

Effect of *Eurycoma longifolia* extract on the Glutathione level in *Plasmodium falciparum* infected erythrocytes *in vitro*

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Abstract. In the present study we examined the effect of *E. longifolia* methanol extract (TA164) on the GSH levels of *P. falciparum* infected erythrocytes and uninfected erythrocytes. Our study on parasite growth shows the IC₅₀ and IC₇₅ values of TA164 to be 0.17 µg/ml and 6 µg/ml respectively while for BSO was 25.5 µg/ml and 46.5 µg/ml respectively. About 95% to 100% growth inhibition of *P. falciparum* infected erythrocyte was observed when treated with TA164 and BSO at 16 µg/ml and 64 µg/ml respectively. The study on GSH contents indicated that non-infected erythrocytes treated with 6 µg/ml (IC₇₅ values) of TA164 at 24 hours incubation showed less GSH content as compared to non-treated erythrocytes. A similar observation was seen on treated trophozoite infected erythrocyte (10% parasitemia) when treated with 6 µg/ml at 3 hours incubation. Analysis of the GSH contents of parasite compartments treated with TA164 at the same concentration (6 µg/ml) for 3 hours incubation indicated a reduction of GSH contents. At the same concentration, TA164 did not affect the GSH contents of enriched trophozoite infected erythrocytes (60-70% parasitemia). TA164 did affect the GSH content of non-infected erythrocyte at 24 hours (accept IC₅₀ value) as well as the parasite compartments (trophozoite infected erythrocyte and parasite itself) but fails to affect the GSH content of enriched trophozoite infected erythrocyte.

INTRODUCTION

Malaria remains a devastating scourge in this earth. This has attracted scientists worldwide to investigate and discover newly potent anti-malarial drug. The latter was encouraged by the spread of drug resistant malarial parasite especially *Plasmodium falciparum* infection (Marsh, 1999). The incidence of drug resistant *P. falciparum* has been increasing at a faster rate than that of the efforts for development of new drugs (Kitua & Malebo, 2004). During intraerythrocytic development of malarial parasite, the *Plasmodium* live in a pro-oxidant environment that contains oxygen and iron which form reactive oxygen species via the Fenton reaction (Muller, 2004).

Haemoglobin is taken up by the parasite into their acid food vacuole which leads to the spontaneous oxidation and the formation of superoxides anion and haem (Muller, 2004) which is detoxified by haem polymerization process (Egan *et al.*, 2002) and in conjugation with glutathione (GSH) (Ginsburg *et al.*, 1999).

Inhibition of GSH synthesis by buthionine sulphoximine (BSO) in *Plasmodium* infected erythrocytes resulted in inhibition of parasite growth (Luersen *et al.*, 1999). A study showed that chloroquine competitively inhibit the GSH conjugation (Ginsburg *et al.* 1999) and simplify the important highly synthesized GSH in drug resistant *Plasmodium*.

In order to discover and evaluate newly potent anti-malarial drugs or

substances, there are several steps that we have to take into consideration. One of them is to produce affordable drugs or substances with low side effect as much as possible. Therefore aiming the right biochemical pathway which is important in *Plasmodium* growth is crucial for the selectivity of new drugs.

The roots of *Eurycoma longifolia* Jack from the Simaroubaceae family, known locally as Tongkat Ali or Pasak Bumi in Indonesia, growing wildy in the jungle slopes of Malaysia, are popularly sought after as an essential ingredient in Malay herbal medicine for intermittent fever or malaria (Gimlette & Thomson, 1997; Perry & Metzger, 1980). The plant contains a series of quasinoids which have been reported as active against *in vitro* culture of malaria parasites (Chan *et al.*, 2004). However, the mechanism of action of this plant extracts remains unknown and is still under investigation.

From this study, we wish to report on the effect of the methanol extracts of *E. longifolia* (TA164) on the GSH content of erythrocytes and *P. falciparum in vitro* where GSH was chosen to be the molecule of interest in this study, as this might be a potential determination of parasite growth inhibition.

MATERIALS AND METHODS

Chemicals and reagent

All chemicals were purchased from Sigma, USA unless indicated otherwise. *E. longifolia* methanol extracts (TA164) was prepared and authenticated by Professor Zhari Ismail from Universiti Sains Malaysia (USM). On the day of test, the brown powdered extract of TA164 was dissolved in 100% DMSO (Fluka, Netherlands) as a stock solution and was further diluted with double distilled water to a working concentration, where the final concentration of DMSO is approximately 1%.

Parasites

The chloroquine-resistant isolate of *P. falciparum* Gombak A (Slamet *et al.*, 1991), was originally isolated from an Orang Asli (aborigine) patient in 1982 from Gombak Hospital, Kuala Lumpur. Gombak A was cultured continuously *in vitro* from cryopreservation and maintained in human 'O' group erythrocytes as described by Trager & Jensen (1976). The Gombak A *P. falciparum* were grown in a 75 cm² culture flask at 2% hematocrit in a complete culture medium containing RPMI 1640 medium (Gibco, USA) supplemented with 25 mM HEPES (Gibco, USA), 2% sodium bicarbonate, 40 mg/ml gentamycin (Atlantic Laboratories Corp. Ltd, Thailand) and 10% human serum at pH 7.2. The parasite were cultured in a candle jar (with gas environment of about 3% O₂, 6% CO₂ and 91% N₂) at 37°C incubator. When the majority of parasite culture were at ring stage and few schizont stage, they were synchronized using 5% sorbitol according to Lambros & Vanderberg (1979). The parasites were then put back into culture and the growth monitored until the parasitemia reached about 5 to 10% with majority at ring stage (for use in the growth inhibition assay) or at trophozoite stages (for use in the determination of GSH contents). The parasites were then harvested and the number of ring or trophozoite infected erythrocytes were counted by using hemacytometer.

For compartment analyses of GSH contents, parasites were isolated from the infected erythrocytes by saponin lysis (Luersen *et al.*, 2000). Briefly, the trophozoite-infected erythrocytes were incubated for 10 minutes at 4°C in Earle's Balanced Salt Solution (EBSS) containing 0.15% saponin before adding 4 volume of EBSS. The reaction mixtures were centrifuged at 1500 g for 5 minutes at 4°C. The supernatants were used for analyzing the GSH content in the erythrocyte cytoplasm. The isolated parasites in the pellet were further washed twice in ice-cold EBSS (Gibco, USA) also for the

purpose of analyzing the GSH contents (Meierjohann *et al.*, 2002).

For the determination of GSH contents of trophozoite infected erythrocyte, the parasite culture with majority at trophozoite stage were harvested. The trophozoite-infected erythrocytes were enriched to 60-70% by performing 60% Percoll gradient centrifugation (Wahlgren *et al.*, 1983). The isolated trophozoite infected erythrocytes were washed twice with EBSS, calculated (using hemacytometer), and recultured with or without the addition of IC75 value of TA164 and BSO at 10% haematocrit for 3 hours incubation period in a candle jar, 37°C. Then the infected cells were harvested, lysed, and processed as mentioned above for analyzing the GSH content of the supernatant (erythrocyte cytoplasm).

Growth inhibition assay

TA164, chloroquine and GSH synthesis inhibitor, D,L-buthionine-(S,R) sulphoximine (BSO) were dissolved and diluted in appropriate solvent and complete culture medium to a final concentration of 64 µg/ml to 0.03 ng/ml. The drug concentration were prepared in a 24 wells culture plate. Hundred and ninety microliter of blood suspension at 2% parasitemia (containing only ring stage) and 2% haematocrit were seeded into each of the 24 wells culture plate containing 10 µl of the drug concentration. The reactions were incubated at 37°C in a candle jar for 36 hours. At the end of the incubation, the erythrocyte cultures were harvested and thin blood smear from each of the well were prepared and stained with 10% Giemsa for 20 minutes. The test positive control (for parasite growth) was 190 µl of *P. falciparum* infected erythrocytes in wells containing 10 µl of distilled water. BSO or inhibitor act as test substance control. The percentage of schizont infected erythrocytes per 1000 of erythrocytes were counted (WHO, 1990). IC50 and IC75 of each drugs or extracts were determined by graph plotted as percentage of parasite inhibition versus log dose.

Determination of GSH content

The determination of GSH contents in treated and untreated non-infected erythrocyte were carried out at concentration of 64 µg/ml, and at IC50 and IC75 values of TA164 (0.17 µg/ml and 6 µg/ml respectively) and BSO (25.5 µg/ml and 46.5 µg/ml), incubated for 24 hours with 10% haematocrit in a candle jar at 37°C. The incubation was for 24 hours, to determine the effect of the drug on the GSH levels over long incubation, which covers one cycle of the parasite life cycle. In a separate reaction, trophozoite infected erythrocyte and parasite compartments were treated in BSO and TA164 at IC75 values for 3 hours in a similar condition. For short term incubation only concentration at IC75 were used (Meierjohann *et al.*, 2002). GSH level of both non-infected and trophozoite infected erythrocytes were determined as described by Luersen *et al.* (2000). Briefly, 5×10^7 cells of erythrocytes or trophozoite infected erythrocytes (after lysis process as mentioned above) or parasite compartment (isolated from infected erythrocyte after lysis process as mentioned above) was added to two volumes of 5% sulphosalicylic acid and mixed for 1 minute. The samples were centrifuged at 4°C for 15 minutes at 10 000 g. At the end of centrifugation, the supernatant was added to 700 µl of 143 mM sodium phosphate (pH 7.5), 6.3 mM EDTA, 0.34 mM β-NADPH, 100 µl of 6 mM 5,5'- dithiobis-(2-nitrobenzoate) (DTNB) and water was added to make up to final volume of 950 µl. The mixture was incubated at room temperature for 10 minutes and the reaction was started by adding 50 µl of glutathione reductase (20 units/ml) (Luersen *et al.*, 2000). The concentration of GSH was determined by observing the rate of change in absorption at 405 nm using spectrophotometer (Multiskan EX, Thermo Electron Corporation, China) at 1 minute interval for 4 minutes. Concentration of GSH were calculated relative to known standard concentration of GSH.

RESULTS

Effect of TA164 and BSO on the growth of *P. falciparum* in vitro

P. falciparum was cultured in the presence of increasing TA164 and BSO concentration for 36 hours. The growth of *P. falciparum* treated with TA164 was 100% inhibited at concentration 16 $\mu\text{g/ml}$ (Figure 1). Only about 95% of growth inhibition exhibited by BSO at highest concentration, 64 $\mu\text{g/ml}$ (Figure 2). As a standard drug, CQ inhibited 100% of *P. falciparum* growth at 4 ng/ml (Figure 3). IC₅₀ and IC₇₅ value of TA164 was 0.17 $\mu\text{g/ml}$ and 6 $\mu\text{g/ml}$ respectively (Figure 1) while both IC₅₀ and IC₇₅ value of BSO determined as 25.5 $\mu\text{g/ml}$ and 46.5 $\mu\text{g/ml}$ (Figure 2).

GSH content of non-infected (fresh) erythrocytes treated with TA164 and BSO

About 5×10^7 erythrocytes were cultured in the presence of 64 $\mu\text{g/ml}$, IC₅₀ and IC₇₅ value of TA164 and BSO for 24 hours or 3 hours incubation period. The cells were harvested and lysed and GSH content of the erythrocyte supernatants were determined as mentioned earlier. As shown

in Table 1, 64 $\mu\text{g/ml}$ of TA164 and BSO decreased 12% and 28% of the erythrocytes GSH content respectively as compared to nontreated erythrocytes. IC₇₅ of TA164 decreased 17% of the GSH content while erythrocytes treated with IC₇₅ BSO only showed less 7% of GSH as compared to nontreated erythrocytes. Less effect was detected in erythrocytes treated with IC₅₀ of either TA164 and BSO (Table 1).

The effect of TA164 and BSO treatment on the *P. falciparum* trophozoite infected erythrocytes and parasite compartment

Trophozoite infected erythrocytes (10% parasitemia) were incubate with IC₇₅ value of both TA164 and BSO for 3 hours in a candle jar as mentioned earlier. Results showed that TA164 decreased 11% of infected erythrocytes GSH content (Table 2), while BSO only exhibited slight decrease in GSH content as compared to nontreated trophozoite infected erythrocytes (Table 2).

However, the results were corresponded to 10% trophozoite infected erythrocyte containing about 90% of non-infected erythrocytes. In order to investigate the exact effect of TA164 to

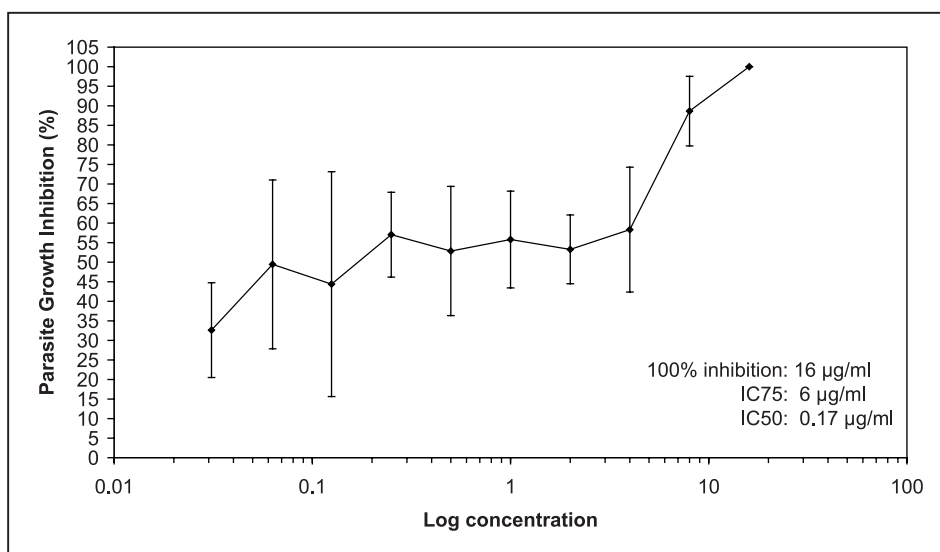


Figure 1. The effect of TA164 on *P. falciparum* Gombak A growth. The result presented was a mean value \pm S.D. from two separated experiments.

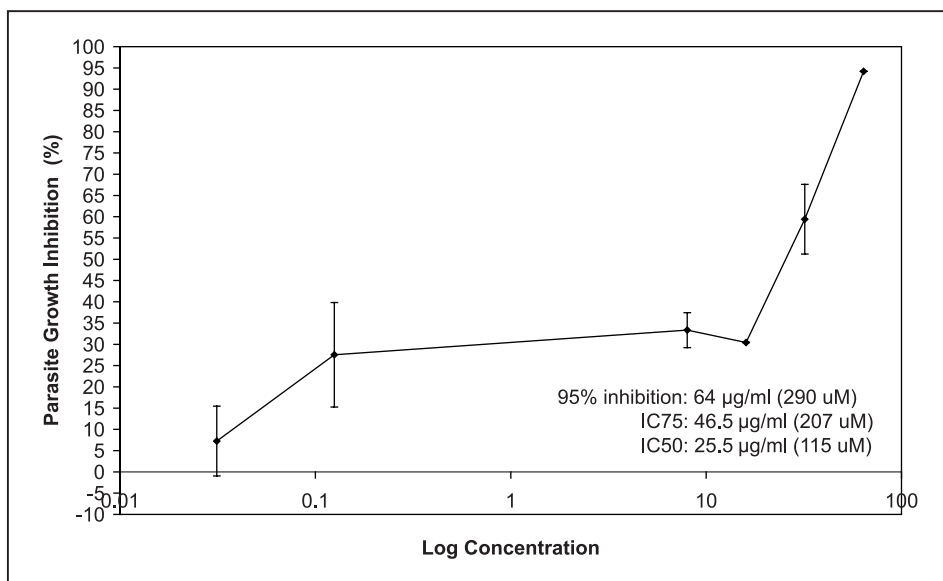


Figure 2. The effect of BSO on *P. falciparum* Gombak A growth. The result presented was a mean value \pm S.D. (n=2).

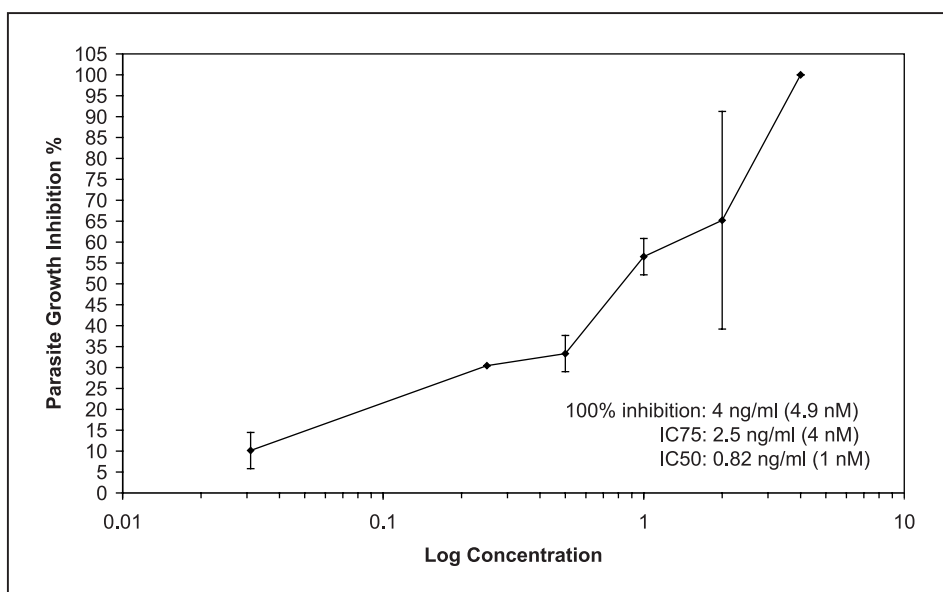


Figure 3. The effect of CQ on *P. falciparum* Gombak A growth. The result presented was a mean value \pm S.D. (n=2).

trophozoite infected erythrocytes, the infected erythrocytes were enriched from 60 to 70 percent by performing Percoll gradient centrifugation where the trophozoite infected erythrocytes were separated and processed as mentioned

earlier. As shown in Table 3, there was no change in GSH content of isolated trophozoite infected erythrocytes cytoplasm after treatment with both TA164 and BSO as compared to nontreated trophozoite infected erythrocytes. Results

Table 1. The effect of TA164 and BSO treatment to GSH content of healthy erythrocytes at 24 hours incubation period. Results are given as means \pm S.D. (n=3)

Treatment	Glutathione (nmol . 10 ⁶ cells ⁻¹)		
	64 ug/ml	IC75	IC50
Nontreated erythrocytes	0.137 \pm 0.007 (100%)	0.137 \pm 0.007 (100%)	0.137 \pm 0.007 (100%)
TA164	0.121 \pm 0.017 (88%)	0.114 \pm 0.019 (83%)	0.139 \pm 0.009 (101%)
BSO	0.099 \pm 0.015 (72%)	0.127 \pm 0.009 (93%)	0.030 \pm 0.002 (103%)

Table 2. The effect of IC75 value of TA164 and BSO on GSH content of trophozoite infected erythrocytes and parasite compartment at 3 hours incubation period. Results are given as means \pm S.D. (n=3)

Cell type	GSH content (nmol.10 ⁶ cells ⁻¹)		
	Control (nontreated infected erythrocytes/nontreated parasite compartment)	3 hours TA164 6 μ g.ml ⁻¹	3 hours BSO 46.5 μ g.ml ⁻¹ (207 μ M)
Trophozoite infected erythrocytes	0.072 \pm 0.003 (100%)	0.064 \pm 0.004 (89 %)	0.069 \pm 0.007 (96%)
Parasite	0.066 \pm 0.019 (100%)	0.058 \pm 0.004 (88%)	0.033 \pm 0.002 (50%)

Table 3. The effect of IC75 value of TA164 and BSO on GSH content of trophozoite infected erythrocytes (enriched by Percoll gradient centrifugation) at 3 hours incubation period. Results are given as means \pm S.D. (n=3)

IC75 value	Glutathione (nmol . 10 ⁶ cells ⁻¹)
Control untreated	0.029 \pm 0.001 (100%)
IC75 TA164/6 μ g.ml ⁻¹	0.030 \pm 0.001 (103%)
IC75 BSO/46.5 μ g.ml ⁻¹ (207 μ M)	0.030 \pm 0.002 (103%)

from this study suggest that the decrease in GSH content of infected erythrocytes detected as shown in Table 2 (before

performing the Percoll gradient centrifugation) could be due to non-infected erythrocytes.

Parasite compartment isolated from infected erythrocytes showed a decrease in GSH content after incubation with the presence of IC75 TA164 and IC75 BSO. TA164 decreased the amount of parasite GSH for about 12% while BSO greatly decreased the amount of GSH to about 50% as compared to nontreated parasite compartment (Table 2).

DISCUSSION

Eurycoma longifolia extract has been extensively studied for its anti-malarial activity. TA164 has been proven for its anti-malarial activity *in vitro* and this study shows the IC50 for TA164 to be 0.17 μ g/ml.

It also shows a good anti-malarial properties *in vivo* tested by 4 Days Suppressive Test (unpublished data). The effect of BSO which inhibits the synthesis of GSH together with the inhibition of parasite growth in this study have already been documented by other studies where the GSH metabolism in *Plasmodium* life cycle is essential (Deharo *et al.*, 2003; Dubois *et al.*, 1995; Luersen *et al.*, 2000; Meierjohann *et al.*, 2002; Safeukui *et al.*, 2004). The interest to know the biochemical effect of TA164 on infected erythrocytes and parasites have directed us to carry out this study focusing on GSH level during *Plasmodium* growth.

Non-infected (fresh) erythrocytes treated with 64 µg/ml and IC75 value of TA164 showed less GSH content as compared to untreated erythrocytes. From this result, TA164 seems to be detrimental to healthy erythrocytes for long period of incubation *in vitro*. The possible way of TA164 in reducing the GSH content is through inhibiting the enzyme responsible for the synthesis and regeneration of GSH. GSH synthesis is controlled by γ -glutamylcysteine synthetase (GCS) and glutathione synthetase (Dickinson & Formen, 2002). It is also regulated by glutathione-S-transferase (GST) and glutathione reductase (GR) (Muftuoglu & Ozer, 1999). GSH depletion may happen if one of these enzymes is deactivated, where the regeneration of GSH is abolished. The study conducted by Muftuoglu & Ozer (1999) showed the effect of primaquine (prooxidant drug) inhibiting the GST and glucose-6-phosphate dehydrogenase (G6PD) at 30 minutes incubation period. For example, inhibition of G6PD will cause less production of NADPH, a cofactor for GSH regeneration, and will result in hemolytic anemia (Anderson, 1998) which is unfavourable for the development of the malaria parasite (Clark & Hunt, 1983; Greene, 1993).

Trophozoite infected erythrocytes (10% parasitemia) showed more susceptibility to TA164 at IC75 value in 3 hours incubation period. TA164 decreased the GSH content

of infected erythrocytes greater than BSO (Table 2). However, after 60% Percoll gradient centrifugation which isolated trophozoite infected erythrocytes from non-infected erythrocytes, GSH content in the isolated trophozoite infected erythrocytes were unaffected by TA164 (Table 3). The results indicate that the reduction observed was not purposely due to trophozoite infected erythrocytes and the non-infected erythrocytes could be the culprit in this reason. In contrast, Luersen *et al.* (2000) reported that the inhibition by BSO had hardly any effect on GSH levels as compared to infected erythrocytes suggesting a higher turnover rate of the tripeptide in the parasite-host unit. The condition of the environment during *Plasmodium* infection *in vitro* can cause the susceptibility of non-infected erythrocytes to TA164 and BSO to increase. Low pH during *Plasmodium* infection has been demonstrated to reduce the glucose utilization of non-infected erythrocytes (Mehta *et al.*, 2005). In addition, glucose is an indirect substrate for G6PD where it was converted to glucose-6-phosphate by hexokinase. Glucose-6-phosphate were oxidized by G6PD and NADP is reduced to NADPH. NADPH will be utilized in regeneration of GSH by GR (Cakir *et al.*, 2004).

Furthermore, the malaria parasite have a mechanism for supplying GSH to its host cell (Atamna & Ginsburg, 1997) resulting in unaffected GSH level in treated infected erythrocytes as mentioned in this study. *P. falciparum* Gombak A was reported to be resistant to chloroquine (Slamet *et al.*, 1991) where the synthesis of GSH was reported to be higher than chloroquine sensitive strain of *Plasmodium* (Meierjohann *et al.*, 2002). It may happen if the delivery system of GSH to host cell by *P. falciparum* Gombak A has the ability to compensate with the inhibitory action of TA164 and BSO.

Parasite compartment isolated from TA164 treated infected erythrocytes show less GSH content as compared to nontreated infected erythrocytes. BSO treatment shows greater inhibition of GSH

synthesis in parasite compartment as also mentioned by Meierjohann *et al.* (2002). BSO plays an important role in inhibition of GSH synthesis. When GSH de novo synthesis is inhibited, 50% of the cellular GSH depleted within 2 hours, suggesting the importance of GSH synthesis to the parasitized cell (Muller, 2004). However, how TA164 react in decreasing the GSH content on both host and parasite compartment remains to be elucidated.

In conclusion, TA164 decreased the GSH content of both infected and healthy erythrocytes at certain dosage and incubation period. GSH content of isolated parasite detected to be affected by TA164 in this study. Both effect of TA164 to GSH content of host or parasite can be the cause of *P. falciparum* growth inhibition *in vitro* and screening the activity of GSH synthesis can be one of the procedure in evaluating the antimalarial properties of herbal products.

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