Tehran University of Medical Sciences Publication
http://tums.ac.ir

Iran J Parasitol

Open access Journal at
http://ijpa.tums.ac.ir

Iranian Society of Parasitology
http://isp.tums.ac.ir

Original Article

In Vitro Antimalarial Activity of Chloroquine-Crocus Sativus Conjugated to Chitosan Nanocomposits against 3D7 and K1 Strains of *Plasmodium falciparum*

Aram Khezri [1], *Mahdi Nateghpour [1,2], *Afsaneh Motevali Haghi [1], Taher Elmi [3], Abbas Rahimi Foroushani [4], Mehdi Shafii Ardestani [5], Haleh Hanifian [1]

1. *Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
2. *Center for Research of Endemic Parasites of Iran, Tehran University of Medical Sciences, Tehran, Iran*
3. *Department of Medical Parasitology and Mycology, School of Medicine, Arak University of Medical Sciences, Arak, Iran*
4. *Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
5. *Department of Radiopharmacy, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

Received 10 Dec 2024
Accepted 18 Mar 2025

Keywords:
Plasmodium falciparum;
Saffron;
Chitosan;
Therapeutic combination;
In vitro;
Nanocomposite

***Correspondence Emails:**
nateghpourm@sina.tums.ac.ir,
amh.mot@gmail.com

Abstract

Background: The use of nanocarriers in combination with other treatments shows significant promise in addressing drug-resistant diseases, particularly malaria. Given the high prevalence of drug-resistant malaria, research into innovative therapies is crucial. This study focuses on a nanoform of chitosan, a biodegradable polymer, combined with *Crocus sativus* (saffron) and chloroquine to enhance their antimalarial effects.

Methods: Saffron extract and chloroquine were separately conjugated with chitosan, followed by confirmation tests to determine conjugation efficiency. Both chloroquine-resistant and sensitive strains of *Plasmodium falciparum* were cultured to calculate the IC50 values of various treatments in vitro. This study was conducted at the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran in 2024.

Results: Confirmation tests (FTIR, DLS, Zeta potential, TEM) verified proper drug conjugation to nanocomposites, with observed nanosize, the percentage of conjugation was 64.4% for chloroquine and 42.9% for saffron. Toxicity and hemolysis tests confirmed safe doses. The IC50s values for Chloroquine, Nanoparticle-Chloroquine, Saffron, and Nanoparticle-Saffron were 0.3, 0.8, 42.5, and 6.24 µg/ml, respectively, for the sensitive strain, and 5, 1, 12.5, and 3.12 µg/ml, respectively, for the resistant strain. Combination therapy with the fixed ratio method showed synergistic effects. Statistical analysis revealed synthesized nanocomposites' superior inhibition of *P. falciparum* growth compared to non-nano. Significant differences were observed in some cases ($P< 0.05$).

Conclusion: Utilizing nanocarriers and combination therapy is an appropriate strategy for addressing drug resistance. Saffron's anti-malarial effects on *P. falciparum* were notably increased when linked to chitosan nanocomposites. Furthermore, employing a fixed ratio technique enhanced the therapeutic effectiveness of saffron when combined with chloroquine and chloroquine-nanocomposites across all concentrations.

DOI: https://doi.org/10.18502/ijpa.v20i2.19027

Introduction

Malaria is a life-threatening disease caused by the *Plasmodium* parasite and transmitted to humans through the bite of infected female Anopheles mosquitoes. Malaria is a major global health concern, especially in tropical and subtropical regions (1). Despite being profusely combated during past years, continues to have a devastating impact on public health and economies worldwide. Numerous efforts are underway to develop effective treatments and vaccines against malaria infection. According to a report published by the WHO in 2024, there will be 263 million cases and 597,000 deaths from malaria in 2023 (2).

P. falciparum poses a major global health threat through malaria transmission and mortality, causing severe issues like cerebral malaria and organ failure. It mainly impacts vulnerable groups, especially children and pregnant women in sub-Saharan Africa. The economic costs are significant, with malaria resulting in productivity losses and increased healthcare costs, potentially reducing GDP by up to 1.3% annually in affected regions (2–4).

Malaria drug resistance is a major global issue. This complex problem arises mainly from the genetic diversity of the *Plasmodium* parasite, allowing it to evolve against treatments (5). Other contributing factors include inadequate drug dosing, poor-quality medications, patient non-compliance, and limited treatment options, which worsen the situation (6).

A review of studies in Iran indicates that chloroquine resistance in *P. falciparum* is significantly associated with the K76T mutation in the chloroquine resistance gene. Additionally, resistance to sulfadoxine-pyrimethamine (SP) in *P. falciparum* is linked to mutations in the dihydrofolate reductase and dihydropteroate synthase genes (7).

Herbal medicines have treated malaria since ancient times, with many effective antimalarial drugs derived from plants. Traditional plants can serve as anti-*Plasmodium* agents (8–10). Saffron, a spice from *Crocus sativus* stigmas, is valued for its color, aroma, and flavor. Used for centuries, it offers antioxidants, boosts mood, enhances cognition, may fight cancer, supports eye health, and has anti-inflammatory properties (11–13).

Chitosan is derived from chitin, which is the second most abundant natural biopolymer after cellulose, commonly found in marine animals like shrimp and crabs (14). This naturally positively charged polysaccharide has gained attention in drug delivery due to its high bioavailability, biodegradability, biocompatibility, and non-toxicity (15). Chitosan's -OH and -NH2 groups enable hydrogen and covalent bonding, while protonation at low pH gives it a positive charge, enhancing mucosal adhesion (16). These properties make chitosan an excellent choice for pharmaceutical applications, particularly in drug encapsulation and delivery (17,18).

Since increasing drug resistance phenomenon among some malaria parasites, administrating combination therapy can postpone such problems (19,20). This widely endorsed method, recommended by WHO, is frequently employed to achieve a more comprehensive and efficacious response to malaria (21).

This study explored the effects of a new chitosan-based nanocomposite with saffron and chloroquine on *P. falciparum*. It evaluated its impact on chloroquine-sensitive (3D7) and resistant (K1) strains through in vitro analysis.

Material and Methods

This study was conducted at the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran in 2024.

Chemicals and reagents

Ethylene diamine tetra acetate (EDTA), Tris buffer, sodium chloride (Nacl), Triton-X 100, KH2PO4, K2HPO4, NaOH, chloroform, potassium hydroxide (KOH), DPA were pro-

cured from Merck Ltd,. Chloroquine diphosphate, Chitosan, DCFDA were purchased from Sigma–Aldrich (St. Louis, MO, USA). Commercially available histopaque 1077, HEPES, were purchased from Sigma Chemical Co., USA. All other chemicals were obtained from Merck Ltd.,

Extraction of whole saffron extract

Saffron, sourced from Saharkhiz Company in Mashhad, Iran, is made from dried stigmas of *Crocus sativus*. Two grams were pounded, then kept cool until extraction. An ultrasonic method enhanced extraction, and the extracts were filtered using Whatman No. 1 paper and freeze-dried with lyophilizer.

Preparation of Nano-chitosan

Chitosan nanocomposites were synthesized via ionotropic gelation using chitosan and sodium tripolyphosphate (TPP). First, 1 g of chitosan was dissolved in 100 ml of 1% hydrochloric acid, and a 0.1% TPP (1.2 mg/ml) solution was prepared in deionized water. The TPP solution was added dropwise to the chitosan solution while stirring at room temperature, resulting in particles of varying sizes based on the chitosan to TPP ratio. The particles were then collected by centrifugation at 12,000 rpm for 10 min, washed with deionized water, and lyophilized (22).

Drug loading on nanocomposits

During nanocomposite synthesis, 1 mg/ml of saffron and chloroquine were prepared in sterile distilled water and mixed with monomerized chitosan for 15 min. TPP was then added to encapsulate the drug in particles.

Determination of encapsulation efficiency (EE) and loading capacity (LC)

A calibration curve for chloroquine and saffron was created by preparing various concentrations and measuring their OD at 343 nm and 440 nm, respectively, using a nanodrop. Drug-loaded polymer nanocomposites were separated from the supernatant by centrifugation at 12,000 rpm for 20 min. The supernatant's absorbance was measured with a UV spectrophotometer to calculate encapsulation efficiency (EE) and drug loading capacity (LC). Calibration curves were generated using blank polymer nanocomposites. EE was determined to be 64.4% for chloroquine and 42.9% for saffron, with LC values of 1.4% for chloroquine and 1.04% for saffron, reported as mean values from triplicate measurements.

$EE = (W_t - W_f)/W_t \times 100\%$ [1]

$LC = (W_t - W_f)/W_n \times 100\%$ [2]

W_t is the total amount of NPs, W_f is the free number of NPs in the supernatant after centrifugation, and W_n is the weight of polymer nanocomposites after freeze-drying.

Release behavior of Nanocomposits

Release of medicinal compounds from 15 ml (1 mg/ml) nanocomposites in 500 ml PBS (pH 7.4, 37 °C) was examined over 24 h. The UV-Vis assay measured drug release at 440 nm (saffron) and 343 nm (chloroquine).

Determination of Physicochemical Properties of Nanocomposits

The morphology, Zeta potential, morphology, distribution, size and distribution of the prepared nanocomposits were determined using Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS). Fourier transform infrared (FTIR) analysis was conducted to verify the synthesis of nanocomposits.

Cell Viability Assay

MTT assay was conducted to evaluate cell viability. PC12 cells were sourced from Iran University of Medical Sciences and seeded into 96-well plates at 100 µL/well, allowing 24 h for adherence. Different concentrations of nanodrugs (100 µL/well) were added and incubated for 48 h at 37 °C in a 5% CO2 humidified environment, with triplicate wells for each sample. After incubation, 10 µL of 5 mg/mL MTT was added and incubated for 3 h, followed by 10 additional minutes at 37 °C.

The optical density was read at a wavelength of 570–630 nm (22,23). The growth inhibition rate was calculated by using the following formula:

$$\text{Cell survival rate } (\%) = \frac{OD\ Treated}{OD\ Control} \times 100$$

Hemolytic Test

To conduct the hemolytic test on synthesized medicinal compounds, 5 ml of fresh whole blood was collected from a healthy volunteer and anticoagulated with heparin. The red blood cells were isolated by centrifugation at 2500 rpm for 10 min and washed three times with PBS to prepare a 1% suspension, added to each well (100 µl). Different concentrations of the medicinal compounds (100 µl) were then added. The first well served as the negative control (PBS), and the last well was the positive control (Triton X-100). After incubating for an hour at 37 °C, samples were centrifuged at 1500 rpm for 10 min, and the supernatant's absorbance was measured at 540 nm. The percentage of drug hemolysis was calculated using the following formula (24):

$$\text{Percent hemolysis}\% = \frac{\text{OD test} - \text{OD Negative Control}}{\text{OD Positive Control} - \text{OD Negative Control}} \times 100$$

Cultivation of the Parasite

Cultivation of *P. falciparum* using the modified Jensen and Trager method took place at the National Malaria Laboratory, Tehran University of Medical Sciences. Chloroquine-sensitive 3D7 and resistant K1 strains were thawed, centrifuged at 1500 rpm, and washed with RPMI 1640 medium to remove freezing media. They were then placed in 25 mm plates with 2250 µL CCM (RPMI 1640, 10% human serum AB+, 50 mg/L hypoxanthine, 50 mg/L gentamycin) and 250 µL washed human O+ red blood cells. Plates were incubated at 37 °C in a candle jar, with medium changes every 48 h and subsequently every 24 h.

In Vitro Assay

P. falciparum was cultivated to 1% parasitemia in the ring stage, and the anti-*Plasmodium* effect of synthesized compounds was assessed using microscopy in 96-well plates. Medicinal compounds were diluted in culture medium (1, 5, 10, 50, 100, 200 µg/ml for Cq and NPCq; 1.6, 3.12, 6.24, 12.5, 25, 50 µg/ml for SAF and NSAF), and added in triplicates to wells, followed by 15 µL of infected RBCs. After incubating for 24 h at 37 °C, blood smears were prepared, and stained with Giemsa, and parasitemia was determined by counting 10,000 red blood cells.

Combination tests

To begin the combination tests, IC50 values of the selected antiplasmodial agents against the cultured parasites were calculated from the fitted curve (dose-response curve). IC50s were mixed at 10, 30, 50, 70, and 90 percent using fixed ratios. A stronger reduction in parasite growth indicates synergism, while adverse or no effects suggest antagonism or additivity, respectively.

Statistical Analyses

Experiments were conducted in triplicate. ANOVA with Post Hoc evaluation using SPSS 27.0 assessed group differences, while independent sample t-tests compared the two strains. A *P*-value ≤ 0.05 indicated statistical significance.

Results

Physicochemical properties analysis of nanocomposits results

The size and charge of the synthesized nanocomposits were analyzed using a transmission electron microscope (TEM) and zeta sizer. The nanocomposits were below 100 nm in size (Fig. 1).

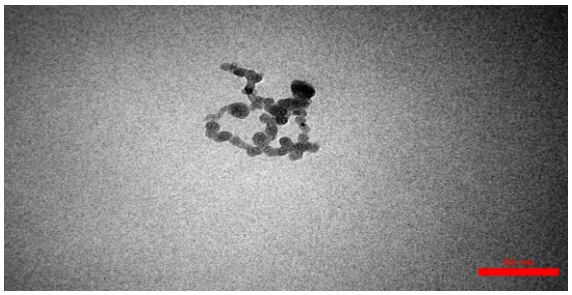

Fig. 1: Transmission Electron Microscopy (TEM) image of chitosan-tripolyphosphate conjugated nanocomposits, scale bar 50 nm

Generally, when zeta potential is higher than +25 mV or less than -25 mV, the system stability increases (25). As shown in Fig. 2, the NPCq and NPSAF nanocomposits exhibited a positive charge within the range of 80-90 mV. Therefore, all of these systems are very stable.

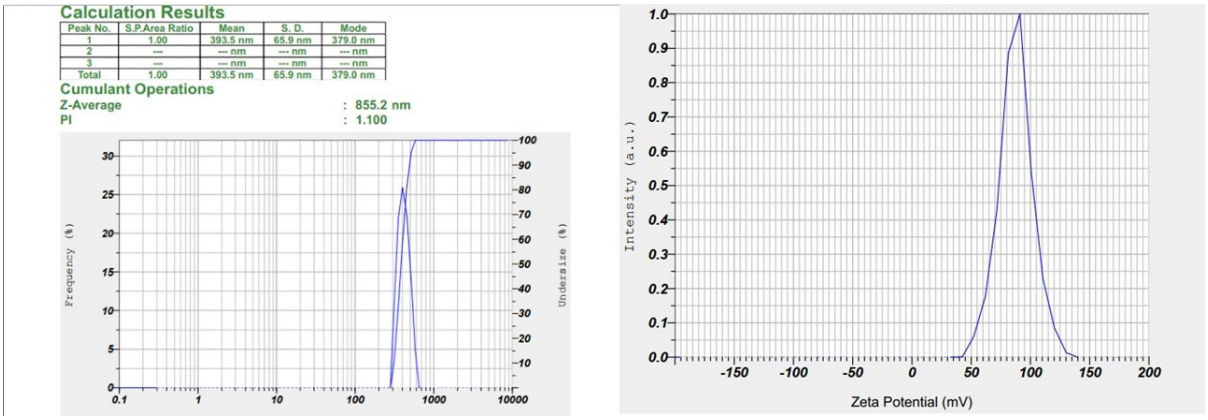

Fig. 2: Particle size, polydispersity index (PDI), and zeta potential of nanocomposits (NPs)

Saffron-loaded nanocomposites exhibit a notable decrease in zeta potential, from +90 to +80. This reduction is due to negatively charged functional groups in saffron, which ionize in the environment. The incorporation of saffron into the chitosan structure confirms the successful loading by lowering the overall charge.

DLS results support the hypothesis that drug loading increases nanocomposite size due to drug filling pores and causing swelling. The rise in zeta potential suggests chloroquine's positive charge transfers to chitosan nanocomposites, likely due to chloroquine's nitrogen elements ionizing by taking hydrogen from water.

Analyzing the results of a Fourier Transform Infrared Spectrometer (FTIR)

Chitosan is a polymer formed by sugar molecules linked with ether bonds, resulting in peaks in the 1200-1150 CM^{-1} range. The hydroxyl group (OH) shows a broad peak at 3400-3300 CM^{-1}. A peak at 1600 CM^{-1} indicates NH amide presence, confirming successful nanocomposite synthesis. Peaks around 2900 CM^{-1} relate to aliphatic hydrogens like NH3, NH2, NH, and methyl.

For saffron, the high crocin content results in increased double bonds in nanocomposites, enhancing aliphatic CHs and OHs. This leads to sharper, stronger peaks at 2900 CM-1 and

3400 CM-1, as well as a peak at 1600 CM^{-1} indicating chitosan presence.

The FTIR spectrum of chloroquine features a peak at 800-600 CM^{-1} from chlorine (Cl) and a carbonyl amide peak at 1600 CM^{-1} associated with chitosan (Fig. 3).

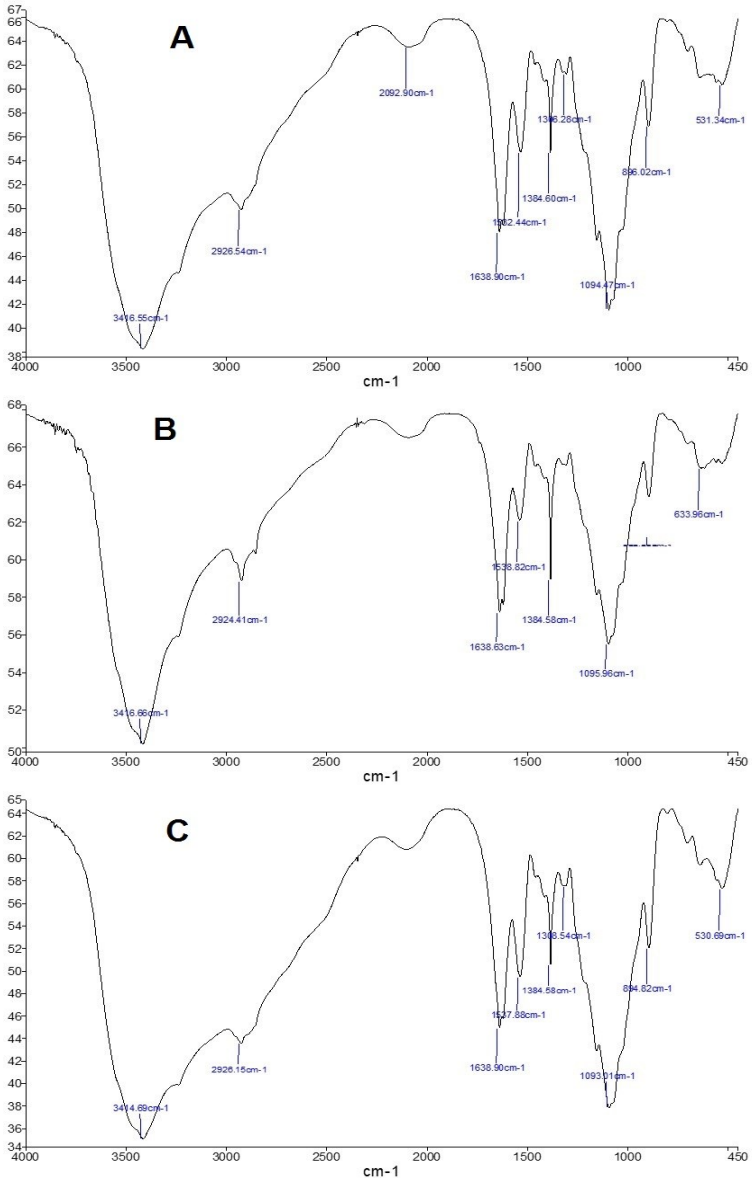

Fig. 3: FTIR spectra: (A) Chloroquine-nanocomposit (NPCq), (B) Saffron-nanocomposit (NPSAF) & (C) nanocomposit (NPs)

Encapsulation Efficiency (EE %) and Loading efficiency (LC %)

In this study, we calculated EE% and LC% for chloroquine loaded in CS nanocomposits (64.4% and 1.4%, respectively) and saffron (42.9% and 1.04%), respectively.

MTT assay

MTT assay evaluated the toxicity of the synthesized nanocomposite (NPC - NPCq - NPSAF) in cell cultures. Results indicated that 500 µg of NPCq killed over 50% of cells.

Therefore, the concentrations of 1, 5, and 10 µg/mg showed no toxicity in the PC12 cell line (Fig. 4). Furthermore, 50 µg/ml NPSAF was non-toxic to PC12 cells, with higher concentrations also displaying low toxicity.

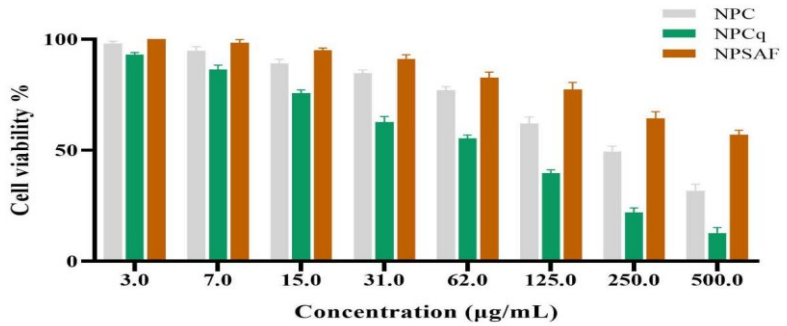

Fig. 4: Bar chart Cell viability% based on MTT assay of nanocomposites (NPC), Chloroquine - nanocomposit (NPCq) and Saffron-nanocomposit (NPSAF)

Hemolytic Activity

In addition to the MTT assay, we performed a hemolysis assay with human red blood cells with the synthesized nanocomposites (Fig. 5). Hemolysis increased with nanocomposite concentration but was not statistically significant compared to the negative control, though it differed significantly from the positive control.

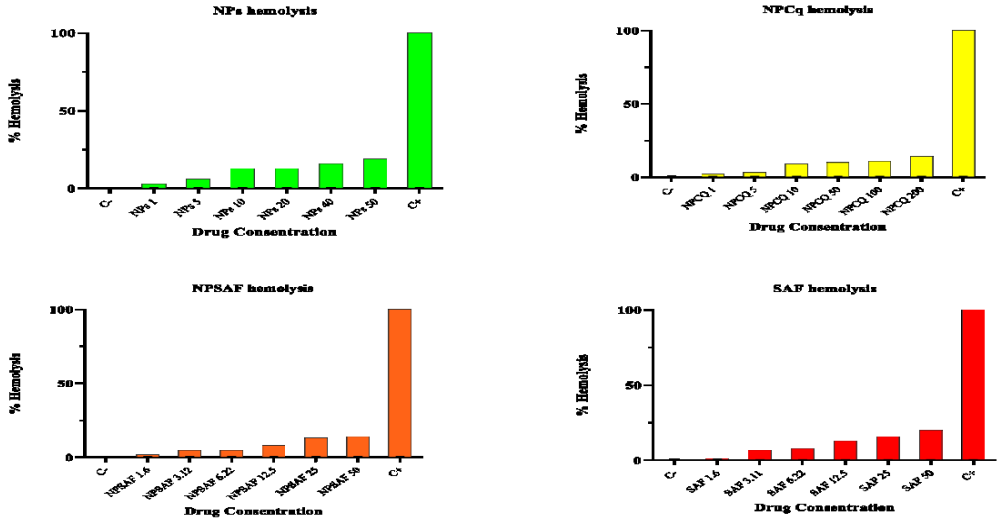

Fig. 5: Bar chart presentation represents the hemolytic activity of nanocomposites (NPs), Chloroquine-nanocomposit (NPCq), Saffron-nanocomposit (NPSAF) and Saffron (SAF). It is represented as a percentage of the amount of released hemoglobin.

Release behavior of Nanocomposit

The release of chloroquine and saffron from chitosan nanocomposites was assessed over 24 h in PBS at pH 7.4. Figure 6 shows that 78% of chloroquine and 81% of saffron were released, while over 90% of each were discharged within 4 h when tested individually.

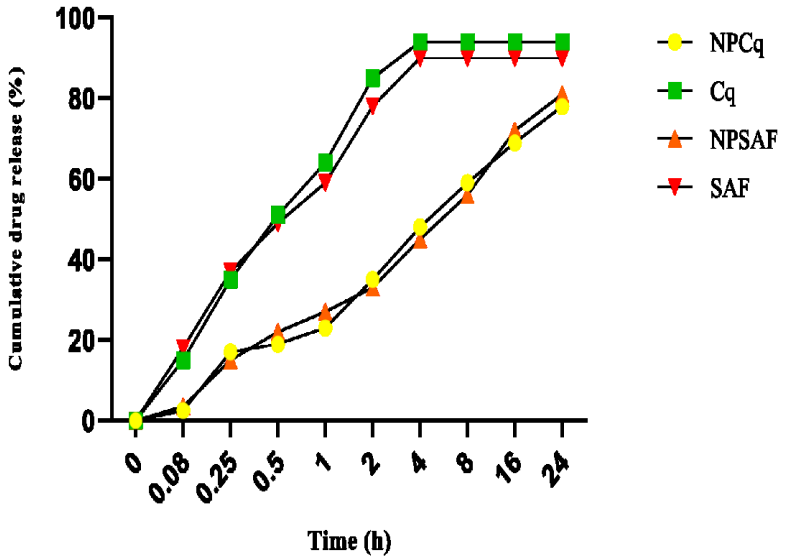

Fig. 6: The release behavior of chloroquine (Cq) and saffron (SAF) alone and in the state of cross-link to chitosan nanocomposites (NPCq & NPSAF), in PBS (pH: 7)

Anti-Plasmodium effect of the synthesized nanocomposite

The synthesized nanocomposites showed an anti-*Plasmodium* effect on both chloroquine-sensitive (3D7) and resistant (K1) strains. Loading chloroquine and saffron enhanced their growth inhibition (Table 1).

Table 1: The mean inhibition effect of synthesized nanocomposite on *P. falciparum* in culture medium. Cq (Chloroquine), NPCq (Chloroquine- nanocomposite), SAF (Saffron), NPSAF (Saffron- nanocomposites), Chit (Chitosan), NPC (Nanocomposites)

Drugs	N	Mean (%)	Std. Deviation	Std. Error
Cq	36	80.10	18.08	3.01
NPCq	36	82.62	15.10	2.51
SAF	36	16.97	31.80	5.30
NPSAF	36	50.26	23.55	3.92
Chit	36	21.90	24.36	4.06
NPC	36	54.70	12.48	2.08
Total	216	51.10	33.38	2.27

The effectiveness of the nanocomposites against the *P. falciparum* strain 3D7 was evaluated using microscopy (Table 2). The chitosan nanocomposites with chloroquine and saffron demonstrated a concentration-dependent growth inhibition. At 200 µg/ml, NPCq achieved 97.88% inhibition, while NPSAF at 50 µg/ml showed 81.89% inhibition. For the

chloroquine-resistant strain (K1), increased concentration also enhanced anti-plasmodium activity, with NPCq at 200 µg/ml inhibiting 92.56% and NPSAF at 50 µg/ml inhibiting 69.46% (Table 2). In the 3D7 strain, the saffron-only group showed a higher level of parasite growth at lower concentrations compared to the control group (P<0.05).

Table 2: The effect of newly synthesized nanocomposits on *P. falciparum* 3D7 & K1 strain in culture medium. Cq (Chloroquine), NPCq (Chloroquine- nanocomposite), SAF (Saffron), NPSAF (Saffron- nanocomposites), Chit (Chitosan), NPC (Nanocomposites)

Drugs	Conc (µg/ml)	Para (%) (3D7)	Inhi (%) (3D7)	Para (%) (K1)	Inhi (%) (K1)
Cq	1	20.16	79.83	59.32	40.67
	5	15.58	84.41	50.53	49.46
	10	6.44	93.55	33.62	66.37
	50	5.20	94.79	18.84	81.15
	100	4.74	94.79	11.59	88.4
	200	4.28	95.71	8.21	91.78
NPCq	1	43.12	56.87	41.73	58.26
	5	10.88	89.11	36.81	63.18
	10	9.43	90.56	13.42	86.57
	50	3.91	96.08	19.51	80.48
	100	2.57	97.42	9.08	90.91
	200	2.11	97.88	7.43	92.56
SAF	1.6	118.31	-18.31	68.88	31.11
	3.12	125.74	-25.74	55.16	44.83
	6.24	122.38	-22.38	56.03	43.96
	12.5	114.38	-14.38	55.07	44.92
	25	89.37	10.61	77.39	22.60
	50	33.37	66.62	79.80	20.19
NPSAF	1.6	94.42	5.57	57.68	42.31
	3.12	84.89	15.1	48.69	51.3
	6.24	54.97	45.02	53.71	46.28
	12.5	34.34	65.65	48.88	51.11
	25	20.93	79.06	41.64	58.35
	50	18.10	81.89	30.53	69.46
Control +		100		100	
Control -		0		0	

The results of combination therapy

IC50 results are shown in Table 3. The combination of chloroquine, NPSAF, NPCq, and NPSAF demonstrated strong effects, as seen in graphs 6 and 7 with the fixed-ratio method. For strain 3D7, the 50/50 Cq-NPSAF ratio had the best outcome, while the 10/90 NPCq-NPSAF ratio achieved the highest growth inhibition. For strain K1, the 50/50 Cq-NPSAF combination also resulted in the greatest growth inhibition, whereas the

70/30 NPCq-NPSAF ratio was the most effective (Fig. 7).

Table 3: IC50s of different treatments used in the study. Cq (Chloroquine), NPCq (Chloroquine- nanocomposite), SAF (Saffron), NPSAF (Saffron- nanocomposites)

Drugs	IC50 (3D7) (µg/ml)	IC50 (K1) (µg/ml)
Cq	0.30	5
NPCq	0.80	1
NPSAF	6.24	3.12
SAF	42.50	12.50

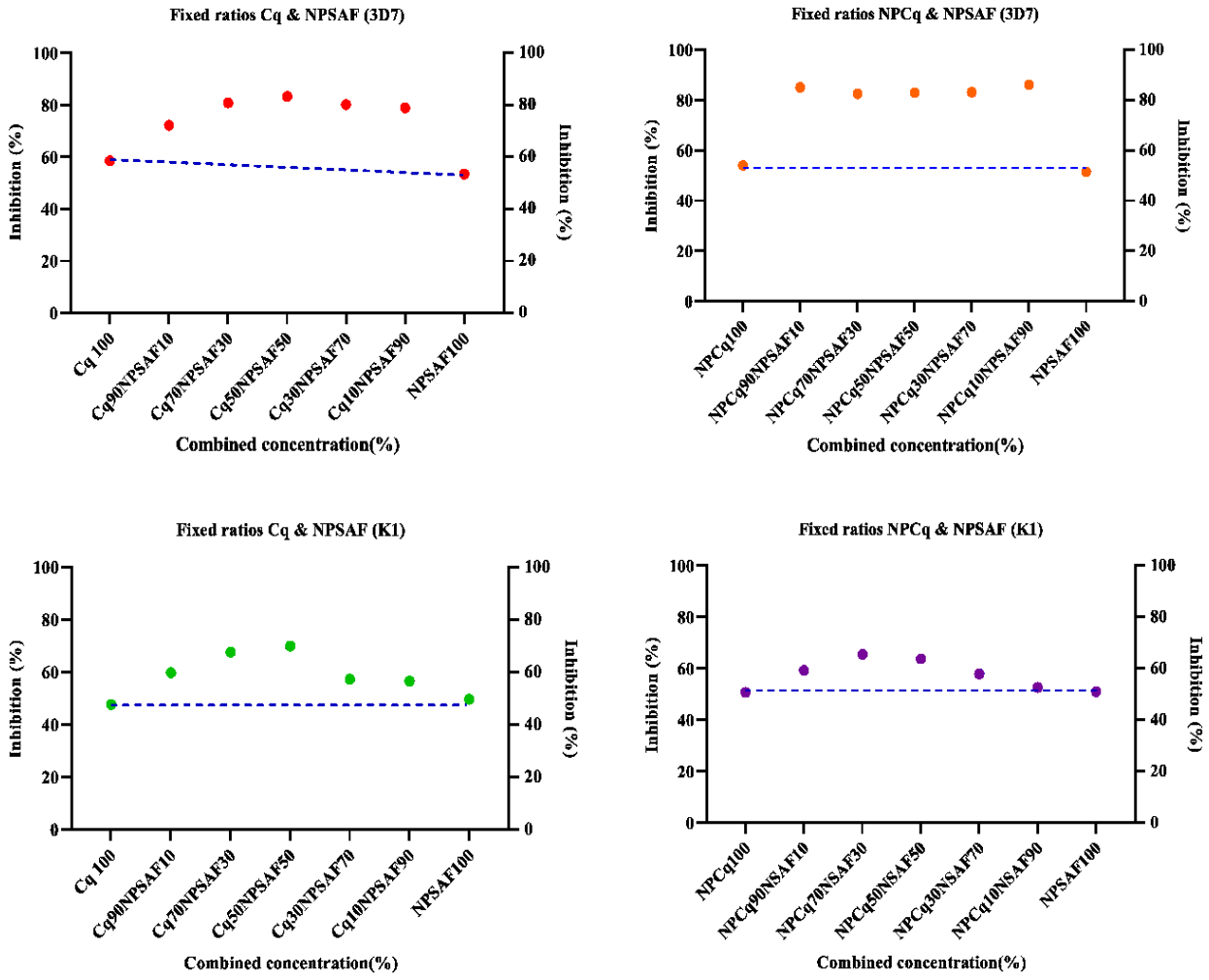

Fig. 7: Interaction between Chloroquine (Cq), Saffron-nanocomposit (NPSAF), Chloroquine-nanocomposit (NPCq), Saffron-nanocomposit (NPSAF) against the chloroquine - sensitive (3D7) and chloroquine- resistant (K1) strains of *P. falciparum*

Discussion

Malaria, a fatal infection, despite extensive combating against the illness still afflicts many people in the world including some death out of them. The resistance of *P. falciparum* to most antimalarial drugs and the absence of a highly effective vaccine give priority to focusing on pharmaceutical research in the field of malaria (26,27).

This study evaluates anti-malarial agents targeting *P. falciparum*. Using therapeutic combinations and nanocarriers may improve treatment for chloroquine-resistant strains. Combining nanocomposite drugs with herbal extracts shows promise for better control of *P. falciparum*. NPCq and NPSAF nanocomposites inhibit *P. falciparum* more effectively than chloroquine and Saffron alone (Table 4).

Table 4: Bonferroni Post Hoc Test for Pairwise Comparisons of the Groups. Cq (Chloroquine), NPCq (Chloroquine- nanocomposite), SAF (Saffron), NSAF (Saffron- nanocomposites), Chit (Chitosan), NPC (Nanocomposites)

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	*P*-value
Cq	SAF	63.13	5.15	<0.001
	NPSAF	29.84	5.15	<0.001
	Chit	58.20	5.15	<0.001
	NPC	25.40	5.15	<0.001
NPCq	SAF	65.65	5.15	<0.001
	NPSAF	32.35	5.15	<0.001
	Chit	60.72	5.15	<0.001
	NPC	27.91	5.15	<0.001
SAF	NPSAF	-33.29	5.15	<0.001
	NPC	-37.73	5.15	<0.001
NPSAF	Chit	28.36	5.15	<0.001
Chit	NPC	-32.80	5.15	<0.001

In Nigeria, 100 *P. falciparum* isolates were tested for drug sensitivity alongside herbal remedies like *Momordica charantia*, *Diospyros Monbuttensis*, and *Morinda lucida* (10). Our research utilized confirmed strains (K1 and 3D7) and achieved better results through nanocomposite synthesis and plant extract conjugation.

Carboxymethyl chitosan composites improved treatment efficacy for *P. berghei*-infected mice by 1.65 times over andrographolide alone (28). Chloroquine and saffron combined with chitosan nanocomposite were more effective against *P. falciparum* in vitro than when used separately.

In a study entitled: Interaction between Chitosan and Chloroquine against *P. berghei* and *P. falciparum* using In-vivo and in-vitro Tests, resulted in IC50s of 1.4 and 5 µg/ml for chloroquine and chitosan, respectively (29). In our investigation, the IC50 of chloroquine against *P. falciparum* 3D7 strain was determined to be 0.3 µg/mL. In our research, based on the fixed ratio method, a synergistic effect was observed across all proportions.

The present research, similar to Zein et al.'s study, observed that in low doses of saffron extract, such as Sambiloto, the parasite showed more growth compared to the control group (30). However, this effect was only seen in the sensitive strain to chloroquine. Saffron has antioxidant properties similar to Sambailoto, it is possible to suggest that in low doses, saffron functions as an antioxidant medication

that eliminates various harmful free radicals for parasites (31,32).

Ionotropic gelation has been used since the 1990s to create chitosan-based polymer micro and nanoparticles for biomedical applications. This method effectively encapsulates various molecules in stable, biocompatible, and biodegradable particles of different sizes (33). We achieved a chloroquine encapsulation efficiency of 64.4% and particle size of about 390 nm. Baharat et al. reported sizes of 410.4, 436.2, and 461.3 nm with entrapment efficiencies of 64%, 69%, and 72%, aligning with our findings (34).

The limitations of this study include the lack of in vivo antiplasmodial evaluation and the lack of investigation of the in vivo toxicity of the synthesized nanocomposites and their effects on different tissues.

Conclusion

This research utilized saffron and chloroquine in nano form within chitosan nanocomposite, alongside their non-nano forms, to test against both chloroquine-sensitive and resistant strains of *Plasmodium falciparum* in vitro. The nano forms showed significantly greater efficacy. The fixed ratio method in combinations demonstrated strong synergistic effects. Release rates of the novel agents were slower than their non-nano counterparts, and toxicity testing confirmed non-toxic doses.

Acknowledgements

The authors would like to acknowledge the experts of the National Malaria Laboratory, School of Public Health, Tehran University of Medical Sciences, Ms. Friver and Ms. Sayad Talai, and the expert of the Central Research Laboratory of the School of Public Health, Tehran University of Medical Sciences, Ms. Dr. Bahri, for their support and contribution to this study.

Funding

This research was financially supported by Tehran University of Medical Science (TUMS), code 57148.

Conflict of interest

The authors declare that there is no conflict of interests.

References

1. Djihinto OY, Medjigbodo AA, Gangbadja ARA, et al. Malaria-transmitting vectors microbiota: Overview and interactions with Anopheles mosquito biology. Front Microbiol. 2022; 13:891573.
2. world-malaria-report-2024. Available from: https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2024
3. Gallup JL, Sachs JD. The economic burden of malaria. CID Working Paper No 52. 2000;
4. White N, Pukrittayakamee S, Hien T, Faiz M, Mokuolu O, Dondorp A. Erratum: Malaria Lancet. 2014; 383(9918):723-35.
5. Mita T, Jombart T. Patterns and dynamics of genetic diversity in *P. falciparum*: what past human migrations tell us about malaria. Parasitol Int. 2015;64(3):238–43.
6. Ataba E, Dorkenoo AM, Nguepou CT, et al. Potential emergence of *Plasmodium* resistance to artemisinin induced by the use of Artemisia annua for malaria and COVID-19 prevention in Sub-African Region. Acta Parasitol. 2022; 67(1):55-60.
7. Heidari A, Keshavarz H. The drug resistance of *Plasmodium falciparum* and P. vivax in Iran: a review article. Iran J Parasitol. 2021;16(2):173-185.
8. Million E, Mulugeta T, Umeta B. Traditional medicine practice and its role in the management of malaria in jimma town, Oromia, Ethiopia. Infect Drug Resist. 2022; 2022:2187–98.
9. Appiah EO, Appiah S, Oti-Boadi E, et al. Practices of herbal management of malaria among trading mothers in Shai Osudoku

District, Accra. PLoS One. 2022; 17(7):e0271669.
10. Olasehinde GI, Ojurongbe O, Adeyeba AO, et al. In vitro studies on the sensitivity pattern of *Plasmodium falciparum* to anti-malarial drugs and local herbal extracts. Malar J. 2014; 13:63.
11. Agarwal N, Kolba N, Jung Y, Cheng J, Tako E. Saffron (*Crocus sativus L.*) flower water extract disrupts the cecal microbiome, brush border membrane functionality, and morphology in vivo (gallus gallus). Nutrients. 2022;14(1):220.
12. Samarghandian S, Borji A. Anticarcinogenic effect of saffron (*Crocus sativus L.*) and its ingredients. Pharmacognosy Res. 2014;6(2):99-107.
13. Khorasany AR, Hosseinzadeh H. Therapeutic effects of saffron (*Crocus sativus* L.) in digestive disorders: a review. Iran J Basic Med Sci. 2016;19(5):455-69.
14. Li B, Elango J, Wu W. Recent advancement of molecular structure and biomaterial function of chitosan from marine organisms for pharmaceutical and nutraceutical application. Appl Sci. 2020;10(14):4719.
15. Rashedi J, Haghjo AG, Abbasi MM, et al. Anti-tumor effect of quercetin loaded chitosan nanoparticles on induced colon cancer in wistar rats. Adv Pharm Bull. 2019;9(3):409-415.
16. M. Ways TM, Lau WM, Khutoryanskiy VV. Chitosan and its derivatives for application in mucoadhesive drug delivery systems. Polymers (Basel). 2018;10(3):267.
17. Baharlouei P, Rahman A. Chitin and chitosan: Prospective biomedical applications in drug delivery, cancer treatment, and wound healing. Mar Drugs. 2022;20(7):460.
18. Li J, Cai C, Li J, et al. Chitosan-based nanomaterials for drug delivery. Molecules. 2018;23(10):2661.
19. Kremsner PG, Krishna S. Antimalarial combinations. Lancet. 2004;364(9430):285–94.
20. Alven S, Aderibigbe B. Combination therapy strategies for the treatment of malaria. Molecules. 2019;24(19):3601.
21. Global Partnership to Roll Back Malaria. (2001). Antimalarial drug combination therapy: report of a WHO technical consultation, 4-5 April 2001. World Health Organization.
22. Elmi T, Rahimi Esboei B, Sadeghi F, et al. In vitro antiprotozoal effects of nano-chitosan on *Plasmodium falciparum*, *Giardia lamblia* and *Trichomonas vaginalis*. Acta Parasitol. 2021; 66:39–52.
23. Kamiloglu S, Sari G, Ozdal T, Capanoglu E. Guidelines for cell viability assays. Food Front. 2020;1(3):332–49.
24. Hajialiani F, Elmi T, Mohamadi M, et al. Analysis of the active fraction of Iranian Naja naja oxiana snake venom on the metabolite profiles of the malaria parasite by 1HNMR in vitro. Iran J Basic Med Sci. 2020;23(4):534-543.
25. Mainardes RM, Evangelista RC. PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution. Int J Pharm. 2005;290(1–2):137–44.
26. Bhattacharyya N, Mukherjee H, Bose D, et al. Clinical case of artesunate resistant *Plasmodium falciparum* malaria in Kolkata: A first report. J Trop Dis. 2014;2(128):2.
27. Roux AT, Maharaj L, Oyegoke O, et al. Chloroquine and sulfadoxine–pyrimethamine resistance in Sub-Saharan Africa—A review. Front Genet. 2021; 12:668574.
28. Retno S, Widyawaruyanti A, Anindita FBT, Astuti SK, Setyawan D. Development of andrographolide-carboxymethyl chitosan nanoparticles: characterization, in vitro release and in vivo antimalarial activity study. Turk J Pharm Sci. 2018;15(2):136-141.
29. Momenfam F, Nateghpour M, Haghi AM, et al. Interaction between chitosan and chloroquine against *Plasmodium berghei* and *P. falciparum* using in-vivo and in-vitro tests. Iran J Parasitol. 2021; 16(2):261-269.
30. Zein U, Fitri LE, Saragih A. Comparative study of antimalarial effect of sambiloto (*Andrographis paniculata*) extract, chloroquine and artemisinin and their combination against *Plasmodium falciparum* in-vitro. Acta Med Indones. 2013;45(1):38-43.
31. Popović-Djordjević JB, Kostić AŽ, Kiralan M. Antioxidant activities of bioactive compounds and various extracts obtained

from saffron. In: Saffron. Elsevier; 2021. p. 41–97.
32. Azghandi Fardaghi A, Es-haghi A, Feizy J, Lakshmipathy R. Antioxidant capacity and chemical composition of different parts of saffron flowers. Journal of Food and Bioprocess Engineering. 2021;4(1):69–74.
33. Sacco P, Pedroso-Santana S, Kumar Y, Joly N, Martin P, Bocchetta P. Ionotropic gelation of chitosan flat structures and potential applications. Molecules. 2021;26(3):660.
34. Patel B, Tripathi Sk, Shukla S, Pandey A. Preparation, characterization and in vitro drug release study of chloroquine phosphate-loaded chitosan nanoparticles as malaria treatment. Oxid Commun. 2021;44(4).