



## **Liver Profile of Artemether-lumefantrine-tinidazole in Healthy and Parasitized Mice**

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### **Authors' contributions**

*This work was carried out in collaboration between both authors. Authors EA and UOG designed the study, performed literature search and statistical analysis, wrote the protocol and wrote the first draft of the manuscript and edited the final manuscript. Both authors read and approved the final manuscript.*

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### **ABSTRACT**

Artemether/lumefantrine/tinidazole (A/L/T) has shown additive antiplasmodial activity; therefore its safety assessment is imperative. This study examined its hepatotoxic effect on healthy and diseased mice. Fifty four Swiss albino mice of n=6 were used. The mice were diseased with *Plasmodium berghei* ( $1 \times 10^7$ ) and treated with T (28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T for 4 days, respectively. Healthy mice were treated with T (28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T for 28 days, respectively. After drug treatment; the mice were weighed and anesthetized. Liver samples were excised, weighed and evaluated for oxidative stress indices and histology. Blood samples were assessed for serum liver function indices. Treatment with T, A/L and A/L/T produced no significant ( $p > 0.05$ ) effects on all evaluated parameters in parasitized mice when compared to control. Significant decrease in body weight with significant increase in liver weight occurred in healthy mice treated with A/L ( $p < 0.05$ ) and A/L/T ( $p < 0.01$ ) when compared to control. Impaired liver function characterized by significantly increased serum aminotranferases, alkaline phosphatase, lactate dehydrogenase, gamma-glutamyl transferase, and bilirubin levels with significantly decreased total protein and albumin levels occurred in healthy mice treated with T ( $p < 0.05$ ), A/L

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( $p < 0.01$ ) and A/L/T ( $p < 0.001$ ) when compared to control. Significantly decreased glutathione peroxidase, superoxide dismutase, glutathione, and catalase levels with significantly increased malondialdehyde levels occurred in healthy mice treated with T ( $p < 0.05$ ), A/L ( $p < 0.01$ ) and A/L/T ( $p < 0.001$ ) when compared to control. A/L/T caused hepatocyte necrosis in healthy mice. The use of A/L/T for malaria treatment seems safe on the liver, but may impair liver function with prolonged use.

**Keywords:** Artemether-lumefantrine; tinidazole; Parasite; mice; liver; toxicity.

## 1. INTRODUCTION

Drug-induced hepatotoxicity is a serious and primary concern in medical practice. It is one of the basic reasons for hospital admissions. Drug-induced hepatotoxicity is one the leading causes of acute liver failure in the world, which may require liver transplantation in some conditions [1]. Dietary supplements, orthodox drugs and herbs have potential to cause hepatotoxicity. In preclinical drug evaluation about 50% of drug candidates show hepatic effects at supra-therapeutic dose and face drug attrition [2]. Hepatotoxicity is one of the primary causes of drug failure in clinical trials, which results in regulatory actions causing drug withdrawal [3]. Apart from the association of genetic factors with hepatotoxicity, the physicochemical properties of drugs and their interactions with hosts and environmental factors are important factors [4].

Artemether/lumefantrine (A/L) is one of the artemisinin based combination therapies recommended by the World Health Organization (WHO) for malaria treatment. It is efficacious for the treatment of uncomplicated and chloroquine resistance *P. falciparum* parasite [5]. Artemether has blood schizontocidal and gametocytocidal activities [6] whereas lumefantrine is a blood schizontocide which prevents heme polymerization [7]. The use of, A/L may be associated with hepatotoxicity marked by elevated serum liver enzymes, [8] and altered liver histology [9].

Tinidazole (T) is a synthetic nitroimidazole and a structural analogue of metronidazole. It is an antiprotozoal agent that has been widely used for more than two decades. T has established efficacy and acceptable tolerability for the treatment of trichomoniasis, giardiasis, amebiasis, and amebic liver abscess. It is well tolerated and seems to be relatively safe [10]. T has shown potential antimalarial property against *P. vivax* and *P. falciparum* infections [11]. It has cured blood and liver stages of of *P. cynomolgi*, infection in macaques [12]. Some studies

suggested the repurposing of T for the treatment of malaria [13]. Previously, we reported antiplasmodial activity of artemether/lumefantrine/ tinidazole on *P. berghei* infected mice [14]. Due to the potential of drug combinations to cause synergistic or additive toxicities, this study assessed the safety of artemether/lumefantrine/ tinidazole on the liver of parasitized and healthy mice.

## 2. MATERIALS AND METHODS

### 2.1 Animals, Malaria Parasite and Drugs

Fifty four adult Swiss albino mice ( $n=6$ ) of both sexes (20-25g) were used. The mice were supplied by the animal facility of the Faculty of Basic Clinical Sciences, University of Port Harcourt, Rivers State, Nigeria. The mice were kept in cages under natural environmental conditions and acclimated for 2 weeks before the experiment commenced. The mice had free access to feed and water. A/L (IPAC Laboratory, India), T (Norvatis). Doses of T (28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T used were obtained from previous antiplasmodial study [14]. CQ sensitive strain of *P. berghei* in donor mice was provided by the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria. Blood sample from the donor mouse was diluted to 2ml with 0.9% saline containing  $1 \times 10^7$  parasitized erythrocytes and transferred from mouse to mouse intraperitoneally (i.p). The parasitemia levels were monitored daily by the examination of thin blood smear using a light microscope.

### 2.2 Parasite Inoculation, Treatment and Animal Sacrifice

Thirty mice grouped into 5 of  $n=6$  were used. Group 1 served as normal control whereas groups II-V served as the experimental groups' and were parasitized i.p with *P. berghei* containing  $1 \times 10^7$  parasitized erythrocytes. After 3 days, the mice were treated orally as follows: Group 1: (Normal control) and group II (Parasitized control) were treated with normal

saline (0.2mL) daily for 4 days. Groups III-V were treated with T, A/L and A/L/T for 4 days, respectively. For the sub-acute study, twenty four healthy mice were grouped into 4 of n=6 and treated orally with drugs as follows: Group 1: (Control) was orally treated with normal saline (0.2mL) for 28 days. Groups II-IV were orally treated with T, A/L and A/L/T for 28 days, respectively. After, drug treatment, the parasitized and healthy mice were weighed. The mice were anesthetized with diethylether and blood samples were collected. The blood samples were centrifuged (2000 rmp for 15 minutes) and sera obtained for biochemical evaluations. Liver samples were obtained, rinsed in saline and homogenized in buffered (pH 7.4), 0.1 M Tris-HCl solution. The homogenates were centrifuged (2000rpm for 20 minutes) and the supernatants were decanted for oxidative stress markers assay.

## 2.3 Assessments of Serum Biochemical Markers

### 2.3.1 Assessments of liver function and oxidative stress markers

Serum alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), lactate dehydrogenase, (LDH) conjugated bilirubin (CB), and total bilirubin (TB) were evaluated using laboratory

test kits according to the manufacturer's specifications. Liver glutathione (GSH) was measured as described by Sedlak and Lindsay [15]. Catalase (CAT) was assessed according to Aebi, [16]. Glutathione peroxidase (GPx) was assayed as described by Rotruck et al. [17]. Superoxide dismutase (SOD) was estimated according to Sun and Zigman [18]. Malondialdehyde (MDA) was measured according to the method reported by Buege and Aust [19].

## 2.4 Histology of the Liver

Liver tissues were cut and placed in 10% formalin saline for 24hr. Liver tissues were dehydrated in graded alcohol concentrations. Liver tissues were routinely processed and embedded in paraffin block. Liver tissues were sectioned (3µm each) and stained with Haematoxylin and Eosin. Stained sections were examined on slides using a light microscope for histological changes.

## 2.5 Statistical Analysis

Data as mean  $\pm$  SEM. Data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. Graph Pad Prism 5 Software (San Diego, CA USA) was used for data analysis. Significance was set at  $p < 0.05$ ;  $p < 0.01$  and  $p < 0.001$ .

**Table 1. Effects of artemether/lumefantrine/tinidazole on body and liver weights of healthy and parasitized mice**

Treatment	Final body weight (g)		Absolute liver weight (g)		Relative liver weight (%)	
	Healthy mice	Infected mice	Healthy mice	Infected mice	Healthy mice	Infected mice
Control	25.66 $\pm$ 3.41	24.21 $\pm$ 2.60	0.93 $\pm$ 0.04	0.94 $\pm$ 0.08	0.36 $\pm$ 0.02	0.39 $\pm$ 0.06
T	23.32 $\pm$ 3.63	25.02 $\pm$ 3.55	0.90 $\pm$ 0.07	0.92 $\pm$ 0.07	0.37 $\pm$ 0.06	0.37 $\pm$ 0.01
A/L	24.05 $\pm$ 3.22	26.15 $\pm$ 2.61	1.39 $\pm$ 0.03*	0.95 $\pm$ 0.06	0.58 $\pm$ 0.08*	0.36 $\pm$ 0.09
A/L/T	22.61 $\pm$ 3.21	24.74 $\pm$ 3.43	1.80 $\pm$ 0.05 <sup>††</sup>	0.96 $\pm$ 0.03	0.80 $\pm$ 0.05 <sup>††</sup>	0.39 $\pm$ 0.04

T: Tinidazole, A/L: artemether/lumefantrine, A/L/T: Artemether/lumefantrine/tinidazole Data as mean  $\pm$  SEM, n= 6, \*  $p < 0.05$ , <sup>††</sup>  $p < 0.01$  compared to control (Healthy mice), SEM: Standard error of mean.

**Table 2. Effect of artemether/lumefantrine/tinidazole on serum liver function markers of parasitized mice**

Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)	LDH (U/L)	TB (g/dL)	T. Protein (g/dL)	Albumin (g/dL)
Control	28.53 $\pm$ 2.33	40.76 $\pm$ 5.41	25.81 $\pm$ 4.32	31.0 $\pm$ 3.65	2.21 $\pm$ 0.32	6.17 $\pm$ 0.10	3.31 $\pm$ 0.10
PC	29.05 $\pm$ 2.46	41.53 $\pm$ 4.33	26.00 $\pm$ 3.11	33.5 $\pm$ 2.64	2.42 $\pm$ 0.55	6.00 $\pm$ 0.21	3.25 $\pm$ 0.18
T	27.71 $\pm$ 2.52	40.92 $\pm$ 4.43	27.46 $\pm$ 3.47	32.9 $\pm$ 3.42	2.39 $\pm$ 0.33	6.05 $\pm$ 0.11	3.20 $\pm$ 0.06
A/L	27.85 $\pm$ 3.11	42.07 $\pm$ 3.76	27.72 $\pm$ 3.33	30.5 $\pm$ 4.10	2.30 $\pm$ 0.27	6.09 $\pm$ 0.72	3.19 $\pm$ 0.08
A/L/T	29.14 $\pm$ 2.62	45.71 $\pm$ 4.61	28.04 $\pm$ 4.12	30.2 $\pm$ 4.52	2.27 $\pm$ 0.42	6.13 $\pm$ 0.19	3.14 $\pm$ 0.07

PC: Parasitized untreated control, T: Tinidazole, A/L: artemether/lumefantrine, A/L/T: Artemether/lumefantrine/tinidazole, AST: Aspartate aminotransferase, CB: Conjugated bilirubin, ALT: Alanine aminotransferase, LDH: Lactate dehydrogenase, TB: Total bilirubin, ALP: Alkaline phosphatase, T. Protein: Total protein, Data as mean $\pm$  SEM, n= 6, SEM: Standard error of mean.

**Table 3. Effect of artemether/lumefantrine/tinidazole on serum liver function markers of healthy mice**

Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)	LDH (U/L)	TB (g/dL)	T.Protein (g/dL)	Albumin (g/dL)
Control	29.6±2.56	41.72±5.63	23.84±2.54	30.02±2.67	2.32±0.16	6.21±0.23	3.44±0.23
T	35.9±3.55*	62.96±6.53*	36.52±3.57*	58.36±5.00*	3.56±0.71*	4.10±0.09*	2.10±0.09*
A/L	57.8±4.86**	86.02±7.22**	55.70±5.44**	77.71±7.34**	4.72±0.60**	4.11±0.19*	2.00±0.07*
A/L/T	95.6±5.53 <sup>‡</sup>	160.7±9.43 <sup>‡</sup>	98.01±7.63 <sup>‡</sup>	110.2±10.2 <sup>‡</sup>	7.63±0.59 <sup>‡</sup>	2.00±0.73 <sup>‡</sup>	1.13±0.73 <sup>‡</sup>

T: Tinidazole, A/L: artemether/lumefantrine, A/L/T: Artemether/lumefantrine/tinidazole, AST: Aspartate aminotransferase, CB: Conjugated bilirubin, ALT: Alanine aminotransferase, LDH: Lactate dehydrogenase, TB: Total bilirubin, ALP: Alkaline phosphatase, T. Protein: Total protein, Data as mean± SEM, n=6, \* p<0.05, \*\*p<0.01, <sup>‡</sup>p<0.001 when compared to control, SEM: Standard error of mean.

**Table 4. Effect of artemether/lumefantrine/tinidazole on serum lipid parameters of healthy mice**

Treatment	TG (g/dL)	CH (g/dL)	HDL-C (g/dL)	LDL-C (g/dL)
Control	53.10±6.00	89.60±7.79	33.10±3.49	45.90±4.32
T	54.01±7.57	90.31±8.47	33.01±3.63	46.53±3.43
A/L	57.10±6.15	93.00±9.19	31.72±4.77	49.92±3.62
A/L/T	60.21±7.41	95.22±9.32	30.13±4.11	51.01±4.71

T: Tinidazole, A/L: artemether/lumefantrine, A/L/T: Artemether/lumefantrine/tinidazole, TG: Triglyceride, CH: Total cholesterol, HDL-C: High density lipoprotein cholesterol, LDL-C: Low density lipoprotein cholesterol. Data as mean± SEM, n=6, SEM: Standard error of mean.

**Table 5. Effect of artemether/lumefantrine/tinidazole on liver oxidative stress markers of healthy mice**

Treatment	MDA nmole/mg protein	GSH μmole/mg protein	CAT U/mg protein	SOD U/mg protein	GPx U/mg protein
Control	0.12 ± 0.02	13.44 ± 0.73	25.61 ± 2.01	14.83± 2.51	15.34 ± 1.00
T	0.25 ± 0.04*	10.03± 0.87*	20.83 ± 2.33*	11.01 ± 2.33*	11.62 ± 0.32*
A/L	0.36 ± 0.06**	6.35± 0.45**	16.32 ± 1.71**	9.00 ± 0.41**	8.14 ± 0.71**
A/L/T	0.50 ± 0.03 <sup>‡</sup>	4.22± 0.27 <sup>‡</sup>	12.84 ± 0.79 <sup>‡</sup>	6.01 ± 2.70 <sup>‡</sup>	5.40 ± 0.26 <sup>‡</sup>

T: Tinidazole, A/L: artemether/lumefantrine, A/L/T: Artemether/lumefantrine/tinidazole, MDA: Malondialdehyde, GSH: Glutathione, CAT: Catalase, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, Data as mean ± SEM, n=6, \* p<0.05, \*\*p<0.01, <sup>‡</sup>p<0.001 when compared to control, SEM: Standard error of mean.

### 3. RESULTS

#### 3.1 Effects of Artemether/Lumefantrine/Tinidazole on Body and Liver Weights of Healthy and Parasitized Mice

Body and liver weights were normal ( $p>0.05$ ) in parasitized mice treated with T, A/L and A/L/T for 4 days, respectively when compared to normal control (Table 1). On the other hand, body weight was significantly decreased whereas liver weight was significantly increased in healthy mice treated with A/L ( $p<0.05$ ) and A/L/T ( $p<0.01$ ) for 28 days when compared to normal control (Table 1).

#### 3.2 Effects of Artemether/ Lumefantrine/ Tinidazole on Serum Liver Function Markers of Healthy and Parasitized Mice

Serum AST, ALT, ALP, GGT, LDH, TB, CB, total protein and bilirubin levels were normal ( $p>0.05$ ) in parasitized mice treated with T, A/L and A/L/T for 4 days, respectively when compared to normal control (Table 2). On the contrary, significant increases in serum AST, ALT, ALP, GGT, LDH, TB and TB levels with significant decreases in serum total protein and albumin levels were observed in healthy mice treated with T ( $p<0.05$ ), A/L ( $p<0.01$ ) and A/L/T ( $p<0.001$ ) for 28 days, respectively when compared to normal

control (Table3). In the healthy mice, treatment with T, A/L and A/L/T had no significant effects ( $p>0.05$ ) on serum TG, CH, HDL-C, and LDL-C levels when compared to control (Table3).

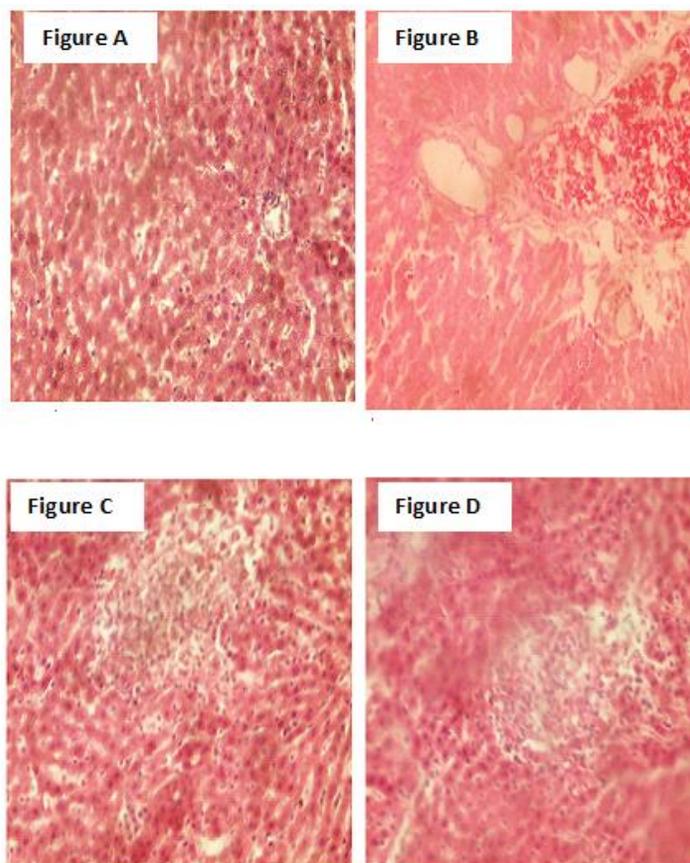
### 3.3 Effects of Artemether/ Lumefantrine/ Tinidazole on Liver Oxidative Stress Markers and Histology of Healthy Mice

Significant decreases in liver antioxidants (SOD, GSH, CAT and GPx) were observed in healthy mice treated with T ( $p<0.05$ ), A/L ( $p<0.01$ ) and A/L/T ( $p<0.001$ ) for 28 days, respectively when compared to control (Table 5). On the other hand, significant increases in MDA levels were observed in healthy mice treated with T ( $p<0.05$ ), A/L ( $p<0.01$ ) and A/L/T ( $p<0.001$ ) for 28 days, respectively when compared to control. (Table 5). The liver of control mice showed normal histology (Fig. A), whereas the liver of healthy

mice treated with T showed vascular congestion (Fig. B). The liver of healthy mice treated with A/L (Fig. C) and A/L/T (Fig. D) showed hepatocyte necroses. X 400.

### 4. DISCUSSION

Synergistic or additive toxicological effect, which may occur as a consequence of concurrent medication use, is of great concern to the public and regulatory authorities [20]. This study assessed the hepatotoxic effect of artemether/lumefantrine/tinidazole on healthy and *P. berghei* infected mice. Body and organ weights measurements are imperatively used in the toxicological studies of chemical substances [21]. In this study, A/L/T had no effects on the body and liver weights of parasitized mice, but caused remarkably decrease in the body weight with increase in the liver weight of healthy mice.



**Fig. A-D. The liver of control mice showed normal histology (Fig. A), whereas the liver of healthy mice treated with T (Fig. B) showed vascular congestion. The liver of healthy mice treated with A/L (Fig. C) and A/L/T (Fig. D) showed hepatocyte necroses. X 400**

A/L/T might have suppressed appetite in the healthy mice causing decreased calorie intake and consequently body weight reduction. The observed increase in liver weight may be attributed to the induction of inflammation by A/L/T. Hepatic function was normal in parasitized mice treated with A/L/T. However, A/L/T caused conspicuous hepatic impairment in healthy mice characterized by elevated serum AST, ALT, ALP, GGT, LDH, TB, and CB levels with decreased serum total protein and albumin levels. In this study, the observed alterations in the aforementioned serum markers in healthy mice treated with A/L/T may be a consequence of the disruption of their hepatic syntheses. A/L/T might have also perturbed hepatic membrane, thus increasing membrane porosity causing exodus of these biochemical markers into the blood [22]. In this study, A/L/T caused visible depletions of hepatic antioxidants and up regulation of hepatic MDA in healthy mice. This finding vividly attests to the induction of hepatic oxidative stress by A/L/T. Antioxidants are complex and sophisticated system that function interactively and synergistically to stabilize, or deactivate excess ROS, thus preventing oxidative stress and cellular damage [23]. Therefore, decreased liver antioxidants in A/L/T treated healthy mice will create a robust scene for ROS attack causing hepatic oxidative stress and consequently hepatic biomolecular damage. Also, increased MDA in A/L/T treated healthy mice symbolizes hepatic lipid peroxidation. In the current study, ALT impacted negatively on the liver structure by causing hepatocyte necrosis. Furthermore, in healthy mice treated with A/L, altered hepatic function was characterized by elevated serum AST, ALT, ALP, GGT, LDH, TB, and CB levels with decreased total protein and albumin levels. This observation is consistent with previous reports [24]. A/L also depleted liver antioxidants, increased MDA level and caused hepatocyte necrosis in health mice which is in agreement with previous study [25]. In the current study, in healthy mice, T caused alterations in serum hepatic markers, depletion of hepatic antioxidants and vascular congestion in the liver.

## 5. CONCLUSION

The use of A/L/T for malaria treatment seems safe on the liver, but may impair liver function with prolonged use.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

Animal Ethic Committee approval has been taken to carry out this study.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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