CHAPTER 3

Results

3.1 Flow cytometric analysis of *P. falciparum* parasite

SYBR Green I is an intercalating cyanine dye that binds to double-stranded DNA and can freely permeate through the cell membrane, so chemical fixing agent such as glutaraldehyde is not necessary (Rengarajan *et al*, 2002). Its DNA staining property can distinguish parasite-infected RBC (PRBC) from non-infected RBC (NRBC). Because mature human erythrocytes do not contain nucleic acids, this technique can detect the presence of parasitic DNA in PRBC and allow for the rapid quantification of PRBC populations by considering flow cytometric profile (Campo *et al*, 2011). Green fluorescence intensity (FI) of SYBR Green I-stained *P. falciparum*-infected RBC was examined by using flow cytometric technique. After the fluorochrome is excited at 488 nm with a laser beam, fluorescence emits light at 529 nm which can be detected at the same channel as fluorescein isothiocyanate (FITC) using 530/30 band pass filter. The bi-dimensional assessment was applied in this study by using the FITC channel/phycoerythrin (PE) channel method (Somsak *et al*, 2012). Flow cytometric profilings of NRBC (A) and PRBC (B) were shown in **Figure 3-1**. Importantly, the SYBR Green I flow cytometric assay is commonly used in antimalarial drug susceptibility test because it is a rapid and precise tool for a high-throughput screening. Regression analysis shows a significant correlation, $R^2 = 0.9890$ (**Figure 3-2**).

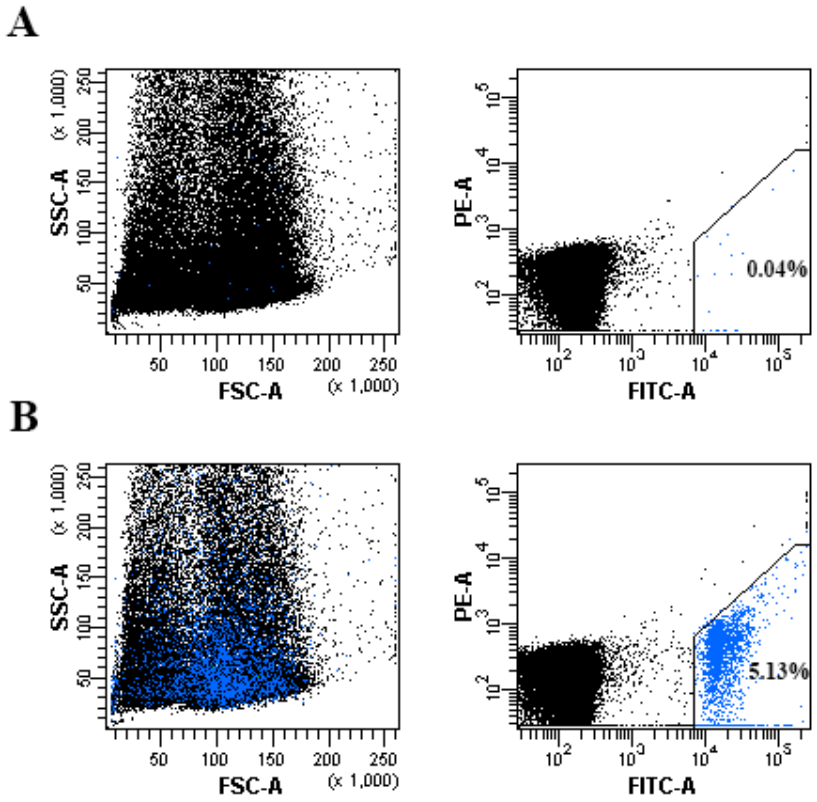

Figure 3-1 Analysis of SYBR Green I-fluorescence intensity by flow cytometry of *P. falciparum*-infected RBC. Dot plot represents population of human red blood cells. Typical flow patterns of human erythrocytes show in a FSC/SSC dot plot (both parameters in linear scale; dot plots on the left side of the figure) and the bi-dimensional analysis method of samples were analyzed using the FITC channel/PE channel method (both parameters in logarithmic scale; dot plots on the right side of the figure). The upper panels are from non-infected controls and show 0.04% of background signal (A). The lower panels are from analysis of *P. falciparum*-infected RBC and show 5.13% of parasitemia (B). The samples were obtained from ring-stages *in vitro* malaria culture of *P. falciparum*-infected RBC with synchronization technique.

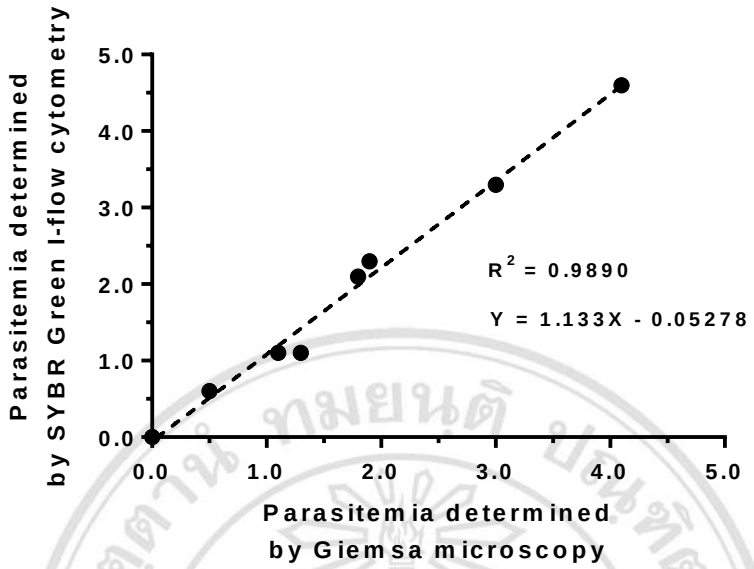

Figure 3-2 Correlation of flow cytometric and microscopic medthods for determination of parasitemia. Blood samples of *P. falciparum*-infected human RBC with a parasitemia between 0.0 and 5.0% were measured for the parasites using both SYBR Green I-based flow cytometric technique and conventional Giemsa staining microscopic method. Regression analysis showed a correlation of $R^2 = 0.9890$ between the two assays.

3.2 *In vitro* drug-susceptibility testing of *P. falciparum*

3.2.1 Single drug treatment

This study was experimentally designed to investigate antimalarial property of a novel chelator CM1 and natural compound GTE compared with other iron chelators and antimalarial drugs *in vitro* model. In the assay, chelators (DFO, DFP, DFX and CM1), GTE and antimalarial drugs (PYR and DHA) were incubated with *P. falciparum* stain 3D7-infected RBC for 48 h and measured by flow cytometric analysis as previously described in **Section 2.3.2**. The dose response curve describes the relationship between response to drug treatment and drug dose or concentration. It was plotted using a linear scale of percentage of parasite growth against a logarithmic scale of dose (concentration) and fitted by non-linear regression to provide IC_{50} values.

As shown in **Figure 3-3**, DFO, GTE (EGCG equivalent concentration), CM1, DFX and DFP had different efficiencies in inhibition of *P. falciparum* growth and development as shown in a percentage inhibition of parasite growth. Their IC_{50} values are 14.09, 21.11, 35.14, 44.71 and 58.25 μM, respectively, as summarized in **Table 3-1**. Indeed, this study showed that DFO has the most antimalarial effect among different chelators. GTE and CM1 both have lower inhibitory effect on parasite growth in comparison with DFO, whereas higher than the others. In comparison, DHA and PYR (IC_{50} = 1.93 and 37.89 nM, respectively, **Table 3-2**) are much more potent than these compounds as they are clinically established antimalarial drugs, with their IC_{50} values in the low nanomolar range, as shown in **Figure 3-4**.

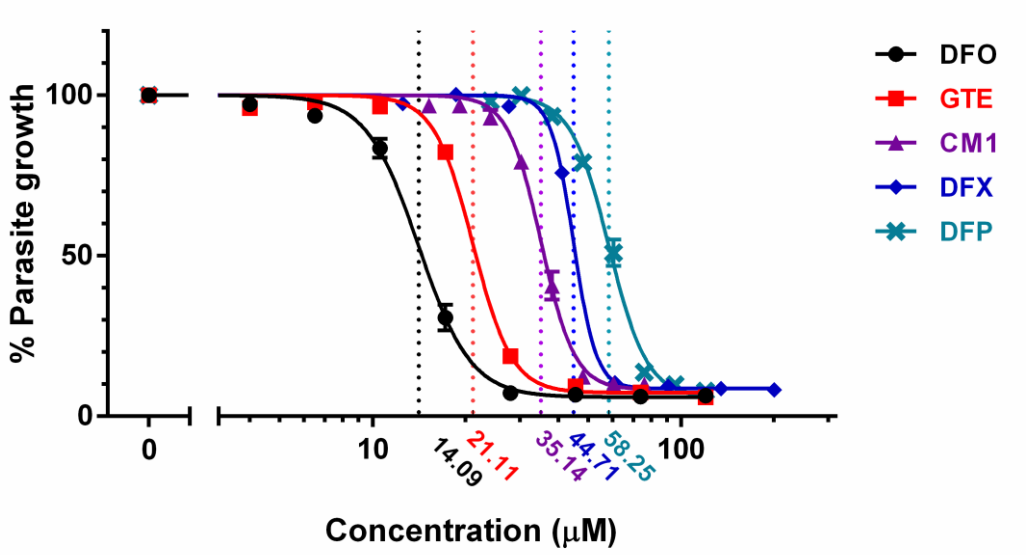

Figure 3-3 Effect of iron chelators and GTE on growth of *P. falciparum*. *P. falciparum*-infected RBC were treated with chelators and GTE (EGCG equivalent concentration) ranging of 0-200 μM for 48 h. The percentage of parasite growth was calculated from parasitemia, determined by using flow cytometry. Data was obtained from three independent experiments performed in triplicate and expressed as mean±SEM.

Table 3-1 *In vitro* susceptibilities of *P. falciparum* against iron chelators and GTE.

	Concentration (μM)	
	IC_{50}	**95% CI**
DFO	14.09	13.58 - 14.60
GTE (EGCG equivalent)	21.11	20.45 - 21.79
CM1	35.14	34.29 - 36.01
DFX	44.71	43.71 - 45.73
DFP	58.25	56.75 - 59.79

Abbreviations: CI = confidence interval; CM1 = 1-(*N*-Acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one; DFP = deferiprone; DFO = desferrioxamine; DFX = eferasirox; GTE = green tea extract; IC_{50} = 50% inhibitory drug concentration.

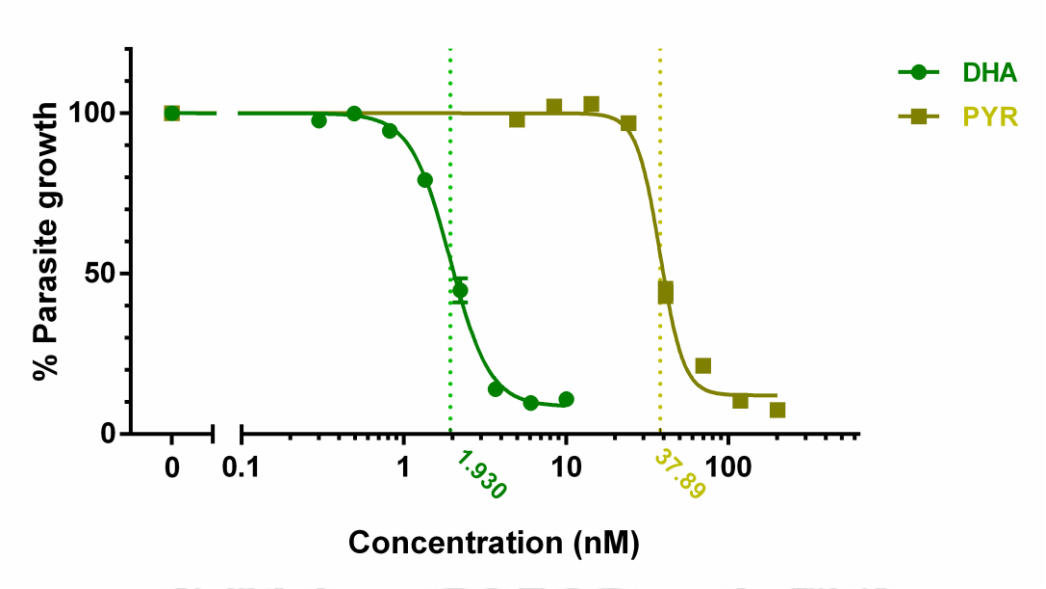

Figure 3-4 Effect of antimalarial drugs PYR and DHA on growth of *P. falciparum*. *P. falciparum*-infected RBC were treated with antimalarial drugs PYR and DHA in the range of 0-200 nM for 48 h. The percentage of parasite growth was calculated from parasitemia, determined using flow cytometry. Data was obtained from three independent experiments performed in triplicate and expressed as mean±SEM.

Table 3-2 *In vitro* susceptibilities of *P. falciparum* against antimalarial drugs.

	Concentration (nM)	
	IC_{50}	**95% CI**
DHA	1.93	1.85 - 2.02
PYR	37.89	36.30 - 39.55

Abbreviations: IC_{50} = 50% inhibitory drug concentrations; CI; confidence interval; DHA = dihydroartemisinin; PYR = pyrimethamine.

3.2.2 Drug combination treatment

Subsequently, we determined agonistic and antagonistic *in vitro* drug interactions with antimalarial agents. In the assay, either CM1 (0-200 µM) or GTE (0-200 µM EGCG equivalent) was combined with fixed concentration of antimalarial drug (PYR, 30 nM) and were incubated with *P. falciparum* stain 3D7-infected RBC for 48 h. Then samples were measured by flow cytometric analysis as previously described in **Section 2.3.2** and analysis of drug interactions was performed and data were expressed in histogram.

Using CM1 in combination with PYR, the percentage of parasite growth has no significant difference between PYR alone group and CM1 alone group. In PYR+CM1 groups, parasite growth seemed to be an upward trend from 5 to 20 µM of CM1 combined with PYR. Thereafter, there was a downward trend and a significant difference between PYR alone group and PYR+CM1 groups, from 50 to 200 µM of CM1 combined with PYR, as shown in **Figure 3-5**.

In GTE combination with PYR, the results were quite similar with PYR-CM1 combination, the percentage of parasite growth has no significant difference between PYR alone group and GTE alone group. In PYR+GTE groups, parasite growth seemed to be an upward trend from 5 to 10 µM of GTE combined with PYR. Thereafter, there was a downward trend and a significant difference between PYR alone group and PYR+GTE groups, from 35 to 200 µM of GTE combined with PYR, as shown in **Figure 3-6**.

It is most likely that 30 nM PYR with low doses of CM1 (5-20 µM) does not enhance the PYR activity whereas high doses of CM1 (35-200 µM) clearly synergized the PYR activity in a concentration-dependent manner. Similar effects could also seen in the PYR-GTE combination.

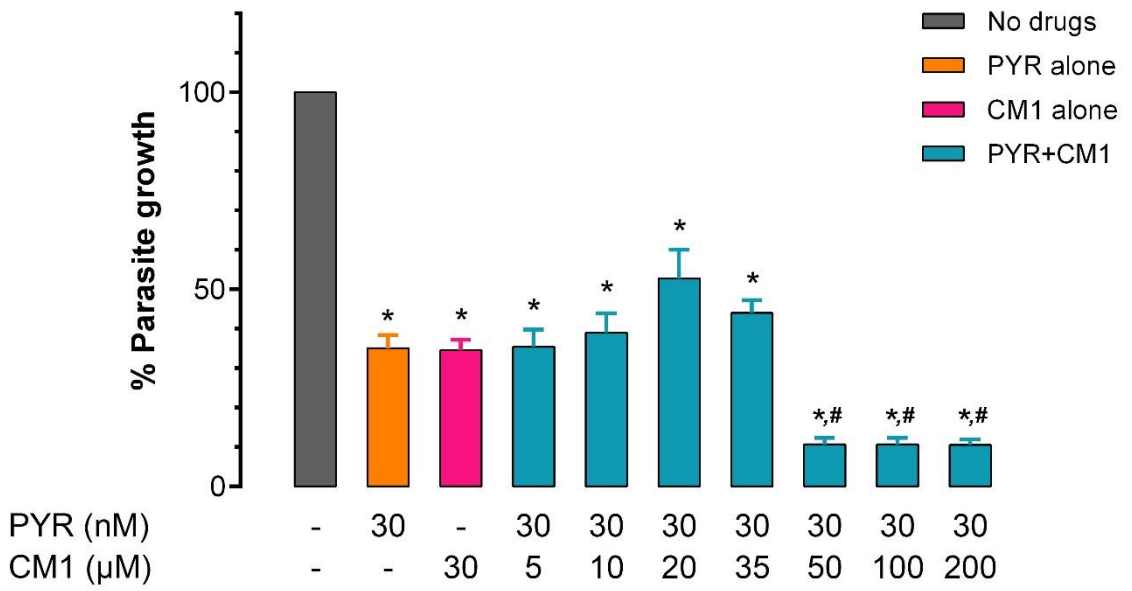

Figure 3-5 Effect of PYR combined with CM1 on growth of *P. falciparum*. *P. falciparum*-infected RBC were treated with antimalarial drug PYR (fixed concentration of 30 nM) combined with CM1 (0-200 μM) for 48 h. The percentage of parasite growth was calculated from parasitemia, determined using flow cytometry. Data were obtained from three independent experiments performed in triplicate and expressed as mean±SEM. $^{*}P<0.05$ compared with non-treatment group; $^{\#}P<0.05$ compared with PYR alone group (one-way ANOVA followed by Tukey's multiple comparison test).

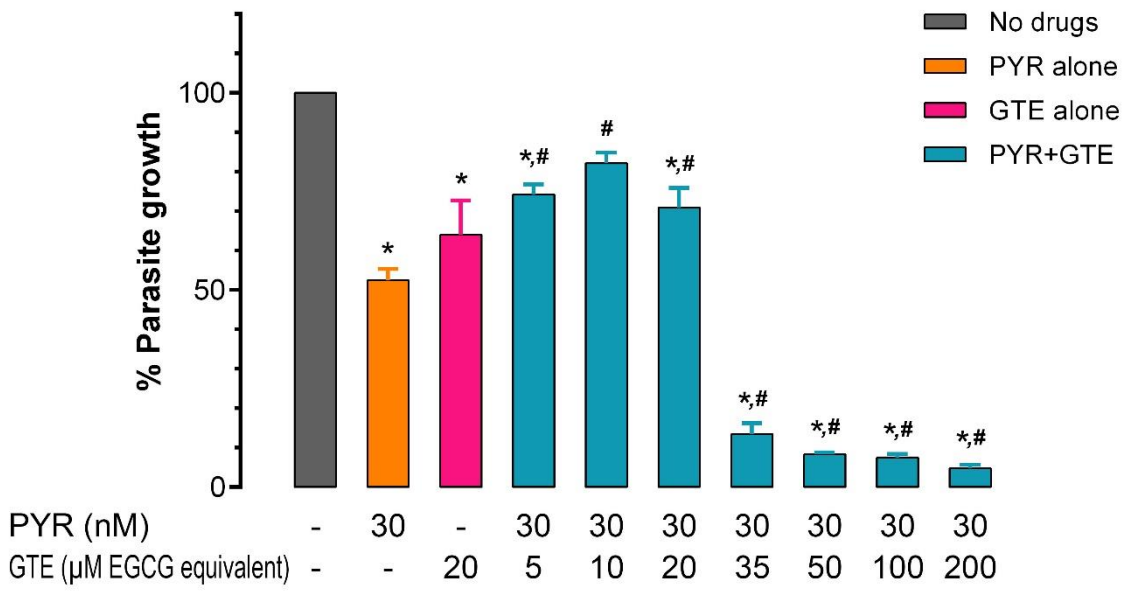

Figure 3-6 Effect of PYR combined with GTE on parasite growth of *P. falciparum*. *P. falciparum* parasites were treated with antimalarial drug PYR (fixed concentration of 30 nM) combined with GTE (EGCG equivalent of 0-200 μM) for 48 h. The percentage of parasite growth were calculated from parasitemia, determined using flow cytometry. Data was obtained from three independent experiments performed in triplicate and expressed as mean±SEM. $^{*}P<0.05$ compared with non-treatment group; $^{\#}P<0.05$ compared with PYR alone group (one-way ANOVA followed by Tukey's multiple comparison test).

3.3 Effect of iron chelators and GTE on intracellular LIP levels in *P. falciparum*-infected RBC

This experiment was designed to investigate whether or not CM1 and GTE were able to remove the intracellular LIP levels in *P. falciparum*-infected RBC and compare it to the standard chelator DFP. Green fluorescent dye calcein which is a product obtained from esterase hydrolysis of non-fluorescent calcein acetomethoxy (Calcein-AM) was used to probe and measure the amount of LIP. Previously, SYBR Green I fluorescent dye was used to probe *P. falciparum* DNA and to distinguish between PRBC and NRBC. However, SYBR Green I (488 nm/529 nm) was unable to be used whereas calcein AM (488nm/530nm) was used simultaneously, due to emitting the same green fluorescence. Instead, red fluorescent SYTO-61 dye was used to distinguish between NRBC and PRBC completely as seen in flow cytometric profile in **Figure 3-7**, which calculated parasitemia could be also obtained.

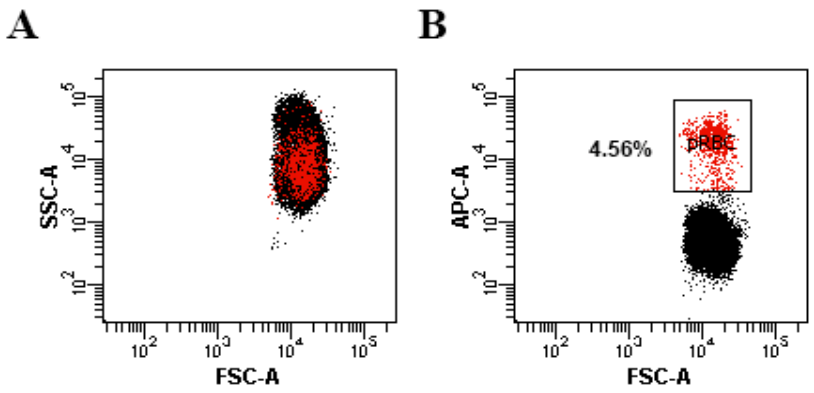

Figure 3-7 Discrimination of PRBC from NRBC by flow cytometry using SYTO-61 dye. (A) Typical flow patterns of human erythrocytes showed in a forward scatter (FSC)/side scatter (SSC) dot plot (both parameters in logarithmic scale). (B) PRBC and NRBC were determined by assessing the fluorescence of SYTO-61 (–) (uninfected) and SYTO-61 (+) (parasitized) RBC, the parasitemia is indicated in its panel.

In the assay, *P. falciparum*-infected RBC was treated with either of the two chelators (CM1 and DFP) or natural compound GTE for 2 h. The treated cells were then washed with PBS, stained with two-color fluorescent dyes, SYTO-61 and calcein-AM, and analyzed with a flow cytometer (488 nm/633 nm; 488 nm/530 nm, respectively). The results showed that CM1 significantly removed intracellular LIP in *P. falciparum*-infected RBCs in a concentration-dependent manner (**Figure 3-8** and **3-9**). DFP treatments (0-100 µM) were not effective in removing LIP in the PRBC; nonetheless, DFP at 200 µM was able to decrease LIP levels in the PRBC (**Figure 3-10**). Notably, GTE had similar result to CM1 (**Figure 3-11**). GTE would probably be more effective in reducing LIP level in the *P. falciparum*-infected RBC than CM1. Moreover, the parasitemia of CM1, DFP and GTE treatments showed a similar trend to decrease, as shown in **Figure S-1, S-2** and **S-3**.

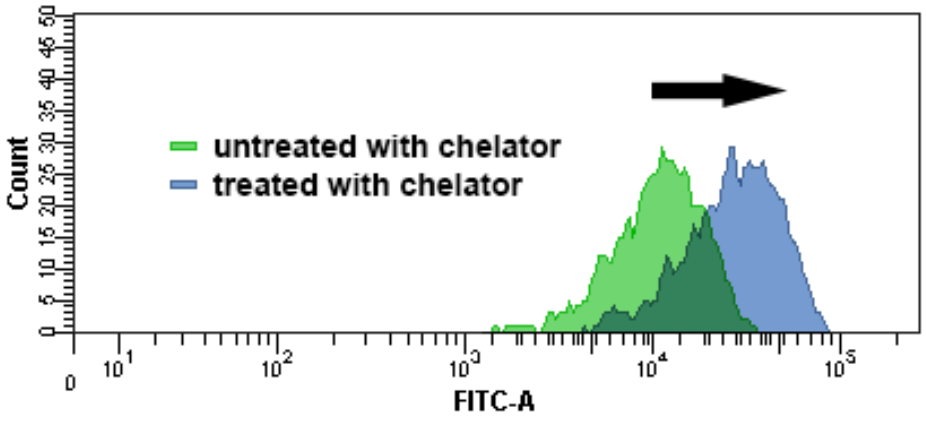

Figure 3-8 Measurement of intracellular LIP in *P. falciparum*-infected RBCs using flow cytometry. The LIP of infected RBCs [SYTO-61 (+)] population, gated from **Figure 3-7B**, was determined by evaluating the change in mean fluorescent intensity (ΔMFI) of calcein-loaded cells achieved in the presence of different iron chelators.

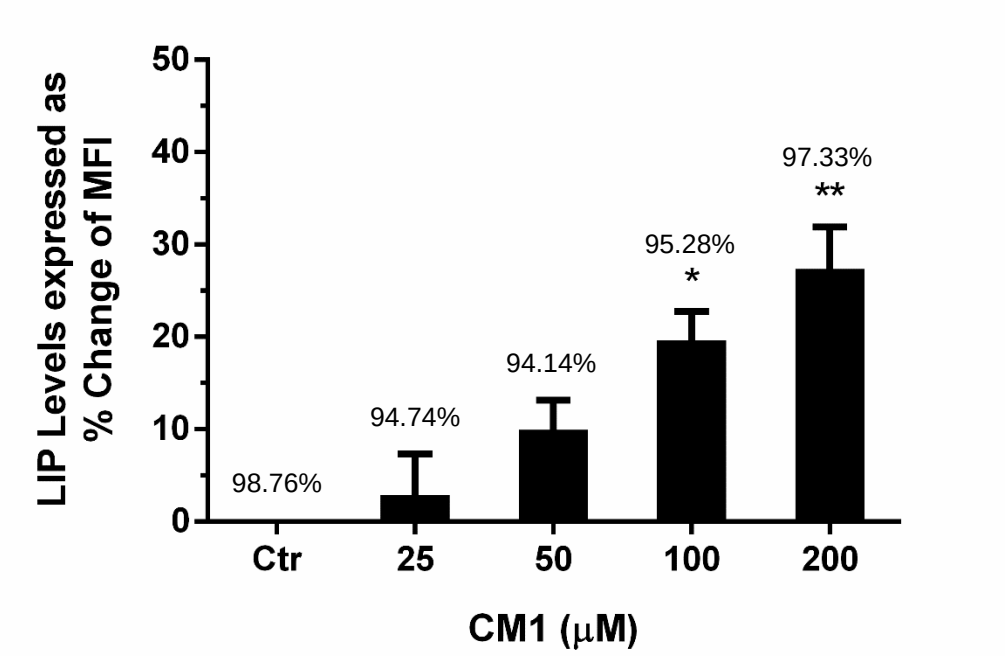

Figure 3-9 Levels of LIP in *P. falciparum*-infected RBC treated with CM1 (0-200 μM) for 2 h. Data was obtained from three independent experiments performed in triplicate and expressed as mean±SEM. $^{*}P$ <0.05 and $^{**}P$ <0.01 compared with the control group (one-way ANOVA followed by Tukey's multiple comparison test). An increase in MFI is related to a decrease in LIP levels. % Parasite growth was indicated above each histogram.

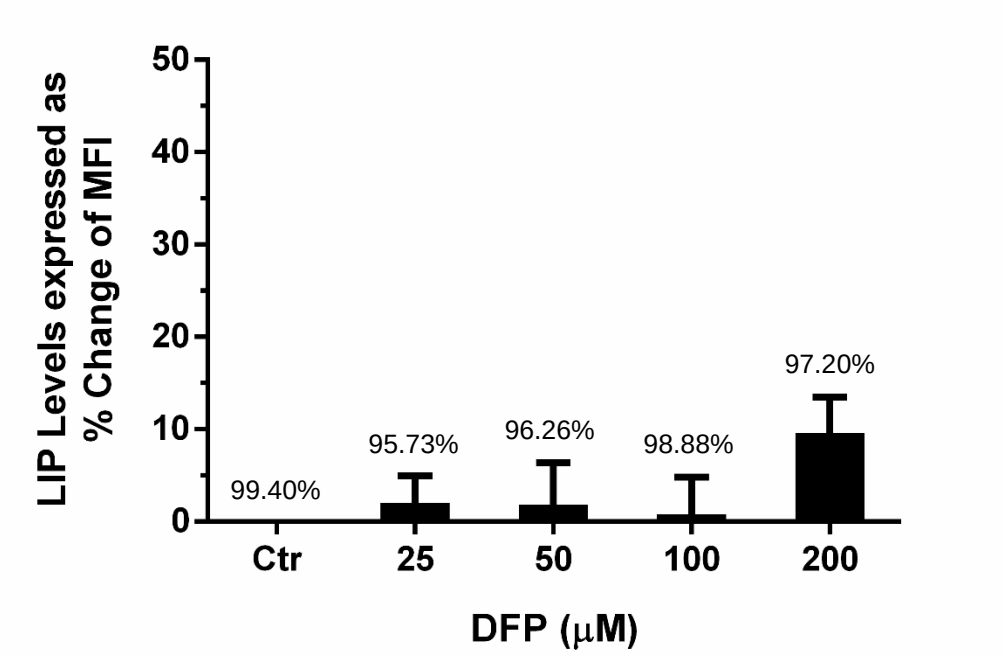

Figure 3-10 Levels of LIP in *P. falciparum*-infected RBCs treated with DFP (0-200 μM) for 2 h. Data was obtained from three independent experiments performed in triplicate and expressed as mean±SEM. **P* <0.05 and ***P* <0.01 compared with the control group (one-way ANOVA followed by Tukey's multiple comparison test). An increase in MFI is related to a decrease in LIP levels. % Parasite growth was indicated above each histogram.

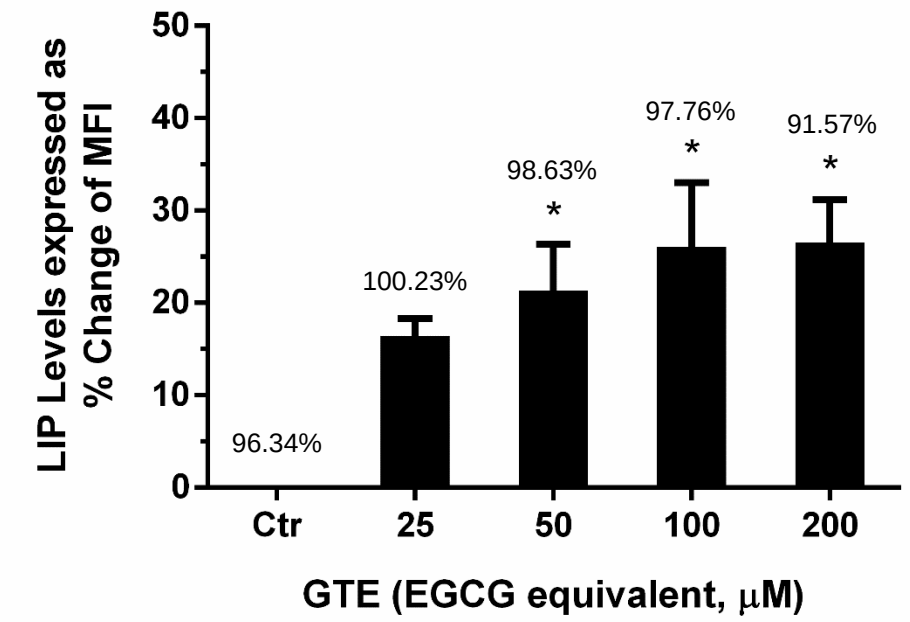

Figure 3-11 Levels of LIP in *P. falciparum*-infected RBC treated with GTE (0-200 μM EGCG equivalent) for 2 h. Data was obtained from three independent experiments performed in triplicate and expressed as mean±SEM. $^{*}P$ <0.05 and $^{**}P$ <0.01 compared with the control group (one-way ANOVA followed by Tukey's multiple comparison test). An increase in MFI is related to a decrease in LIP levels. % Parasite growth was indicated above each histogram.

3.4 Effect of chelators and GTE on intracellular ROS levels in *P. falciparum*-infected RBC

Other previous studies suggested that EGCG mediated generation of H_2O_2, triggering Fe(II)-dependent formation of highly toxic radicals, which in turn induced oxidative cell damage in various cell types (Suh *et al*, 2010; Tao *et al*, 2014). Some iron chelators are also able to induce reactive oxygen species (ROS) production (Agriesti *et al*, 2013; Pullarkat *et al*, 2012).

This experiment was designed to measure whether GTE and chelators were able to increase the intracellular ROS levels in *P. falciparum*-infected RBC affecting parasite growth or not. *P. falciparum*-infected RBC was treated with either chelators (CM1 and DFP) or natural compound GTE for 2 h. Then, the treated cells were stained with two-color fluorescent dyes. The red fluorescent SYTO-61 dye was used to distinguish between NRBC and PRBC completely by flow cytometry and the parasitemia could be calculated, as described in **Figure 3-7**. Also dichlorofluorescein (DCF) diacetate dye, which is oxidized in the presence of intracellular ROS into the green fluorescent DCF, was used to probe and measure the amount of intracellular ROS, similar to calcein-based LIP assay as shown in **Figure 3-8**.

DFP and CM1 at any dose did not affect the levels of intracellular ROS in the PRBC (**Figure 3-12** and **13-13**). Surprisingly, GTE treatment was able to decrease the intracellular ROS level significantly in the PRBC in a concentration-dependent manner, rather than increase ROS production (**Figure 13-14**). Possibly, GTE is anti-oxidative natural product that can scavenge persisting ROS and free radicals while DFP and CM1 are not.

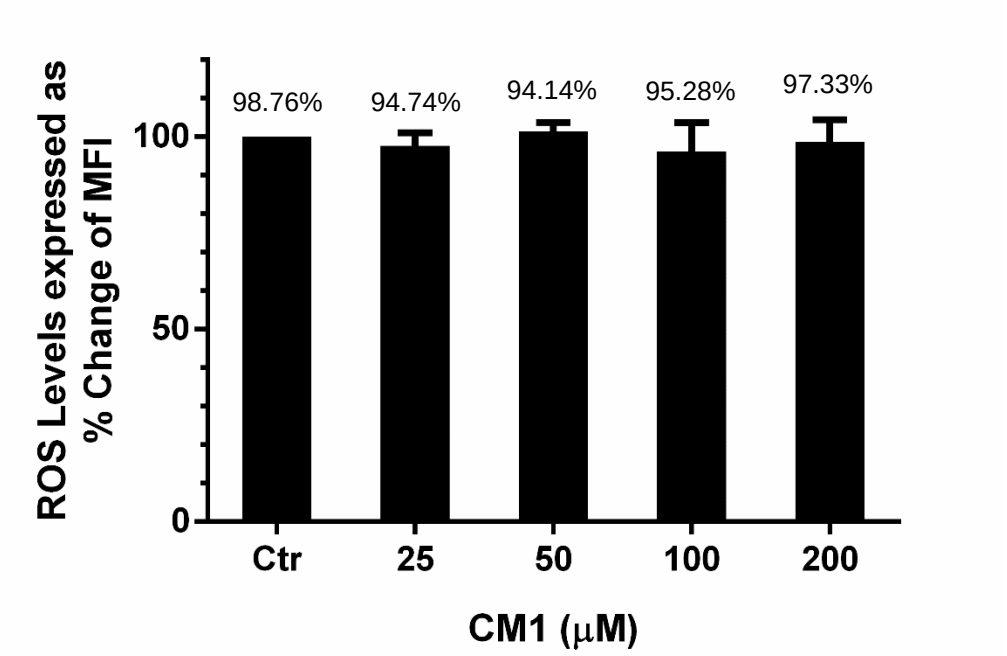

Figure 3-12 Levels of ROS in *P. falciparum*-infected RBC treated with CM1 (0-200 μM) for 2 h. Data was obtained from three independent experiments performed in triplicate and expressed as mean±SEM. % Parasite growth was indicated above each histogram.

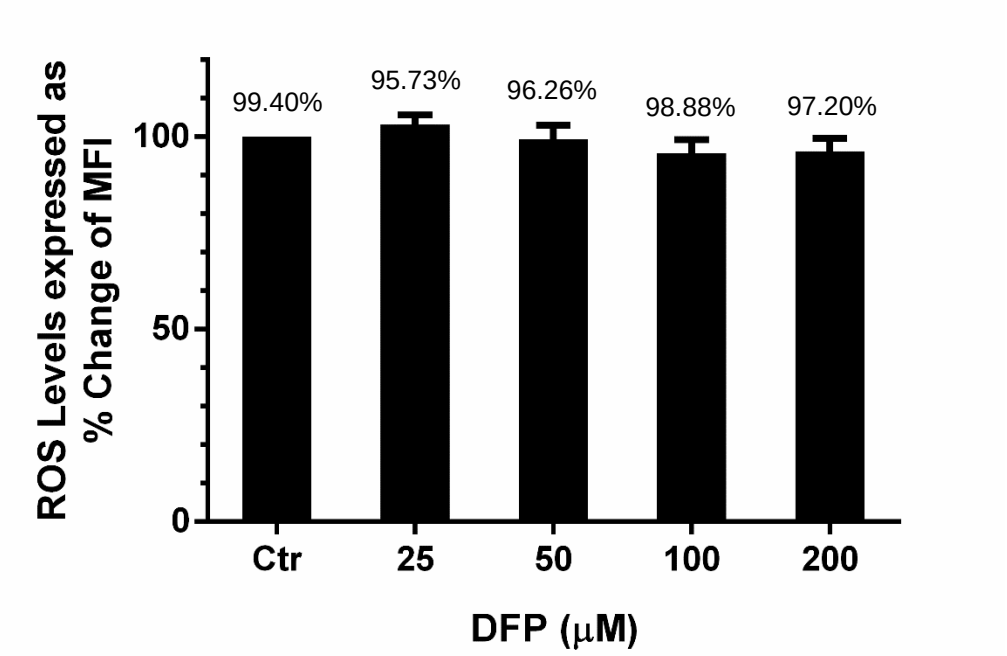

Figure 3-13 Levels of ROS in *P. falciparum*-infected RBC treated with DFP (0-200 μM) for 2 h. Data was obtained from three independent experiments performed in triplicate and expressed as mean±SEM. % Parasite growth was indicated above each histogram.

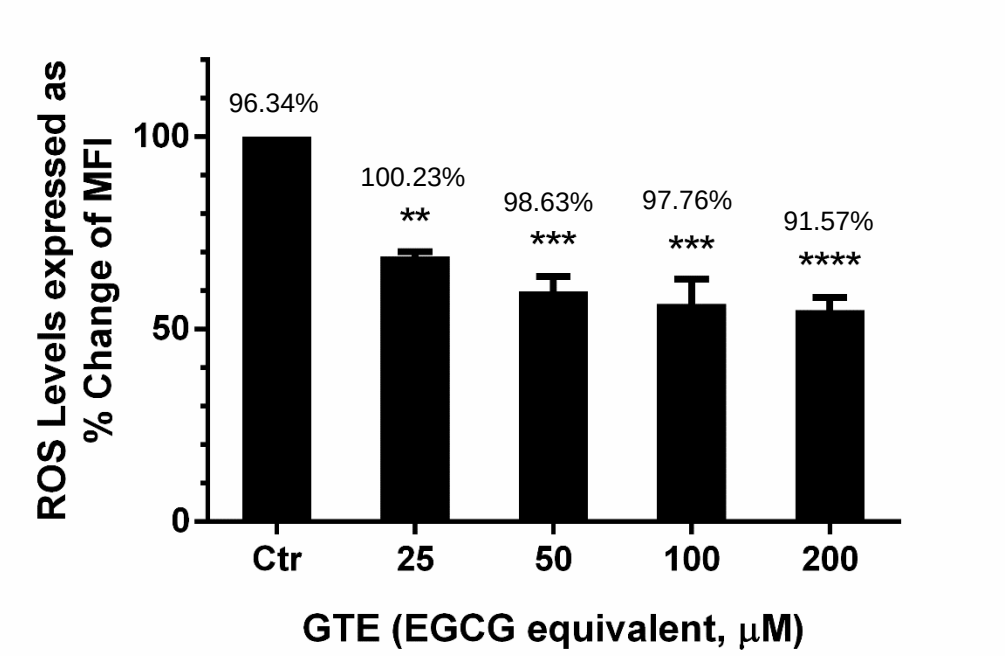

Figure 3-14 Levels of ROS in *P. falciparum*-infected RBC treated with GTE (0-200 μM EGCG equivalent) for 2 h. Data was obtained from three independent experiments performed in triplicate and expressed as mean±SEM. $^{**}P$ <0.01; $^{***}P$ <0.001 and $^{****}P$ <0.0001 compared with the control group (one-way ANOVA followed by Tukey's multiple comparison test). % Parasite growth was indicated above each histogram.

3.5 *In vivo* drug-susceptibility testing of *P. berghei*

3.5.1 Single drug treatment

Certainly, PYR is an established antimalarial drug, and showed antimalarial property against *P. berghei in vivo* model with ED_{50} of 0.76 mg/kg (95% CI = 0.62 to 0.92 mg/kg) in this study (**Figure 3-15**). Surprisingly, CM1 also showed antimalarial property against *P. berghei* in mouse model with ED_{50} of 56.91 mg/kg (95% CI = 47.98 to 67.50 mg/kg), similar effect to *in vitro* drug-susceptibility testing of *P. falciparum* experiment (**Figure 3-16**). Conversely, GTE did not inhibit *in vivo P. berghei* parasite growth. Moreover, the percentage of parasitemia was increased up to 158.41% by GTE treatment at EGCG equivalent of 12.5 mg/kg, which was a significant difference when compare with non-treatment group, as shown in **Figure 3-17**.

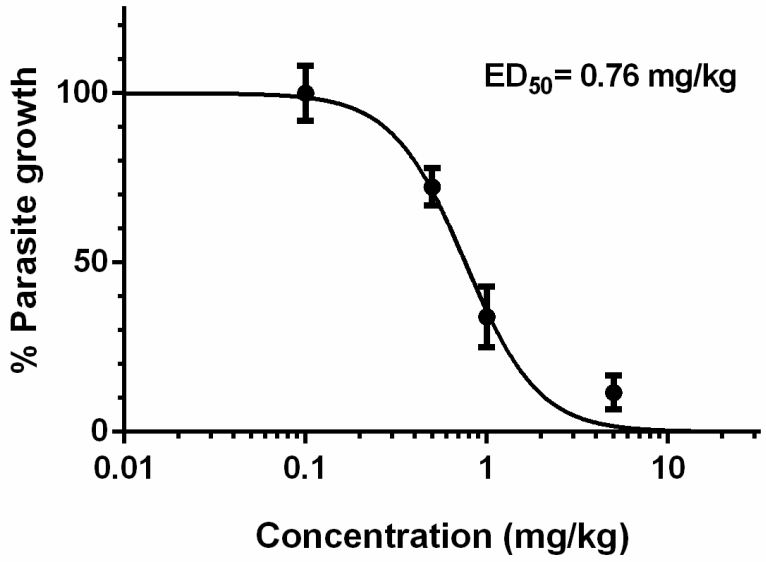

Figure 3-15 Effect of PYR on *P. berghei* growth in infected mice. *P. berghei*-infected mice were treated with PYR (0 - 5 mg/kg) by oral gavage for 4 consecutive days. The percentage of parasite growth were calculated from parasitemia, determined by Giemsa staining method. Data were obtained from 4 mice per group compiled from two independent experiments and expressed as mean±SEM.

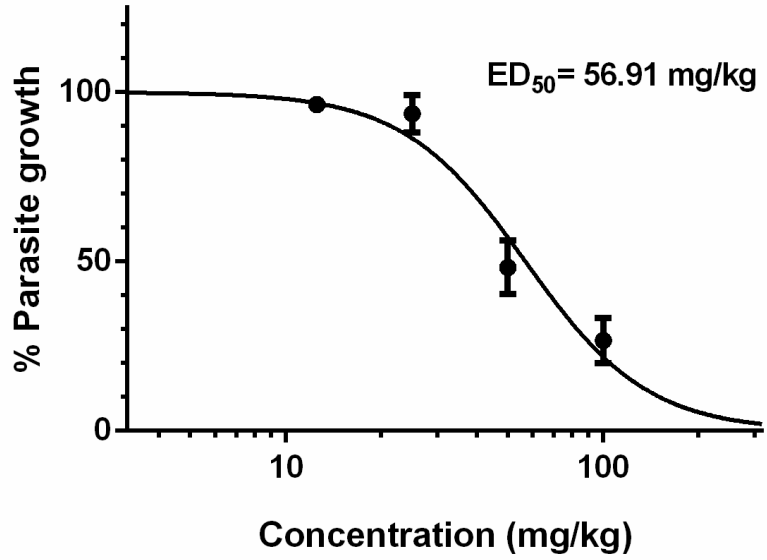

Figure 3-16 Effect of CM1 on *P. berghei* growth in infected mice. *P. berghei*-infected mice were treated with CM1 (0-100 mg/kg) by oral gavage for 4 consecutive days. The percentage of parasite growth were calculated from parasitemia, determined by Giemsa staining method. Data were obtained from 4 mice per group compiled from two independent experiments and expressed as mean±SEM.

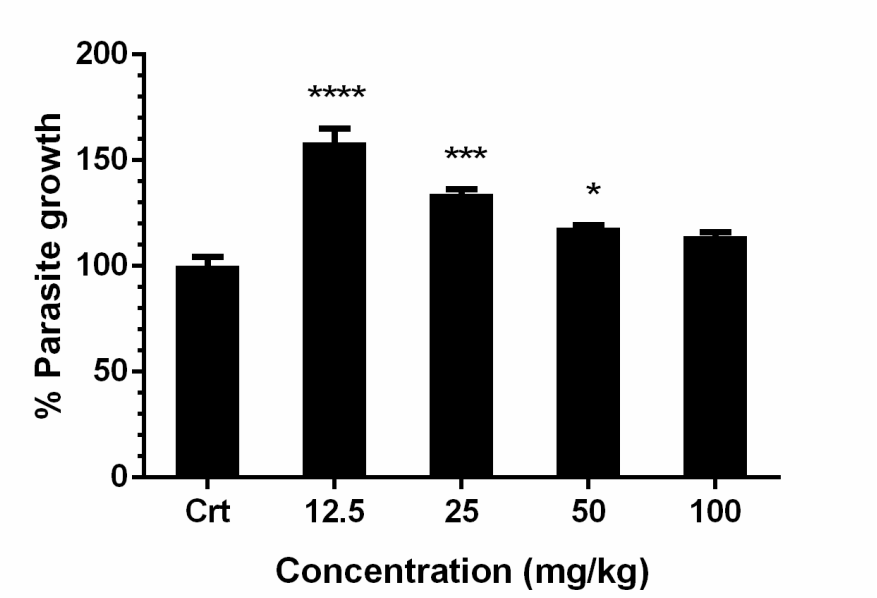

Figure 3-17 Effect of GTE on *P. berghei* growth in infected mice. *P. berghei*-infected mice were treated with GTE (EGCG equivalent of 0 - 100 mg/kg) by oral gavage for 4 consecutive days. The percentage of parasite growth were calculated from parasitemia, determined by Giemsa staining method. Data were obtained from 4 mice per group compiled from two independent experiments and expressed as mean±SEM. $^{*}P$ <0.05; $^{***}P$ <0.001 and $^{****}P$ <0.0001 compared with the control group (one-way ANOVA followed by Tukey's multiple comparison test).

3.5.2 Drug combination treatment

In CM1 combination with PYR, the percentage of parasite growth had a significant difference between non-treatment group and PYR alone group, but not between non-treatment and CM1 alone group. In PYR+CM1 groups, parasite growth was decreased by combination with PYR and it seemed to be an upward trend from 12.5 to 25 mg/kg of CM1 combined with PYR. Thereafter, there was a downward trend from 50 to 100 mg/kg of CM1 combined with PYR and a significant difference when compared with 0.6 mg/kg PYR + 25 mg/kg CM1 group, as shown in **Figure 3-18**.

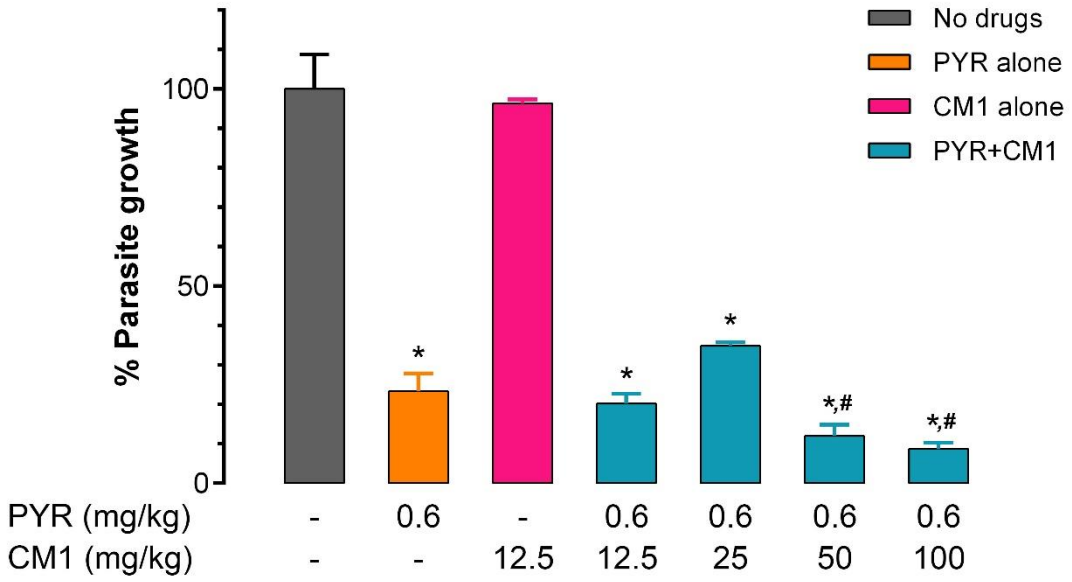

Figure 3-18 Effect of PYR combined with CM1 on *P. berghei* growth in infected mice. *P. berghei*-infected mice were treated with antimalarial drug PYR (fixed concentration of 0.6 mg/kg) combined with CM1 (0-100 mg/kg) by oral gavage for 4 consecutive days. The percentage of parasite growth were calculated from parasitemia, determined by Giemsa staining method. Data were obtained from 4 mice per group compiled from two independent experiments and expressed as mean±SEM. $^{*}P$ <0.05 compared with non-treatment group; $^{\#}P$ <0.05 compared with 0.6 mg/kg PYR + 25 mg/kg CM1 group compared with the control group (one-way ANOVA followed by Tukey's multiple comparison test).

In GTE combination with PYR, the results were quite similar to PYR-CM1 combination. In GTE alone group, the parasitemia shot up to about 150% which had a significant difference when compared with non-treatment group. In PYR+CM1 groups, parasite growth was decreased by combination with PYR and had no significant difference from PYR treatment alone, as shown in **Figure 3-19**.

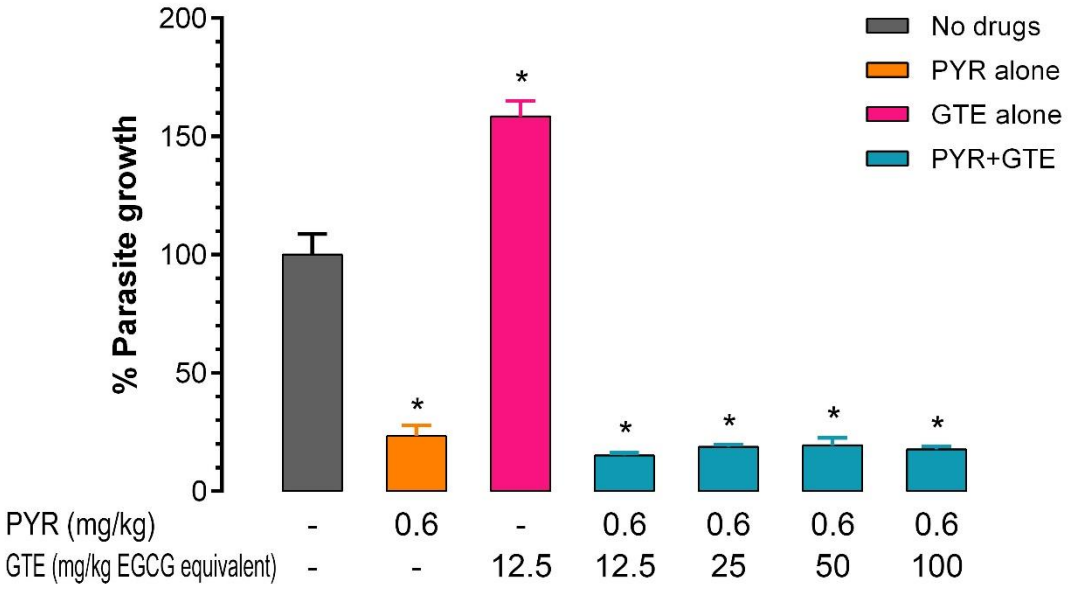

Figure 3-19 Effect of PYR combined with GTE on *P. berghei* growth in infected mice. *P. berghei*-infected mice were treated with antimalarial drug PYR (fixed concentration of 0.6 mg/kg) combined with GTE (EGCG equivalent of 0-100 mg/kg) by orally gavage for 4 consecutive days. The percentage of parasite growth were calculated from parasitemia, determined by Giemsa staining method. Data were obtained from 4 mice per group compiled from two independent experiments and expressed as mean±SEM. $^{*}P$ <0.05 compared with non-treatment group compared with the control group (one-way ANOVA followed by Tukey's multiple comparison test).