as substitute of artemisinin.

CONCLUSION

The outcome of this research is that doxorubicin can reduce the number of lysed RBC due to *P. falciparum* and inhibit the development

of *P. falciparum* with the greatest effect at a concentration of 0.4 μ M. These results indicate that doxorubicin has potential as an alternative drug candidate for malaria.

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Conflict of Interest

There is no conflict of interest.

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