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RESEARCH ARTICLE

In vivo anti-plasmodial activity of alpha-onocerin and artesunate combination against *Plasmodium berghei* infected mice

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Abstract

Alpha-onocerin is a triterpenoid derived from *Huperzia phlegmaria* which is used in ethnomedicine for the treatment of fever, headaches, pains and malaria. This study was conducted to evaluate the safety, antipyretic and anti-plasmodial activity of alpha-onocerin and artesunate (ART) co-administration in ICR mice for use in traditional medicine.

The anti-plasmodial effects of alpha-onocerin (10, 30, 100, 300 mg/kg) and ART (1, 2, 4, 8, 16 mg/kg) were assessed in *P. berghei*-infected mice. Alpha-onocerin /ART were administered with a fixed dose combination of their median effective doses (ED_{50s}) to determine the experimental ED_{50} (Z_{exp}). An isobologram was developed to identify the nature of the interaction by comparing Z_{exp} with the theoretical ED_{50} (Z_{add}).

Alpha-onocerin (300 mg/kg) showed a similar chemosuppression (93.51 ± 2.15%) to ART (2 mg/kg, i.p.) of 97.02 ± 0.27% in the 4-day suppressive test as well as in the prophylactic test with chemosuppression at 54.94% and 69.76% for alpha-onocerin (300 mg/kg) and artesunate (2 mg/kg, i.p.) respectively. All doses of alpha-onocerin significantly ($p < 0.05$) reduced pyrexia in 1 h and 2 h after their administration in the baker's yeast test. ED_{50}s for ART and alpha-onocerin were 1.33 ± 0.11 and 13.64 ± 0.22 mg/kg, respectively. The Z_{add}, Z_{exp} and interaction index for alpha-onocerin /ART co-administration were 7.49 ± 3.46, 1.61 ± 0.78 and 0.22 respectively. The Z_{exp} for alpha-onocerin /ART laid below the additive isobole indicating significant ($p < 0.001$) synergistic activity with ART.

Alpha-onocerin showed analgesic effects, antipyretic and synergistic anti-plasmodial effects in *P. berghei*-infected mice.

Keywords Anti-plasmodial · Alpha-onocerin · Combination therapy · Antimalarial · *Plasmodium berghei*

Abbreviations

AUC	Area under curve
ART	Artesunate
CNS	Central nervous system
MST	Mean survival time
NSAIDS	Non-Steroidal Anti-inflammatory Drugs
ONO	alpha-onocerin
PCV	Packed cell volume
P. berghei	Plasmodium berghei
P. falciparum	Plasmodium falciparum

Introduction

Malaria is among the most common infectious diseases and continues to be prevalent in various parts of the world. Despite a decrease in the overall global rates of malaria, Africa continues to face a significant challenge with the

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disease, with a 93% prevalence rate in 2017 and approximately 395,000 deaths reported in 2015 (Nkumama et al. 2017; World Health Organization 2018; World Health Organization 2019). *Plasmodium falciparum* is the parasite causing most of the malaria cases. However, there are other species, such as *Plasmodium malariae*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium knowlesi*, that are attributable to malaria in humans (World Health Organization 2019).

Malaria is caused by strains of Plasmodium, and it exhibits signs and symptoms like; pain in the abdomen and muscles, chills, fatigue, fever, night sweats, shivering, diarrhoea, nausea, vomiting, headaches, mental confusion and pale skin (Caraballo and King 2014). Malaria is capable of causing long-term underdiagnosed and under-reported cardiovascular complications such as myocarditis, endocarditis, and ischemic heart disease. These discreet cardiac pathologies could result in valve failure and ultimately heart failure, requiring valve repair or replacement with annuloplasty (Sidiki et al. 2020, 2022).

Population dynamics and increasing resistance are challenges to controlling malaria. However, the World Organization believes that malaria can be eliminated with at least a 75% efficacious vaccine (World Health Organization 2019). Despite advancements in technology, plant constituents provide important compounds for drug development. In malaria pharmacotherapy, a wide variety of antimalaria drugs have been developed since the inception of cinchona alkaloids in the 1800's to modern artemisinin-based combined therapies (ACTs) (Rawe and McDonnell 2020).

Over the past decades, the control and treatment of malaria have faced obstacles due to the development and recurrence of *P. falciparum* resistance to antimalarial medications (Hanboonkunupakarn and White 2016). Several studies have been conducted aiming to identify novel antimalarial drugs to improve treatment and prevent parasite resistance to treatment. Plant-derived products have gained attention as promising candidates for new antimalarial drugs (Osei et al. 2021; Zeleke et al. 2017). For instance, quinine, which comes from the bark of *Cinchona rubra*, and artemisinins, from *Artemisia annua*, are very potent against malaria parasites and are now important parts of many malaria treatments (Andrade-Neto et al. 2003; White 2008). One such plant product is alpha-onocerin.

Alpha-onocerin is a triterpenoid obtained from *Huperzia phlegmaria*, *Lycopodium clavatum* and *Ononis spinosa L.* among other plants. *Huperzia phlegmaria* is used as a decoction applied externally to wounds and ulcers. Pulverized leaves of *Huperzia phlegmaria* are mixed with plantain and milk in small doses to treat diarrhea and dysentery (Al-Snafi 2020). Furthermore, the whole plant is employed as an insecticide to drive away mosquitoes and other harmful insects. The roots and aerial parts are used in the treatment of malaria, inflammation, bacterial and urinary tract infections (Al-Snafi 2020; Spiegler et al. 2020). *Huperzia phlegmaria* has the ability to cure headaches, inflamed lips and to induce vomiting after food poisoning or acute stomach pain (Al-Snafi 2020; Chen et al. 2020). This study aims at investigating the antipyretic activity, safety and efficacy of alpha-onocerin in the treatment of *Plasmodium berghei* Malaria in ICR mice.

Materials and methods

Preparation of plant material and compound

Roots of *Ononis spinosa L.* were purchased from Caesar and Loretz Hilden, Germany. The powdered root material (250 g) was extracted in a Soxhelt apparatus for 4 h with 2 L of dichloromethane. The dichloromethane extract of 10.0 g was dissolved in petroleum ether, and activated charcoal was added and filtered. The filtrate yielded crystals after evaporation. Approximately 5 mg of crystals were obtained and the identification of alpha-onocerin in these crystals was achieved by GC-MS analysis.

Chemicals and drugs

Sodium chloride (0.9%) manufactured by Atlantic Life Science, artesunate (30 mg Combi Pack) manufactured by India Bharat Parental Ltd., aspirin (250 mg), indomethacin (500 mg), paracetamol (500 mg), and methanol (1 L) were obtained from Ernest Chemist Limited, Santasi Roundabout, Kumasi. Immersion oils (500 ml), microscope slides (frosted), vacuum tubes (1–5 ml), Syringes (1–5 ml), acetic acid (100 ml) and 10% Giemsa stain were obtained from Diacon Medical Laboratory Diagnostics, Bantama-Kumasi.

Experimental animals

ICR mice (18–29 g) of both sexes were used for the experiment. These animals were obtained from the Animal House of the Department of Pharmacology, KNUST. The animals were allowed to acclimatize for a week before every experiment and were given free access to feed and water *ad libitum*. All experimental animals were housed in aluminium laboratory cages ($34 \times 47 \times 18$ cm) with wood shaving as bedding and maintained under standard laboratory conditions of 12 h light/dark cycles, a mean temperature of 22 ± 2 °C and 41 ± 4% humidity. All procedures and techniques in the care of these animals were in line with National Institute of Health Guidelines for the Care and Use of Laboratory Animals (1985).

Rodent parasite

Fresh frozen parasitized erythrocytes containing *Plasmodium berghei* (ANKA) strains were purchased from the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon and kept in the research laboratory of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana.

Acute toxicity

Twenty (20) ICR mice (18–25 g) were randomized into four groups ($n=5$). One group served as control and received 1 ml of normal saline and the remaining three groups received 30, 100, and 300 mg/kg of alpha-onocerin. The mice were placed in their respective motor activity cages. The motor activities of these mice were monitored and scored at 15-, 30-, 50- and 90-min intervals except for the analgesic activities. The analgesic effects were also monitored using the tail pinch method. Animals were kept and monitored for signs of toxicity after 24 h (Baah et al. 2020).

Four-day suppressive test

The in vivo antimalarial activity of alpha-onocerin was assessed using the 4-day suppressive test as described previously (Belay et al. 2018). Thirty (30) ICR mice of both sexes (18–29 g) were divided into six groups ($n=5$) and injected intraperitoneally (i.p.) with 0.2 ml of saline suspension of parasitized erythrocytes. Three hours after inoculation with parasitized erythrocytes, the mice in their groups were treated as follows:

- Group I: received 1 ml of 0.9% sodium chloride solution (negative control, i.p.) for 4 days,
- Group II: received artesunate (2 mg/kg, i.p.) for 4 days,
- Group III: received 10 mg/kg of alpha-onocerin (i.p.) for 4 days,
- Group IV received 30 mg/kg of alpha-onocerin (i.p.) for 4 days,
- Group V: received 100 mg/kg of alpha-onocerin (i.p.) for 4 days and.
- Group VI: received 300 mg/kg of alpha-onocerin (i.p.) for 4 days.

On the fifth day, 24 h after the last doses were administered, thin blood smears were made from the tail of each mouse, fixed with methanol and stained with 10% Giemsa stain. The parasitemia level was determined by counting the number of parasitized erythrocytes out of 100 erythrocytes in 8 random fields of microscopic view.

Anti-plasmodial curative test

This test evaluated the schizontocidal activity of alpha-onocerin and artesunate (as a reference drug) in an established malaria infection. The test procedure was carried out according to the methods of Okokon et al. (2017). A volume of 0.2 ml of parasitized erythrocytes, which contained 1×10^7 cell/mm^3 of *Plasmodium berghei (ANKA)* strains, were intraperitoneally infected into thirty (30) ICR mice with weights between 18 g and 25 g on the first day. Three days infecting with *P. berghei* parasites, the mice were randomly divided into six groups ($n=5$) as follows:

- Group I: received 10 ml/kg (i.p.) of phosphate buffered normal saline (negative control) once daily for 7 days,
- Group II: received 5 mg/kg (i.p.) artesunate (positive control) for 7 days,
- Group III: received 10 mg/kg of alpha-onocerin (i.p.) for 7 days,
- Group IV received 30 mg/kg of alpha-onocerin (i.p.) for 7 days,
- Group V: received 100 mg/kg of alpha-onocerin (i.p.) for 7 days and.
- Group VI: received 300 mg/kg of alpha-onocerin (i.p.) for 7 days.

Giemsa-stained thin smears were prepared using the tail blood collected on microscopic slides for analysis. These blood samples were collected each day of treatment in order to monitor the parasitemia levels (Okokon et al. 2017).

Prophylactic anti-plasmodial test

The repository activity of the compound (alpha-onocerin) and artesunate was assessed using the methods described previously (Belay et al. 2018; Okokon et al. 2017). Thirty (30) ICR mice weighing 18–25 g were randomly divided into six groups ($n=5$) and treated as follows:

- Group I: received 10 ml/kg (i.p.) of phosphate buffered normal saline (negative control) once daily for 3 days,
- Group II: received 5 mg/kg (i.p.) artesunate (positive control) for 3 days,
- Group III: received 10 mg/kg of alpha-onocerin (i.p.) for 3 days,
- Group IV received 30 mg/kg of alpha-onocerin (i.p.) for 3 days,
- Group V: received 100 mg/kg of alpha-onocerin (i.p.) for 3 days and.
- Group VI: received 300 mg/kg of alpha-onocerin (i.p.) for 3 days.

On the fourth day, the mice were all inoculated with 0.2 ml of parasitized RBCs which contained 1×10^7 cells/mm^3 of *Plasmodium berghei (ANKA)* strains. The parasitemia level of each mouse was assessed by taking a tail blood smear on a microscope slide. Thin films of blood smears were prepared and stained with 10% Giemsa. The slides were prepared 72 h post-*Plasmodium berghei* infection and viewed under the microscope. Parasitemia was evaluated as follows, using the equation:

$$\%Parasitemia = \frac{Number\ of\ Parasitized\ RBC's}{Total\ number\ of\ RBC's\ counted}\ x\ 100$$

Average chemosuppression was calculated as:

$$\%Mean\ parasitemia = \left[\frac{A-B}{A}\right]\ x\ 100,$$

Where A is the average percentage parasitemia in the negative control and B is the average percentage parasites.

Packed cell volume and body weight measurements

As described previously (Osei et al. 2021) the packed cell volume (PCV) was measured to predict the effectiveness of the test compound in preventing hemolysis resulting from increased parasitemia. Heparinized capillary tubes were used to collect blood from the tail of each mouse. The capillary tubes were filled with blood up to 3/4th of their volume and sealed at the dry end with sealing clay. The tubes were then placed in a micro-hematocrit centrifuge with the sealed end outwards and centrifuged at 12,000 rpm for 5 min. The tubes were then taken out of the centrifuge and PCV was determined using a standard micro-hematocrit reader. Measurement of PCV was carried out before inoculating the parasite and after treatment. PCV which is a measure of the proportion of RBCs to plasma was calculated using the following relationship:

$$PCV = \frac{Volume\ of\ erythrocytes\ in\ a\ given\ volume\ of\ blood}{Total\ blood\ volume}$$

The body temperature of each mouse was determined before infection on day 0 and after treatment on day 4 (in the 4-day suppressive test), using a digital thermometer to assess changes in temperature due to the infection. The body weights of mice were also taken before the start of each experiment and at the end of some experiments, as described. The weights were taken using a top-pan balance (Baah et al. 2020).

10 Mean Survival Time

In both suppressive and curative assay models, mortality was monitored and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups for 30 days (Fentahun et al. 2017). The mean survival time (MST) for each group was then calculated as follows:

$$MST = \frac{sum\ of\ survival\ time\ for\ all\ mice\ in\ a\ group\ (in\ days)}{total\ numbers\ of\ mice\ in\ that\ group}$$

In vivo anti-plasmodial interaction assay

The ED_{50} of both artesunate and alpha-onocerin was estimated using the model described previously (Tallarida 2011). Briefly, 0.2 ml of prepared parasitized erythrocytes were inoculated into 50 mice. These 50 mice were randomly assigned to one of 10 groups ($n = 5$). These groups were the negative control group (normal saline), the positive control groups (1, 2, 4, 8, 16 mg/kg of artesunate) and the test groups (10, 30, 100 and 300 of alpha-onocerin). After 72 h post-parasite inoculation, all 50 mice were given their various drug doses as described above for five consecutive days. On each day of treatment, blood smears were collected and monitored for parasitemia levels under the microscope. To obtain the combination potency of co-administered alpha-onocerin and artesunate drug samples, the two drugs were assayed for their anti-malarial activity with methods (Osei et al. 2021). The ED_{50}s of both alpha-onocerin and artesunate were combined in fixed ratios of 1:1, 1/2: 1/2, 1/4: 1/4, 1/8: 1/8 and 1/16: 1/16. The ED_{50}s from the combination fractions (Z_{exp}) were determined. Furthermore, the type of interaction existing between the co-administered substances was determined by drawing an isobologram consisting of alpha-onocerin (ED_{50}s) on the x-axis and artesunate (ED_{50}s) on the y-axis. Thus, the Z_{exp} was plotted and compared to the Z_{add} to determine the kind of interaction between artesunate and alpha-onocerin.

Antipyretic effect of alpha-onocerin

The antipyretic effect of alpha-onocerin was evaluated using procedures as described by Baah et al. (2020). Thirty (30) mice were randomly assigned into six groups ($n = 5$). The mice were denied feed but allowed free access to water. The baseline rectal temperatures were taken before the administration of yeast solution (20% w/v) at 10 mg/kg *i.p*. The anal temperatures of each animal in their respective groups were measured 3 h post-pyrexia induction and mice showing a rise in temperature at least 1 °F (0.6 °C) above the baseline were used for the study. The animals were then kept in six

groups ($n=5$) and treated with four doses of alpha-onocerin (10, 30, 100 and 300), paracetamol (200 mg/kg), or normal saline (10 ml/kg) orally. Rectal temperatures were recorded every hour for up to 3 h after the various drug administrations (Baah et al. 2020).

Statistical analysis

The data generated were analyzed using GraphPad Prism 8.4.2, and the results were expressed as mean ± standard error of mean (SEM). Each sample was run in triplicate. An analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used to compare treated groups with the control group. Values were considered significant at $p<0.05$. A two-way ANOVA, followed by Bonferroni's multiple comparison tests for suppressive and curative tests, was employed. An isobologram, which consists of the ED_{50} of alpha-onocerin on the x-axis and the ED_{50} of artesunate on the y-axis, joined with a line of additivity, was constructed. The ED_{50}s of the drugs were determined using linear regression analysis of the log dose-response curves. A t-test comparison of Z_{exp} to the theoretical additive ED_{50}, i.e., Z_{add} was computed. The Z_{add} was computed with the formula:

$$Z_{add} = (f)\, ED50\ of\ ART + (1-f)\ \ ED50\ of\ alpha-onocerin$$

Where *f* is the fraction of each component in the mixture while *Var* (variance of Z_{add}) was also computed as: Var (Z_{add}) = *f2 (VarED50 of* alpha-onocerin*) + (1-f) VarED50 of ART)*.

Results

Acute toxicity testing

After 15 min of administering alpha-onocerin, all the mice in the three groups (30, 100 and 300 mg/kg) showed crouching, irritability, tremors and piloerection as well as a decrease in activity and eyelid ptosis, which was absent in the control group. Crouching was again observed in all three groups, however, tremors, eyelid ptosis and piloerection, which were observed after 15 min, were absent after 30 min. There was a more pronounced irritability and decrease in activity after 30 min in the mice that received 100 and 300 mg/kg. Conversely, analgesia was observed in all the treatment groups after 15 min of administering alpha-onocerin to the mice, which was absent in the control group (Table 1).

Four-day suppressive test

The percentage suppression analysis of alpha-onocerin showed a significant ($p<0.001$) decrease in parasitemia at all dose levels (10–300 mg/kg) compared to the control. No significant change in parasitemia levels was seen with treatment with alpha-onocerin (100 and 300 mg/kg) as compared to artesunate control. Longer mean survival times (MST) of 20–24 days were recorded for higher doses of alpha-onocerin (100–300 mg/kg) which was similar to 25 days for ART which is the positive control (Table 2).

Anti-plasmodial curative test

ICR mice were treated with 10, 30, 100 and 300 mg/kg of alpha-onocerin and 5 mg/kg of artesunate (positive control). After the 3rd and 7th days of treatment, the positive control (artesunate 5 mg/kg) demonstrated a percentage suppression of 53.8 ± 0.5% and 64.4 ± 3.1%, respectively. Alpha-onocerin also demonstrated a significant decrease in parasitemia at all dose levels. The highest dose estimated on day 7 was 300 mg/kg with chemosuppression of 93.83 ± 1.22%, which was significantly higher ($p<0.01$) compared to the ART positive control (Table 3).

Prophylactic anti-plasmodial test

Similarly, 2 mg/kg (i.p.) of artesunate recorded the highest chemosuppression compared to the negative control in the prophylactic test. At 300 mg/kg of alpha-onocerin, a chemosuppression of 54.94% was recorded, which was greater than 10, 30 and 100 mg/kg of the same test drug. Prophylactic activity in both 2 mg/kg (artesunate) and alpha-onocerin (10–300 mg/kg), parasitemia was significantly ($p<0.001$) decreased compared to the negative control (Table 4).

Packed cell volume and body weight measurements

Alpha-onocerin prevented the reduction of body weight on day 4, as observed in the negative control (32% weight loss). A dose-dependent prevention of body weight loss was seen in the alpha-onocerin treatment groups (Table 5). The negative control group showed a significant loss ($p<0.05$) of PCV on day 4 compared to the positive control. Furthermore, in treated groups, no significant change in PCV was recorded (Table 5).

Mean survival time

The survival times of all animals were assessed over a period of 30 days, as shown in Fig. 1. Non-treated mice died on the 7th to the 9th day after infection, and the mean survival was

Table 1 Acute toxicity screening of alpha onocerin carried out after administering mice with different doses (0–300 mg/kg) and observing them different time intervals for 90 min

Observation	Control				30 mg/kg				100 mg/kg				300 mg/kg			
	15	30	50	90	15	30	50	90	15	30	50	90	15	30	50	90
Crouch					+	+	+	+	+	+	+	+	+	+	+	+
Tremors					+				+				+			
Activity Increase								+				+				+
Activity Decrease					+	+	+		++	++	++		+++	+++	+++	
Irritability					+	+	++	+	+	++	++	++	+	+++	+++	++
Increased Respiration					+				+				+			
Eyelid Ptosis					+				++	++			+++	+++		
Piloerection					+				+				+			
Analgesia						+	+	+		+	+	+		+	+	+
Fixed Posture																
Ataxia																
Mydriasis																
Miosis																
Lacrimation																
Salivation																
Vasodilitation																
Righting Reflex Loss																
Corneal Reflex Loss																
Tail Erection																
Diarrhoea																
Skin Colour																

Control is 1mL normal saline, (-) absence of effect, (+) presence of effect, (++) fairly pronounced effect and (+++) highly pronounced effect

Table 2 Four-day suppressive activity of alpha-onocerin and Artesunate (2 mg/kg) on *P. berghei*-infected mice

Treatments	% Suppressions	Mean survival times (days)
Control (NS)	4.25 ± 0.2	12.1 ± 1.10
ART (2 mg/kg)	97.2 ± 0.1***	25.8 ± 0.1
alpha-onocerin (10 mg/kg)	65.6 ± 0.1***	10.8 ± 1.8
alpha-onocerin (30 mg/kg)	72.10 ± 1.2***	15.8 ± 2.2
alpha-onocerin (100 mg/kg)	84.9 ± 2.3***	24.0 ± 0.1
alpha-onocerin (300 mg/kg)	93.5 ± 2.5***	20.2 ± 2.1

Values are expressed as means ± SEM ($n = 5$), ***$p < 0.001$ as compared to the normal saline control compared to the control (normal saline) by Tukey's multiple comparison test

Table 3 Percentage suppression of *P. berghei* by four dose points of alpha-onocerin and Artesunate (5 mg/kg) from day 3 to day 7

Treatments	Day 3 (%)	Day 7 (%)
ART (5 mg/kg)	53.8 ± 0.5	64.4 ± 3.1
alpha-onocerin (10 mg/kg)	27.9 ± 2.3	42.8 ± 2.2
alpha-onocerin (30 mg/kg)	39.5 ± 1.2	74.7 ± 1.4
alpha-onocerin (100 mg/kg)	49.1 ± 3.4	86.6 ± 3.9
alpha-onocerin (300 mg/kg)	51.2 ± 0.5	93.8 ± 1.2**

Values are expressed as means ± SEM ($n = 5$), **$p < 0.01$ compared to the ART control by Bonferroni's multiple comparison test

Table 4 Prophylactic activity of alpha-onocerin against *Plasmodium berghei* infection in mice

Treatments	% Suppressions	% Parasitemia
Control	-	0.50 ± 0.25
ART (2 mg/kg)	69.76 ± 0.62	12.25 ± 0.25***
alpha-onocerin (10 mg/kg)	36.73 ± 0.93	25.63 ± 0.38***
alpha-onocerin (30 mg/kg)	49.03 ± 0.31	20.63 ± 0.13***
alpha-onocerin (100 mg/kg)	39.82 ± 0.93	24.38 ± 0.38***
alpha-onocerin (300 mg/kg)	54.94 ± 2.47	18.25 ± 1.00***

Values are expressed as means ± SEM ($n = 5$), ***$p < 0.001$ as compared to the negative control by ANOVA followed by Tukey's multiple comparison test. ART is artesunate

8.60 days. Mice treated with 2 mg/kg, artesunate died on the 22nd to the 30th day after treatment and had an average survival time of 25.80 days. Alpha-onocerin-treated groups (10, 30, 100 and 300 mg/kg) remained alive for 10.8, 15.8, 24.0 and 20.2 days respectively. All doses of alpha-onocerin significantly enhanced ($p = 0.0022$) the survival time for 30 days (Fig. 1).

In vivo anti-plasmodial interaction assay

Alpha-onocerin produced a significant dose-dependent reduction in parasitemia levels, similar to the ART-treated group (positive control). The potencies of alpha-onocerin and ART were 13.64 ± 0.22 mg/kg and 1.33 mg/kg, respectively.

The combination of alpha-onocerin and ART produced a significant reduction in parasitemia from day 1 to day 5. Alpha-onocerin and ART combinations at all dose levels produced high percentage suppressions compared to alpha-onocerin alone as monotherapy. The lowest dose ratio combination (1/16:1/16) even showed high chemosuppression on the 4th and 5th days compared to the negative control (Table 6).

The theoretical ED_{50} (Z_{add}) of the alpha-onocerin/ART combination was 7.49 ± 3.46 mg/kg. The experimental ED_{50} (Z_{exp}) of the mixture was 1.61 ± 0.78 mg/kg. The interacting index was extrapolated to be 0.22 (Table 7). The Z_{exp} (open circle) was significantly below the line of additivity as well as the Z_{add} (closed circle) on the isobologram, indicating synergism (Fig. 2).

Antipyretic effect of alpha-onocerin

All doses of alpha-onocerin (10–300 mg/kg) significantly reduced ($p < 0.05$) the baker's yeast-induced fever in mice. Paracetamol (150 mg/kg, i.p.) used as the reference drug caused a greater reduction in the rectal temperatures of the ICR mice at the onset, which was significantly different ($p < 0.001$) compared to the negative control but showed a similar effect with 100 mg/kg and 300 mg/kg of alpha-onocerin (Fig. 3).

Table 5 Effect of alpha-onocerin on the body weights and packed cell volume of *P. berghei* infected mice in a 4-day suppressive test

Treatment	Mean body weight (g)			Mean Packed cell volume (PCV)		
	Day 0	Day 4	% Change	Day 0	Day 4	% Change
Negative control	25.4 ± 1.6	23.3 ± 2.5	-8.27	54.0 ± 1.2	48.0 ± 0.9	-11.1
Artesunate (2 mg/kg)	21.2 ± 1.7	20.3 ± 1.7	-4.25	52.0 ± 3.4	52.0 ± 4.2	0.0
alpha-onocerin (10 mg/kg)	23.8 ± 1.3	22.6 ± 0.9	-5.04	53.0 ± 0.6	50.0 ± 0.3	− 5.7
alpha-onocerin (30 mg/kg)	23.8 ± 1.1	23.3 ± 1.8	-2.10	54.0 ± 2.3	52.0 ± 4.1	− 3.7
alpha-onocerin (100 mg/kg)	20.4 ± 1.5	20.0 ± 1.4	-1.96	52.0 ± 2.6	51.0 ± 2.3	− 1.9
alpha-onocerin (300 mg/kg)	22.2 ± 1.8	21.9 ± 1.6	-1.40	51.0 ± 0.9	50.0 ± 1.5	− 1.9

Data are expressed as mean ± SEM ($n = 5$)

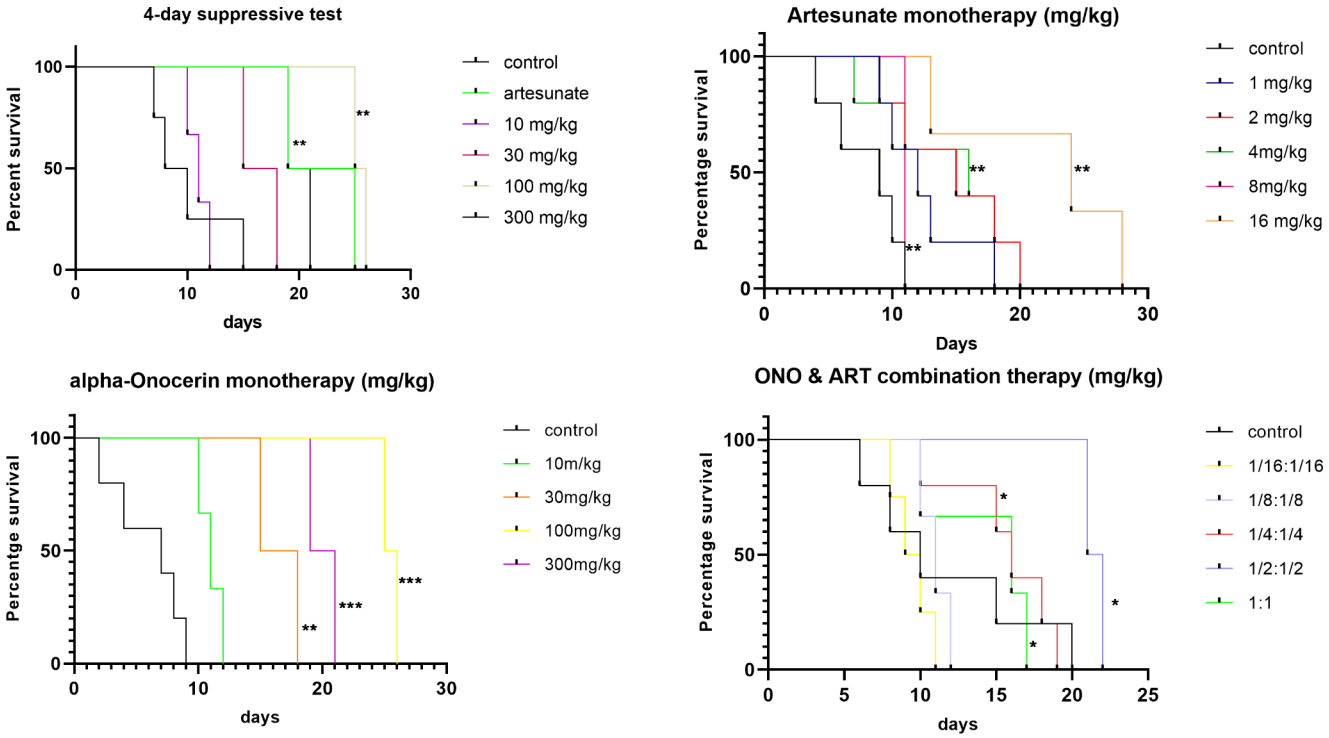

Fig. 1 Kaplan-Mier survival curves comparing the 30 days post treatment of *P. berghei*-infected mice treated with alpha-onocerin/artesunate combination ($n=5$). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ as compared to the negative control by ANOVA followed by Tukey's multiple comparison test. ART is artesunate and ONO is alpha-onocerin

Table 6 Percentage chemosuppressions with different doses of alpha-onocerin and Artesunate combination for 5 days

Treatment (ED_{50} mg/kg)	% Chemosuppression		
	Day 1	Day 2	Day3
Negative control	-	-	-
ART (2 mg/kg)	60.7 ± 0.4	89.7 ± 0.2	96.5 ± 0.8
alpha-onocerin (100 mg/kg)	49.3 ± 3.2	86.6 ± 3.9	69.7 ± 1.2
(alpha-onocerin/ART)/1	82.8 ± 1.2	94.1 ± 1.6	96.2 ± 0.77
(alpha-onocerin/ART)/2	80.3 ± 1.9	93.8 ± 1.2	97.7 ± 0.9
(alpha-onocerin/ART)/4	71.67 ± 1.3	80.15 ± 1.3	97.6 ± 1.5
(alpha-onocerin/ART)/8	63.80 ± 3.4	92.1 ± 6.6	95.6 ± 1.6
(alpha-onocerin/ART)/16	75.84 ± 0.8	83.7 ± 1.8	89.8 ± 4.1

Data are presented as Mean ± SEM ($n=5$). ART is artesunate

Table 7 Theoretical (Z_{add}) and Experimental (Z_{exp}) ED_{50} of alpha-onocerin and Artesunate co-administration in the anti-malarial assay

ED_{50} (alpha-onocerin/ART)	Antimalarial activity
Z_{add} (mg/kg)	7.49 ± 3.46
Z_{exp} (mg/kg)	1.61 ± 0.78
Interaction index	0.22

Data are presented as Mean ± SEM ($n=5$)

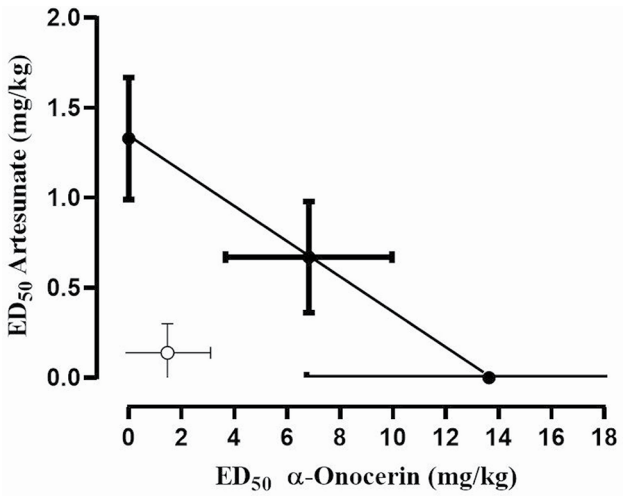

Fig. 2 Isobologram of the coadministration of alpha-onocerin and artesunate. Filled circle shows theoretical $ED_{50} \pm$ SEM, while open circle shows experimental $ED_{50} \pm$ SEM. The line of additivity joins the ED_{50} of alpha-onocerin on the abscissa to that of artesunate on the ordinate

Discussion

There is evidence of the current resistance of *P. falciparum* to the classical antimalaria regimen (Gupta et al. 2019). The problem of parasite resistance to antimalarial agents poses an urgent need for more efficient treatment options. Combination therapy offers a promising alternative to ease the burden of parasite resistance to antimicrobial drugs (Burrows et al. 2017). The mechanism of action of different compounds when they act synergistically prevents the development of parasite resistance. This study examined the effectiveness

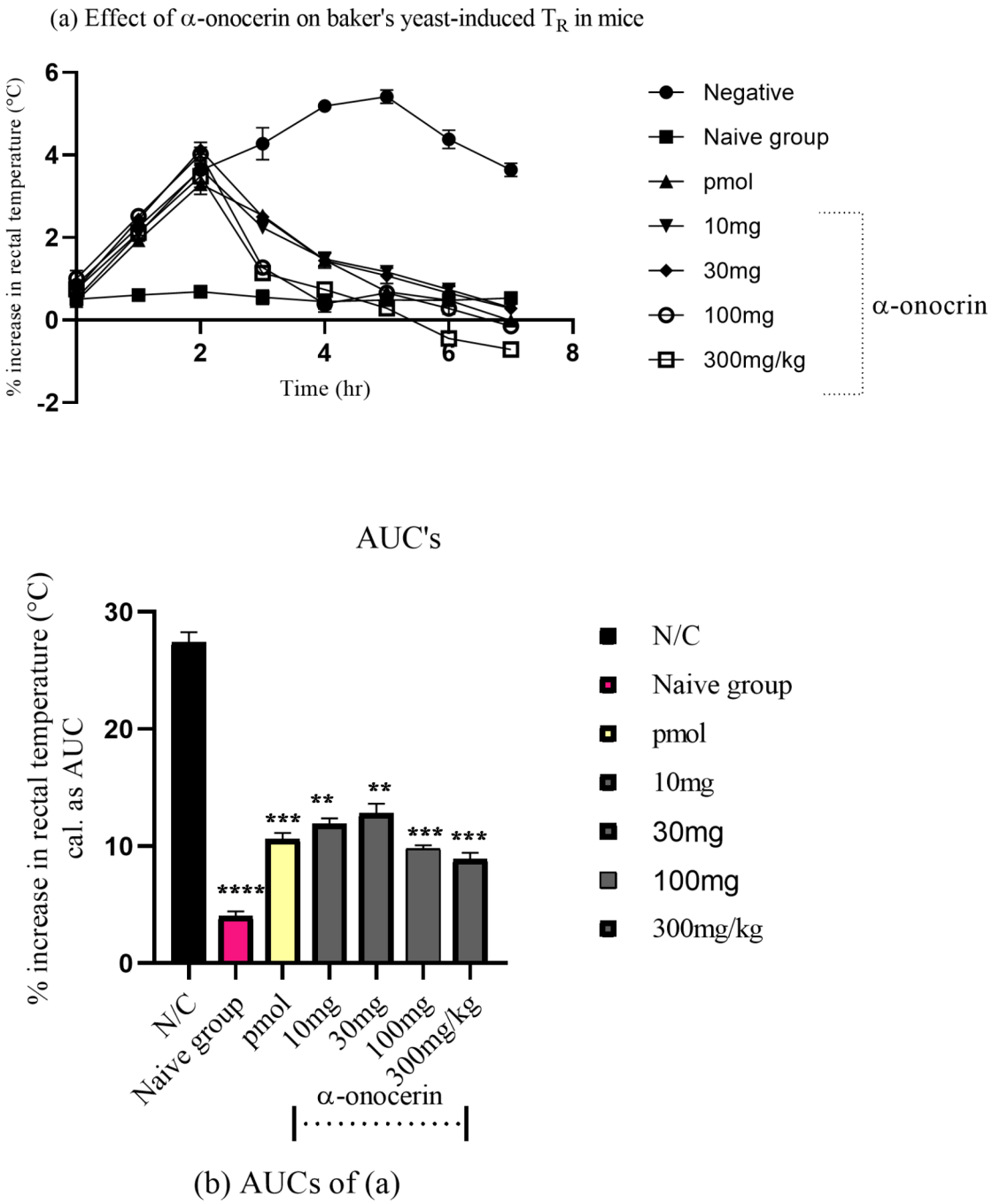

Fig. 3 Antipyretic effect of alpha-onocerin (10–300 mg/kg0 and paracetamol (150 mg/kg) on time-course curve (A) baker's yeast-induced changes in rectal temperatures in mice. Naïve represents animal without yeast treatment and (B) total increase in temperature calculated as area under curve (AUC). Values are expressed as mean ± SEM ($n = 5$), $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ as compared to the negative control by ANOVA followed by Bonferroni's multiple comparison test

and safety of using alpha-onocerin as monotherapy and artesunate/alpha-onocerin as combination therapy.

Alpha-onocerin was derived from the roots of *Ononis spinosa* by Hlasweitz in 1885. *Lycopodium clavatum* species in Africa called *Hurpezia* and leguminous *Ononis spinosa L.* have folkloric uses in the treatment of coughs, constipation, diuresis, malaria, wounds, and eczema, although there is no pharmacologically tested evidence on these effects mentioned. More so, there are no recent reports claiming the synergistic effects of alpha-onocerin used in combination with other antimalarials such as artesunate. Therefore, the tested pharmacological effects of alpha-onocerin in this

research work confirm the folkloric use of the agents in the past in managing symptoms of malaria as well as other diseases. The antimalarial efficacy of alpha-onocerin in this research work declares alpha-onocerin a potent antimalaria monotherapeutic agent as well as an ideal candidate for antimalaria combinational therapies.

In the acute toxicity study, all three groups of mice showed a decrease in activity. Also, the drug showed analgesic activity in all three groups of mice. There was a dose-dependent decrease in the locomotor activities of the mice and eyelid ptosis after the first 15 min. The activity of the animals rose again in the last 40 min. Thus, the decrease in activity lasted only for 50–60 min. This showed that the drug may have some central nervous system (CNS) depressant activity (Diniz et al. 2013). Most CNS depressants cause a decrease in the activity of the brain by increasing the activity of the major inhibitory neurotransmitter in the brain which is gamma-aminobutyric acid (GABA) and acting on its receptors (Della Vecchia et al. 2022). The actual mechanism by which alpha-onocerin caused this CNS depressant effect in the mice was, however, unknown.

The drug started showing analgesic effects in the animals 30 min after administration. The analgesic effect persisted until it was lost during the last 40 min towards the end of the experiment. In effect, the analgesic activity of the drug lasted only for 20–30 min. Modulation of pain occurs through complex physiological pathways. Non-steroidal anti-inflammatory drugs (NSAIDS) act as analgesics by inhibiting the formation of prostaglandins from arachidonic acid via the inhibition of cyclooxygenase I and II, which are enzymes in the arachidonic acid pathway (Van Rensburg and Reuter 2019). Examples of NSAIDS are indomethacin, piroxicam, diclofenac and celecoxib. Other agents, such as opioids, may also mediate pain inhibition through both central and peripheral mechanisms. Opioid analgesics act by binding and activating specific receptor sites in the central and peripheral nervous systems. Once these receptor sites are activated, pain signal transmission is blocked through several mechanisms, producing analgesia. The first line opioid analgesics, such as morphine, fentanyl, hydromorphone, and oxycodone, are μ (mu) receptor agonist opioids because they bind primarily to the μ opioid receptors to produce both wanted (analgesia) and unwanted (adverse) effects (Almeida et al. 2004). The mechanism by which analgesia was produced in the mice after treatment with alpha-onocerin was, however, not clear through this study but may be identified in further studies. From the time the drug was administered to the end of the experiment, all the animals in the three groups showed behaviors of irritability, which may be due to the CNS depression that might have been caused (Almeida et al. 2004).

Alpha-onocerin has been shown to have cholinesterase inhibitory activity and for that reason, it was expected that there would be some cholinergic side effects such as lacrimation, salivation, and diarrhea as a result of the accumulation of acetylcholine at the synapses. However, no cholinergic side effect was seen at any point in the experiment. This may be due to the drug doses being too high or too low to elicit anticholinesterase activity (Hosseinzade et al. 2019).

The four-day parasitemia suppression study demonstrated that treatment of *P. berghei*-infected mice with alpha-onocerin reduced the erythrocytic stage development of the parasite. The suppression was done in a dose-dependent manner. The highest dose of alpha-onocerin (300 mg/kg) had the highest chemosuppression ($93.51 \pm 2.15\%$) compared to artesunate (2 mg/kg, i.p.) of $97.02 \pm 0.27\%$ in the 4-day suppressive test. The suppressive effect observed might be due to the inhibition of targeted pathways or boosting of immune cells in destroying these parasites (Hosseinzade et al. 2019). The mean survival time is important in assessing the antimalarial properties of drugs (Osei et al. 2021). Alpha-onocerin at all doses enhanced the survival time, which is associated with parasite suppression. Plants with antimalaria activity must prevent body weight loss in infected mice owing to increasing parasitemia. Hence, alpha-onocerin prevented a significant reduction in body weight on day 4 compared to day 0 at all dose levels. The malaria infected mice died as a result of a drop in internal body temperature as well as a decreased metabolic rate (Bantie et al. 2014; Cumnock et al. 2018). Thus, drugs with antimalaria activity must prevent rapid falling in body temperature and restore an increment in metabolic rate (Baah et al. 2020; Cumnock et al. 2018). The antipyretic effects of alpha-onocerin may be due to the decrease in concentration of the brain's prostaglandin E2 as well as the action of alpha-onocerin on COX-3. Alpha onocerin may also be contributing to the synthesis of vasopressin and arginine. These substances reduce the vast numbers of proinflammatory mediators released during an infection. Alpha-onocerin at 100 mg/kg doses worked just like paracetamol in preventing drops in temperature. Malaria causes the fall in PCV, which happened after 48 h post-infection (Bantie et al. 2014). *Plasmodium-berghei* infected mice suffered from anemia because of red blood cell destruction either through parasite density or by phagocytic action of the spleen reticuloendothelial cells against abnormal erythrocytes (Wang et al. 2021). It was seen that alpha-onocerin prevented a reduction in PCV at all dose levels in between days 0 and 4. PCV reduction and body weight loss preventive effects of alpha-onocerin were in line with the toxicity profile carried out in healthy, non-infected mice. This confirms that alpha-onocerin suppressed parasitemia levels without affecting the measured parameters. Alpha-onocerin showed comparable

efficacy to artesunate in the prophylactic test. This shows the non-selectivity of alpha-onocerin to intracellular receptors at different stages of the parasite's life cycle. Although it has not been documented how alpha-onocerin exerts prophylactic activity on *P. berghei* infection, it may be due to the prevention of pre-invasion, and active echinocytosis (parasite's mechanism of erythrocytic invasion), as well as the prevention of parasite mediated vesicular structures (EVD's, Jdots, MAHRP-2) that facilitate transport of proteins into the host cell. Artesunate used in this study exhibited prophylactic activity by generating free radicals, which interfere with protein and nucleic acid synthesis during the emergent asexual blood stages of development (Adebayo et al. 2020; Wild et al. 2020). In vivo antiplasmodial activity of a compound can be classified as moderate or good, if the compound exhibited a chemosuppression of 50% or greater at doses 500, 250 and 100 mg/kg (World Health Organization 2019). Alpha-onocerin displayed very good antiplasmodial activity, even at doses below 100 mg/kg.

Conclusions

This study shows that alpha-onocerin derived from *Ononis spinosa* is safe and efficacious in the ICR mice and demonstrates suppressive, curative and prophylactic anti-plasmodial activity. It can also be used in antimalaria combinational therapy to combat parasite resistance. Alpha-onocerin possesses an appreciable antipyretic property at doses tested in the baker's yeast-induced pyrexia in ICR model. The study, therefore, confirms the folkloric use of the plant in treating and ameliorating the signs and symptoms of malaria.

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Authors' Contribution JHKO, KBM, AM and ADF conceived the research idea and designed the experiment; JHKO performed the experiment and analyzed the data together with ADF and AOA. JNAA provided the compound for the study. KBM, ADF and PKTAG supervised the study. JHKO SIA and AM drafted the paper. All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during the study are included in this published article.

Declarations

Ethics approval and consent to participate The activities described here did not include human subjects and all animal experiments were approved by the Faculty of Pharmacy and Pharmaceutical Sciences ethics committee (PHARM/ETR/0421). All animals were gently handled in all experimental procedures per Animal Welfare Regulations (USDA 1985; US Code, 42 USC § 289d) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002).

Consent for publication Not applicable.

Competing of interests There are no competing interests on the side of the authors.

References

Adebayo JO, Tijjani H, Adegunloye AP, Ishola AA, Balogun EA, Malomo SO (2020) Enhancing the antimalarial activity of artesunate. Parasitol Res 119:2749–2764

Al-Snafi AE (2020) The traditional uses, constituents and pharmacological effects of *Ononis spinosa*. IOSR J Pharm 10:53–59

Almeida TF, Roizenblatt S, Tufik S (2004) Afferent pain pathways: a neuroanatomical review. Brain Res 1000:40–56

Andrade-Neto VF, Brandão M, Stehmann JR, Oliveira LA, Krettli AU (2003) Antimalarial activity of Cinchona-like plants used to treat fever and malaria in Brazil. J Ethnopharmacol 87:253–256

Baah MK, Mensah AY, Asante-Kwatia E, Amponsah IK, Forkuo AD, Harley BK, Adjei S (2020) *In vivo* antiplasmodial activity of different solvent extracts of *Myrianthus libericus* stem bark and its constituents in *Plasmodium berghei*-infected mice. Evidence-Based Complementary and Alternative Medicine 2020

Bantie L, Assefa S, Teklehaimanot T, Engidawork E (2014) In vivo antimalarial activity of the crude leaf extract and solvent fractions of Croton macrostachyus Hocsht.(Euphorbiaceae) against Plasmodium berghei in mice. BMC Complement Altern Med 14:1–10

Belay WY, Endale Gurmu A, Wubneh ZB (2018) Antimalarial activity of stem bark of *Periploca linearifolia* during early and established plasmodium infection in mice. Evidence-Based Complementary and Alternative Medicine 2018

Burrows JN, Duparc S, Gutteridge WE, van Hooft R, Kaszubska W, Macintyre F, Mazzuri S, Möhrle JJ, Wells TN (2017) New developments in anti-malarial target candidate and product profiles. Malar J 16:1–29

Caraballo H, King K (2014) Emergency department management of mosquito-borne illness: malaria, dengue, and West Nile virus. Emerg Med Pract 16:1–23 quiz 23

Chen Y, Yang Q, Zhang Y (2020) Lycopodium japonicum: A comprehensive review on its phytochemicals and biological activities. Arab J Chem 13:5438–5450

Cumnock K, Gupta AS, Lissner M, Chevee V, Davis NM, Schneider DS (2018) Host energy source is important for disease tolerance to malaria. Curr Biol 28:1635–1642e3

Della Vecchia A, Arone A, Piccinni A, Mucci F, Marazziti D (2022) GABA system in depression: impact on pathophysiology and psychopharmacology. Curr Med Chem 29:5710–5730

Diniz TC, Araújo CdS, Silva JC, Oliveira-Júnior Rd, Lima-Saraiva Sd, Quintans-Júnior LJ, Nunes XP, Almeida J (2013) Phytochemical screening and central nervous system effects of ethanolic extract of *Annona vepretorum (Annonaceae)* in mice. J Med Plant Res 7:2729–2735

Fentahun S, Makonnen E, Awas T, Giday M (2017) In vivo antimalarial activity of crude extracts and solvent fractions of leaves of *Strychnos mitis* in *Plasmodium berghei* infected mice. BMC Complement Altern Med 17:1–12

Gupta P, Singh L, Singh K (2019) The hybrid antimalarial approach, in: Anonymous, Annual Reports in Medicinal Chemistry. Elsevier, pp. 73–105

Hanboonkunupakarn B, White NJ (2016) The threat of antimalarial drug resistance. Tropical diseases, travel medicine and vaccines 2, 1–5

Hosseinzade A, Sadeghi O, Naghdipour Biregani A, Soukhtehzari S, Brandt GS, Esmaillzadeh A (2019) Immunomodulatory effects of flavonoids: possible induction of T CD4 regulatory cells through suppression of mTOR pathway signaling activity. Front Immunol 10:51

Nkumama IN, O'meara WP, Osier FH (2017) Changes in malaria epidemiology in Africa and new challenges for elimination. Trends Parasitol 33:128–140

Okokon JE, Augustine NB, Mohanakrishnan D (2017) Antimalarial, antiplasmodial and analgesic activities of root extract of *Alchornea laxiflora*. Pharm Biol 55:1022–1031

Osei SA, Biney RP, Obese E, Agbenyeku MA, Attah IY, Ameyaw EO, Boampong JN (2021) Xylopic acid-amodiaquine and xylopic acid-artesunate combinations are effective in managing malaria in *Plasmodium berghei*-infected mice. Malar J 20:1–13

Rawe SL, McDonnell C (2020) The cinchona alkaloids and the aminoquinolines, in: Anonymous, Antimalarial Agents. Elsevier, pp 65–98

Sidiki AI, Faybushevich AG, Lishchuk AN, Koltunov AN, Roshchina EA (2020) The Carpentier-Edwards Classic and Physio Annuloplasty Rings in Repair of Degenerative Mitral Valve Disease: A Retrospective Study. J Saudi Heart Assoc 32(2):224–232

Sidiki AI, Akulova AA, Hussein MH, Al-Ariki MK, Donsov VV, Iluhin MA, Limeshkin AA, Ananko VA (2022) Physio and Physio II rings: beyond the annular physiology. J Cardiovasc Surg (Torino) 63(4):529–535

Spiegler V, Gierlikowska B, Saenger T, Addotey JN, Sendker J, Jose J, Kiss AK, Hensel A (2020) Root extracts from *Ononis spinosa* inhibit IL-8 release via interactions with toll-like receptor 4 and lipopolysaccharide. Front Pharmacol 11:519045

Tallarida RJ (2011) Quantitative methods for assessing drug synergism. Genes cancer 2:1003–1008

Van Rensburg R, Reuter H (2019) An overview of analgesics: NSAIDs, paracetamol, and topical analgesics Part 1. South African Family Practice

Wang H, Li S, Cui Z, Qin T, Shi H, Ma J, Li L, Yu G, Jiang T, Li C (2021) Analysis of spleen histopathology, splenocyte composition and haematological parameters in four strains of mice infected with Plasmodium berghei K173. Malar J 20:1–12

White NJ (2008) Qinghaosu (artemisinin): the price of success. Science 320:330–334

Wild M, Bertzbach LD, Tannig P, Wangen C, Müller R, Herrmann L, Fröhlich T, Tsogoeva SB, Kaufer BB, Marschall M (2020) The trimeric artesunate derivative TF27 exerts strong anti-cytomegaloviral efficacy: Focus on prophylactic efficacy and oral treatment of immunocompetent mice. Antiviral Res 178:104788

World Health Organization (2019) WHO malaria policy advisory committee (MPAC) meeting, October 2019: meeting report. WHO malaria policy advisory committee (MPAC) meeting, October 2019: meeting report

World Health Organization (2018) Update on the E-2020 initiative of 21 malaria-eliminating countries: report and country briefs. World Health Organization

Zeleke G, Kebebe D, Mulisa E, Gashe F (2017) In vivo antimalarial activity of the solvent fractions of fruit rind and root of Carica papaya Linn (Caricaceae) against Plasmodium berghei in mice. Journal of parasitology research 2017