

Effect of α -tocopheryloxy acetic acid on the infection of mice with *Plasmodium berghei* ANKA in vivo and humans with *P. falciparum* in vitro

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Research Article

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Abstract

Background

Malarial parasites are susceptible to oxidative stress. The effects of α -tocopheryloxy acetic acid (α -TEA), a vitamin E analog, on infection by *Plasmodium berghei* ANKA and *P. falciparum* in mice and human red blood cells (RBCs), respectively, were examined in this study.

Methods

For *in vivo* studies in mice, RBCs infected with *P. berghei* ANKA were inoculated via intraperitoneal injection and α -TEA was administered to C57BL/6J male mice after infection. The blood–brain barrier (BBB) permeability was examined by Evans blue staining in experimental cerebral malaria at 7 days after infection. The *in vitro* inhibitory effect of α -TEA on *P. falciparum* 3D7 (chloroquine-sensitive strain) and K1 (multidrug-resistant strain) was tested using a SYBR Green I-based assay.

Results

When 1.5 % α -TEA was administered for 14 days after infection, 88 % of *P. berghei* ANKA-infected mice survived during the experimental period. Nevertheless, all the control mice died within 12 days of infection. Furthermore, the Evans blue intensity in α -TEA-treated mice brains was less than that in untreated mice, indicating that α -TEA might inhibit the destruction of the BBB and progression of cerebral malaria. The *in vitro* experiment revealed that α -TEA inhibited the proliferation of both the 3D7 and K1 strains.

Conclusions

This study showed that α -TEA is effective against murine and human malaria *in vivo* and *in vitro*, respectively. Although α -TEA alone has a sufficient antimalarial effect, future research could focus on the combined effect of α -TEA and already existing drugs. The prophylactic antimalarial activity of premedication with α -TEA may also be an interesting perspective in the future.

Background

It is well known that malaria parasites are sensitive to oxidative stress, since reactive oxygen species (ROS) lead to the hemolysis of host erythrocytes and parasite death [1, 2]. One vitamin E derivative, α -tocopheryl succinate (α -TOS), which is synthesized by esterification of α -tocopherol, leads to the production of ROS and exhibits anticancer activity in mice [3–6]. Previous studies have demonstrated that α -TOS inhibits mitochondrial complex II, resulting in the generation of ROS, which triggers selective apoptosis in malignant cells, while it appears to be non-toxic to normal cells [7–15]. In addition, cells lacking mitochondrial respiratory chain activity are not sensitive to α -TOS toxicity [10–13]. In murine malaria infection, administration of α -TOS after infection with *Plasmodium yoelii* 17XL or *P. berghei* ANKA resulted in a significant increase in host survival [16]. However, because α -TOS is decomposed by

an esterase, its low stability and clinical application is challenging. Recently, significant effects of α -tocopheryloxy acetic acid (α -TEA), a stable vitamin E derivative [17], on *P. yoelii* 17XL infection have been reported in mice [18]. The α -TEA compound has an ether bond and is not degraded by esterase, making it suitable for oral administration [19, 20]. Alpha-TEA stimulates mitochondria to generate ROS and induces apoptosis in tumor cells [21–24], while also enhancing the anti-tumor activity of trastuzumab against breast cancer [25, 26]. Since α -TEA has a more pronounced inhibitory effect on breast cancer than α -TOS and is more stable in plasma [17], it was predicted that the stimulatory effect of α -TEA on mitochondria [27] and the associated responses, such as ROS generation, apoptosis induction, and autophagy stimulation [22], could have some effect on malaria infection [18].

In this study, to determine whether α -TEA is an appropriate anti-malarial drug candidate, C57BL/6J male mice were infected with *P. berghei* ANKA, a lethal strain of murine malaria and experimental cerebral malaria, and treated with α -TEA. In addition, to analyze whether the antiprotozoal effects were the result of modification of the physiology of the host or a direct effect on the protozoan, the effect of α -TEA on the growth of *P. falciparum* *in vitro* was also examined.

Methods

Experimental infection with *P. berghei* ANKA was carried out using C57BL/6J male mice (Japan CLEA, Tokyo, Japan) at biosafety level 2 in a specific pathogen-free facility. The room temperature ($24 \pm 1^\circ\text{C}$) and humidity ($50 \pm 10\%$) were adjusted, and the lighting was controlled (lights on from 7 AM to 7 PM). The mice were allowed free access to water and a diet (CA-1; CLEA Japan, Tokyo, Japan). The animals used in this study were handled and cared for in accordance with the “Guiding Principles for the Care and Use of Research Animals” established by Obihiro University of Agriculture and Veterinary Medicine, Japan. All animal experimental procedures were approved by the Obihiro University of Agriculture and Veterinary Medicine Institutional Animal Ethics Committee (ethical approval number #29-86, April 14, 2017; #18-112, May 16, 2018; #19-130, May 23, 2019).

Alpha-TEA (molecular weight 488.75 g/mol) distributed by Eisai (Eisai Co., Ltd., Tokyo, Japan) was mixed with the diet [0.75% and 1.5% (w/w) of α -TEA] and fed to 8-weeks old C57BL/6J male mice (23-25 g body weight) for 14 days after infection. The day of infection was defined as Day 0. The mixed diet was outsourced to Oriental Yeast Co. Ltd. (Tokyo, Japan).

For parasite infection, 4×10^4 *P. berghei* ANKA-infected red blood cells (iRBCs) were intraperitoneally injected into mice and their survival rates and parasitemia were monitored. On day 4 post-infection, 2 μL of blood was collected from the tip of the tail, smeared on a glass slide, and stained with Giemsa (Sigma-Aldrich, Tokyo, Japan). The percentage of infected erythrocytes to total erythrocytes (parasitemia) was determined using a phase contrast microscope (DIAPHOTO-TMD300, Nikon, Tokyo, Japan). More than 1,000 red blood cells (RBCs) were counted in each mouse to assess parasitemia. On day 7 after infection, the permeability of the blood-brain barrier (BBB) was evaluated by Evans blue staining as follows: 0.1 mL of 2% (w/v) Evans blue (056-04061; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) dissolved

in PBS was injected into infected mice with or without treatment with α -TEA through the tail vein. Two hours after injection the mice were sacrificed, and brain samples were collected.

Survival rates were analyzed using log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests implemented in GraphPad Prism 5. Parasitemia was analyzed using a one-way analysis of variance and Tukey's test. A value of $P < 0.05$ was considered statistically significant.

Experimental *in vitro* infections with *P. falciparum* 3D7 and K1 were performed using human O+ RBCs (Japanese Red Cross Society, Hokkaido, Japan). Both parasites were maintained in complete Roswell Park Memorial Institute-1640 medium (RPMI-1640, Sigma-Aldrich, MO, USA), containing, per liter, 6 g HEPES (Sigma-Aldrich, MO, USA), 25 mg hypoxanthine (Wako, Osaka, Japan), 2 g NaHCO_3 (Wako, Osaka, Japan), 250 μL of 50 mg/ml gentamicin solution (Gibco, CA, USA), and 5 g AlbuMax™ II Lipid-Rich BSA (Gibco, CA, USA). The medium was changed daily and parasitemia was monitored using Giemsa-stained thin blood smears (Merck, Darmstadt, Germany). Malarial parasite culture in human blood was approved by the ethical committee of Obihiro University of Agriculture and Veterinary Medicine (#2013-04-3).

In vitro growth inhibition of *P. falciparum* 3D7 and K1 was performed using the SYBR Green I-based fluorescence assay (SYBR® Green I Nucleic Acid Stain 10,000 \times , ME, USA) as previously described [28, 29]. Briefly, test compounds were prepared in complete media at eight final concentrations (two-fold serial dilution) ranging from 100 to 0.78 μM . Chloroquine diphosphate (molecular weight 515.86 g/mol; Sigma-Aldrich, MO, USA) was used as the reference drug. Before use, the parasites' life cycle was synchronized by treatment with 5% D-sorbitol to obtain $\geq 90\%$ ring-stage parasites. Synchronous parasites (50 μL) at 0.5% parasitemia and 2% hematocrit were seeded in a 96-well plate containing 50 μL of the test compounds. The 96-well plates were incubated for 72 h at 37°C in 5% CO_2 , 5% O_2 , and 90% N_2 . Next, 100 μL of lysis buffer containing 1 \times SYBR Green I was added to each well, mixed by pipetting, and then incubated in the dark at room temperature for 2 h. Fluorescence intensities were measured using a Fluoroskan Ascent instrument (Thermo Scientific, MA, USA) at excitation and emission wavelengths of 485 and 518 nm, respectively. Cells treated with 1% (v/v) DMSO were used as a negative control, and wells containing only test compounds and erythrocytes were used to correct the background signals. The inhibition assays were performed in quadruplicate for each concentration and repeated three independent times. The half-maximal inhibitory concentration (IC_{50}) values were analyzed using nonlinear regression analysis implemented in GraphPad Prism 8.

Human foreskin fibroblast cells (HFFs) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution ($\times 100$, Wako, Osaka, Japan) at 37°C and 5% CO_2 in air. A cell viability assay was used to evaluate the cytotoxic effects of the tested compounds, as previously described [28]. Briefly, the cell suspension was seeded in a 96-well plate at a density of 1×10^4 cells/well in DMEM with 10% FBS and incubated for 48 h at 37°C and 5% CO_2 in air. Then, two-fold dilutions (total eight concentrations) of the test compounds in DMEM were added in quadruplicate to each well and incubated for an additional 72 h. Cell Counting Kit-8 (CCK-8) was added and incubated for an additional 3 h at 37°C and 5% CO_2 in

air, and the absorbance was measured at 450 nm. The tests were independently repeated three times. The half-maximal cytotoxic concentration (CC₅₀) values were analyzed using nonlinear regression analysis implemented in GraphPad Prism 8.

The human RBC hemolysis assay was performed as previously described [30]. Briefly, each test compound was prepared in 1× phosphate-buffered saline (PBS) at a desired concentration in a 96-well plate, and 3% RBCs suspension in PBS was added. The plate was incubated for 3 h at 37°C in 5% CO₂, 5% O₂, and 90% N₂ and then centrifuged at 1,300 × *g* for 5 min. Finally, 100 μL of the supernatant of each mixture was transferred to a new 96-well plate, and the absorbance was recorded at 540 nm. PBS (with 1% DMSO) and RBC lysis buffer (0.83% NH₄Cl; 0.01 M Tris-HCl, pH 7.2) were used as negative and positive controls, respectively. The hemolysis rate of RBCs was calculated using the following formula: $Hemolysisrate = [(A_{sample} - A_{negative\ control}) / (A_{positive\ control} - A_{negative\ control}) \times 100]$, where A stands for absorbance. The experiments were performed in technical quadruplicates, and independently repeated three times.

Results

As shown in Figure 1A, the effect of the administration of α-TEA on *P. berghei* ANKA infected mice was remarkable. Although the parasitemia of 0.75% α-TEA treated mice was found to be significantly lower than that of the untreated group on day 10 after infection (Figure 1B), both control and 0.75% α-TEA-treated mice died within 12 days after infection (Figure 1A). Remarkably, when 1.5% α-TEA was mixed with the diet for the 14 days following infection, 88% of the *P. berghei* ANKA-infected mice survived the experimental period. In fact, the proportion of parasitemia in this group of mice was significantly lower than that of the control group on days 4, 8, and 10 following infection (*P* < 0.05). Seven days after infection, Evans blue staining was observed to be maximal in the mice from the untreated group, while staining intensity was dramatically attenuated by 0.75 and 1.5% α-TEA treatment (Figure 2).

The *in vitro* effects of α-TEA are summarized in Tables 1 and 2 and are shown in Figure 3. The proliferation of *P. falciparum* was inhibited by α-TEA in both 3D7 (IC₅₀ 13.89 ± 1.16 μM) and K1 (IC₅₀ 15.51 ± 0.56 μM) strains. The cytotoxicity of α-TEA against human foreskin fibroblast (HFF) cells showed the cytotoxic concentration CC₅₀ value of 80.41 ± 17.24 μM, resulting in the selectivity indexes (SI) of 5.79 and 5.18 for *P. falciparum* 3D7 and K1, respectively. Furthermore, at a concentration of 100 μM, α-TEA exhibited an RBC hemolysis rate of 14.70 ± 1.12%.

Table 1

In vitro examinations of α -TEA and chloroquine against *P. falciparum*, HFF cells, and RBC hemolysis rate*

Compound	IC ₅₀ <i>P. falciparum</i> (μ M)		CC ₅₀ HFF Cells (μ M)	SI		RBCs Hemolysis Rate (%) at 100 μ M
	3D7	K1		3D7	K1	
α -TEA	13.89 \pm 1.16	15.51 \pm 0.56	80.41 \pm 17.24	5.79	5.18	14.70 \pm 1.12
Chloroquine	0.027 \pm 0.002	0.740 \pm 0.055	26.69 \pm 6.58	988.52	36.07	0.71 \pm 0.35

* Values were mean \pm SD from three independent experiments.

Table 2

The RBCs hemolysis rate of α -TEA at various concentrations*

Concentration (μ M)	400	200	100	50	25	12.5	6.25	3.125
Hemolysis Rate (%)	55.23 \pm 7.80	24.09 \pm 0.70	14.70 \pm 1.12	5.76 \pm 1.41	2.15 \pm 0.37	0.75 \pm 0.18	0.19 \pm 0.22	0.20 \pm 0.78

* Values were mean \pm SD from three independent experiments.

Discussion

This study reports a significant effect for α -tocopheryloxy acetic acid (α -TEA) on the treatment of infection by *P. berghei* ANKA in mice. To date, α -TEA has been studied mainly for its antitumor effect [21–26, 31], but the results of this study showed an anti-protozoal effect for this compound on *P. yoelii* 17XL infection in mice [18]. When α -TEA was mixed with the diet and orally administered to *P. yoelii* 17XL-infected mice, their survival was almost completely maintained and the parasites were eradicated from their blood cells [18]. In *P. berghei* ANKA-infected mice, a lower dosage of α -TEA (0.75%) showed similar kinetics to control mice in both survival rate and parasitemia (Figure 1). However, when the infected mice were treated with a higher dose of α -TEA (1.5%) for 14 days after infection, their survival was significantly higher and parasitemia was significantly lower than those in the control and 0.75% α -TEA-treated groups (Figure 1). This data suggests that it may be possible to maintain low levels of parasitemia by increasing the α -TEA concentration in the blood stream immediately before the elevation of parasitemia after the moment of infection. It has been reported that treatment with 50 mM α -TOS after *P. berghei* ANKA infection significantly increased host survival in mice [16]. The median survival time of the solvent- and 50 mM α -TOS-treated mice infected with *P. berghei* ANKA was 8.0 and 11.5 days after infection, respectively [16]. The effect of α -TEA on *P. berghei* ANKA infection was greater than that of α -TOS. In our study, approximately 90% of the infected mice treated with of 1.5% α -TEA survived throughout the experimental period (Figure 1A). As α -TEA is not hydrolyzed and has good stability [19, 20], it may be a candidate anti-malarial drug. In the pharmacokinetic experiment, in which a 0.25% α -TEA-mixed diet was

fed to C57BL/6J mice for three weeks, the average food intake of the mice was 3.6 g/head/day. The daily food intake was comparable to that of mice fed a normal chow diet. Based on this value, the mean α -TEA intake was 9 mg/head/day. After a single oral dose of α -TEA (10 mg/head) administered to mice, the elimination half-life ($T_{1/2}$) in plasma was 61.3 h; the maximum concentration (C_{max}) was 25.7 μ g/mL and the area under the plasma concentration-time curve (AUC) was 1,780 μ g·h/mL. Furthermore, in mice for which 10 mg/day α -TEA was orally administered for three days, the plasma α -TEA concentration was higher than 10 μ g/mL for six days after the start of treatment [18].

To investigate if the antiprotozoal effect of α -TEA was the result of modifying the host physiology or a direct impact on the protozoan, the effect of α -TEA on the *in vitro* growth of *P. falciparum* was examined. The resulting IC_{50} values against 3D7 ($13.89 \pm 1.16 \mu$ M) and K1 ($15.51 \pm 0.56 \mu$ M) strains' infection were close to those previously reported for plasma α -TEA concentration (10 μ g/ml, which is equal to 20.46 μ M) on the mice orally administered with 10 mg/day for 3 days [18], indicating that α -TEA was affecting the parasite proliferation in mice. Notably, from the HFF cytotoxicity data, an *in vitro* α -TEA concentration below 25 μ M (Table 2) was considered to be safe, showing a 2.15% hemolysis rate, as recommended by the American Society for Clinical Pathology [32].

Collectively, our results indicate that the antimalarial effect of α -TEA was caused by its direct impact on parasites (Table 1 and Figure 3). Parasites may consume α -TEA from plasma and erythrocyte membranes. Similarly, in preliminary experiments, the *in vitro* proliferation of trypanosomes after α -TEA treatment was significantly inhibited compared with that after control treatment (no α -TEA), suggesting that α -TEA has a direct effect on trypanosomes. In addition, α -TEA induces the production of ROS, such as hydroxyl radicals and peroxynitrite, in protozoans [33]. Oxidative stress may inhibit parasite growth. We hypothesize that parasites exposed to α -TEA accumulate ROS by interference of this molecule with the mitochondrial redox chain and by stimulation of the intrinsic apoptotic pathway. Endothelial cells lacking mitochondrial DNA are resistant to α -TEA, both in the accumulation of ROS, induction of apoptosis, and maintenance of their angiogenic potential [34]. Furthermore, α -TEA has been shown to be a potent anti-cancer agent with a safe toxicity profile in mice [35] and dogs [36].

Conclusion

This study showed that α -TEA is effective against murine and human malaria *in vivo* and *in vitro*, respectively. Although α -TEA alone has a sufficient antimalarial effect, future research could focus on the combined effect of α -TEA and already existing drugs. The prophylactic antimalarial activity of premedication with α -TEA may also be an interesting perspective in the future.

Declarations

Consent for publication: Not applicable.

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Conflicts of interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics approval: The animals used in this study were treated and cared for based on the Guiding Principles for the Care and Use of Research Animals established by Obihiro University of Agriculture and Veterinary Medicine. All animal experimental protocols were approved by the Institutional Animal Ethics Committee, Obihiro University of Agriculture and Veterinary Medicine.

Availability of data and material: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions: The research was designed by H.S., A.K., Y.N., and S.K. Laboratory experiments were performed by N.A. and A.K. The manuscript was written by H.S., T.T., Y.N. and R.U-S. All the authors have read and agreed to the published version of the manuscript.

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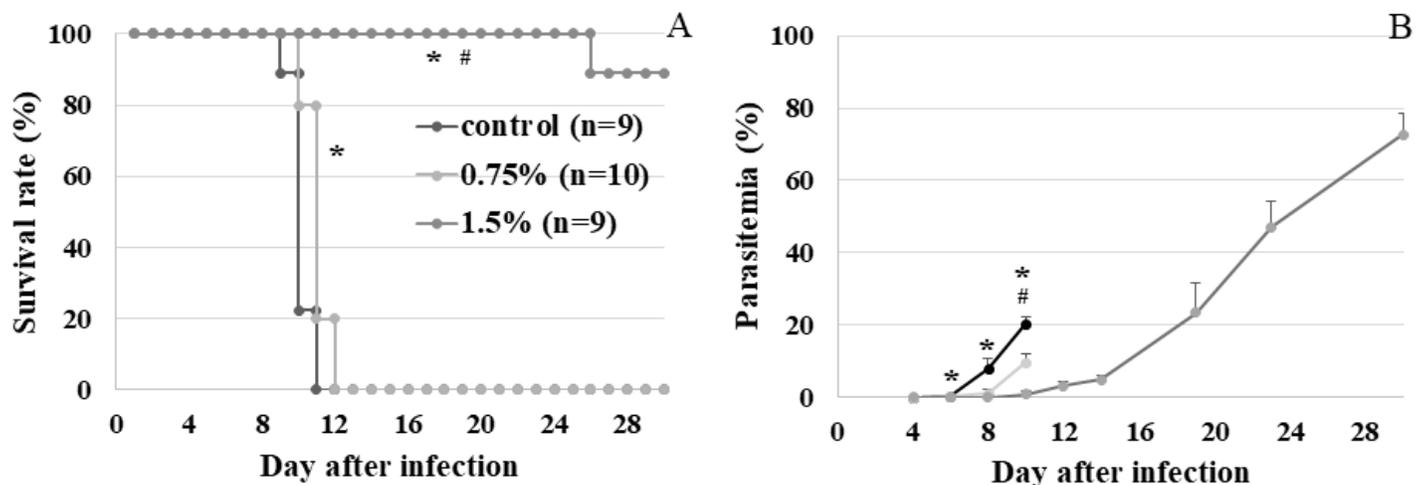
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Figures



vs control * $p < 0.05$
vs 0.75% # $p < 0.05$

Fig. 1

Figure 1

Effect of α -TEA mixed with diet on mice survival (A) and parasitemia (B) following *P. berghei* ANKA infection. A concentration of 0.75 % and 1.5 % (w/w) α -TEA was mixed with diet and fed to C57BL/6J mice for 14 days after infection with 4×10^4 *P. berghei* ANKA infected red blood cells (RBCs.) Error bars indicate standard error. An * indicates $P < 0.05$ on 1.5 % (w/w) α -TEA vs untreated group; A # indicates $P < 0.05$ on 1.5 % (w/w) vs 0.75 % (v/v) α -TEA group.

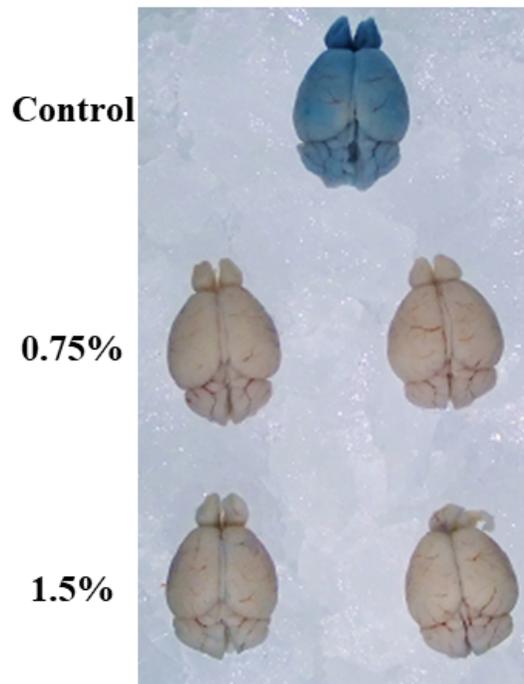


Fig. 2

Figure 2

Permeability of blood–brain barrier (BBB) in mice infected with *P. berghei* ANKA assessed by Evans blue staining. Evans blue solution was injected to infected mice through tail vein on day 7 following infection. After 2 h following injection, mice were sacrificed and dissected. Brains were taken from untreated mice infected with *P. berghei* ANKA (control; upper panel) and from 0.75 % and 1.5 % (w/w) α -TEA-treated mice infected with *P. berghei* ANKA (middle and lower panels, respectively).

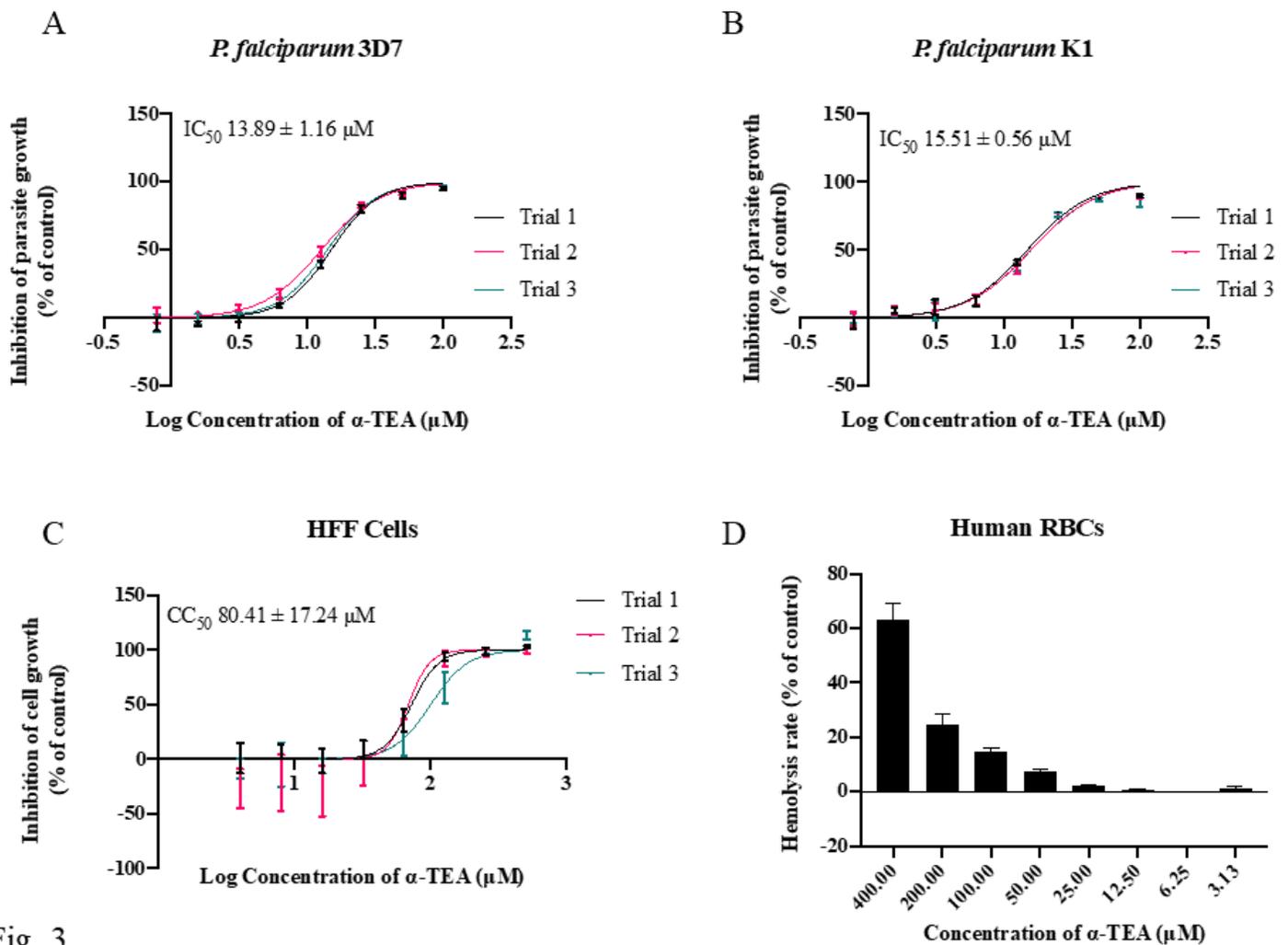


Fig. 3

Figure 3

In vitro effect of different concentrations of α-TEA against *Plasmodium falciparum* 3D7 (A), *P. falciparum* K1 (B), and human foreskin fibroblast (HFF) cells (C) at 72 h culture. In each graph, three curves are represented, indicating three independent trials. The bar graph shows the representative hemolysis rate of human red blood cells (RBCs) (D). Each data point (A-C) is the mean of four technical replicates, and error bars indicate the standard deviations.