

***Eurycoma longifolia* extract-artemisinin combination: parasitemia suppression of *Plasmodium yoelii*-infected mice**

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Received 12 February 2007; received in revised form 17 April 2007; accepted 18 April 2007.

Abstract. *Eurycoma longifolia*, locally known as ‘Tongkat Ali’ is a popular local medicinal plant that possess a lot of medicinal properties as claimed traditionally, especially in the treatment of malaria. The claims have been proven scientifically on isolated compounds from the plant. The present study is to investigate the anti malaria properties of *Eurycoma longifolia* standardized extract (root) (TA164) alone and in combination with artemisinin *in vivo*. Combination treatment of the standardized extract (TA164) with artemisinin suppressed *P. yoelii* infection in the experimental mice. The 4 day suppressive test showed that TA164 suppressed the parasitemia of *P. yoelii*-infected mice as dose dependent manner (10, 30 and 60 mg/kg BW) by oral and subcutaneous treatment. By oral administration, combination of TA164 at 10, 30 and 60 mg/kg BW each with artemisinin respectively showed a significant increase in the parasitemia suppression to 63, 67 and 80 percent as compared to artemisinin single treatment (31%). Using subcutaneous administration, at 10 mg/kg BW of TA164 in combination with 1.7 mg/kg BW of artemisinin gave a suppression of 80% of infection. This study showed that combination treatment of TA164 with artemisinin gives a promising potential anti malaria candidate using both oral and subcutaneous route, the later being the most potent.

INTRODUCTION

Multidrug resistant parasite is the biggest therapeutic challenge to health care in the most malaria endemic areas. This phenomenon necessitates research and development of new antimalarial drug involving the current and new potential drug (Kremsner & Krishna, 2004). Combination of drugs with synergistic action resulted in increase in efficacy, shorten duration of treatment, increasing compliance, and decrease the risk of resistant parasites (Kremsner & Krishna, 2004).

Recent studies on combination therapy to treat malaria have been shown to have a promising antimalarial activity (Perez

et al., 1994; Mohd Ridzuan *et al.*, 2006; Nandakumar *et al.*, 2006). These studies highlight the use of combination of natural compounds and extracts with standard antimalarial drugs such as artemisinin and chloroquine.

Antimalarial properties of *Eurycoma longifolia* or ‘Tongkat Ali’ root extracts and compounds have been widely studied *in vitro* and *in vivo* (Ang *et al.*, 1995; Satayavivad *et al.*, 1998; Kuo *et al.*, 2004; Chan *et al.*, 2005; Guo *et al.*, 2005; Mohd Ridzuan *et al.*, 2005). In addition, quassinoids, one of the major bioactive groups in this plant has been reported to play an important role in affecting *Plasmodium* growth (Ang, 2004) by inhibiting

the protein synthesis process (Guo *et al.*, 2005).

Artemisinin are derived from a plant called sweet wormwood, *Artemisia annua*. It is a sesquiterpene lactone structure that play an important role in malaria parasite killing. Poor cure rate and neurotoxic effect have been reported by the monotherapy of artemisinin (Woodrow *et al.*, 2005).

Thus, the aim of this study is to prove the effectiveness of *E. longifolia* standardized methanol extract (TA164) in promoting the antimalarial activity of artemisinin in mouse model at lower treatment dosage. This is the first study where the TA164 extract is used for combination treatment *in vivo* and not as a single compound.

MATERIALS AND METHODS

The parasite

Plasmodium yoelii MRA 312 was obtained from American Type Culture Collection (ATCC), Malaria Reference Reagent Resource Center (MR4). The cryopreserved parasite was thawed and inoculated into donor mice for further use in this experiment.

Animal husbandry and maintenance

The usage of the laboratory animals and their studies were approved by the Institutional Care and Used Committee (IACUC) of Institute for Medical Research (IMR) Kuala Lumpur, with ACUC number ACUC/KKM 4/2004. The handling of experimental mice was according to the guidelines of 'Handling of Laboratory Animals' by Ministry of Health Malaysia (MOH, 2000). The mice were acclimatized in their cages for 5 days prior to the start of the study. They were maintained at an environmental condition of $\pm 27^\circ\text{C}$ with 12 hours of light and 12 hours of dark. They were fed with 'Animal foodstuff: Specially Feeds Rat and Mouse Cubes' with food content of protein (9%), fat (4.6%), crude fiber (4.6%), calcium (0.77%), phosphorus (0.57%), salt (0.5%), and digestible energy

(14.3 MJ/kg) from Glen Forrest Western, Australia. The mice were given unlimited supply of water supplemented with 1 parts per million (ppm) of para-aminobenzoic acid (PABA) (ICN, Biomedicals, Inc., USA).

Chemical preparation

Eurycoma longifolia standardized extract, TA164, was obtained from Professor Zhari Ismail, Universiti Sains Malaysia, Penang, Malaysia. The TA164 is a brown free flowing powder prepared using methanol extraction method. Artemisinin was purchased from Aldrich, USA.

Artemisinin and TA164 were dissolved in 100% dimethyl sulfoxide (DMSO). The different doses for the experiment drugs were obtained by dilution of the stock with distilled water which resulted in 5% of DMSO at final concentration. The amount of drug given was calculated based on the body weight.

The fixed dose of artemisinin at 1.7 mg/kg body weight (BW) was chosen based on an *in vivo* antimalarial ED₅₀ values (Peters *et al.*, 2002). The selection of the doses for TA164 was based on its ED₅₀ values obtained from both routes, oral and subcutaneous in a prior study in the laboratory (unpublished data). The doses for the extracts and the standard drug, artemisinin, were summarized in Table 1 and were administered by oral and subcutaneous route.

In vivo schizonticidal activity

The schizonticidal activity of TA164 was carried out using the classical 4 day suppressive test (Peters *et al.*, 1975) optimized by Mohd Ridzuan *et al.* (2006). Briefly, on day 0, the donor mouse with parasitemia of about 60 to 80 percent was sacrificed and blood withdrawn by cardiac puncture and adjusted with normal saline to the required number. The mice were inoculated intravenously with 0.2 ml saline solution containing 2×10^6 *P. yoelii*-infected red blood cells. The mice were randomly assigned into control and treatment groups with 5 mice in each group. Two control groups were used in the experiment, one treated with 1.7 mg/

kg BW of artemisinin, while the other group was untreated control given water in the same volume. The single and combination formula were administered by oral and subcutaneous route at 2 hours post inoculation. The drugs administration was continued on day 1 to day 3 post inoculation. On day 4 post inoculation, blood smears from tail vein of all experimental mice, fixed with methanol and stained with 10% Giemsa solution. The percentage of parasitemia was calculated by using light microscopy with 1000 times magnification.

Parasitemia and percentage of suppression

Parasitemia of infected mice was calculated by the following formula:

$$\frac{\text{Number of infected red blood cell}}{\text{Total red blood cell}} \times 100 = \% \text{ parasitemia}$$

Percentage of parasitemia suppression was calculated by the following formula:

$$\frac{\text{Parasitemia in control} - \text{Parasitemia with drugs or extracts}}{\text{Parasitemia in control}} \times 100 = \% \text{ suppression}$$

Data analysis

The significance of treatment effect was evaluated by Tukey, multiple comparison test.

RESULTS

In vivo antimalarial activity of TA164 alone and combination with artemisinin by oral administration

TA164-treated *P. yoelii* infected mice showed a decrease percentage in their parasitemia as compared to control mice (untreated *P. yoelii* infected mice). By oral administration, TA164 suppressed the parasitemia development at dose-dependant manner (Figure 1). At 10 mg/kg body weight (BW) of TA164, parasitemia of *P. yoelii*-infected mice was suppressed to 25 percent as compared to control mice

(Figure 1). The parasitemia was significantly suppressed to 41 percent ($p < 0.05$) and 51 percent ($p < 0.05$) at 30 mg/kg BW and 60 mg/kg BW of TA164 respectively (Figure 1). *P. yoelii*-infected mice treated orally with artemisinin alone resulted in suppression of parasitemia about 31 percent ($p < 0.05$) as compared to control mice (Figure 1).

Combination treatment by oral administration yielded more significant parasitemia suppression of *P. yoelii*-infected mice as compared to artemisinin treatment alone. Oral treatment at 10 mg/kg BW TA164 in combination with 1.7 mg/kg BW artemisinin suppressed 63 percent of parasitemia as compared to control mice (Figure 1). As mentioned earlier, artemisinin alone only yielded 1 fold lower in suppression of parasitemia, which is less effective than latter treatment (Figure 1). Similar treatment with 30 mg/kg BW TA164 in combination with 1.7 mg/kg BW artemisinin yielded 67 percent suppression of parasitemia (Figure 1). An extremely low parasitemia development was observed when *P. yoelii*-infected mice were treated with 60 mg/kg BW TA164 in combination with 1.7 mg/kg BW artemisinin, which resulted in 80 percent of parasitemia suppression as compared to control (Figure 1). Above results were statistically significant ($p < 0.05$) as compared to artemisinin treatment alone.

In vivo antimalarial activity of TA164 alone and combination with artemisinin by subcutaneous administration

By subcutaneous administration, 3 mg/kg BW of TA164 significantly suppressed 48 percent of *P. yoelii*-infected mice parasitemia as compared to control mice ($p < 0.05$) (Figure 2). The parasitemia of *P. yoelii*-infected mice was also suppressed significantly to 67 percent at 10 mg/kg BW of TA164 ($p < 0.05$) (Figure 2). A similar treatment with artemisinin alone resulted 64 percent suppression of parasitemia ($p < 0.05$) (Figure 2).

About 75 percent to 80 percent of parasitemia suppression was observed on treatment with 3 mg/kg BW and 10 mg/kg

BW of TA164 in combination with 1.7 mg/kg BW artemisinin respectively (Figure 2). However, the combination treatment showed no significant suppression of parasitemia as compared to artemisinin treatment alone (Figure 2).

DISCUSSION

The 4 day suppressive test showed that *E. longifolia* standardized extract, TA164 alone and its combination with artemisinin resulted in a suppression of *P. yoelii* growth in infected mice. Treatment with TA164 alone given orally and subcutaneously to mice infected with *P. yoelii*, showed a dose dependant percentage of suppression.

The treatment with TA164 alone and its combination was more effective by subcutaneous route. For example, by subcutaneous route, 10 mg/kg BW of TA164 alone and its combination (10 mg/kg BW TA164-1.7 mg/kg BW artemisinin) suppressed the parasitemia of *P. yoelii* infected mice 1 to 2 fold higher than oral route (Figure 1 & Figure 2). Parenteral drug administration such as subcutaneous route is normally a rapid treatment where the drugs is directly exposed and absorbed to the bloodstream with the rapid detection of drugs in plasma (Ritter *et al.*, 1999; Galbraith *et al.*, 2001). The high concentration of antimalarial drug in plasma was associated with high anti-malarial activity (Ezzet *et al.*, 2000; Silamut *et al.*, 2003). Oral administration has some

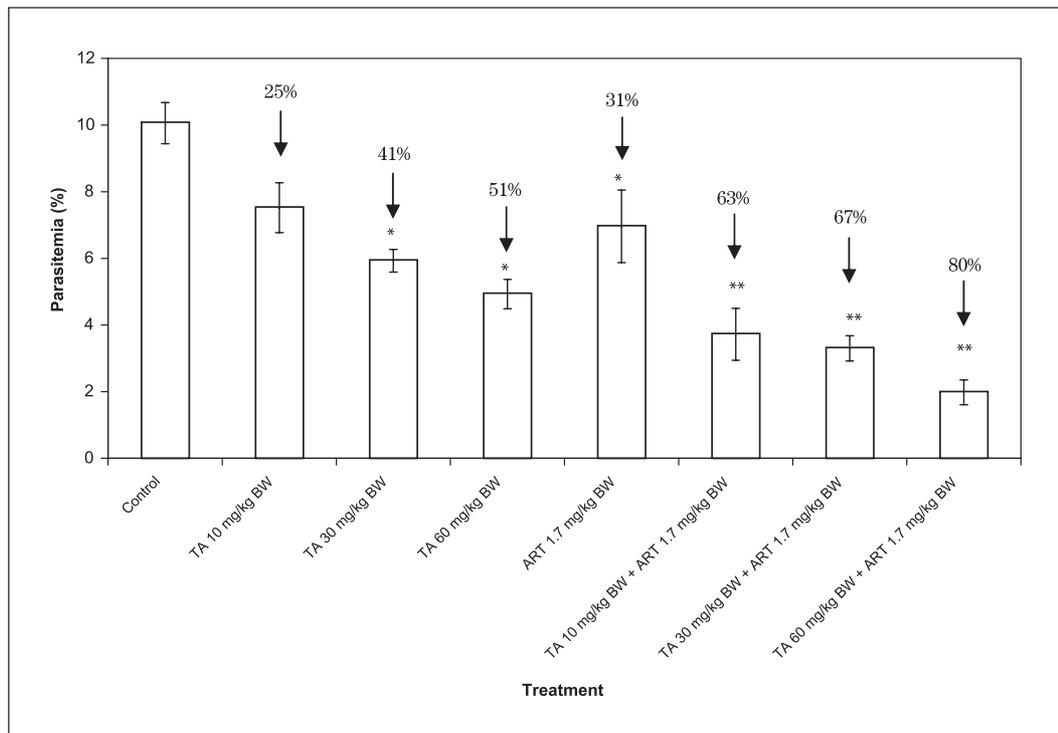


Figure 1. Effect of *E. longifolia* extract-artemisinin combination therapy to *P. yoelii* infected mice by oral administration. The results presented are in mean value \pm S.E.M. (n=5).

Note: ↓ = suppression of parasitemia

* = significant difference as compared to control ($p < 0.05$)

** = significant difference as compared to control and artemisinin alone ($p < 0.05$)

TA: *Eurycoma longifolia* (Tongkat Ali)

ART: Artemisinin

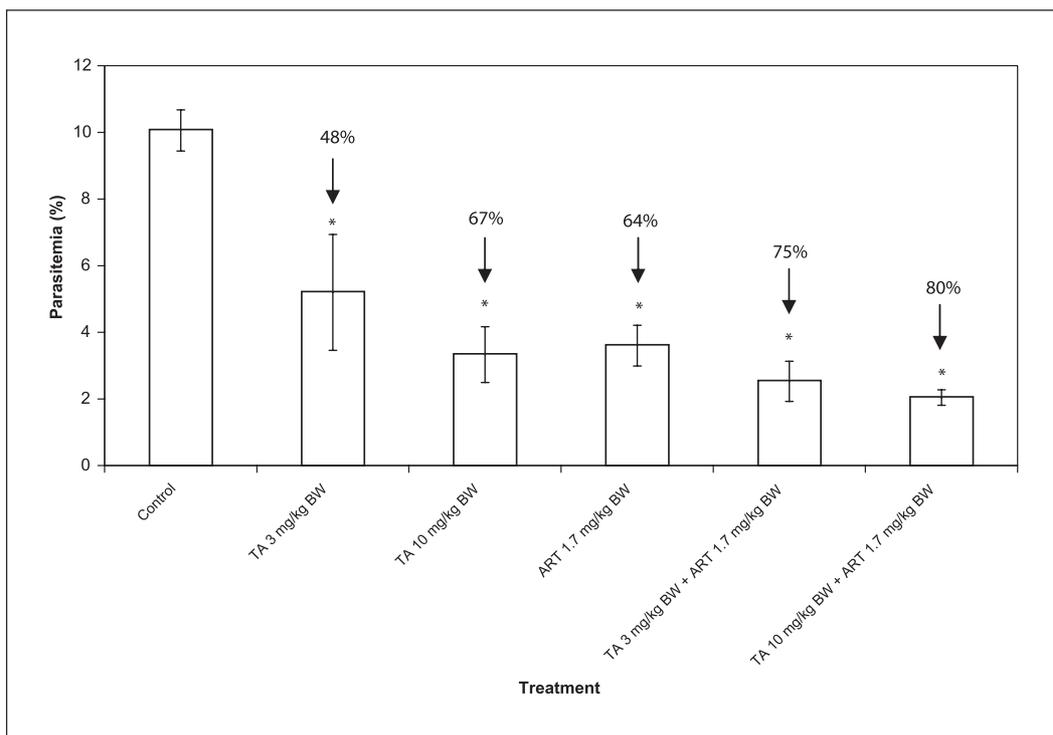


Figure 2. Effect of *E. longifolia* extract-artemisinin combination therapy to *P. yoelii* infected mice by subcutaneous administration. The results presented are in mean value \pm S.E.M. (n=5).

Note: ↓ = suppression of parasitemia

* = significant difference as compared to control (p<0.05)

** = significant difference as compared to control and artemisinin alone (p<0.05)

TA: *Eurycoma longifolia* (Tongkat Ali)

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limitation because the drug is absorbed through the digestive tract and passed through the liver before it is transported via the bloodstream. Latter can cause the inactivation and incomplete absorption of the drugs given (Ritter *et al.*, 1999; Galbraith *et al.*, 2001). Due to this, both TA164 and artemisinin could be chemically altered to its less active metabolite or incompletely released to the blood stream. The polarity of the extract and drug also has to be considered as the polar drugs reported to be well absorbed through parenteral administration (Ritter *et al.*, 1999; Galbraith *et al.*, 2001). Experiment performed by Peters *et al.* (2002) has also showed that antimalarial drugs given subcutaneously yielded lower ED₅₀ than

those given orally. However, the specific effect on extract or drug absorption still remain to be elucidated.

The amount of TA164 used in this study was not in the range of toxic dose. In acute toxicity study conducted by our lab it had already been shown that the LD₅₀ of the extract was more than 5000 mg/kg BW (data not shown). Another study performed by Satayavivad *et al.* (1998) showed that the LD₅₀ of 34% alcohol extract of this plant was 1500 mg/kg BW to 2000 mg/kg BW while water extract was more than 3000 mg/kg BW when given orally.

The *E. longifolia* extracts, TA164, is a potential and promising candidate for new antimalarial drug development. It showed

Table 1. Doses of administration for single and combination treatments

Treatment group	Route of administration (mg/kg body weight)	
	Oral (in 0.2 ml)	Subcutaneous (in 0.2 ml)
Control	Double distilled H ₂ O	Double distilled H ₂ O
Artemisinin alone	1.7	1.7
TA164 alone	10, 30, and 60	3 and 10
Combination TA164-artemisinin	10 (TA164) +	3 (TA164) +
	1.7 (Artemisinin)	1.7 (Artemisinin)
	30 (TA164) +	10 (TA164) +
	1.7 (Artemisinin)	1.7 (Artemisinin)
	60 (TA164) +	
	1.7 (Artemisinin)	

better parasitemia suppression on rodent malaria than other plant extracts. In a similar study by Tona *et al.* (2001), 200 mg/kg BW (given by oral route) of ethanolic and dichloromethane extracts of *Cassia occidentalis* root bark and *Phyllanthus niruri* whole plant produced a 60 percent parasitemia suppression while *Morinda morindoides* leaves extracts produced 30 percent parasitemia suppression on mice infected with *P. berghei*. Oral treatment with 500 mg/kg BW of *Cinchona officinalis* and *A. annua* extracts showed 67 percent and 53 percent parasitemia suppression respectively (Bertani *et al.*, 2005). Other methanol extract from different plant species such as *Annona senegalensis* (Annonaceae) against *P. berghei* yielded 59.2 percent of parasitemia suppression at the dose of 200 mg/kg BW (Ajaiyoba *et al.*, 2006). According to Deharo *et al.* (2001), antimalarial activity at 200 mg/kg BW of an extract is considered good if percentage of parasitemia suppression is more than 50 percent and 100 mg/kg BW of an extract is

considered very good when the parasitemia suppression is equal to 50 percent. Thus, based on the latter, TA164 has showed a very good antimalarial activity against *P. yoelii*.

For combination formula, low dose of artemisinin was chosen to assess the actual effect of TA164 and the advantage effect of combination formula. There was statistically significant difference between the percentage of parasitemia suppression yielded by artemisinin treatment alone and the combination formula (TA164-artemisinin) ($p < 0.05$) (Figure 1). In contrast, by subcutaneous route, the percentage of parasitemia suppression achieved was not significant as compared to artemisinin treatment alone (Figure 2). Combination of artemisinin with other natural sources had been proven to act synergistically by *in vitro* and *in vivo* (Nandakumar *et al.*, 2006). For example, curcumin, which was isolated from the roots of *Curcuma longa* (turmeric) shows an additive interaction in killing *P. falciparum in vitro* and prevents

recrudescence due to curcumin-artemisinin combination therapy (Nadakumar *et al.*, 2006). Other combination study on natural sources with different standard antimalarial drug such as chloroquine with goniiothalamine, isolated from *Goniothalamus schortechinii* ('Selada Putih') (Mohd Ridzuan *et al.*, 2006) and ajoene, derived from *Allium sativum* (garlic) (Perez *et al.*, 1994) demonstrated a good antimalarial activity. In conclusion, combination of *Eurycoma longifolia* with artemisinin is possible and this study suggested the combination treatment can be a promising antimalarial chemotherapy candidate.

Acknowledgement. We thank the Director, Institute for Medical Research, Kuala Lumpur, Malaysia for the encouragement and permission to publish this paper. This work received funding from the Malaysia Government Research and Development Fund, Malaysian-MIT Biotechnology Partnership Program (MMBPP), and SEAMEO-TROPMED.

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