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Ex vivo Study of Antimalarial Activity of *Canarium Odontophyllum* Leaf Extracts Against *Plasmodium Berghei* NK65

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Abstract

Background: Malaria is a parasite that is transmitted to human through the bite of a female Anopheles mosquito. Every year human was exposed to the threat of malaria infection. This disease becomes more fatal as these parasites show resistance towards the drug available. Thus, searches for new antimalarial drug are crucial. This study was carried out to evaluate the antimalarial activity in Canarium odontophyllum leaf extracts (methanol, acetone and aqueous) against erythrocytes infected with Plasmodium berghei NK65 using Plasmodium Lactate Dehydrogenase (pLDH) Assay and SYBR green I fluorescence Assay. Method: Three types of solvents were used to extracts Canarium odontophyllum leaf according to increasing polarity index; acetone, methanol and aqueous. These extracts were made into eight-fold serial dilution; concentrations ranging from 0.00001µg / ml as the lowest concentration until 100 µg / ml as the highest concentration and further tested on Plasmodium berghei NK65 infected erythrocytes via ex-vivo. The IC50 (inhibition concentration) 50 readings were taken at the point of 5% parasitemia level and in the synchronization process. Both PLDH assay and SYBR green I fluorescence assay were being carried out simultaneously. Result: The One-way ANOVA showed that there is no significant difference between extracts at 5% parasitemia level, even so methanol was further tested on synchronization process as it showed the lowest reading of IC50 among the three extracts for PLDH assay and SYBR green I fluorescence assay respectively, (IC50 0.00045µg / ml, 0.002 µg / ml). For synchronization stages, the One-way ANOVA result showed there is no significant difference between stages of morphology. However, methanol extracts showed the most potent on schizont, (1.16x10-5 µg / ml) and young trophozoite, (0.00195 µg / ml) stages for each method respectively. Conclusions: All three extracts of Canarium odontophyllum leaf were effective on Plasmodium berghei NK65, however methanol showed most promising results and further research on the fractions were required for proper drug development. The Ministry of Higher Learning funded this project, Government of Malaysia, under the Fundamental Research Grant Scheme Code No. FRGS/2/2014/SG05/UKM/02/3.

Key word: canarium odontophyllum, plasmodium berghei NK 65, antimalarial, pLDH Assay, SYBR green 1 fluorescence Assay.

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Introduction

Globally, there are 3.2 billion estimations of people in 95 countries and territories are at risk of being infected with malaria and developing disease and about 1.2 billion are at risk which means that more than 1 in 1000 people have a chance of getting malaria in a year. Based on the World Malaria Report in 2015, there were 214 million cases of malaria globally reported in 2015 and 438 000 malaria deaths (range 236 000–635 000), representing a decrease in malaria cases and deaths of 37% and 60% since 2000, respectively [1]. Recently, a significant reduction in Malaria cases has been observed and achieved in Malaysia, and as a mission that Malaysia is now striving to be malaria-free in the Peninsular in the past 2 years; 2015 and in Malaysian Borneo by 2020. However, there is a significant increment in Plasmodium knowlesi cases in Sabah between 2004 and 2011 and this trend threatens malaria elimination [2]. It is predicted that clinical infections and deaths will begin to increase due to the rapid spread of parasite resistance toward drugs [3]. The example of resistance cases was by P. *knowlesi*, a zoonotic monkey malaria parasite that infects humans in forest fringe areas of Southeast Asia is fully susceptible to chloroquine and other currently used medications [4]. In the need for new or novel antimalarial drugs developing, it is essential to establish the efficacy and safety of traditional medicinal plants which are used to fight the disease. The use of plants for malaria treatment extends to at least three continents including several countries in Africa, the Americas, and Asia [5].

There were several studies done on local plants available here in Malaysia, for example *Zerumbone* extract, however the result showed that on 5% parasitemia level the IC50 reading (0.05 μg/ml) via pldh assay and 0.31 μg/ml via SYBR green 1 fluorescence assay.[6] Another study on *Psidium guajava* leaf extracts showed that using methanol extracts resulted in 4.27 mg/ml, while on 5% parasitemia level (> 10 mg/ml),[7] which according to WHO standard of biological active substances as poor. Antimalarial activity using *Zingiber zerumbet* extracts on erythrocytes resulted in 0.091 μg/ml on a 5% parasitemia level.[9] Taking this into consideration, thus *Canarium odontophyllum* leaf extracts were chosen as in previous research by Basri and Nor in 2014, the phytochemical analysis showed that acetone, methanol, and aqueous extract of C. *odontophyllum* have contained a high level of total tannin, flavonoid, terpenoid, and phenol [10]. This fruit is rich in minerals, protein, carbohydrates, and fat with a high level of total phenolic, flavonoid, and anthocyanin which is related to its antioxidant activity which is one of the factors that contributes to the treatments of malaria [11]. In this research, the IC50 of the 3 types of C. *odontophyllum* leave extraction (methanol, acetone, and aqueous) were measured and compared with the positive control (chloroquine) at a 5% parasitemia level. The extraction that gave the lowest value of IC50 indicated that the ex vivo treatment given was effective towards the infected erythrocytes with Plasmodium *berghei* NK 65. Then, the best extraction was proceeded to the synchronization test to determine IC50 at different parasite's morphological stages such as young trophozoite, mature trophozoite, and schizont and compared with the IC50 value of chloroquine as a positive control.

Plasmodium lactate dehydrogenase (pLDH) is a soluble glycolytic enzyme expressed at high levels in asexual stages of malaria parasites found in all four human malaria species. pLDH activity is correlated with the level of parasitemia found in in vitro cultures of malaria. A specific measurement of pLDH from Plasmodium can be measured by using the substrate 3-acetylpyridine adenine dinucleotide (APAD), an analog of NAD, in an immunocapture assay (IC assay) [12] made it as the chosen technique in this study. Meanwhile, SYBR Green 1 Fluorescence Assay was used to measure the percentage of antimalarial activity as its ability to detect the presence of DNA double helices of *Plasmodium berghei* NK 65 with high sensitivity in the screening of antimalarial drugs in vitro [13]. The value percentage of the fluorescence relative unit acted as an indicator in order to get the inhibition DNA activity of the *Plasmodium berghei* NK 65 parasite. Calculation of the IC50 is used to estimate the inhibition concentration of the 50% *Plasmodium berghei* NK 65 population in this study.

Materials and methods

Canarium odontophyllum leaf extracts preparation

Three organic solvents sequentially extracted the powdered *Canarium odontophyllum* leaves; hexane, acetone, and methanol, based on the different polarity orders based on Basri & Nor, 2014 method [10]. Dissolved a stock solution with 1 mg of *Canarium odontophyllum* leaf extract in DMSO solution (Merck, New York, USA) and complete RPMI 1640 media (Gibco by life technologies, New York, USA) using a vortex (Scientific Industries. Inc, USA). A total volume of 1000 μl (100 μg/ml) were then undergone eight-fold serial dilutions of concentration with the last concentration of 0.00001 μg/ml.

Plasmodium berghei NK65 infested in mice

Twenty mice were injected with Plasmodium berghei NK65 with a standard inoculum of 106 parasite erythrocytes (Unit Malaria, UKM Bangi). All mice were placed in an animal house UKM with temperature 25-30°C with five mice per cage. All procedures described were reviewed and approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) (FSK/2016/AKMAR/28-SEPT./792-SPET.-2016-FEB.-2017).

5% Parasitemia Level Estimation

In this study, a 5% parasitemia level is used as an indicator for low-level severity in malaria infection and also as a parameter associated with the morphological development of P. *berghei* NK 65. Thin blood films were made to determine the level of parasitemia by snipping the tails until a drop of blood was visible and immediately smeared on a glass slide.

Blood processing

After confirming that the mice have reached 5% parasitemia level, the infected and normal mice (as control) were sacrificed via diethyl ether inhalation method. As the heart beat stopped, mice were dissected and blood were withdrawn through cardiac puncture and collected into EDTA tube.

Before the filtration process started, cellulose fibre (CF) was first wet with incomplete RPMI 1640 media with 6 ml, and the blood was aliquot into wet CF. The purpose of using CF was to filter and remove white blood cells and platelet, leaving only red blood cells. After the filtration process was complete, blood was centrifuge by adding incomplete RPMI 1640 media and spin for 5 minutes, 1600 rpm at 24°C to obtain blood pellet.

The process repeated at least three times until the media appeared clear before removing the media completely, leaving only the blood pellet. Infected red blood cells were observed using the thin blood film.

Ex-vivo *Canarium odontophyllum* leaf extracts antimalarial treatment

90 µl of extracts solution of 8 serial dilution concentrations were mixed with 10µl of blood pellet into 96 microtitre plate well. For positive control, mixed 10 µl of infected blood pellet in 90 µl of chloroquine solution of 8 serial dilution concentrations. For negative control, a triplicate sample of uninfected blood pellet and infected blood mixed with complete RPMI 1640 media. For normal control, normal blood pellets were used with the same steps as mentioned above. Incubated the plates for 24 hours for the antimalarial treatment process on P. *berghei* infected blood.

After 24 hours of incubation, plates were removed from the incubator and the freeze-thaw process begins according to Makler & Hinrich (1993) to get the pLDH enzyme of plasmodium cell from P. *berghei* infected erythrocytes. The freeze-thaw process started from the -80°C freezer for 10 minutes and thawing in 37°C incubators for 10 minutes, the process repeated for four cycles to enable red blood cells to lyse and thus releasing parasite enzyme, pLDH, and the parasite itself. Lysed blood was used in pLDH assay to determine the antimalarial activity of C. *odontophyllum* leaf extracts on 5% parasitemia level infected blood. Determination of the antimalarial activity of C. *odontophyllum* leaf extracts on infected blood by morphology continued in the synchronization process.

Synchronization process

Synchronization was a process to obtain the same Plasmodium stage with modification of the study done by Roncalés et al. (2015) [14], based on the synchronization method described by Lambros and Vanderberg, 1979. Once the mice infected with the P. *berghei* parasitemia level reached 5%, they were sacrificed together with non-infected mice as normal control. Blood was pooled and withdrawn through the punctured cardiac technique. Blood was filtered using a cellulose fibre column and centrifuge the blood suspension at 1600 rpm for 5 minutes. Next, added sorbitol solution into erythrocyte sediment with ratio 1:5 with one ratio equal to hematocrit and 5; sorbitol. Incubated the mixture for 10 minutes at 37°C and shook it for 5 minutes before centrifuged at 1600 rpm for 5 minutes.

After centrifuge, removed the supernatant and added the same volume of sorbitol. This mixture was then incubated again at 37°C for 10 minutes and shook for every 2 minutes. The mixture was centrifuged again and removed the supernatant. Repeated these steps twice, with RPMI 1640 media, leaving rupture erythrocyte with a sorbitol solution.

Ex-vivo *Canarium odontophyllum* leaf extracts antimalarial treatment (synchronization)

Blood obtained from the synchronization process was cultured in RPMI 1640 complete media solution in 6 microtitre plate well. Next, mixed 200 µl blood pellets with 20 ml RPMI 1640 complete media solution before transferring equally into six microtitre plate well and incubated at 37°C. The Plasmodium parasite exists in cultured media according to the morphology stages desired. The culturing process to obtain the trophozoite stage started 6 hours after the incubation process, 10 hours later for the mature trophozoite stage, and the next 6 hours for the schizont stage. As the time reached the desired stages, centrifuged the cultured blood at 1600 rpm for 5 minutes. Removed the supernatant and transferred blood sediment into 96 microtitre plate well. Then aliquoted 10µl of blood pellet into 96 microtitre well plate contained 90µl extract solution. Plates were then incubated at 37°C for 24 hours before pLDH Assay and SYBR Green 1 Fluorescence assay performed.

PLDH assay
Preparation of PLDH reagents
Malstat reagent preparation

120 mg natrium-L-lactate, 33 mg tris base buffer and 3-acetylpyridine adenine dinucleotide (APAD) were added in 6 ml deionized water. The mixture was kept in 4°C before use.

NBT-PES Salt solution preparation

1.6 mg NBT (Nitroblue tetrazolium) and 1 mg of phenazine ethosulfate were dissolved in 10 ml distilled water in dark room condition. Solution was wrapped in aluminium foil, 4°C before use.

Antimalarial activity determination via PLDH assay

10 µl lysed red blood cells were mixed with 20 µl of Malstat reagent and 30 µl of NBT-PES into a new 96 microtiter plate well. The addition of NBT-PES needs to be done in a dark place as it is photosensitive. Every sample was run in triplicate in order to get a legit average reading via ELISA reader at 655 nm wavelength. The IC_{50} readings were obtained through Graph Pad version 7.2. PLDH activities were measured using the formula below:

$$\frac{(infected\ erythrocytes + extracts) - (non - infected\ erythrocytes + complete\ RPMI\ 1640\ media)}{(infected\ erythrocytes + complete\ RPMI\ 1640\ media) - (non - infected\ erythrocytes + complete\ RPMI\ 1640\ media} \ x\ 100$$

SYBR Green 1 Fluorescence assay

Similar technique applied for SYBR Green 1 Fluorescence assay following the manual kit provided. Every sample was run in duplicate via ELISA reader at wavelength at 497 nm and the emission wave at 520 nm. Readings were obtained in similar manners too.

Statistical analysis

The reading of IC50 for both 5% prasitemia level and morphology level were done in triplicate. By using the formula stated above, the values were then plotted using GraphPad Prism version 7.2 in order to obtain a graph and IC50 value. A one-way ANOVA was used to determine the difference of mean between extracts (IC50) and the difference mean between different morphology of malarial stages (IC50) with significant value $p < 0.05$ (Statistical Package for the Social Sciences (SPSS) Version 18).

Materials and methods

Determination of parasitemia level

Figure 1 shows a thin blood film stained with FIELD Stain. The figure showed the malaria parasites stained in deep red chromatin and pale blue cytoplasm, leucocyte, purple nuclei, and a pale blue background. Lysed red cells revealed only background stroma.[15] In Figure 2, young trophozoite and schizont were detected in the thin blood film. Usually, ring forms were common in 5% parasitemia, where trophozoites and a few schizonts appeared in the thin blood film. Infected erythrocytes were counted using a cell counter and calculated using the formula stated below:

$$Parasitemia\ percentage\ (\%) = \frac{Total\ infected\ erythrocytes}{Total\ of\ erythrocytes}\ x\ 100$$

Fig. 1: 5% parasitemia of infected erythrocytes with P. berghei NK65 using FIELD stain under 100x magnification using light microscopy. Malaria parasites stain deep red chromatin and pale blue cytoplasm. Leucocyte stained purple nuclei and pale blue background. Red cells that are lysed showed only background stroma remains

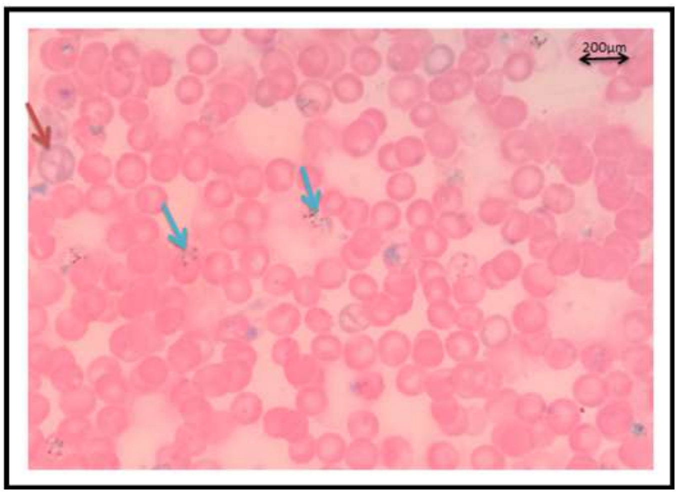
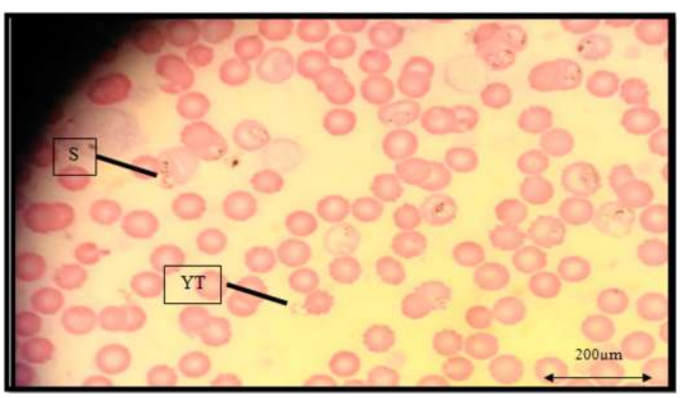


Fig. 2: Thin Blood film stain by FIELD stain showed the morphology of young trophozoite (YT) and schizont (S) stage of *Plasmodium berghei* under 100x magnification light microscope



Ic_{50} reading on 5% parasitemia using pLDH assay

The IC_{50} readings of C. *odontophyllum* leaf extracts (acetone, methanol, and aqueous) and chloroquine as positive controls against P. *berghei* NK65 at 5% parasitemia level were determined by two methods: the pLDH Assay and the SYBR Green-1 fluorescence Assay, which are presented in Table 1. From the table, it showed that methanol extracts (0.00045 µg/ml) were more potent when compared to the other two extracts, acetone (0.017 µg/ml) and aqueous (0.0092 µg/ml) and chloroquine (0.0011 µg/ml). The results were further interpreted as in Graph 1, where it showed that chloroquine inhibits the malarial parasite at 50% at the lowest concentration of 0.00001 µg/ml, methanol extracts at the lowest concentration of 0.0001 µg/ml, acetone extracts at 0.1 µg/ml, and aqueous at 0.01 µg/ml. The graphs were plotted using the x-axis as log-concentration (log-µg/ml) starting from 1 µg/ml and pLDH activity (%) as the y-axis. The pLDH activity was calculated using the formula from [16], as stated.

$$\frac{(infected\ erythrocytes + extracts) - (non - infected\ erythrocytes + complete\ RPMI\ 1640\ media)}{(infected\ erythrocytes + complete\ RPMI\ 1640\ media) - (non - infected\ erythrocytes + complete\ RPMI\ 1640\ media}\ x\ 100$$

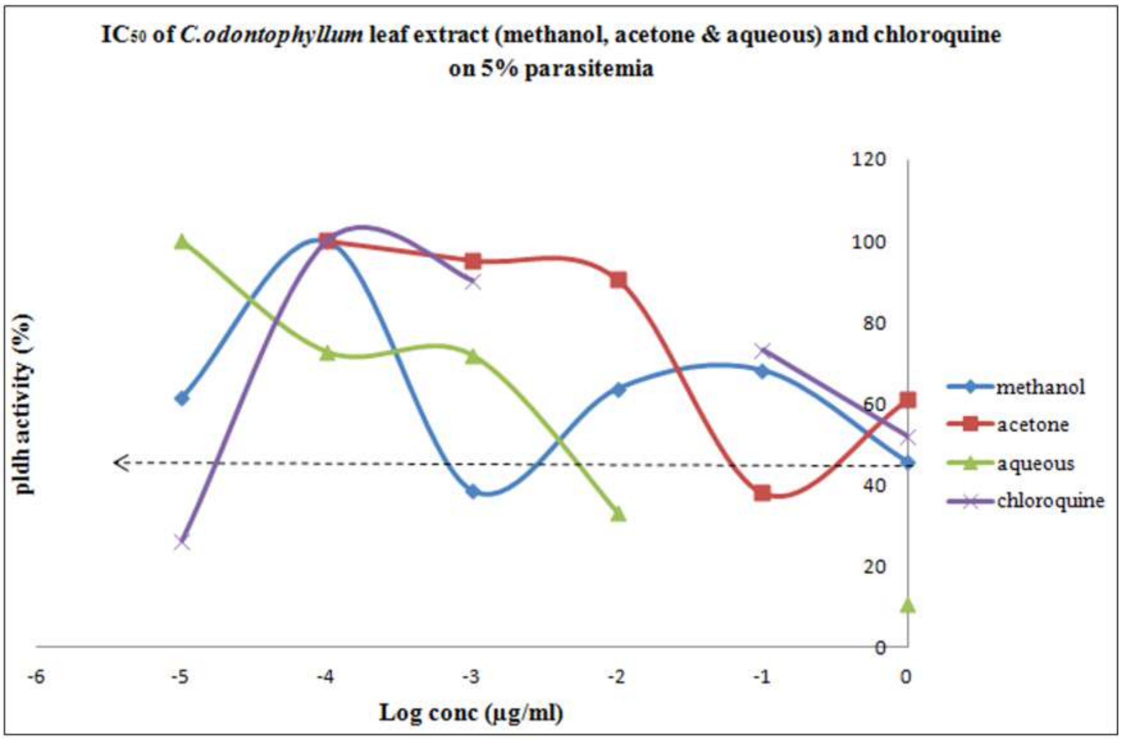
Ic_{50} reading on 5% parasitemia using SYBR Green I fluorescence Assay

Table 1 stated that the value of inhibition of IC_{50} of malaria parasite with the methanol extraction of C. *odontophyllum* was at 0.002 µg/ml, which is the lowest value compared to other types of extracts. The value of inhibition of IC_{50} for aqueous extraction of C. *odontophyllum* is 0.0068 µg/ml, and the acetone extraction value of IC_{50} is 4.371 µg/ml. The methanol extract showed the value of IC_{50} was ten times more inhibition rates than the value of IC_{50} chloroquine as a positive control compared to acetone and aqueous. Graph 2 showed the graph of inhibition DNA activities of the P. *berghei* parasite with three types of C. *odontophyllum* extraction (methanol, acetone, and aqueous) at a 5 % level of parasitemia. Graph 2 showed the value of Inhibition Concentration (IC_{50}) of three types of extraction; methanol (0.0002 µg/ml), acetone (4.37 µg/ml), and aqueous (0.0068 µg/ml) and positive control, chloroquine (0.0293 µg/ml).

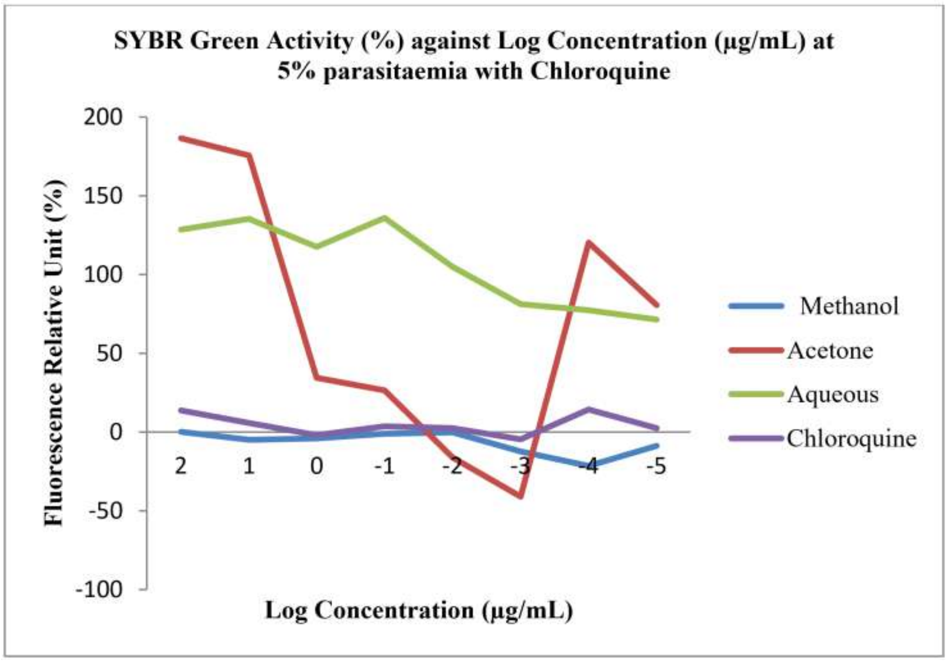
Table 1: The results of the IC50 of acetone, methanol, and aqueous extracts from C. *odontophyllum* leaf extracts and chloroquine on 5% parasitemia level using two different methods; PLDH Assay &SYBR Green 1 Fluorescence assay

Type of extracts/test	IC_{50} (µg/ml)	
	PLDH Assay	SYBR Green 1 Fluorescence assay
Chloroquine (positive control)	0.0011	0.0293
Acetone	0.017	4.371
Methanol	0.00045	0.002
Aqueous	0.0092	0.0068

Graph 1: IC50 of C. *odontophyllum* leaf extracts (Aqueous, Methanol, and Acetone) and chloroquine on 5% parasitemia by using plasmodium lactate dehydrogenase (pLDH)



Graph 2: DNA inhibition of Plasmodium *berghei* parasite towards *C. odontophyllum* extraction of methanol, acetone, and aqueous extract with the positive control of chloroquine at 5% parasitemia by using SYBR Green I Fluorescence Assay

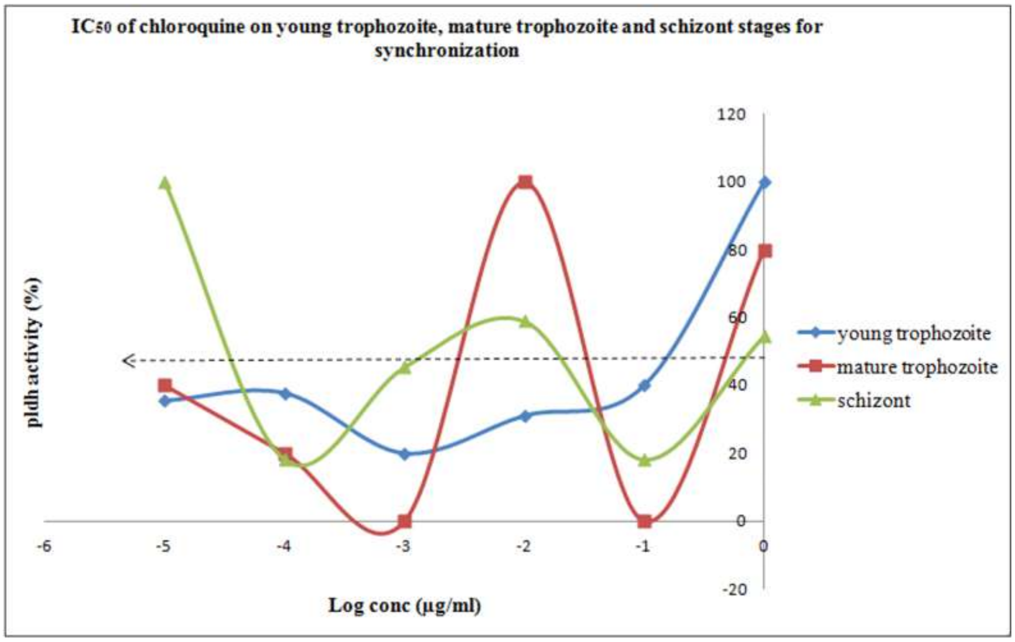


Synchronisation process (PLDH Assay and SYBR Green 1 Fluorescence Assay). Table 2 showed the IC50 reading of methanol leaf extract of C. *odontophyllum* and chloroquine on the synchronisation process via PLDH Assay and SYBR Green I fluorescence assay. Based on the table, the IC50 reading of methanol extracts for the PLDH Assay showed a lower IC50 reading at schizont stages (1.16×10^{-5} µg/ml), followed by the young trophozoite stages (6.27×10^{-5} µg/ml) and mature trophozoite stages (0.00031 µg/ml). IC50 reading for chloroquine, as stated according to stages, with mature trophozoite at 2.534×10^{-5} µg/ml, followed by schizont at 0.0004323 µg/ml and young trophozoite at 0.282 µg/ml. Based on the reading of IC50 obtained, the methanol extracts from C. *odontophyllum* leaf were shown to be more potent compared to chloroquine, thus proving their potential as an antimalarial drug. These results were summarised in Graph 3 and 4, where it was shown that the PLDH activity (%) was plotted against log-concentration (log µg/ml) of chloroquine and methanol extracts, respectively, starting from the highest concentration of 1 µg/ml to 0.00001 µg/ml, as the lowest concentration.

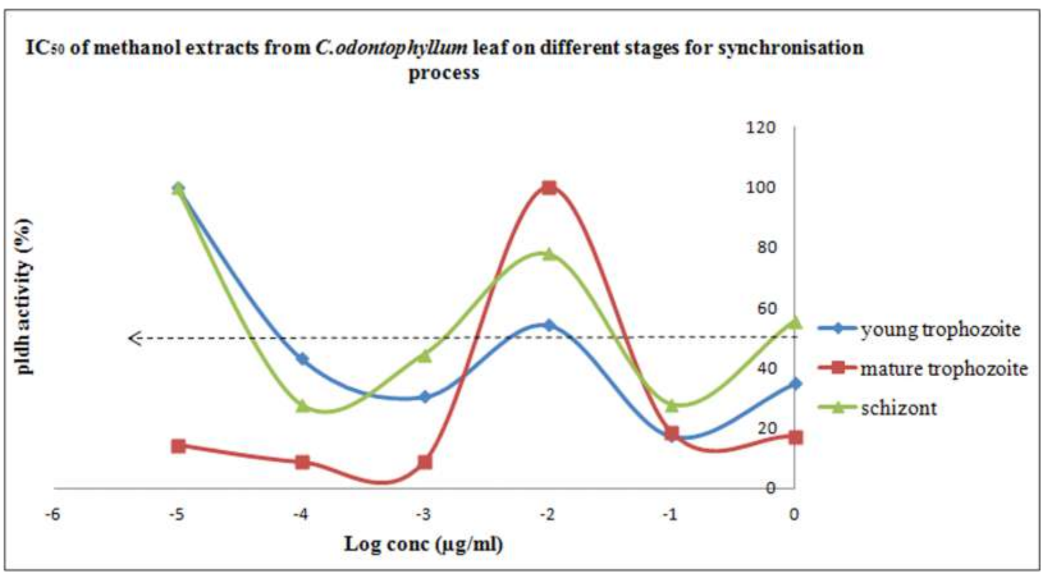
Table 2 The results of IC_{50} of methanol extracts from C. *odontophyllum* leaf and chloroquine (as positive control) on different morphology (young trophozoite, mature trophozoite, and schizont based on two methods, PLDH Assay and SYBR Green-1 fluorescence assay

Stages	Methanol Extract of C. *odontophyllum* (IC_{50})µg/mL		Positive control (Chloroquine) (IC50)µg/mL	
	PLDH Assay	SYBR Green-1 fluorescence assay	PLDH Assay	SYBR Green-1 fluorescence assay
Young Trophozoite	6.27×10^{-5}	0.00195	0.282	0.03219
Mature Trophozoite	0.00031	0.07231	2.534×10^{-5}	0.4567
Schizont	1.16×10^{-5}	0.5373	0.0004323	0.9646

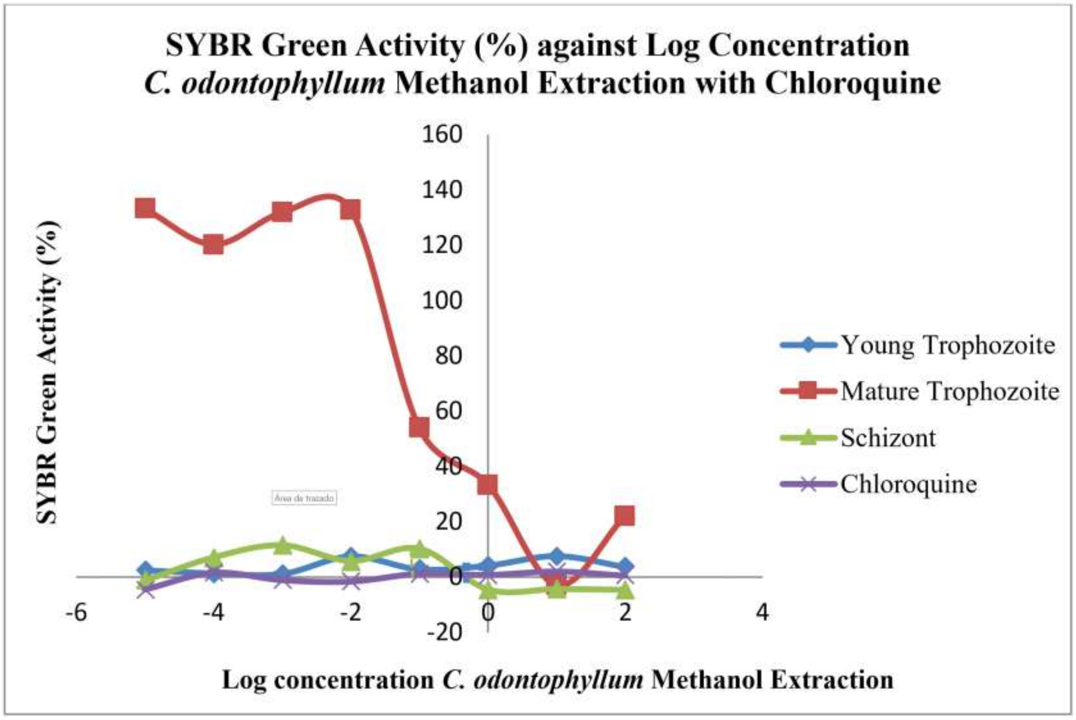
Graph 3: IC_{50} of Chloroquine (positive control) on young trophozoite, mature trophozoite, and schizont stages showed few points of young trophozoite and schizont from the lowest concentration of 0.00001µg/ml to 1µg/ml by using plasmodium lactate dehydrogenase (pLDH). The dotted line showed the 50% PLDH activity of Chloroquine on different morphology of plasmodium



Graph 4: IC_{50} readings of methanol extracts from *C.odontophyllum* leaf on young trophozoite, mature trophozoite, and schizont stages using pLDH) assay. The dotted line showed the 50% PLDH activity of *C.odontophyllum* leaf on different morphology of plasmodium



Graph 5: Graph of DNA inhibition activity by *Canarium odontophyllum* methanol extraction at the different morphology stages of *Plasmodium berghei* with the chloroquine (positive control). The chloroquine used is a positive control for young trophozite stage



In Table 2, the IC_{50} reading of methanol leaf extracts of C. *odontophyllum* on the young trophozoite stage (0.00195 µg/ml), which was the most potent, was followed by the mature trophozoite (0.07213 µg/ml), and the schizont stage (0.5373 µg/ml). For the positive control chloroquine, the value of the IC_{50} reading at the young trophozoite stage is the most potent, which is 0.03219 µg/ml, followed by the mature trophozoite at 0.4567 µg/ml and lastly, at the schizont stage with a value of 0.9646 µg/ml. Graph 5 shows the DNA inhibition activity with methanol leaf extracts of C. *odontophyllum* at different P. *berghei* morphology stages with chloroquine as a positive control. The chloroquine used as a positive control plot in the graph is from a young trophozoite's positive control. For chloroquine, the young trophozoite stage was picked as the benchmark because of the IC_{50} values that were close to those of the young trophozoite in methanol extracts. Graph 5 showed the IC_{50} values of DNA inhibition of methanol leaf extracts of C. *odontophyllum* at different P. *berghei* morphological stages with chloroquine as a positive control by the SYBR Green assay.

Discussion

Malaysia recorded a total of 1875 of confirmed cases in 2015 with a total death of 8 and estimate death less than 10 according to WHO,2015 report. Despite all the effort to control the spread of malaria, the number of cases was high. Reported malaria cases in Malaysia as of 2015 were only 1875 cases which showed so much decline compared to back in 1994 where the cases recorded were approximately 10,000 in Peninsular Malaysia and about 49,192 cases in Sabah state and with Sarawak state with annually cases between 1000 to 3000 cases since 19990s. [17] This showed how Sabah and Sarawak state still faces malaria with a high number of cases.

Dabai (Canarium odontophyllum) is locally known in Sarawak as Sibu olives. It had been widely planted in Kapit, Sibu, Sarikei and Limbang. It commands a good market price and commercial planting using superior selected quality material is promising. The export market is currently limited to Brunei and the state of Sabah. Studies involving leaves have proof to have an anticancer property [18] , antioxidant [19] while the fruits too believe to have anti-microbial and anti-inflammatory substances.[20] This prove that this plant has high potential to be explored more for its medicinal value.

A research done by Fredalina Basri, 2015 proved the existence of the total phenolics and extractable condensed tannins in the leaves, twigs and stem bark of Canarium album.[18] Although the research was for anticancer but in most malaria treatment drug, flavonoid and terpenoid played an important role as an antimalarial agent. However there are a few studies were other active compounds were as important as these two.

Phytochemical compounds such as alkaloids are commonly implicated in the antiplasmodial activity of many plants. Terpenes or terpenoids have been identified as active antiprotozoal and antimalarial agents in many pharmacological studies.[21] Flavonoid is believed in inhibiting the fatty acid biosynthesis (FAS II) of the parasite. Some flavonoids have also been shown to inhibit the influx of L-glutamine and myoinositol into infected erythrocytes.

The experiment started out with 5% parasitemia level of P. *berghei* NK65 harvested inside mice. Through thin blood film, 5% of parasitemia levels were determined before preceding the antimalarial treatment process. Figure 1 and Figure 2 showed a thin blood film of erythrocytes infected by P. *berghei* NK65. The blood film was stained at 5% of the parasitemia level with FIELD stain and observed via 100x magnification using light microscopy. In this study, 5% of the parasitemia levels were determined before the antimalarial treatment process. A 5% parasitemia level is calculated based on the formula stated in the result section.

It was chosen as it was considered an indicator of risk for severe malaria in a low-transmission area.[23] Parasite load estimation has been an objective measure of less than 5% parasitemia in drug testing. For testing the sensitivity and specificity of new technologies such as magnetic resonance relaxometry (MRR), a low parasitemia level was considered as low as 0.0001%.[24] However, for antimalarial drug testing, a 5% level of parasitemia is preferable as it indicates low parasite density.[25]

The IC_{50} (inhibition concentration) means the concentration of an inhibitor at which the response (or binding) is reduced by half. In other words, the lower the IC50, the more potent the extractions are. All IC_{50} readings, starting from 1 µg/ml concentration point to the lowest concentration, 0.00001 µg/ml, were recorded as shown in Table 1. All extracts showed good IC_{50} readings throughout this study. However, methanol extracts stood out from all three extracts of C. *odontophyllum* leaf extracts on P. *berghei* NK65; it was chosen to be tested further on different parasite morphological stages.

Based on previous studies by Satish et al. (2015),[26] the IC50 readings for inactive substances are 100 µg/ml for the in vitro antiplasmodial activity. Overall, the IC_{50} of C. *odontophyllum* leaf extracts (aqueous, methanol, and acetone) on 5% parasitemia was less than 5 µg/ml, which is classified as non-toxic substances.

The IC_{50} readings of extracts from C. *odontophyllum* leaf (aqueous, methanol, and acetone) and chloroquine via the pLDH assay were expressed in Graph 1. The x value indicated the reading of the IC_{50} of all three types of extractions and the positive control (chloroquine). Methanol extracts at the lowest concentration of 0.0001 µg/ml, acetone extracts at 0.1 µg/ml and aqueous at 0.01 µg/ml. Chloroquine inhibits the malarial parasite at 50% at the lowest concentration of 0.00001 µg/ml. Through this, it was indicated that the lowest concentration was the most potent.

Graph 2 showed the IC_{50} readings of the P. *berghei* parasite with three types of C. *odontophyllum* extraction (aqueous, methanol, and acetone) tested using the SYBR Green 1 Fluorescence Assay at a 5% level of parasitemia. Based on the IC_{50} values obtained, only methanol extract showed an inhibition value closer to chloroquine's positive control value. The methanol value of IC50 was 0.0020 µg/ml and the chloroquine (positive control) was 0.0293 µg/ml, aqueous (0.0068 µg/ml) and acetone (4.3710 µg/ml).

On top of that, for the synchronization process, methanol extracts are used among the three extracts. Synchronization was a process to obtain the same Plasmodium stage based on the different permeabilities of the parasitized RBC membrane using sorbitol. RBCs are naturally impermeable to sorbitol, while infected RBCs with mature stages have a permeable membrane due to the modifications in the parasites' structural. This property is used to kill mature forms of the parasite by osmotic shock without affecting uninfected RBCs or RBCs parasitized by ring stages.[14] pLDH activities were measured to see the effectiveness of C. *odontophyllum* leaf methanol extracts on each stage.

Based on Graph 3, the IC_{50} reading of chloroquine on young trophozoite, mature trophozoite, and schizont stages showed few points at the young trophozoite and schizont stages. The readings consist of the lowest concentration of 0.00001 µg/ml to 1 µg/ml via the pLDH assay. The readings were parallel to the 50% point, and only one point was for mature trophozoite, at approximately 0.1µg/ml.

Graph 4 showed the IC_{50} readings of methanol extract from C. *odontophyllum* leaf on young trophozoites, mature trophozoites, and schizont stages using pLDH assay. The points fall on the 50% inhibition point; few were seen on schizont, followed by mature trophozoites and young trophozoites.

This result showed that the extracts are more potent on the schizont stage than on mature trophozoite and young trophozoite stages, meaning they can be developed as schizonticidal. Chloroquine and quinine act as schizonticidal among the drugs used in antimalarial treatment. Quinine is a derivative from a natural plant cinchona tree bark.[27] As first-line drugs for malaria treatment, they were administered after the diagnosis, or even suspected, in severe disease.[28] These drugs destroyed the blood schizonts and prevented erythrocytic schizogony from happening and terminating the malarial fever attack.[29] This explained why chloroquine produced its low IC_{50} reading at schizont and mature trophozoite stages. However, nowadays these drugs were not used anymore as part of the treatment regime of malarial infection.[8]

Based on the synchronization result on the different morphological stages of the P. *berghei* NK 65 treated by methanol extracts of C. *odontophyllum* leaf. The lowest IC50 value indicated the most potent and the highest rate of DNA inhibition activity. It happens at the young trophozoites stage compared to the mature trophozoites and schizont stage. The highest value of inhibition IC50 from the population is the higher population of the parasites present in the erythrocytes.

According to Graph 5, the process of DNA duplication of parasites happened at the young trophozoites stage, where the DNA replication is followed by nuclear division and leads to the binuclear parasite, where it replicates the DNA and divides its nuclei several times, forming a syncytial cell with 8-24 nuclei. Nuclear division is endomitotic, a common feature in unicellular eukaryotes, and the segregating chromosomes and the spindle apparatus remain within the nuclear envelope throughout the process. The individual chromosomes do not condense into tight, visible structures like what happens in the common mitosis process. *Plasmodium berghei* has a plastid-like organelle that contains a circular, extra-chromosomal genome of ~30 kb (apicoplast genome), similar to P. *falciparum*.

This DNA shows 70-95% homology with the 30 kb apicoplast genome of P. *falciparum*, and the arrangement of characterized genes is similar to those found on the P. *falciparum* apicoplast genome. Also, rodent parasites have a ~6 kb extra-chromosomal mitochondrial DNA, homologous to P. *falciparum's* mitochondrial genome. The total duration of the asexual blood-stage development is 22-24 hours. Mature schizonts in mature erythrocytes usually contain fewer merozoites (8-12) than schizonts in reticulocytes (16-18 nuclei). In the mature schizonts, the pigment granules (hemozoin granules) become compacted in a single 'food vacuole' as a single, dense, rounded mass.[30] The precision with which fluorescence assay resolves multiply-infected erythrocyte peaks require the culture to be at the ring-stage. The culture transitions from late rings to early trophozoites; the parasite begins to replicate its DNA. However, ex vivo parasites are almost always ring-stage parasites as later stages of parasite development. Late-stage trophozoites and schizonts sequester *in vivo*, and blood collected from malaria patients consist almost exclusively of ring-stage parasites.[31]

Based on the principle of SYBR Green-1 Assay, more reactions happen between the intercalation dye with DNA double helices of P. *berghei* NK65. The indication of the parasite population is when more action of the inhibition of the DNA activity will give a higher value of IC_{50}. The lowest value of IC_{50} means that the most effective treatment of the methanol towards the different morphological stages of the parasites. In this study, the young trophozoite stage shows the lowest value of IC_{50} compared with other stages. Generally, the main principle behind the SYBR Green 1 Assay is through intercalation dye with DNA double helices of P. *berghei* NK 65, then will be formed SYBR Green DNA complex.[32] In molecular biology, SYBR Green 1 was used as a substitute for ethidium bromide for several years. It is an asymmetrical cyanine dye, binding to double-stranded DNA, preferring G and C base pairs.[33] When intercalated into DNA, it is highly fluorescent, absorbing light at a wavelength between 390 and 505 nm, with a peak at 497 nm and a secondary peak near 254 nm. Emitting lights at 505 to 615 nm, with a peak at 520 nm. The results obtained by Bacon and others, who used a mean starting 0.75% parasitemia level for their comparison of the SYBR Green I and the HRP2 Assay, showed that the SYBR Green I assay had an excellent performance in detecting higher parasite densities.[34] SYBR Green I is a highly sensitive indicator of DNA. Its inability to discriminate parasitical and non-parasitical DNA, such as WBCs, makes it an unspecific assay and also highly susceptible to contamination.[35] Therefore, in this study, blood filtration using the cellulose fiber column is essential to get only the red blood cells as a filtrate. From the study, the process of synchronization for the lowest value in IC50 was at the young trophozoite stage at the ring stage.

As an enzyme assay for P.*falciparum* detection, the Plasmodium lactate enzyme (pLDH) worked as the potential molecular target for antimalarial. By converting lactate to pyruvate, the lactate dehydrogenase (LDH) enzyme used up APAD as a co-enzyme instead of NAD in this reaction. Lactate dehydrogenase is named as the most abundant enzyme in Plasmodium species. The measured development of APADH leads to the formation of a product that could establish an assay that detects P. *falciparum's* presence from in vitro cultures. A correlation between parasitemia levels and the activity of parasite LDH is helpful as LDH does not remain in the blood, but clears about the time as the parasite, following successful treatment.[36] They showed a good correlation between parasitemia level and the level of pLDH enzyme activity and protein. These proved that methanol leaf extracts from C. *odontophyllum* have a potential value as an antimalarial drug. These two studies confirm that different method used in measured inhibition concentration of the parasite population based on the morphological stages gives different results. The principle behind the methods itself has a different impact on the outcome. For the SYBR Green-1 Fluorescence assay, the process adhered only to double-stranded DNA, and any fluorescence that is detected is attributable to parasite DNA.[37]

Conclusion

In conclusion, the methanol leaf extract of C.*odontophyllum* can further develop into an antimalarial drug, as it can inhibit the Plasmodium at 5% parasitemia level and on different morphology stages, young trophozoite, mature trophozoite, and schizont. Future studies in identifying the mechanism of action of C.*odontophyllum* leaf extracts might be helpful in future development of antimalarial drug.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Authors' contributions

All authors have contributed in all stages of the investigation and have approved the final version.

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