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Original Article

Synergistic Antimalarial Activity of Alpha-Mangostin Chitosan Alginate Nanoparticles with Chloroquine in Mice Infected with *Plasmodium berghei*: The Potential of a New Antimalarial Drug Combination

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What's Known

- Malaria is resistant to the standard drug regimen and chloroquine. Chloroquine has shown re-sensitizing signs in recently *ex vivo* studies. Drug combinations with different mechanisms of action can prevent resistance.
- Alpha-mangostin as antimalaria *in vitro*. It has a different mechanism of action. Alpha-mangostin in alpha-mangostin chitosan alginate nanoparticles (ACANs) has a good antimalarial activity *in vivo*.

What's New

- Combination of ACAN with chloroquine has a good antimalarial activity. ACANs work very synergistically with chloroquine as antimalaria.
- ACAN-chloroquine is a potential candidate for a new antimalarial drug combination to be studied further to support the malaria elimination program.

Abstract

Background: Malaria drug resistance is one of the leading causes of malaria-related morbidity and mortality worldwide. Alpha-mangostin exhibits antimalarial and antioxidant activity *in vitro*. The soluble alpha-mangostin chitosan alginate nanoparticles (ACAN) exhibit proper antimalarial activity *in vivo*. This study aimed to explore the antimalarial activity of the chloroquine-ACAN combination and the interaction between them in various ratios.

Methods: A 4-day suppressive test, according to Peter's test, was conducted using *P. berghei*-inoculated Swiss Webster mice in Bandung, 2024. It was done for six different concentrations (in triplicate) of three kinds of the combinations respectively i.e.: ½ effective dose 50 (ED_{50}) ACAN:½ED_{50} chloroquine (ratio-1, ACAN and chloroquine were used in a weight ratio of 189:1), ¼ ED_{50} ACAN:¾ ED_{50} chloroquine (ratio-2, weight ratio of 63:1), ¾ ED_{50} ACAN:¼ ED_{50} chloroquine (ratio-3, weight ratio of 567:1) to find out growth inhibitory percentage of each concentration. ED_{50} of each combination was determined using probit analysis in IBM SPSS Statistics 27 software. The sum of fractional effective dose 50 ($\sum FED_{50}$) was determined using a specific formula. $\sum FED_{50}$ indicates the kind of interaction: <1, >1, or =1 means synergistic, antagonistic, or additive.

Results: ED_{50} of ratio-1 (ACAN/Chloroquine weight ratio 189/1), ratio-2 (weight ratio 63/1), ratio-3 (weight ratio 567/1) is 9.196, 7.626, 82.13 mg/Kg BW (<100 mg/Kg BW). $\sum FED_{50}$ of ratio-1, ratio-2, and ratio-3 is 0.069, 0.113, and 0.414 (far below 1).

Conclusion: ACAN-chloroquine exhibits good and marked synergistic antimalarial activity, especially in a ratio-1. It offers a glimmer of hope for future research to combat and ultimately eliminate malaria.

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Keywords ● Chloroquine ● Xanthones ● Nanoparticles ● Antimalaria

Introduction

Human malaria is still a problem in several countries because of its severity and the problem of therapy, especially for

malaria falciparum. In many countries of tropical and subtropical regions, it is still a leading cause of disease and death.[1] In 2020, because of malaria, it was reported that 241 million cases with 627,000 deaths occurred globally.[2] P.falciparum resistance against chloroquine was first found in four areas in Southeast Asia, Oceania, and South America in the late 1950s / early 1960s, and then this resistance spread to nearly all over the world where P.falciparum was transmitted. P.falciparum has also developed resistance against other antimalarial drugs such as sulfadoxine/pyrimethamine, mefloquine, halofantrine, and quinine to develop multidrug-resistant parasites.[3] Artemisinin-based combination therapy (ACT) is the treatment of choice because of the parasite's resistance against conventional drugs. Nowadays, signs of parasite resistance, especially partial resistance against the ACT is also starting to develop in several countries, and its impact is a slow response against the drug.[2,4] Parasite resistance against ACT happens against the artemisinins themselves as well as against artemisinin partner drugs with various mechanisms of action.[5] This problem becomes an obstacle to reaching the target for the malaria elimination program. That's why it is important to find new antimalarial drugs. Fortunately, *ex vivo* stable persistence susceptibility of *Plasmodium falciparum* against chloroquine after discontinuation has been reported in Northern Uganda, followed by the same evidence in other endemic areas of this country.[6] Other study in Ghana also indicates the expansion of chloroquine-sensitive *P. falciparum* markers.[7] This evidence might open channels to wisely reuse chloroquine in the future to combat the disease and might become a glimmer of hope.[6] It is wise to prevent further re-resistance of this drug against malaria. In the future, using this drug in combination with other drugs with different mechanisms of action, hopefully, might decrease the progression of this resistance.[8] Chloroquine acts as an antimalarial drug through inhibition of the toxic heme polymerization, and chloroquine can also insert into *Plasmodium* DNA double helix, which inhibits *Plasmodium* DNA replication and transcription to inhibit *Plasmodium* growth and reproduction.[9]

Garcinia mangostana L rind has antioxidant activity,[10] and it has also antimalarial activity *in vitro*.[11] Alpha-mangostin is one of the most abundant xanthones found in *G. mangostana* L rind,[12] and alpha-mangostin, besides, has antioxidant activity,[13] also has strong antimalarial activity and can inhibit cysteine protease enzyme of *P. falciparum*.[14] Xanthone (alpha-mangostin) can also inhibit *P. falciparum* lactate dehydrogenase enzyme involved in the parasite glycolysis process.[15] On the other side, there might have special benefit of using antioxidants as antimalaria because malaria disease itself causes oxidative stress in the host.[16] However, because of the very poor solubility of alpha-mangostin in various safe solvents, it becomes a problem, especially for *in vivo* study, as the low solubility is one of the most important factors that causes low bioavailability, including for oral administration.[17]

Chitosan-eudragit nanoparticles enhance the aqueous solubility of alpha-mangostin, offering a promising approach to improve its oral bioavailability, especially for oral administration.[18] According to Wathoni and others, alpha-mangostin nanoparticles—specifically alpha-mangostin chitosan–alginate nanoparticles (ACAN)—not only exhibit improved water solubility but also show greater potential for enhancing the performance of alpha-mangostin, as well as its physicochemical properties.[19] Moreover, chitosan itself has been studied as an anti-microbial agent by several suspected mechanisms which were not clear. Chitosan also has shown antimalarial activity, but the effect is not linear with the dose.[20] According to our previous study, ACAN also exhibits good *in vivo* antimalarial activity orally in mice with *Plasmodium berghei* malaria.[21]

Berghei malaria in mice caused by *P. berghei* is usually used as a P.falciparum model in humans. This study aimed to explore the antimalarial activity of chloroquine combined with the ACAN using several ratios and determine the interaction between these substances as antimalarial in mice with *P. berghei* malaria.

Materials and Methods

Frozen Anka strain *P. berghei* was obtained from The Eijkman Institute for Molecular Biology, while alpha-mangostin was from The Biopurify Phytochemicals Ltd., Chengdu, China. Male Swiss Webster mice aged 7-8 weeks and 20-25 g body weight were from The School of Life Science and Technology, Bandung Institute of Technology (ITB). Chloroquine diphosphate (98-100% purity), polyethylene glycol, chitosan, alginate, sodium tripolyphosphate (TPP), ethanol 96%, and citric acid were from Sigma Aldrich, United States of America.

Alpha-Mangostin Chitosan Alginate Nanoparticles Production

The ACAN used in this experiment was the same product that had been used in the previous experiment, which had shown spherical shape

with several particle sizes included: 50, 100, and 500 nm according to scanning electron microscopy (SEM) and transmission electron microscopy (TEM) examination. Each of alpha-mangostin, chitosan, and alginate was used in 0.1% solution, while TPP was used in 1%. alginate and TPP were diluted in aquadest while alpha-mangostin was diluted in alcohol 96%, and chitosan in acetic acid 1%. Alpha-mangostin, chitosan, TPP, and alginate were used in 1:10:2:1 volume ratio, mixed consecutively and slowly one by one with stirring for each step, sonicated, and sprayed with pyrolysis.[22]

The chloroquine was diluted in a solution of hydroxy propyl methyl cellulose 0.5% in aquadest and the ACAN was diluted in 0.5% sodium carboxymethyl cellulose solution.

This study was ethically approved by the Ethical Committee of the Faculty of Medicine, Maranatha Christian University, with certificate number: 144/KEP/XI/2022. It was carried out in Bandung in 2024 using a 4-day suppressive test (Peter's Test).[23]

Adaptation Period

Animal care was done ethically.[24] Initially, the mice were adapted for 1 week at 23-24 °C, 60-70% relative humidity, and cycles of 12 hours light/ dark. The mice were given pellets and water *ad libitum* and treated ethically in clean cages, one cage for each treatment group, until the end. Sixty mice were included in the control group.

The Procedure for Obtaining a Donor Mouse as a Source of Parasites

Frozen parasites were thawed and inoculated intraperitoneally and aseptically into the mouse. Parasitemia was examined every day by making thin blood smear from its tail aseptically, fixed it in methanol (Sigma Alrich, USA), and stained with Giemsa (Sigma Aldrich, USA) then parasitized red blood cells (pRBC) among 1000 red blood cells (RBC) were counted under light microscope (Olympus cx-23, Japan) to find out the parasitemia percentage. After the minimal parasitemia of 5-10% was reached, the mouse was terminated ethically using carbon-dioxide (CO2) and cardiac puncture was done to obtain the blood.

The Procedure of the Experiment

Each of the experimental mice was injected intraperitoneally and aseptically with 10^7 pRBC from the donor mouse in 200 µL phosphate buffer saline (PBS) (Biosharp, China). After reaching parasitemia of around 2%, the treatment was started. The treatment consisted of three kinds of ratios of ACAN-chloroquine combination according to each effective dose 50 (ED_{50}): ratio-1 (½ ED_{50} ACAN: ½ ED_{50} chloroquine), ratio-2 (¼ ED_{50} ACAN: ¾ ED_{50} chloroquine), and ratio-3 (¾ ED_{50} ACAN: ¼ ED_{50} chloroquine). The ED_{50} of ACAN is 264.5 mg/Kg BW, which contains 15.87 mg of alpha-mangostin[21] and the ED_{50} of chloroquine is 1.4 mg/Kg BW.[25] We studied three kinds of ratios with the intention of knowing the range in general. Six concentrations were used for each type of treatment (ratio), in triplicate.

Four-day suppressive test *in vivo* was done according to Peter's test for each concentration, briely: in triplicate, the *P. berghei*-infected mice were treated with the drug orally every day for 4 days starting from day 0 (D0) until day 3 (D3) and at day 4 (D4), the parasitemia percentage was counted microscopically from blood smears to determine the percentage of parasite growth and parasite growth inhibition of each concentration comparing to the control *P. berghei*-infected mice without treatment. The formula of percentage of parasite growth is

$$\%\ \text{of parasite growth} = \frac{\text{Parasitemia percentage of treatment group at D4}}{\text{Parasitemia percentage of control group at D4}} \times 100\%$$

The percentage of parasite growth inhibition of each concentration is equal to the percentage of parasite growth of the control group (=100%) minus the percentage of parasite growth of the treatment group.[23] The effective dose 50 (ED_{50}) of the ratio was calculated using the growth inhibition percentages at various concentrations by probit analysis in IBM SPSS Statistics 27 software.

The type of interaction of ACAN-chloroquine combinations at each ratio was determined by the sum of fractional ED_{50} ($\sum FED_{50}$). $\sum FED_{50}$=ED_{50} ACAN in combination/ ED_{50} ACAN in non-combination regimen+ED_{50} chloroquine in combination/ ED_{50} chloroquine in non-combination regimen. If the $\sum FED_{50}$ is <1, the interaction is synergistic; if it is >1, it is antagonistic; and if it is equal to 1, it means an additive effect.[26]

Results

The ACAN was produced accordingly,[22] and it was found that the alpha-mangostin contained in the ACAN was 6%.

Table 1, table 2, and table 3 show the parasitemia percentage, the percentage of parasite growth, as well as the parasite growth inhibition in ratio-1, ratio-2, ratio-3, and the ED_{50} of each ratio.

Table 1: Average daily parasitemia percentage, parasite growth, and growth inhibition percentage on day 4 (the 5th day), total active substances given at each concentration of the ACAN-chloroquine in ratio-1, and its ED_{50}

Groups	Average parasitemia (%)					PG %	PGI %	TCAS (mg)/ Kg BW	ED_{50}
	D0	D1	D2	D3	D4				
NC	2.10±0.26	7.60±0.75	15.53±0.71	18.03±1.06	19.67±0.61	100	-		9.196
Ratio1x10^{0}	2.03±0.47	4.67±1.03	3.57±0.81	1.77±0.57	2.57±1.10	13.05	86.95	133	
Ratio1x10^{-1}	2.10±0.26	6.77±0.26	10.60±0.60	8.93±1.16	9.80±1.35	49.83	50.17	13.3	
Ratio1x10^{-2}	2.17±0.21	6.37±0.61	11.27±2.75	11.87±3.38	13.10±2.66	66.61	33.39	1.33	
Ratio1x10^{-3}	2.13±0.06	10.43±4.84	22.47±3.40	21.97±7.72	16.73±0.68	85.08	14.92	0.133	
Ratio1x10^{-4}	2.17±0.35	12.23±1.72	20.03±3,42	20.30±0.36	17.03±1.18	86.61	13.39	0.0133	
Ratio1x10^{-5}	2.03±0.31	13.17±0.55	16.33±1.01	17.80±2.51	17.90±0.46	91.02	8.98	0.00133	

NC: Normal control; D0: day 0; D1: Day 1; D2: Day 2; D3: Day 3; D4: Day 4; Ratio-1×10^{0} means no dilution (consists of 132.25 mg ACAN+0.7 mg chloroquine), ratio-1×10^{-1} means 10 times dilution, etc. PG: Parasite growth; PGI: Parasite growth inhibition; TCAS: Total active substance content given at each concentration; ED_{50}: Effective dose 50 (mg/Kg BW)

Table 2: Average daily parasitemia percentage, parasite growth, and growth inhibition percentage on day 4 (the 5th day), total active substance given at each concentration of the ACAN-chloroquine in ratio-2, and its ED_{50}

Groups	Average parasitemia (%)					PG %	PGI %	TCAS (mg)/ Kg BW	ED_{50}
	D0	D1	D2	D3	D4				
NC	2.05±0.35	7.15±0.64	15.6±0.85	18.5±0.85	19.15±1.06	100	-		7.626
Ratio2x10^{0}	2.50±0.37	3.74±1.26	1.86±0.62	0.70±0.24	0.16±0.09	0.84	99.16	67.18	
Ratio2x10^{-1}	2.10±0.36	6.70±1.25	12.53±2,33	11.87±2.57	9.80±3.05	51.17	48.83	6.718	
Ratio2x10^{-2}	2.47±0.21	7.83±1.42	15.50±7.20	15.90±6.15	12.37±2.05	64.58	35.42	0.6718	
Ratio2x10^{-3}	2.17±0.25	11.83±0.75	16.17±2.20	17.87±2.22	15.27±1.67	79.72	20.28	0.06718	
Ratio2x10^{-4}	2.05±0.21	6.30±2.83	11.30±0.71	12.35±1.06	17.60±3.82	91.91	8.09	0.006718	
Ratio2x10^{-5}	1.95±0.21	6.85±0.92	11.15±0.78	14.00±0.71	20.45±0.92	106.79	-6.79	0.0006718	

NC: Normal control; D0: Day 0; D1: Day 1; D2: Day 2; D3: Day 3; D4: Day 4; Ratio-2x10^{0} means no dilution (consists of 66.13 mg ACAN+1.05mg chloroquine), ratio-2x10^{-1} means 10 times dilution, and so on. PG: Parasite growth; PGI: Parasite growth inhibition; TCAS: Total active substance content given at each concentration; ED_{50}: Effective dose 50 (mg/Kg BW)

Table 3: Average daily parasitemia percentage, parasite growth, and growth inhibition percentage on day 4 (the 5th day), total active substance given at each concentration of the ACAN-chloroquine in ratio-3, and its ED_{50}

Groups	Average parasitemia (%)					PG %	PGI %	TCAS (mg)/ Kg BW	ED_{50}
	D0	D1	D2	D3	D4				
NC	2.00±0.28	13.00±0.42	16.30±0.57	18.75±1.63	20.75±0.21	100	-		82.13
Ratio3x10^{0}	2.13±0.47	6.43±1.10	8.80±1.15	7.90±1.14	9.43±1.91	45.46	54.54	198.73	
Ratio3x10^{-1}	1.45±0.21	8.50±1.98	15.95±4.45	11.55±2.62	12.80±0.28	61.69	38.31	19.873	
Ratio3x10^{-2}	1.80±0.30	9.93±1.71	16.23±2.43	19.90±4.40	21.33±7.57	102.81	-2.81	1.9873	
Ratio3x10^{-3}	2.23±0.31	13.50±2.95	17.20±1.25	24.13±1.10	23.70±3.02	114.22	-14.22	0.19873	
Ratio3x10^{-4}	2.07±0.93	12.90±1.74	18.47±1.12	25.87±3.40	24.30±3.83	117.11	-17.11	0.019873	
Ratio3x10^{-5}	2.03±0.06	13.80±0.92	18.53±1.00	26.93±4.16	25.60±1.57	123.37	-23.37	0.0019873	

NC: Normal control; D0: Day 0; D1: Day 1; D2: Day 2; D3: Day 3; D4: Day 4; Ratio-3x10^{0} means no dilution (consists of 198.4 mg ACAN+0.35 mg chloroquine), ratio-3x10^{-1} means 10 times dilution and so on. PG: Parasite growth; PGI: Parasite growth inhibition; TCAS: Total active substance content given at each concentration; ED_{50}: Effective dose 50 (mg/Kg BW)

Ratio-1

Ratio-1 contained ACAN+chloroquine in ratio ½ED_{50} ACAN: ½ED_{50} Chloroquine (ACAN and Chloroquine were used in a weight ratio of 189:1).

According to table 1, in the ratio-1×10^{0} (no dilution), the TCAS was 133 mg/Kg BW, which represents the sum of ½ED_{50} of ACAN and ½ED_{50} of chloroquine. Each dilution was prepared serially tenfold, so that each subsequent concentration contained one-tenth of the previous total dose. The TCAS for each dilution, in correlation with the parasite growth inhibition at the corresponding concentration, was analyzed using probit analysis to determine the ED_{50} of the combination. Based on the ED_{50} of the combination, the amount of each active compound was calculated according to the 189:1 ratio to estimate the individual ED_{50} values of ACAN and chloroquine in the combination.

According to probit analysis, the ED_{50} of the ratio-1 ACAN-chloroquine combination was 9.196 mg TCAS/Kg BW (<100 mg/Kg BW), which contained 9.148 mg ACAN+0.048 mg chloroquine/Kg BW.

The $\sum FED_{50}$ of ratio-1 was 0.069 (<<1). It means that ACAN -chloroquine in ratio-1 (189:1) exhibits a very synergistic drug interaction.[26]

Ratio-2

Ratio-2 contained ACAN+chloroquine in ratio ¼ ED_{50} ACAN: ¾ ED_{50} chloroquine (ACAN and chloroquine was used in a weight ratio of 63:1).

According to table 2, in the ratio-1×10^0 (no dilution), the TCAS was 67.18 mg/Kg BW, which represents the sum of ¼ ED_{50} of ACAN and ¾ ED_{50} of chloroquine. Each dilution was prepared serially tenfold, so that each subsequent concentration contained one-tenth of the previous total dose. The TCAS for each dilution, in correlation with the parasite growth inhibition at the corresponding concentration, was analyzed using probit analysis to determine the ED_{50} of the combination. Based on the ED_{50} of the combination, the amount of each active compound was calculated according to the 63:1 ratio to estimate the individual ED_{50} values of ACAN and chloroquine in the combination.

According to probit analysis, the ED_{50} of ratio-2 ACAN-chloroquine was 7.626 mg TCAS/Kg BW (<100 mg/Kg BW), which contained 7.507 mg ACAN+0.119 mg chloroquine/Kg BW.

The $\sum FED_{50}$ of ratio-2 was 0.113. It means also that ACAN-chloroquine in ratio-2 (63:1) exhibits a very synergistic drug interaction, but less synergistic than ratio-1 (189:1).[26]

Ratio-3

Ratio-3 contained ACAN+chloroquine in a ratio of ¾ ED_{50} ACAN: ¼ ED_{50} Chloroquine (ACAN and chloroquine were used in a weight ratio of 567:1).

According to table 3, in the ratio-1×10^0 (no dilution), the TCAS was 198.73 mg/Kg BW, which represents the sum of ¾ ED_{50} of ACAN and ¼ ED_{50} of chloroquine. Each dilution was prepared serially tenfold, so that each subsequent concentration contained one-tenth of the previous total dose. The TCAS for each dilution, in correlation with the parasite growth inhibition at the corresponding concentration, was analyzed using probit analysis to determine the ED_{50} of the combination. Based on the ED_{50} of the combination, the amount of each active compound was calculated according to the 567:1 ratio to estimate the individual ED_{50} values of ACAN and chloroquine in the combination.

According to probit analysis, the ED_{50} of the ratio-3 ACAN-chloroquine combination was 82.13 mg TCAS/Kg BW (<100 mg/Kg BW), which contained 81.985 mg ACAN + 0.145 mg chloroquine/Kg BW. All three ratios (ratio-1, ratio-2, and ratio-3) exhibit good antimalarial activity, with ED_{50} values below 100 mg/Kg body weight.[27]

The $\sum FED_{50}$ of ratio-3 was 0.414. It also means that ACAN-chloroquine in ratio-3 exhibits a synergistic drug interaction but less synergistic than ratio-2 (63:1).[26]

Discussion

This study demonstrated that all ACAN-chloroquine combinations exhibited good *in vivo* antimalarial activity. The ED_{50} values of ratio-1, ratio-2, and ratio-3 were 9.196, 7.626, and 82.13 mg/Kg BW, respectively. All of these ED_{50} values were less than 100 mg/Kg BW. According to the ED_{50} values, all of these combinations can be categorized as good antimalarial drugs.[27] Moreover, these drugs worked very synergistically at all ratios, including the $\sum FED_{50}$ values of ratio-1, ratio-2, and ratio-3 were 0.069, 0.113, and 0.414, respectively ($\sum FED_{50}$ <1).[26] It is interesting because chloroquine is one of the safest low-cost antimalarial drugs.[28] Although chloroquine resistance has been reported in many regions,[3] a recent *ex vivo* study demonstrated the re-emergence of chloroquine-sensitive *P. falciparum* in Africa after the drug had been discontinued for many years.[6] The use of a malaria drug as monotherapy is one of the causes of drug resistance, so it is wise to protect against chloroquine re-resistant malaria by combining the chloroquine with a partner drug with different mechanisms of action and different drug targets. The same condition is also valid for using a drug combination for rifampicin for tuberculosis, to decrease the drug resistance against the microbes.[8]

Kuncoro and others have shown that alpha-mangostin exhibits strong antimalarial activity *in vitro,* and one of the proposed mechanisms of its action is the inhibition of the parasite's cysteine protease enzyme.[14] Alpha-mangostin can also inhibit heme polymerization, thereby inhibiting hemozoin formation.[29] The *in vivo* alpha-mangostin as an antimalarial drug has been improved by using nanoparticle preparation (ACAN) because the nanoparticle formulation is much more soluble than non-nano formulation, which is very insoluble in various safe solvents. This nano formulation has shown promising proper antimalarial activity with low alpha-mangostin content in its ED_{50}.[21]

Plasmodium can detoxify heme as a byproduct of hemoglobin digestion in the parasite food vacuoles by heme polymerization to form hemozoin as a non-toxic product. If some heme escapes from this polymerization, *P. falciparum* exported protein 1 (PfEXP1) enzyme, which is located at the membrane of parasite food vacuoles, can act as a glutathione S transferase and detoxify this heme. Chloroquine targets these two mechanisms of detoxifying heme: indirectly by inhibiting heme polymerization and

directly by inhibiting the process of detoxifying free heme through inhibition of PfEXP1 enzyme.[30] As mentioned before, chloroquine can also insert into the *Plasmodium* DNA double helix structure, forming a stable chloroquine-DNA complex to inhibit DNA replication, translation process, growth, and reproduction of the parasites.[9] Alpha-mangostin can also disrupt heme polymerization to suppress hemozoin production.[29] Other mechanisms of action of alpha-mangostin as an antimalarial agent is through inhibition of cysteine protease enzyme, which is needed for globin digestion, so that the parasites have less or no nutrition for growth.[14] There are different targets between chloroquine and alpha-mangostin as antimalaria: DNA and PfEXP1 enzyme versus cysteine protease enzyme. This difference may have a benefit in preventing further drug resistance against this combination in the future.[31]

Inhibition of heme polymerization, which works for chloroquine as well as for alpha-mangostin as an antimalaria may possibly produce a synergistic effect and it is needed to be studied further. Another study reported that mangostin has antimalarial activity but it cannot substitute chloroquine. Mangostin can increase the sensitivity of chloroquine against *Plasmodium* because it improves the intravacuolar accumulation of chloroquine.[32] This report also supports the synergistic interaction between these drugs.

In this study, alpha-mangostin in ACAN also exhibited synergistic activity with chloroquine as antimalaria *in vivo*. The synergistic activities were shown in all of these combinations (ratio-1, ratio-2, and ratio-3), where $\sum FED_{50}$-s were <<1, and the most synergistic activity was found in ratio-1 with the lowest $\sum FED_{50}$. Why the ratio-1 exhibited the most synergistic interaction is not clear. It may be able to be explained as follows: oxidative stress is produced during malaria by the host response against the infection[33] as well as by the chloroquine action as an anti-PfEXP1 enzyme ("anti-glutathione S transferase") to eliminate the parasites.[30] That's why it is useful to maintain the oxidative balance and at a high level of antioxidant (alpha-mangostin has antioxidant activity), the combination may accelerate parasite growth. The optimal ratio is needed.[16] However, this evidence may serve as a glimmer of hope, warranting further investigation to determine the optimal dose of this ratio required to completely eliminate parasitemia and, potentially, cure the disease in the future.

Moreover, there may be another benefit of these combinations. Oxidative stress happens in malaria by the activity of phagocytes of the host to eliminate parasites, as well as by the haemoglobin degradation by parasites producing heme as a free radical, and oxidative stress in malaria may contribute to the development of severe malaria and its complications, which can be fatal.[16] The use of alpha-mangostin as antimalaria may reduce this oxidative stress because this compound has antioxidant activity[13, 14, 33] and it may be considered as another benefit for its usage.

This synergistic interaction of these substances as an antimalarial drug *in vivo* may also be caused by the chitosan in the ACAN, although the mechanism of antimalarial activity of the chitosan is still not clear. Moreover, its antimalarial activity does not show a linear relation with its dose.[20] According to Momenfam and others, the considerable potentiation of chloroquine and chitosan as antimalarial *in vitro* and *in vivo* against P.falciparum is shown in a certain ratio by subcutaneous administration.[34] In our study, a marked synergistic interaction was observed across all dose ratios, which may be attributed to the treatment acting similarly to a triple-drug combination with antimalarial effects, and this approach may also reduce the likelihood of resistance development in *Plasmodium*, offering hope for more sustainable antimalarial therapy.[31]

This study needs to be continued to explore more about this ACAN-chloroquine combination, especially for ratio-1 to determine the optimal dosage required to completely eliminate parasitemia, and to explore the safety of this combination.

A limitation to consider is that antioxidant activity may benefit both the host and the parasites. This is important because host immune cells use free radicals to kill parasites, and antioxidants could potentially interfere with this process.[33] This issue should be acknowledged and explored further in future studies.

Conclusion

It can be concluded that the combination of alpha-mangostin chitosan alginate nanoparticles–chloroquine has a good antimalarial activity, and it exhibits a marked synergistic interaction as an antimalarial agent *in vivo* in several ratios of combination, especially in ratio-1 (1:1 based on ED_{50}; 189:1 by weight). This finding may provide a glimmer of hope for future research and development efforts to combat malaria.

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Authors' Contribution

S.T: Conceptualization, study design, data collecting, data analysis, and drafting; F.H: Conceptualization, data collecting, data analysis, and reviewing the manuscript; M.M: Data analysis, data acquisition, and reviewing the manuscript; DL.A: Data analysis and reviewing the manuscript; FA.H: Data collecting, data analysis, writing review and reviewing the manuscript; All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of AI:

The authors used ChatGPT version 5.3 (OpenAI, San Francisco, USA) to improve the clarity and grammar of some sentences. All conceptual content, data analysis, and interpretation were conducted solely by the authors.

Conflict of Interest: None declared.

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