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Therapeutic potential of selected micronutrients in malaria: an in vivo study in *Plasmodium berghei* infected mice

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Abstract
The study involves an in vivo evaluation of the role of some antioxidant micronutrients in the therapeutics of malaria. Rodent malaria model using *Plasmodium berghei* NK-65 strain (chloroquine sensitive) was used. In the first stage of the experiment, a 4 day suppressive test was conducted using 40 mice of either sex weighing 20.05 ± 0.02 g which were inoculated intraperitoneally with 1×10^7 million *P. berghei* infected erythrocyte and were administered with 0.2 ml of distilled water, 0.2 ml of vehicle, Tween 80 (control and vehicle group), chloroquine 25 mg/kg (standard drug group), vitamin A 60 mg/kg, vitamin E 100 mg/kg, selenium 1 mg/kg, zinc 100 mg/kg, and vitamin C 200 mg/kg (test groups D, E, F, G, and H respectively) 3 hours post-inoculation. Similarly, 35 mice of either sex were used to conduct a 4 day curative test after the initial screening test. Selenium demonstrated significant ($p < 0.05$) chemosuppressive (82.01%) and schizonticidal activity (76.16%) when compared with negative control during the 4 day suppressive and 4 day curative test respectively. Mean parasitemia was significantly reduced ($p < 0.05$) in the micronutrient treated groups after the 4 day curative test when compared with negative control. This was also significant between groups ($F = 7.04$; $p \leq 0.05$). Conclusively, antioxidant micronutrients have potential antimalarial activity and may be of benefit in malaria therapeutics.

Keywords: Antioxidant micronutrients; antimalarial activity; therapeutic potential.

Introduction

Malaria has been estimated to cause 2.3% of global disease and 9% of disease in Africa (WHO, 1997), it ranks third among major infectious disease threats in Africa after pneumococcal acute respiratory infections (3.5%) and tuberculosis (TB) (2.8%). Cases in Africa account for approximately 9% of malaria cases in the world (WHO, 1997). Between 1994 and 1996, malaria epidemics in 14 countries of Sub-Saharan Africa caused an unacceptably high number of deaths, of which many occurred in areas previously free of the disease (Anderson *et al.*, 1996). Air travel has brought the threat of the disease to the doorsteps of industrialized countries, with an increasing incidence of imported cases and deaths from malaria by visitors to endemic-disease regions. The estimated annual direct and indirect cost of malaria was 800 million US dollars in 1987, this was expected to exceed 1.8 billion US dollars by 1995 (Anderson *et al.*, 1996). The interaction between malaria and nutrition is complex (Mc Gregor, 1982) and has been the subject of controversy since the early 1950s. Several studies have shown associations between malaria and protein energy malnutrition, poor growth, and certain micronutrient deficiencies among children (Mc Gregor *et al.*, 1956; Marsden, 1964; Rowland *et al.*, 1977; Nyakeriga *et al.*, 2004). Despite clear evidence of the impact of malaria on the nutritional status of affected individuals, the effect of nutritional status on host resistance to the acquisition and progression of malaria is still not clearly defined. Earlier studies suggested that poorly nourished individuals were to some extent protected against malaria (Edington, 1954; Hendrickse, 1971; Murray *et al.*, 1975,

1976, 1978a, 1978b). A more recent review of the malaria–nutrition literature (Shankar, 2000), concluded that the earlier findings of a protective effect of malnutrition against malaria were mainly based on studies with several methodological shortcomings. Reappraisal of the data together with recent literature indicates that the effect of nutrition on host susceptibility to malaria is more complex and, in many cases, poor nutritional status predisposes the host to an increased risk of infection, symptomatic clinical malaria attacks, and a higher likelihood of mortality from malaria. Several factors influence malaria treatment decisions. These include the severity of the infection, the child's age, degree of background immunity, other host factors that may impair immune function such as malnutrition or advanced HIV infection, local patterns of antimalarial drug resistance, availability of drugs, and the cost of antimalarial drugs (White, 1996). Hence, this study aims to determine the therapeutic potential of some antioxidant micronutrients in malaria using *P. berghei* mouse model.

Materials and Methods

Materials

Chemicals and equipments
Heparinised capillary tubes, light microscope (Olympus, Japan), EDTA bottles, feeding trochars, syringes (1 ml, 5 ml), cotton wool, microscopic slides (Olympus, China), hand gloves, Giemsa stain (Sigma), 98% methanol (Sigma), and Tween 80 (Sigma).

Drugs
Vitamin A (Clarion Medical Pharmaceuticals, Nigeria), vitamin E (Clarion Medical Pharmaceuticals, Nigeria), vitamin C (Emzor Pharmaceuticals, Nigeria), zinc gluconate (Mason Vitamins Incorporated, USA), selenium-organic (Mason Vitamins Incorporated, USA), chloroquine (Emzor Pharmaceuticals, Nigeria), pyrimethamine (Glaxo Smith Klime, Nigeria), and artesunate (Emzor Pharmaceuticals, Nigeria).

Preparation of animals
Seventy five in bred pure Swiss albino mice of either sex weighing between 18–25 g was used for the study. They were obtained from the animal house of the Nigerian Institute of Medical Research, Yaba Lagos State and housed in stainless steel cages with wire screen top. The animals were about 7–8 weeks old and were maintained on commercial feeds (Vital feeds, Jos) and tap water *ad libitum* for the entire duration of the study. The mice were allowed to acclimatize for 1 week in the laboratory environment under a controlled temperature of 20°C and at optimum humidity before being subjected to the experiment (Obernier and Baldwin, 2007). Good hygiene was maintained by constant cleaning and removal of faeces and spilled feeds from the cages daily.

Preparation of inoculum of chloroquine sensitive strain of P. berghei
P. berghei NK-65 strain maintained in the laboratory of Nigerian Institute of Medical Research, Yaba by serial blood passage from mouse to mouse was used for the study. Donor mouse with a rising parasitemia of 20–30% confirmed by thin and thick blood film microscopy was used. Blood (0.2 ml) was collected in a heparinized tube from the auxiliary plexus of veins in the donor mouse using heparinized capillary tubes. The blood was diluted with 5 ml of phosphate buffer solution (PBS) pH 7.2 so that each 0.2 ml contained approximately 1×10^7 infected red cells (Peter *et al.*, 1975; David *et al.*, 2004). Each animal received inoculu of about 10 million parasites per kilogram body weight, which is expected to produce a steadily rising infection in mice.

Preparation of drugs

Chloroquine
50 mg of powdered chloroquine sulphate were dissolved in 20 ml of distilled water. So that 1 ml will contain 2.5 mg of chloroquine sulphate. Dosage administered to the animals in the standard drug group (A) was 25 mg/kg. Hence the 0.2 ml of solution administered contained 0.5 mg of chloroquine sulphate.

Vitamin A
200,000 IU of vitamin A caplet, which is equivalent to 60 mg of vitamin A, was used to prepare the dose administered (60 mg/kg). The drug was dissolved in 0.2 ml of Tween 80 used as a vehicle and distilled water in a ratio of 0.2:0.2:9.6. To make up a total volume of 10 ml. The final volume of drug administered was 0.2 ml, which is equivalent to 0.495 mg of vitamin A.

Vitamin E
100 mg of vitamin E caplet was dissolved in 0.2 ml of Tween 80 and distilled water in a ratio of 0.2:0.2:9.6 making up a total volume of 10 ml. The

dose administered to the animal was 100 mg/kg. Hence the final volume of drug administered to the animal was 0.2 ml, which is equivalent to 1.6 mg of vitamin E.

Selenium
1 mg of selenium was dissolved in 10 ml of distilled water in its powdered form. A dose of 1 mg/kg body weight was administered to the animals. The final volume of drug administered was 0.2 ml equivalent to 0.0145 mg of selenium.

Zinc
The dose of zinc administered was 100 mg/kg. 100 mg of zinc was dissolved in 10 ml of distilled water in its powdered form. 0.2 ml of the solution was administered which is equivalent to 1.91 mg of zinc.

Vitamin C
200 mg of the powdered form of vitamin C was dissolved in 10 ml of distilled water. Dosage administered to the animals was 200 mg/kg body weight. A final volume of 0.2 ml of solution was prepared which is equivalent to 1.8 mg of vitamin C.

Drugs/micronutrient administration
In the first stage of this study, a 4 day suppressive test was performed using the methods of (Peters, 1965; Peter *et al.*, 1975; David *et al.*, 2004). Certain parameters were assessed during the 4 day suppression test; these included blood % parasitemia on day 4 after infection, the base line parasitemic profile and average survival time in the control group. Chloroquine sulphate at a dose of 25 mg/kg was used as a reference drug (Tekalign *et al.*, 2010). The antioxidant micronutrients were administered orally as follows: vitamin A (60 mg/kg), vitamin E (100 mg/kg), vitamin C (200 mg/kg), zinc (100 mg/kg), selenium (1 mg/kg) using doses based on LD_{50} values as reported by Gerhard (2000), Oncu *et al.* (2002), Oreagba and Ashorobi (2006). Tween 80 (0.2 ml) was used, as vehicle for fat-soluble vitamins A and E. Agents, which demonstrated activity in the 4 day assay, went through a curative test (Rane test) using the method described by Agbaje and Onabanjo (1994), David *et al.* (2004), Adzu *et al.* (2007). In this test, mice were administered a single daily dose of test agent by oral route on day 3 (72 hours) post-infection followed by daily monitoring of parasitemia.

In the 4 day suppressive test, infected mice were randomly divided into standard drug group (A), control group (B), vehicle group (C), and treatment groups (D, E, F, G, and H). The treatment group, sub-divided into sub groups D, E, F, G, and H, received vitamin A (60 mg/kg), vitamin E (100 mg/kg), selenium (1 mg/kg), zinc (100 mg/kg), and vitamin C (200 mg/kg) respectively (Table 1). Group C received the vehicle (0.2 ml of Tween 80); while group A received chloroquine 25 mg/kg, daily for 4 days, starting on the same day as that of the parasite inoculation. The drugs/micronutrients were administered via oral route using a feeding trochar coupled to a 1 ml syringe. All were given daily for 4 consecutive days starting 3 hours after infection, i.e., from day 0 (D0) to day 3 (D3), receiving a total of 4 oral doses. Thin smears of blood films were obtained from the peripheral blood by nipping the tail of each mouse on day 4 after infection. The smears were placed on microscopic slides, fixed with methanol and stained with 5% Giemsa at pH 7.2, for parasitemia. The numbers of parasitized erythrocytes in each of the 10 fields were counted and the average was calculated to give the % parasitemia. Parasitemia was monitored over 15 days. Percentage suppression was calculated by using the formulae by (Peter *et al.*, 1977; Peter and Anatoli, 1998; David *et al.*, 2004).

$$\% \text{ Suppression} = \frac{\text{Parasitemia in negative control} - \text{Parasitemia in study group}}{\text{Parasitemia in negative control}} \times 100$$

In the 4 day curative test animals were weighed and divided into 7 groups (A, B, C, D, E, F, and G) of 5 mice each (Table 1). They were inoculated intraperitoneally with 1×10^7 *P. berghei* infected red blood cells on day 0 according to previous schedule. The animals in group A were then administered a single oral dose of chloroquine at 25 mg/kg. Group B, which served as the negative control group was treated with 0.2 ml of distilled water. Group C animals were treated with 0.2 ml of the vehicle Tween 80, group D animals were treated with a single oral dose of vitamin A (60 mg/kg), group E animals with a single oral dose of vitamin E (100 mg/kg), group F animals with a single oral dose of selenium (1 mg/kg), and group G animals with a single oral dose of zinc (100 mg/kg) administered daily for 4 days consecutively. On day 4 post-infection, daily smears for thin blood film were made to ascertain % chemosuppression,

Table 1: Dosing schedule in 4 day suppressive and curative test.

Test	Groups	Dose of drugs/micronutrients administered							
		Chloroquine (mg/kg)	Distilled H_2O (ml)	Tween 80 (ml)	Vitamin A (mg/kg)	Vitamin E (mg/kg)	Selenium (mg/kg)	Zinc (mg/kg)	Vitamin C (mg/kg)
4 DST	A	25	–	–	–	–	–	–	–
	B	–	0.2	–	–	–	–	–	–
	C	–	–	0.2	–	–	–	–	–
	D	–	–	–	60	–	–	–	–
	E	–	–	–	–	100	–	–	–
	F	–	–	–	–	–	1	–	–
	G	–	–	–	–	–	–	100	
	H	–	–	–	–	–	–	–	200
4 DCT	A	25	–	–	–	–	–	–	–
	B	–	0.2	–	–	–	–	–	–
	C	–	–	0.2	–	–	–	–	–
	D	–	–	–	60	–	–	–	–
	E	–	–	–	–	100	–	–	–
	F	–	–	–	–	–	1	–	–
	G	–	–	–	–	–	–	100	–

parasite clearance time (PCT), recrudescence time (RT), and mean survival time.

Results

As shown in Figure 1, the parasitemic profile in the control group during the 4 day suppressive test peaked at 54.3% prior to the death of inoculated mice with an average mean survival of (16.00 ± 1.48 days).

As shown in Figure 2, antioxidant micronutrients in this study exhibited significant shizonticidal activity in the early phase of *P. berghei* infection ($p < 0.05$). However, this was more marked in selenium treated group (82.01%) and insignificant in the vitamin C treated group when compared with control ($p > 0.05$).

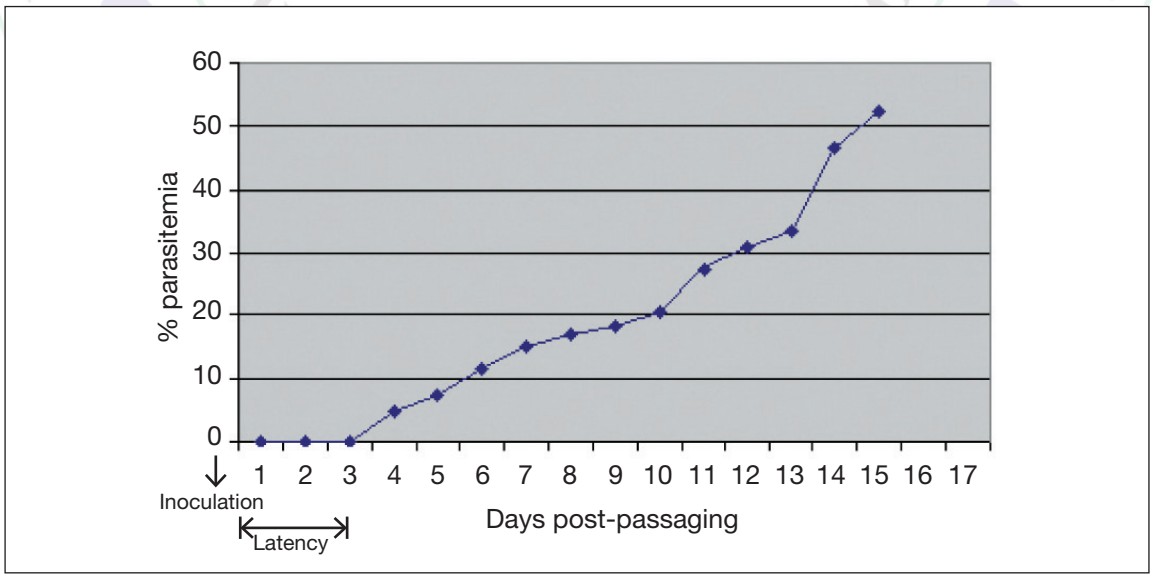

Figure 1: Parasitemic profile in negative control group 15 days post-passaging in 4 day suppressive test.

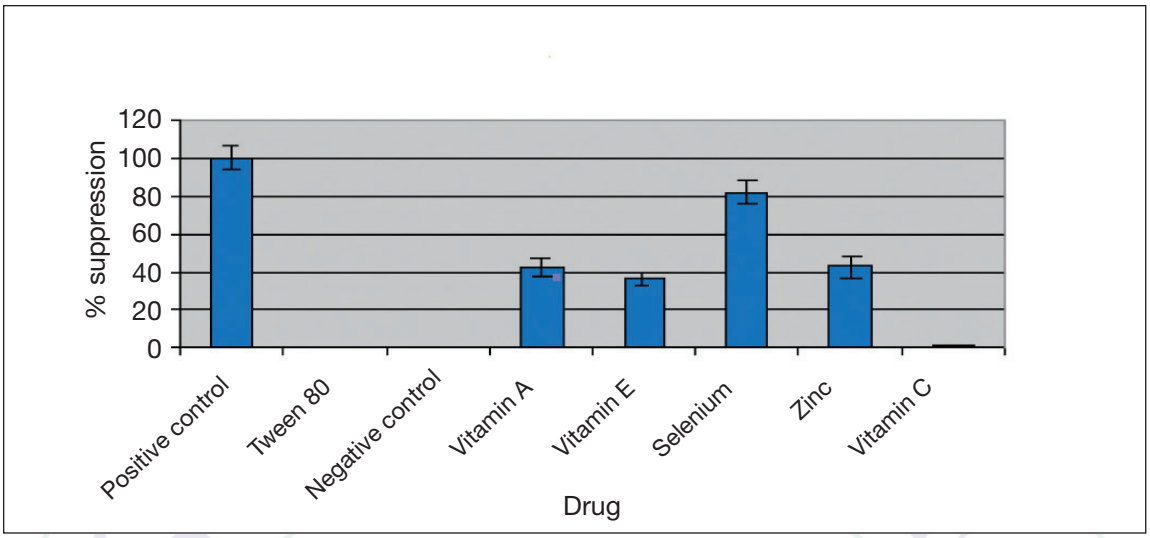

Figure 2: % suppression of different test agents in 4 day suppressive test.

Additionally, average % parasitemia was markedly reduced in the selenium treated group (0.86 ± 0.70%) when compared to the other micronutrient groups after 4 days suppressive treatment of *P. berghei* infection (Figure 3). This was significant ($p < 0.05$) when compared to control.

Mean parasitemic levels after 4 days curative treatment was 5.82 ± 1.48% in the selenium treated group and 9.95 ± 2.81% in the vitamin A treated group respectively, when compared with the control group (24.42%). This was significant when compared with negative control ($p < 0.05$) and significant between micronutrient treated groups ($F = 7.04$; $p < 0.05$), though chemosuppression was not as marked when compared to the chloroquine treated group (100%) as shown in Table 2. Additionally, average

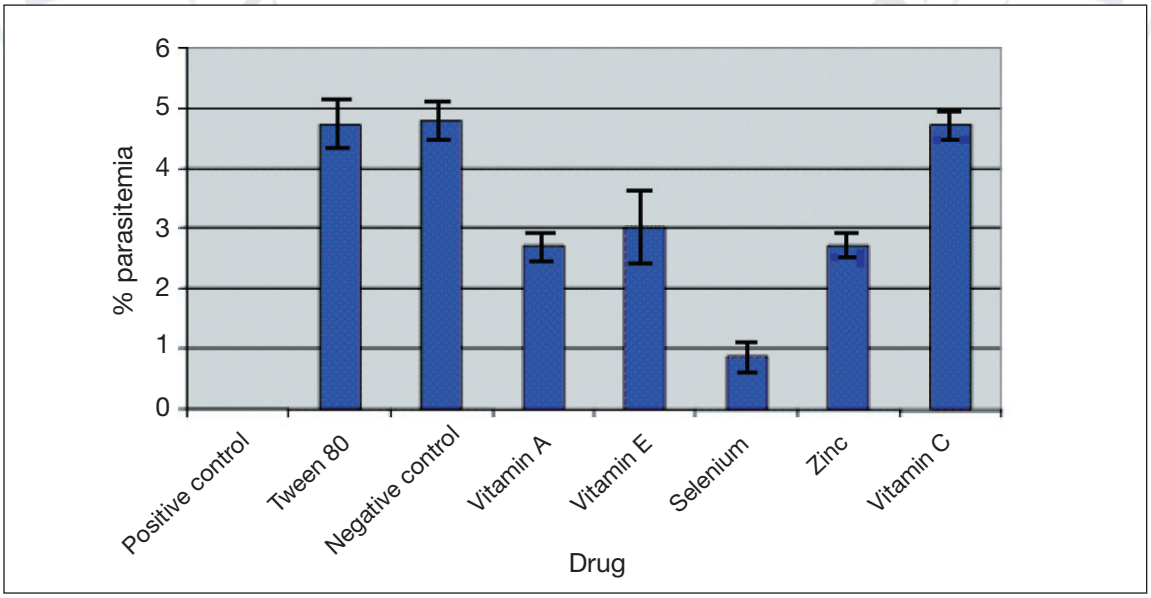

Figure 3: % parasitemia of different test agents in 4 day suppressive test.

Table 2: Mean parasitemic levels of established *P. berghei* infection after 4 days of treatment (curative test). $N = 5$ mice per group.

Groups	Dose (mg/kg)	Average % parasitemia	% Suppression	MST (days)
Positive control (Chloroquine)	25	0.00 ± 0.00	100	55.00 ± 3.16
Negative control (Distilled H_2O)	0.20 ml	24.42 ± 3.84	–	16.00 ± 1.48
Tween 80	0.2 ml	24.15 ± 2.08	0.70	15.80 ± 1.20
Vitamin A	60	*9.95 ± 2.81	59.19	*22.70 ± 0.86
Vitamin E	100	*15.98 ± 2.60	34.56	*21.60 ± 1.17
Selenium	1	*5.82 ± 1.48	76.16	*24.00 ± 0.63
Zinc	100	*15.33 ± 2.63	37.22	*22.40 ± 0.81
One way ANOVA		$F = 7.04$; $p \leq 0.05$		$F = 374.31$; $p \leq 0.05$

Results are expressed as mean ± SEM, d.f. = 4.
*$p < 0.05$ is significant when compared with control.

mean survival time (MST) was significantly prolonged in the entire micronutrient treated group when compared to control ($p < 0.05$).

As shown in Figure 4, the parasitemic profile of *P. berghei* in micronutrient treated mice during the curative monotherapy test showed an initial decline in parasitemia after the first day of treatment in the selenium group. However, this effect was not sustained when compared to the chloroquine treated group as there was a steady rise in parasitemia after the 4th day of treatment.

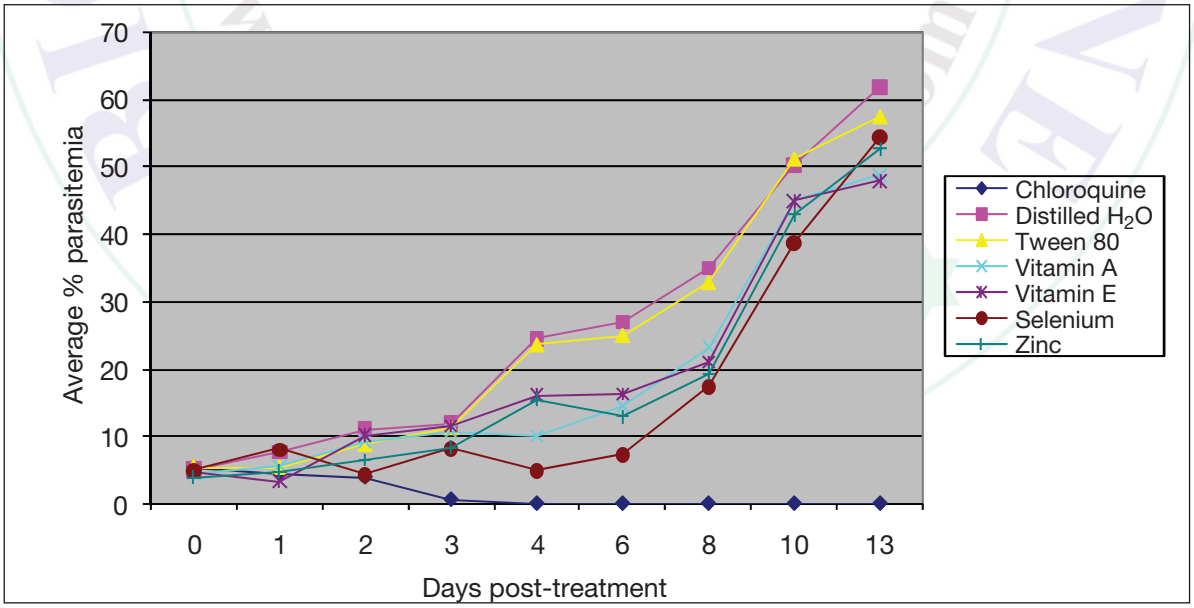

Figure 4: Parasitemic profile of *P. berghei* 13 days post-treatment in 4 day curative test.

Discussion

Results from the in vivo study revealed that vitamin A has antimalarial activity. It caused 43.1% chemosuppression during a 4 day suppressive test. This finding is in agreement with the finding of (Oreagba and Ashorobi, 2006), which demonstrated a similar antimalarial activity against *P. berghei berghei* in rodents and stated that the antimalarial activity of retinol became stronger with chronic administration. However, no complete parasite clearance was recorded after a 4 day curative treatment which also corroborates the findings of Oreagba and Ashorobi (2006). The antimalarial activity of vitamin A as demonstrated in this study is supported by the study of Hamzah *et al.* (2004) which revealed that retinol inhibits the growth of cultured *P. falciparum*. The present study also revealed that vitamin C has little or no antimalarial activity. Results from the present study revealed that parasitemic levels after a 4 day suppression test were not significantly different from the control (4.70 $\pm$ 0.33% versus 4.78 $\pm$ 0.65%). Results from other studies in mice have been mixed. One study by Godfrey (1957), showed that large doses of vitamin C abrogated the protective effect of cod liver oil in mice. Levander and Ager (1993), concluded that vitamin C supplementation did not modify the course of parasitemia in normal mice. This finding supports the present study. However, the study by Oreagba *et al.* (2008), revealed that extremely high doses of ascorbic acid and grape juice has antimalarial activity in *P. berghei* infected mice. Vitamin E administration also demonstrated moderate antimalarial activity as evidenced by its chemosuppressive activity in the 4 day suppressive and the 4 day curative test (36.82% and 34.56% respectively). This finding was however in agreement with two previous studies. In one of such studies, the morbidity and outcome of avian malaria infection with *P. spartani* was more severe in ducklings fed with vitamin E and selenium deficient diets than in ducklings fed with vitamin E and selenium adequate diets (Yarrington *et al.*, 1973). From the study conducted, selenium was found to exhibit a marked chemosuppressive activity against *P. berghei* infected mice in the 4 day suppressive test and curative test (82.01% and 76.16% respectively). Apart from the study done by Yarrington *et al.* (1973), no other in vivo study has been done till date to corroborate these findings. The work by Yarrington *et al.*, (1973) was inconclusive as it suggested that ducks fed with vitamin E and selenium deficient diet had a more severe manifestation of avian malaria compared with ducks fed with vitamin E and selenium supplemented diet. However, he noted that vitamin E and selenium supplemented diet did not influence the survival time in avian malaria. In a separate experiment he stated that selenium deficient duck were more susceptible to avian malaria (Yarrington *et al.*, 1973). In this same study the author noted that vitamin E and selenium deficiency in swine was associated with a high incidence of microbial infection and that studies in mice fed a diet deficient in factor 3, vitamin E, and cystine had diminished natural resistance to *Schistosoma mansoni* infection. The findings of this study revealed that zinc supplements have similar chemosuppressive activity with vitamin A (43.51% versus 43.10%) after a 4 day suppressive test. This antimalarial activity is supported by the study of Shankar *et al.* (1995) who indicated that moderate zinc deficiency resulted in an increased mortality from normally non-lethal rodent malaria from *P. yoelii* 17X-NL strains. Other studies reveal that zinc is essential for a variety of lymphocyte function implicated in resistance to malaria including production of Immunoglobulin G, IF-γ, TNF-α, and enhances the microbicidal activities of macrophages (Shankar and Prasad, 1998).

Conclusion

The present study has shown that antioxidant micronutrients have potential antimalarial activity that may be of benefit in the management of uncomplicated *falciparum* malaria.

Ethical Approval

The study was approved by the ethics committee of the College of Medicine, University of Lagos, Nigeria.

Conflict of Interests

None declared.

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