

# **Antiplasmodial Test of *Tinospora crispa* Stem Extract against *Plasmodium falciparum* 3D7 Strain In Vitro**

## **Uji Anti Plasmodium Ekstrak Batang *Tinospora crispa* terhadap *Plasmodium falciparum* Galur 3D7 secara In Vitro**

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### **ABSTRACT**

This study aimed to prove the existence of anti-plasmodial activity from *Tinospora crispa* (*T. crispa*) stem extracts and to determine the  $IC_{50}$  values as well as the best *T. crispa* stem extract concentration in inhibiting the growth of *P. falciparum* 3D7 strain in vitro. The degree of parasitemia was observed by counting the number of infected red blood cells in Giemsa stained blood films under a light microscope with a magnification of 1000x. The Plasmodium DNA concentration was measured using flow cytometry with Propidium Iodide (PI) staining. Analysis of fourier transform infrared (FTIR) spectrophotometer showed that the methanol extracts of *T. crispa* stem contained tinocrisposide compound. From the quantitative test results of Thin Layer Chromatography (TLC), it was obtained 0,22 % alkaloids that might contain compounds of berberine and palmatine. One way ANOVA statistical analysis showed that the degree of parasitemia and the concentration parasite DNA of treatment group of dose of 2,0mg/ml was significantly lower compared to the control group after 48 hours ( $p=0,001$ ) and 72 hours ( $p=0,001$ ) of incubation. The *T. crispastem* methanol extract has anti-plasmodium activity, with  $IC_{50}$  values between 0,27 mg/ml and 0,29mg/ml, and the effective dose to inhibit the growth of *P. falciparum* 3D7 strain is 2,0 mg/ml with reducing parasitemia degree by 47,12% and 56,83% after 72 hours of incubation. From this study shown the methanol extract of *T. crispastem* was able to reduce the parasitemia degree of *P. falciparum* 3D7 strain in vitro and could to be a potential candidate for anti malarias.

**Keywords:** Antiplasmodium, methanol extract, *T. crispa*, *Plasmodium falciparum*

### **ABSTRAK**

Penelitian ini bertujuan untuk menguji adanya aktivitas anti plasmodium dari ekstrak batang *T. crispa*, dan mengetahui nilai  $IC_{50}$  serta konsentrasi ekstrak batang *T. crispa* paling baik dalam menghambat pertumbuhan *P. falciparum* galur 3D7. Derajat parasitemia diamati menggunakan mikroskop cahaya dengan perbesaran 1000x dengan cara menghitung jumlah sel darah merah yang terinfeksi melalui pengecatan Giemsa dan densitas DNA Plasmodium diukur menggunakan *flow cytometri* dengan pewarnaan Propidium Iodium (PI). Analisis spektrofotometer *fourier transform infrared* (FTIR) menyimpulkan bahwa ekstrak batang *T. crispa* mengandung senyawa tinokrisposid dan hasil uji kuantitatif Kromatografi Lapis Tipis (KLT) diperoleh 0,22% alkaloid sehingga di dalam ekstrak tersebut diduga mengandung senyawa berberin dan palmatin. Hasil analisa statistik *one way* ANOVA menyimpulkan, kelompok perlakuan menunjukkan perbedaan yang signifikan dibandingkan kontrol, (nilai  $p=0,000$ ) setelah inkubasi 48 dan 72 jam. Dapat disimpulkan bahwa ekstrak metanol batang *T. crispa* memiliki aktivitas anti plasmodium, dengan nilai  $IC_{50}$  sebesar 0,29mg/ml dan 0,27mg/ml, dan dosis yang efektif untuk menghambat pertumbuhan *P. falciparum* galur 3D7 adalah 2,0mg/ml dengan menurunkan derajat parasitemia sebesar 47,12% dan 56,83% setelah diinkubasi 72 jam. Pada penelitian ini diketahui, ekstrak metanol batang *T. crispa* mampu menurunkan derajat parasitemia *P. falciparum* galur 3D7 secara *in vitro* dan merupakan salah satu kandidat untuk pengobatan malaria.

**Kata Kunci:** Antiplasmodium, ekstrak metanol, *T. Crispa*, *Plasmodium falciparum*

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## INTRODUCTION

Malaria is an infectious disease caused by Plasmodium parasite (*Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*) that is naturally transmitted through the bite of female Anopheles mosquito (1). Until today, malaria is still to be the cause of high morbidity and mortality (2). Malaria parasite such as *P. falciparum* has been known to be resistant to anti-malarial drug such as chloroquine and sulfadoxine-pyrimethamine (SP) (3,4). Due to the development of drug-resistance by the malaria parasite since 2004, malaria eradication programs have not used chloroquine anymore as the primary drug for *P. falciparum*. World Health Organization (WHO) has recommended the use of combination anti-malarial drug containing artemisinin derivatives (Artemisinin-based Combination Therapy/ACT) (5).

The emergence of *Plasmodium sp* resistance to antimalaria has encouraged researchers to look for new antimalarial drugs through research on plants that have been traditionally used by people to treat malaria. We investigated the usefulness of a traditional medicine *Tinospora crispa* (*T. crispa*), a climber plant commonly grown in the garden, locally called Brotowali. *Tinospora crispa* contain; alkaloid, soft resin, starch, glycosides, bitter substances, picroretin and anti-malaria of quaternary alkaloid derivative in the form of berberine and palmatine. *Tinospora crispa* also contains compounds such as glycosides furanoditerpen in the form of tinocrisposide (6,7). Thus, it is supposedly able to suppress the development of Plasmodium. Previous study showed mice that inoculated with *Plasmodium yoelii* then treated with the crude extract of *T. crispa* at doses of 40 mg/kg showed a surprising and interesting result, one mouse from the group in which the dose of 80 mg/kg was administered was still alive and the parasite was cleared from the blood stream (8).

## METHODS

This study was conducted from September 2012 until July 2013 at Central Laboratory of Biomedicine, Faculty of Medicine, Brawijaya University. This study was an experimental study. The subjects used in this study were culture of *Plasmodium falciparum* divided into control group (*P. falciparum* cultures without treatment), group that exposed by artemisinin  $10^{-8}$  M (an  $IC_{50}$  dose of artemisinin *in vitro*) and groups that exposed by methanol extract of *T. crispa* stem with the dose of 0,5mg/ml, 1,0mg/ml, 1,5mg/ml, 2,0mg/ml, 2,5mg/ml, and 3,0mg/ml respectively, each of group was repeated 2 times (in duplicates well) (9).

### Preparation of Methanol Extract of *Tinospora crispa* stem

Brotowali (*Tinospora crispa*) was collected from its UPTD Materia Medica in Batu, the stems of *T. crispa* were minced into small pieces then dried in sunlight for a day and ground into powder with a traditional grinder. The powdered stem was macerated for 24 hours using 90 % methanol solvent. Maceration process was carried out 3 times by replacing the methanol solvent every 24 hours, then evaporated and concentrated using N<sub>2</sub> gas. Extract was made in the organic laboratory of Faculty of Mathematics and Natural Sciences of Brawijaya University, Indonesia.

### Preparation of *Tinospora crispa* Extract

The methanol extract of *T. crispa* stem was made as stock solution, 10 mg extract was dissolved in 1000µl of RPMI 1640 medium (concentration of stock solution was 10

mg/ml). Then, it was added with 10 % complete medium as a diluent to get the concentration of 0,5mg/ml, 1,0mg/ml, 1,5mg/ml, 2,0mg/ml, 2,5mg/ml, and 3,0mg/ml. Dose of artemisinin used was  $10^{-8}$  ( $IC_{50}$ )

### Antiplasmodial activity test of *T. crispa* extract

Isolates of *P. falciparum* 3D7 strain was diluted and cultured in a medium containing the erythrocytes of O blood group with 5 % hematocrit and 10 % O blood group serum in the incubator with 5% CO<sub>2</sub> at 37°C. Culture medium was replaced every day and culture was continued to obtain the degree of parasitaemia >10 %. To test the antimalarial activity of methanol extract of *T. crispa* stem, we put a 200µl culture medium containing methanol extract of *T. crispa* stem with concentrations appropriate to treatment into sterile micro-plate (96 wells), each well contained 20µl malaria culture pellet and incubated for 48 and 72 hours. The thin blood smear was made by giemsa staining and observed under a microscope. Next, the number of erythrocytes infected by *P. falciparum* 3D7 strain parasite was counted in 1000 erythrocytes. *Parasites Staining and Flow cytometric Analysis*

Pellets was suspended in 1ml PBS containing 0,25% glutaraldehyde and incubated at room temperature for 20 minutes and centrifuged at 450xg for 5 minutes. The pellet was washed using cold PBS 2 times. Cells were suspended in 0,5ml PBS containing 0,01 % saponin and incubated at room temperature for 5 minutes and centrifuged at 450xg for 5 minutes. Then, the pellet was mixed with 40µl RNase A and incubated in the dark at a temperature of 37°C for 40 minutes. The cells were suspended into 500µl propidium iodide and incubated in the dark at a temperature of 37°C for 60 minutes. Drug activity against malarial parasites was determined by measuring incorporation of PI. Flow cytometric data analyses were performed on a FACS caliber (Becton-Dinkinson) by using the CellQuestPro program. Data were analyzed with normality and homogeneity test. The statistical analysis was conducted using SPSS software version 20 with one-way ANOVA. If significant results were obtained, it would be conducted further testing using LSD.  $IC_{50}$  values analyzed using probit quadratic analysis with SPSS software version 20.

## RESULTS

### Analysis of *Tinospora crispa* Anti-malarial Compounds

Identification results of anti-malarial compound of furanoditerpen glycoside in the form of tinocrisposide using *fourier transform infrared* (FTIR) spectrophotometer can be seen in Figure 1.

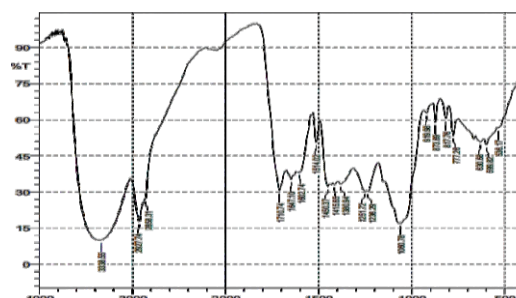


Figure 1. Infrared spectrum of methanol extract of *T. crispa* stem in Potassium Bromide (KBr) pellet

The data of infrared spectrum of methanol extract of *T. crispastem* (Figure 1) shows the absorption at wave number  $3338,55\text{ cm}^{-1}$  suspected as absorption for the OH group, and the absorption at wave number region  $2927,74\text{ cm}^{-1}$  suspected as absorption for the C-H group and the absorption at wave number region  $1710,74\text{ cm}^{-1}$  suspected as absorption for the ester C=O group.

#### Parasitemia Degree of Parasite Culture

The activity of methanol extract of *T. crispa* stem antiplasmodium was obtained by calculating the degree of parasitemia examined at 0 hour, just after *T. crispa* extract was exposed. Next, the degree of parasitemia was examined at 48 and 72 hours after the administration of *T. crispa* extract. The research results show that the degree of parasitemia for all treatments were lower than the positive control. The examination results of parasitemia degree are presented in Table 1.

The data in Table 1 below shows that over the observation time accretion (48 and 72 hours), it is followed by the average reduction trend in the degree of parasitemia. The results of one way ANOVA test showed a difference in the degree of parasitemia among groups with p value of 0,000, the results of statistical analysis of LSD indicated that after incubation for 48 hours, several treatment groups were significantly different compared to the positive control group ( $p<0,005$ ) and after 72 hours of incubation, all treatment groups showed significantly different compared to the positive control group ( $p<0,005$ ).

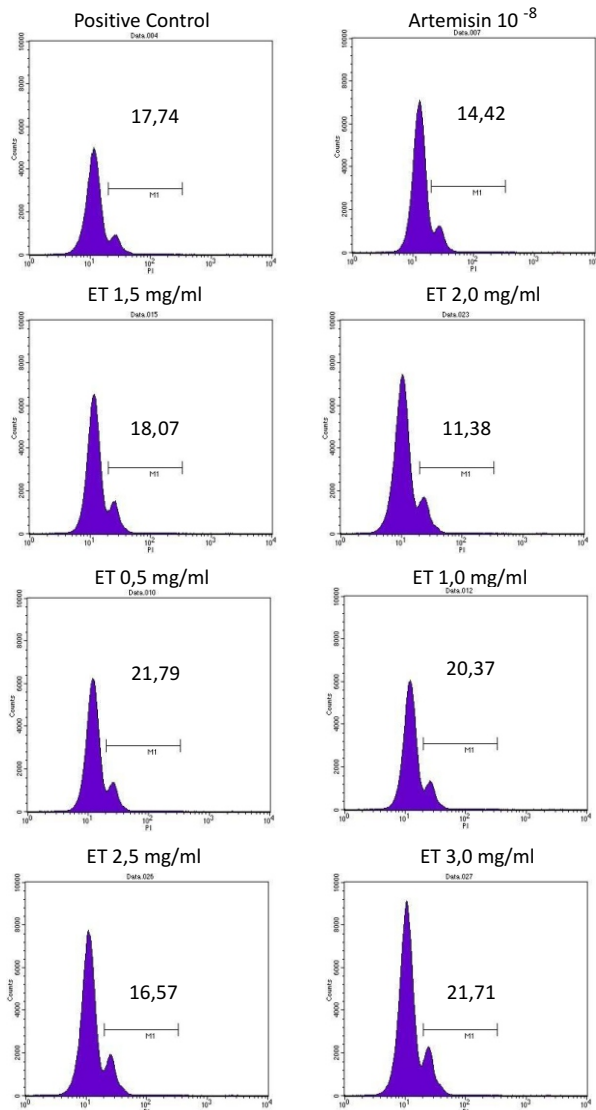
**Table 1. The mean of parasitemia degree (%) the methanol extract of *T. crispa* stem**

No	Treatments	Degree of Parasitemia	
		Mean + SD (%) 48 hours	Mean + SD (%) 72 hours
1	0 Hour	11,18±0,57	11,18±0,57
2	Positive Control	12,12±0,54	13,55±0,45
3	Artemisin $10^{-8}$	8,77±0,78*	6,24±0,13*
4	ET 0,5 mg/ml	11,97±0,64	10,66±0,39*
5	ET 1,0 mg/ml	10,99±0,40	10,79±0,03*
6	ET 1,5 mg/ml	8,33±0,31*	8,27±0,56*
7	ET 2,0 mg/ml	7,62±0,58*	5,85±0,54*
8	ET 2,5 mg/ml	9,31±0,26*	9,03±0,20*
9	ET 3,0 mg/ml	10,95±0,11*	10,11±0,36*

Based on the data in Table 1 above, it can also be seen that treatment with methanol extract of *T. crispa* stem administration with the dose of 2,0mg/ml provides the best result or the average degree of parasitemia was the lowest compared to other treatments with the percentage of parasitaemia degree equal to 7,62% (48 hours) and 5,85% (72 hours) with reducing parasitemia degree by 56,83% after 72 hours of incubation.

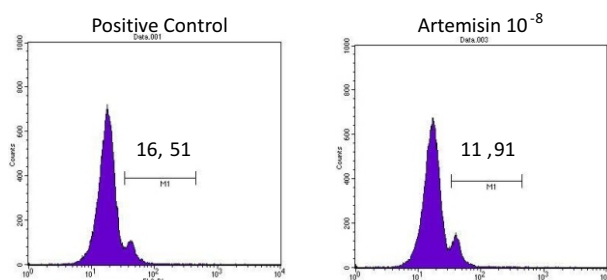
#### Concentration of parasite DNA

Flow cytometry analyzes using *propidium iodide* (PI) was conducted to determine the concentration of parasite DNA. Flow cytometry examination was conducted at 48 and 72 hours after treatment. The analysis results of flow cytometry are presented in Figure 2 and 3



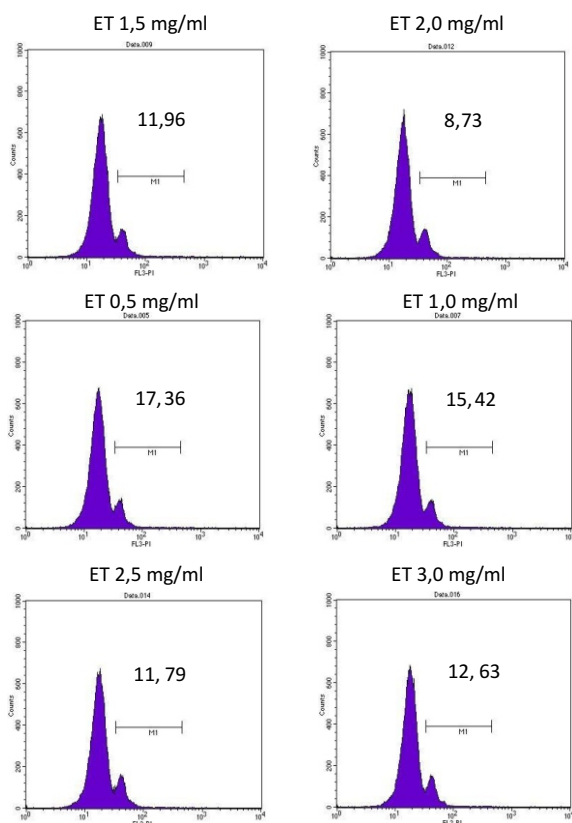
**Figure 2. Concentration of parasite DNA as the results of analysis of flow cytometry 48 hours after treatment.**  
Note : ET=Extract of *Tinospora crispa*

The research results showed that the concentration of Plasmodium DNA for all treatments were lower than the positive control. Treatment with methanol extract of *T. crispa* stem administration with the dose of 2,0mg/ml provided the best result or the average DNA concentration was the lowest compared to other treatments with the percentage of DNA concentration equal to 11,38%



**Figure 3. Concentration of parasite DNA as the results of analysis of flow cytometry 72 hours after treatment.**  
Note: ET= Extract of *Tinospora crispa*





**Figure 3. Concentration of parasite DNA as the results of analysis of flow cytometry 72 hours after treatment.**  
**Note: ET= Extract of *Tinospora crispa*. (Continued)**

The research results showed that the concentration of Plasmodium DNA for all treatments were lower than the positive control. Treatment with methanol extract of *T. crispa* stem administration with the dose of 2,0 mg/ml provided the best result or the average DNA concentration was the lowest compared to other treatments with the percentage of DNA concentration equal to 8,73%

Similar to the microscopic analysis, flow cytometric analysis results showed significant differences in mean DNA concentration among groups with the value of  $p=0,002$  (48 hours) and the value of  $p=0,000$  (72 hours). Of probit test results based on the observation of parasitemia degree using light microscope, it is obtained  $IC_{50}$  value equals to 0,29mg/ml, and of probit test results of concentration of DNA parasites based on flow cytometric analysis, it is obtained  $IC_{50}$  value equals to 0,27mg/ml.

## DISCUSSION

The identification of *T. crispa* above indicate the same pattern of absorption similar with the results of research conducted which is suspected as the absorption of OH, CH and ester  $C=O$  (7). It is supported by the statements that OH group absorption is in the range of wave numbers of  $2700-3800cm^{-1}$ , CH group absorption is in the range of wave numbers of  $2850-3000cm^{-1}$  and  $C=O$  group is in the range of wave numbers of  $1600-1850cm^{-1}$  (10). Thus, it might be that the methanol extract of *T. crispa* stem contains the antimalarial compounds of glycoside furanoditerpen in form of tinocrisposide.

Quantitatively conducted Thin Layer Chromatography test results indicate that the methanol extract of *T. crispa* stem contains alkaloid compounds equal to 0,22%, in line with several studies concluding that *T. crispa* contains several alkaloid derivatives compounds such as aporfin, pikoretin, berberine and palmatine, pikroretosid tinocrisposide and triterpenoids (11,12).

Reduction in the percentage of parasitemia degree of *P. falciparum* 3D7 equal to 47,12% and parasite DNA concentration to 56,83% after 72 hours of incubation parasite culture in the treatment of administration methanol extract of *T. crispa* stem is suspected to be caused by the active ingredient of *T. crispa* as an anti-malarial compound form; berberine, palmatine and tinocrisposide. Similar research data were revealed by previous study using *Plasmodium falciparum* treated with the crude extract of *Tinospora crispa* at doses of 2,5 mg/ml showed reducing parasitemia degree equal to 100% after 72 hours treatment with methanol extract of *T. crispa* stem in vitro (9).

Berberine constitutes quaternary alkaloid compound, which is compound structure containing quaternary nitrogen which is known to be able to inhibit the growth of Plasmodium by blocking/inhibiting intracellular transport of choline (13). Choline compounds are required for phospholipid biosynthesis in the parasite membrane formation to close the parasitophorous vacuole, the cytosol and various subcellular compartment. Thus, inhibition of choline causes the failure of the formation of a new parasite. The choline transport blocking has been used as one of malaria treatment strategies (14). Berberine is an inhibitor of nucleic acids and proteins biosynthesis of *P. falciparum*, which is in vitro showed a strong interaction with DNA (15). Similar with berberine, palmatine also constitutes quaternary alkaloid compound and quinoline whose chemical structure only differs in their methoxyl and hydroxyl groups (16). Therefore, the two active ingredients are thought to have similar activities, mechanism, and inhibition target as antimalaria. According to tinocrisposide, it is a compound that has structure of glycosides furanoditerpen (7). This structure is similar to the structure of nimbold compound that has the effect of antimalaria. Although up to recently, the inhibition mechanism of the two compounds on the growth of Plasmodium is still unknown, but due to the similarity of molecular structure between the two compounds, it is assumed that these two compounds have the same mechanism in inhibiting the growth of *P. falciparum*.

Neem seed extract contains antimalarial compounds of azadirachtin and nimbold that are able to reduce the degree of parasitemia, and the number of *P. falciparum* hemozoin is better than chloroquine (17). It is also explained that although the mechanism of inhibiting the formation of hemozoin is unknown, it is suspected that nimbold compound interact with heme such as artemisinin, due to the similarity between the molecular structure of artemisinin active substance (*sesquiterpene lactone*) and molecular structure of nimbold compound (*triterpenoid*) (17).

Based on the average degree of parasitemia (Table 1) and the concentration of parasite DNA (figure 2 & 3), it can be seen that the inhibitory effect of *T. crispa* extract on the growth of *P. falciparum* tends to follow the magnitude of the dose, at doses more than 2.0 mg/ml, an increase in the

inhibitory effect is no longer visible, even tends to rise although still lower than positive control. Moreover, the correlation test results between the magnitude of *T. crispa* methanol extract dose and inhibition percentage and concentration of parasite DNA have no significant relationship. This phenomenon is expected because of the role of antioxidants contained in *T. crispa*. Brotowali (*Tinospora crispa* (L.) Miers), besides containing antimalarial compound, also contains antioxidant compound. A previous research showed that the ethanolic extract of brotowali stem has an effect as an antioxidant (18). Other research showed that brotowali plant contains flavonoid compound, epigenin, which has an activity as antioxidant (19). Antioxidant has an important role for parasite survival in particularly at high concentrations or under certain conditions.

Another possibility is the influence of metabolic enzymes. Tinocrisposide in small dose has not been able to induce metabolic enzymes so that it remains active against Plasmodium growth, whereas larger dose of tinocrisposide will stimulate metabolic enzymes, resulting in tinocrisposide compound that will be decomposed, oxidized or conjugated, thus, the anti malaria activity will be reduced or become inactive (7). It has been explained that it is predicted due to biphasic nature of tinocrisposide compound, of which the interaction with the receptor is not directly proportional to the concentration (20). It also may be due to the physical nature of tinocrisposide itself constituting a compound group of diterpene glycosides.

The artemisinin treatment group can reduce the parasitemia degree of parasite culture, it is presumably because the artemisinin is known to have the bridge structure of peroxide (C-O-O-C), which plays an important role in the mechanism of drug action. This peroxide bridge structure would bind to the ion  $\text{Fe}^{2+}$  (iron II ion) so that it breaks the bond of peroxide bridge and generates free radicals which are highly reactive, thus inhibiting and

modifying a wide range of molecules in the parasite cause the parasite dies (21). In addition, the mechanism of parasite growth inhibition by artemisinin is due to the covalent bond between drugs and the parasite protein will damage the parasite membrane (22).

The use of single drug, including artemisinin, can lead to Plasmodium resistance on anti-malarial drugs. The use of artemisinin as a single drug is only weakening but not killing the parasites. To prevent this, WHO (World Health Organization) recommends the use of the antimalaria drug of artemisinin in combination with other antimalaria drugs through Artemisinin Combination Therapies (ACTs) method which has been shown effective to achieve cure up to 95% (23).

The curve of parasitemia degree and the concentration of parasite DNA (the data are not shown) show that they are in line with the increase of observation time (48 and 72 hours), it is followed by the downward trend in the average degree of parasitemia and parasite DNA concentration although the correlation test results provide insignificant correlation.

Based on the results above, it can be concluded that the methanol extract of *T. crispa* stem has anti-plasmodium activity, with  $\text{IC}_{50}$  value between 0,27mg/ml and 0,29mg/ml, and the dose effective to inhibit the growth of *P. falciparum* 3D7 strain is 2.0 mg/ml by decreasing parasitemia degree equal to 47,12% and DNA concentration to 56,83% after 72 hours of incubation. In this research, it can be concluded that the methanol extract of *T. crispa* stem is able to reduce the parasitemia degree of *P. falciparum* 3D7 Strain in vitro and could be a potential candidate for anti malarias.

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