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Original Article

Targeting and Inhibiting *Plasmodium berghei* Growth in Balb/c Mice Using Kojic Acid-Solid Lipid Nanoparticles and Kojic Acid-Nanostructured Lipid Carriers

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Abstract

Background and Aim: Malaria is a life-threatening infection in the world. The emergence of strains of *Plasmodium* species that are resistant to anti-parasitic drugs, and the lack of licensed high-performance malaria vaccines have raised serious concerns worldwide. In recent years, new treatment strategies such as nanoformulations have been suggested as effective drug delivery systems to enhance the therapeutic efficiency of various drugs.

Materials and Methods: In this study, kojic acid-solid lipid nanoparticles (KA-SLNs) and kojic acid-nanostructured lipid carriers (KA-NLCs) were synthesized using high-speed homogenization and ultra-probe sonication methods to improve their antiplasmodial activities. The obtained nanoformulations were evaluated against the *Plasmodium berghei* malaria parasite in mice. Anti-plasmodium activities and cytotoxicity of the nanoparticles were assed. Fifty percent effective dose (ED50) was calculated as well. Moreover, ex vivo human red blood cells (RBCs) hemolysis was assessed.

Results: Kojic acid solution was significantly effective in all concentrations on the seventh day (D7) and the tenth day (D10) (P. value <0.05). The toxicity test revealed no toxic impact on the subjects. ED50 was obtained at 150 mg/kg concentration for KA-NLCs and 400 mg/kg concentration for KA-SLNs on D10. The results of the evaluation of KA nanoformulations and KA solution on RBCs indicated that KA nanoformulations could reduce the lysis of RBCs. These results also showed that the lysis of RBCs increased with raising drug concentration in KA nanoformulations, and KA-NLCs (100 mg/kg) gave the least lysis. KA nanoformulations (especially KA-NLCs) and KA solution significantly reduced parasite growth.

Conclusion: These results revealed that the KA solution was safe and had no side effects on the subjects in the range of evaluated concentrations. Moreover, the results of this study showed that KA nanoformulations had a better therapeutic effect on *Plasmodium berghei* in Balb/c mice compared with KA solution.

Keywords: Malaria, *Plasmodium berghei*, Kojic acid, Nanoparticles, Nanostructure

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Introduction

The malaria parasite is known to be one of the lethal pathogens whose victims are mostly children and pregnant women. This disease is caused by attacks and reproduction of parasites inside red blood cells. *Plasmodium falciparum* is known as the most dangerous form of malaria. According to the World Health Organization (WHO), in 2020, about 228 million malaria cases occurred, of which 409,000 people died. It was announced in this report that African regions had the highest rate (about 94%) (1).

One of the deadliest types of malaria, which mostly infects mice, is *P. berghei.* This species has many metabolic and pathogenic properties as to human plasmodia. That is why *P. berghei* can be used in many in vivo laboratory studies as a model for human malaria studies (2,3). Resistance of malaria parasites to chemical drugs (first to chloroquine and then to pyrimethamine, sulfadoxine, and others) is one of the major challenges that has claimed many people's lives in communities (4). This drug resistance has prompted many communities to seek alternative drugs. Herbal extracts and natural remedies can be perfect choices for developing countries due to their cost-effectiveness and low side effects (5).

Kojic acid (5-hydroxy-2-hydroxymethyl-4H-4-pyranone), discovered by K. Saito in 1907, is an antibiotic secreted by several species of fungi such as Aspergillus and Penicillium. It has its anti-microbial, anti-inflammatory, anti-cancer, anti-viral, anti-parasitic, anti-diabetic, insecticidal, and detoxification properties. Studies on KA have shown that it can help activate macrophages and phagocytic processes, and can increase the production of superoxide, reactive oxygen species, and nitric oxide (6, 7). Due to the poor biopharmaceutical properties of KA and storage instability, applications of KA in traditional drug formulations and its use in clinical trials are limited (8, 9). To eliminate the problems associated with the biological and clinical challenges of various drug formulations, new drug delivery systems called nanoparticles have been designed that have better properties than traditional drug formulations. Indeed, nanoformulations are valuable tools for drug delivery applications to achieve specific therapeutic targets. Lipid materials are used in many pharmaceutical industries for preparation of different kinds of formulations such as lotions, ointments, emulsions and gelatins. A review of literature on nanoformulations such as solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) showed that these nanosystems could have promising therapeutic perspectives in various fields of medicine due to their non-toxic and biocompatible properties and also because they contribute to the easy production of formulations (10-16).

The aim of this study was to investigate whether SLN and NLC nanoformulations could be promising alternative approaches to target and inhibit *Plasmodium berghei* growth in Balb/c mice. However, many studies have been conducted in various regions of the world to determine the effect of KA on various parasites such as *Toxoplasma* and *Giardia lamblia* and some bacteria and viruses (6, 17-21). It should be noted that this is the first *in vitro*, *ex vivo*, and *in vivo* study on the effect of KA on *Plasmodium berghei*.

Materials and Methods

Oleic acid (OA), absolute methanol, absolute ethanol, and KA were purchased from Merck Co. Germany with catalogue No. 8184610010. Giemsa stain, chloroquine, cholesterol (Chol), and Tween 20 were supplied from Merck (Merck Co., Germany). Span 60 was obtained from Daejung (Daejung Chemicals & Metals Co., Ltd. Korea). Glyceryl monostearate (GMS) was received by Gattefossé (France). Deionized water was produced using a Human power 2 system (human Co., Korea). Alanine aminotransferase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) were assessed using commercial kits from Pars Azmoon Company (Tehran, Iran).

Preparation of KA-SLNs & KA-NLCs

KA-SLN & KA-NLC nanoformulations were prepared by high-speed homogenization and ultra-probe sonication methods, which have been described in our previous papers (8, 9). Since the aim of the present study was to evaluate the anti-plasmodium activity of the synthesized KA-SLNs & KA-NLCs nanoformulations, further details on the preparation of KA-SLNs & KA-NLCs nanoformulations were not described here. Briefly, the lipid phase was prepared by mixing GMS, Cho, and Span 60 by heating at 95 °C in a closed container. Meanwhile, a mixture of KA and 2/3 of the aqueous phase containing deionized water and Tween 20 was stirred continuously on a heater-stirrer at 95 °C until KA was dissolved entirely in it, and a clear and homogenous solution was formed. Subsequently, it was slowly added to the lipid phase and stirred for 2 minutes. Then, an ultrasonic processor was performed immediately using a high-intensity sonicator probe (Bandelin, 3100, Germany) with this mixture for 5 min (100% amplitude). The resulting emulsion was then transferred to an ice-water bath. At this stage, the remaining one-third of the aqueous phase, including deionized water and Tween 20, was added to the emulsion and placed in an ice-water bath for 30 minutes. It was then transferred to the ultrasonic device under sonication (100% amplitude) for 5 min. Finally, the resulting emulsion was transferred to an ice bath for 30 min to form KA-SLNs. In all steps in the synthesis of KA-NLCs nanoformulations, oleic acid was added to solid lipids (GMS and Chol) in a certain amount as liquid lipids.

Determination of Physicochemical Characterizations

DLS (dynamic light scattering) analyses, including the mean particle size, polydispersity index (PDI), and zeta potential of KA-SLNs & KA-NLCs were performed using a Zetasizer Nano ZS (Malvern Instruments, UK).

Entrapment efficiency percent (EE%) and drug loading percent (DL%) values of KA-SLNs & KA-NLCs were monitored by UV-visible spectroscopy (UV-Vis) and were calculated using the following equations:

$$E\% = \frac{WInitial\ drug - WFree\ drug}{Winitial\ drug} \times 100$$

$$DL\% = (WInitial\ drug - WFree\ drug)/WLipid \times 100$$

where $W_{Initial\ drug}$ is the total amount of KA incorporated in the KA-SLNs & KA-NLCs nanoformulations and $W_{Free\ drug}$ is the amount of KA observed in the supernatant after centrifugation of the KA-SLNs & KA-NLCs nanoformulations.

Morphology

Transmission electron microscope (TEM) imaging method (912AB, Leo, England) was used to assess the size, morphological structure, and dispersion quality of the KA-SLNs & KA-NLCs within the matrix.

Attenuated Total Reflectance-Fourier Transform Infra-Red Spectroscopy (ATR-FTIR)

FTIR spectrophotometer (Agilent CARY 630 ATR-FTIR) method was used to detect intermolecular interactions between excipients and KA and to evaluate the amorphous state of the nanoparticles.

Differential Scanning Calorimetry (DSC)

DSC method was used to ensure the encapsulation of KA in SLN& NLC and to evaluate matrix structure and thermal behavior studies, including the enthalpy and melting temperature changes, and recrystallization behavior of KA-SLNs & KA-NLCs nanoformulations.

Powder X-Ray Diffraction (PXRD)

PXRD analysis (PHILIPS-PW1730; Netherland) (40 kV; 30 mA) was performed on the samples to investigate the crystal lattice structure and polymorphic behavior in the component structure after the preparation of KA-SLNs & KA-NLCs nanoformulations.

Drug Release

In vitro release studies from KA-SLNs & KA-NLCs nanoformulations were conducted to evaluate the KA release rate profile of SLNs & NLCs and pure KA for 24 h at 37 °C.

Experimental Procedure

Laboratory Animals

Fifty male BALB / c mice weighing approximately 20 g (7-8 weeks old) were used in this study. These mice were purchased from Razi Institute in Karaj and divided into 10 groups (n = 5 each) for in vivo studies. This study was conducted based on the organizational Animal Research Center of Tehran University of Medical Sciences guidelines. The experimental protocols for usinganimals in this study were approved

by the Ethics Committee of Tehran University of Medical Sciences (Permit number IR.TUMS.VCR.REC.1398.869).

Parasites

The parasite used in this study was *Plasmodium berghei* (Indian strain) NICD (amplified in the National Malaria Laboratory, School of Public Health, Tehran University of Medical Sciences).

Infecting the Studied Mice with *P. berghei* Parasite

First, 10^6 parasitized erythrocytes were intraperitoneally injected as a suspension in physiological serum with a final volume of 0.2 ml. After a few days, blood was collected from plasmodium-infected mice using a syringe containing sodium citrate, and then the percentage of parasitemia was calculated. It was then diluted with physiological saline. In fact, with this dilution, 10^6 erythrocytes of the parasite were present in every 0.2 ml of the suspension, which was intraperitoneally injected into the studied mice.

Treatment of the Infected Mice

In this study, KA solution at a concentration of 400 mg/kg, and KA-SLNs & KA-NLCs nanoformulations at the concentrations of 100, 200 and 400 were used to treat the infected mice. Physiological serum was used to dilute the studied drugs.

Five mice were specified for each concentration of drugs. Treatment started two hours after the parasite injection and lasted for 4 days. To inject the drug into the studied mice, 0.2 cc of each drug concentration was subcutaneously injected into the mice using a syringe, and the treatment of the mice continued for up to four days. In this study, to evaluate the cytotoxic effect of the studied drugs, a concentration of 800 mg/kg KA was injected into the mice until the fourteenth day, and then the cytotoxic effect of the drug was determined by examining the length of the liver and spleen as well as the number of liver enzymes.

Different Types of Treatments Provided for Each Group of the Mice

Treatment was pursued subcutaneously for 4 days using concentrations of 100, 200 and 400, mg/kg of nanostructured liquid carriers of kojic acid to groups 1 to 3, and 100, 200 and 400 Solid Lipid Nanoparticles of KA to groups 4 to 6, respectively. Moreover, solid (non-Nano) KA in the concentration of 400 mg/kg was injected into group 7, and 20 mg/kg chloroquine was injected into group 8 as the positive control (gold standard), respectively. Group 9 received physiological serum as a placebo and remained the negative group (untreated group). Group 10 was left in the animal house without infection and treatment (Mortality group).

Preparing Blood Blides

In this study, after anesthesia, blood samples were taken from the mice infected with *P. berghei* in two ways, i.e. blood sampling from the tail of the mouse and using a 2 mm syringe. Blood sampling was performed on the seventh, tenth, and fourteenth days. Then, a drop of the desired blood was poured on the slide and a thin smear was prepared. At this stage, the features of every mouse were recorded on the slide with a pencil, and then the slides were stained with Giemsa stain.

Staining Method of the Blood Slides

This method uses concentrated Giemsa dye in a ratio of 3-5% with water and pH 7-7.2 for dilution. Five ml of diluted Giemsa solution was poured on each slide to completely cover the slide. Staining time by Giemsa stain was 30-45 minutes. Afterwards, the solution of Giemsa stain was washed with a gentle stream of water from the slide and was placed diagonally on the grooved board to dry. The parasite was then detected in the blood using immersion oil and microscopy methods.

Parasite Counting

To determine the degree of parasitemia, the number of parasites was counted as a percentage against 10000 red blood cells, and all the infected red blood cells were counted according to the following equation:

$$\text{Parasite } \% = \frac{\text{number of infected erythrocytes}}{10000 \text{ RBCs}} \times 100$$

Assessing the Therapeutic Activity of KA Nanoformulation on *P. berghei*

The therapeutic effect of KA on *P. berghei* mice was evaluated using the peters method, and the most effective drug concentration was determined according to the degree of toxicity, reduction of parasitaemia, and increase in growth inhibition percentage compared with chloroquine (22).

Measurement of Liver and Spleen Length and Liver Enzymes of the Mice

Blood samples were taken from all the mice to evaluate liver enzymes on day D14, and serum was isolated from the cells for 3 minutes using a centrifuge at 2500 rpm. The serums were refrigerated at – 20 °C. During the analysis of enzymes, serums were removed from the refrigerator and the values of serum liver parameters, including AST, ALT, and ALP were tested using commercial kits of Pars Azmoon Company (Tehran, Iran) and a biochemical analyzer (Biotecnica, Targa 3000, Rome, Italy).

To measure the length of the mice's liver and spleen, we used scissors to open the mice's chest, and the mice's liver as well as spleen were separated. Then, their length was measured using a caliper.

Evaluation of Human Blood Hemolysis Using the Studied Drugs

To determine the safety of the nanoformulations and KA solution, a hemolysis test was performed on red blood cells. For this purpose, we washed 5 ml of human venous blood in the laboratory three times using the physiological serum. Seven ml of physiological serum was added to the blood and after mixing, it was centrifuged at 600 rpm for 5 minutes, and this process was repeated three times. Two hundred microliters of counted red blood cells were added to 96 well plate wells. The studied drugs were added to different wells at different concentrations of 200 µl. Standard hemolytic drug and PBS solution were added to the wells of the positive and negative control groups, respectively. The plate was placed at 37 ° C for 24 h, and then the number of red blood cells was counted. The number of red blood cells before and after the effect of different drug concentrations was counted using a neobar slide.

ED50 Calculation

The fifty percent effective dose (ED50) was used to evaluate the volume dose required for KA nano-formulation to kill 50% of parasites in mice or inhibit 50% of parasite growth in *in-vitro* compared with the control group. The percentage of parasite growth was calculated according to the following equation:

$$\text{Inhibition of Parasite Growth\%} = \frac{\text{Parasitemia rate in control group} - \text{test group}}{\text{Parasitemia rate in control group}} \times 100$$

To determine the effective dose of fifty (ED50), the required concentrations of KA nanoformulation (100, 200 and 400 mg/kg) were prepared and nano drugs were applied for the bioassay on *P. berghei*-infected mice.

***In vitro* Biocompatibility and Cytotoxicity Assays**

The mice in the cytotoxic group were treated with the highest dose of nanoformulations (800 mg/kg nanoformulation) once up to 14 days. The health status of the mice (in terms of diarrhea, weight loss, clarity and opacity, necrosis at the injection site, and mortality) was regularly assessed. Then, the animals were dissected at the end of the work. To find any toxic effect of the drug, the size of the liver, spleen, and liver enzymes in the mice were measured.

Statistical Analysis

All the statistical data, including Kolmogorov-Smirnov Z, one-way analysis of variance (abbreviated one-way ANOVA), Post Hoc analysis, and Kruskal–Wallis test, were statistically computed using SPSS software (version 16).

This research was conducted according to institutional animal ethics guidelines of the Animal Research Center, Tehran University of Medical Sciences. The experimental protocols for animal use in this study were approved by the Ethics Committee of Tehran University of Medical Sciences (Permit number IR.TUMS.VCR.REC.1398.869).

Results and Discussion

Characterization of KA-SLNs & KA-NLCs Nanoformulations and Morphology Evaluation

Selection of the appropriate lipid/s for KA-SLNs & KA-NLCs nanoformulations was the key step for obtaining the desired qualities of KA nanoformulation, including particle size, PDI, zeta potential, entrapment efficacy, and drug loading.

The optimized KA-SLNs had a particle size of 156.97 ± 7.15 nm, polydispersity index (PDI) of 0.388 ± 0.004, zeta potential (ZP) of -27.67 ± 1.89 mV, entrapment efficiency of $59.02 \pm 0.74\%$, and drug loading $14.755 \pm 1.63\%$.

The optimized KA-NLCs had a particle size of 172.9 ± 7.1 nm, polydispersity index (PDI) of 0.3 ± 0.1, zeta potential (ZP) of -39.1 ± 2.7 mV, entrapment efficiency of $76.4 \pm 0.1\%$, and drug loading $17.6 \pm 1.3\%$ (**Table 1**).

The morphology of KA-SLNs (A) & KA-NLCs (B) nanoformulations was considered under TEM. TEM

FTIR

Table 1: Nano-formulation ingredients and KA-SLNs & KA-NLCs characteristics (% w/w). The data have been represented as mean ± SD. Adapted from [8, 9].

Nano-Formula	KA-SLNs & KA-NLCs characteristics				
	Size (nm)	PDI	ZP (mv)	EE (%)	DL %
KA-NLC$_S$	172.9±7.1	0.3±0.1	-39.1±2.7	76.4±0.1	17.6± 1.3
KA-SLN$_S$	156.97±7.15	0.388±0.004	-27.67 ±1.89	59.02±0.44	14.755± 1.63

Nano-Formula	Nano-formulation ingredients (g)						
	KA (%)	Chol (%)	OA (%)	GMS (%)	T 20 (%)	Sp 60 (%)	Water (%)
KA-NLC$_S$	0.2	0.1	0.3	0.8	0.2	0.4	98.4
KA-SLN$_S$	0.2	0.05	-	0.75	0.2	0.4	98.4

KA: kojic acid; Chol: cholesterol; OA: oleic acid; BW: beeswax; GMS: glycerol monostearate; T 20: tween 20; Sp 60: span 60; PDI: polydispersity index; ZP: zeta

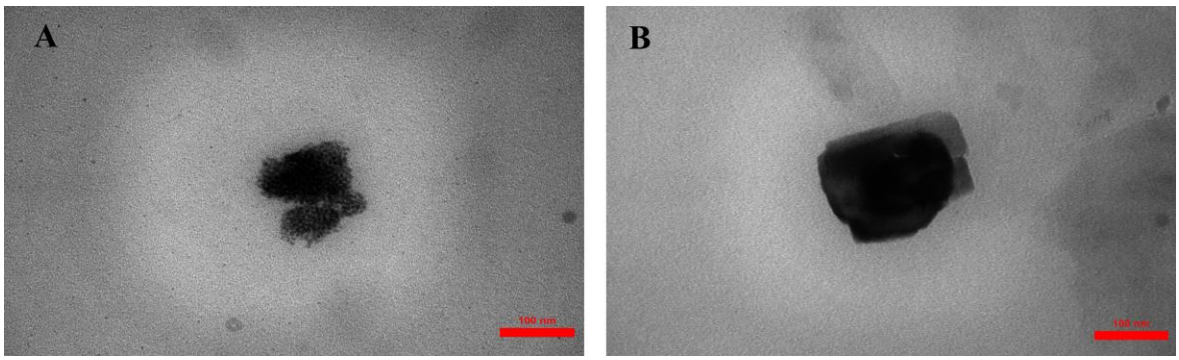

Figure 1: TEM micrographs of KA-SLNs (A) & KA-NLCs (B) nanoformulations

images showed that the KA-SLNs & KA-NLCs nanoformulations had colloidal particle size and were uniform in shape (almost spherical) with narrow size distribution. TEM images indicated that nanoformulation ingredients and preparation methods had developed stable nanoformulations (**Figure 1**).

ATR-FTIR technique is a rapid and new analytical method that can be successfully applied to evaluate loaded drugs in nanoparticles and investigate any interaction between the drug and various ingredients of nanoparticles. According to the spectra obtained of the method, no interaction was observed between the drug and its components in nanoformulations. Previous investigations have shown that in the ATR-spectrum of KA-SLNs & KA-NLCs nanoformulations, the main peaks of KA (C=O and C=C groups) were still observable, and there was no shift in these peaks, which were valid evidence of good drug encapsulation in nanoformulation. DSC thermograms and the XRD diffract grams of the pure KA and ingredients of KA-SLNs & KA-NLCs nanoformulations exhibited sharp and distinct peaks, which corroborated their intense crystalline nature. The disappearance of the crystalline state of KA and its appearance in an amorphous state in XRD and DSC patterns of both nanoformulations are strong reasons that the KA is dispersed molecularly in the structure of nanoformulations (8, 9).

According to the results of DSC, XRD, and ATR-FTIR

patterns, it can be concluded that KA had a good loading capacity in the lipid matrix of KA-SLNs & KA-NLCs nanoformulations without any chemical interaction with the ingredients of these nanoformulations. Moreover, these nanoformulations showed that the SLN & NLCs released KA at a sustained and slower release rate for 24 h and could considerably enhance the *in vivo* antiplasmodial activity of KA (8, 9).

Kolmogorov-Smirnov Test in Mice Treated with KA

In this test, a P-value > 0.05 shows that the data distribution is normal, and a P-value < 0.05 indicates that the data distribution is abnormal. According to the data obtained from the Kolmogorov-Smirnov test (**Table 2**), the data distribution of the length of the mice's liver and spleen was normal in D7 parasitemia and data distribution of liver enzymes (AST, ALT, and ALP) was abnormal in D10 parasitism.

The levels of parasitemia and growth inhibition in the nine groups of mice studied with different concentrations of KA nanoformulations and KA solution have been presented in **table 3**. The 10th group was kept in the animal house without infection or treatment (Mortality group). There was not any mortality rate in this group.

Table 2: Kolmogorov-Smirnov Test.

group	Kolmogorov Smirnov	P-value
Parasitism on the seventh day	0.102	0.2
Parasitism on the tenth day	0.139	0.026
Liver length	0.099	0.2
Spleen length	0.095	0.2
ALT	0.261	< 0.001
AST	0.170	0.01
ALP	0.199	0.001

The data that have been illustrated in Table 3 indicate that there were remarkable distinctions among the studied groups on D7 (P-value <0.05). According to the post-hoc test, there was a substantial relationship between the groups without treatment and those treated with KA (P-value <0.05), which showed that the rate of parasitemia in the groups treated with KA on D7 was less than the groups without treatment.

Due to non-parametric data, statistical analysis of parasitemia in the mice treated with KA on the 10th day was performed using *Kruskal-Wallis* statistical test. There was a significant difference between the groups without treatment and those treated with KA (P-value <0.05). According to the post-hoc study, there was a meaningful relationship between the untreated group and other groups (P-value <0.05). It can be concluded that all drug concentrations of D10 had positive effects on parasite reduction.

In the present study, ED50 was determined in the mice treated with different concentrations of KA nanoformulations on D10. According to the test results, ED50 for KA-NLCs and KA-SLNs were determined at 150 mg/kg and 400 mg/ kg, respectively. The data for this report have been presented in **figure 2** for KA-NLCs and in Table 3 for KA-SLNs. This amount of nanoformulations can decrease 50% of parasites in the host.

As **Figure 3** shows, there was a significant difference between the liver and spleen lengths in the studied mice (P-value <0.05). According to the post-hoc test, there was a significant relationship between the negative control group and most groups treated with drug concentrations (P<0.05). Moreover, there was no significant relationship between the cytotoxic group

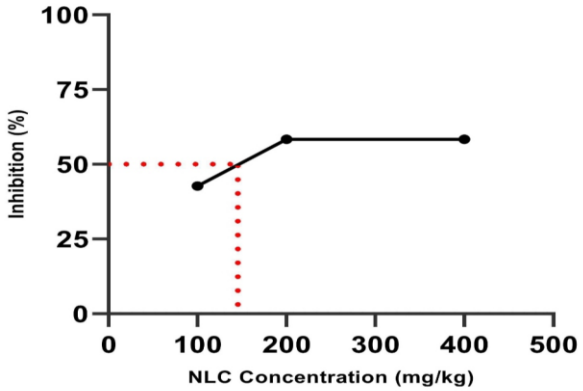

Figure 2: ED50 on D10 for KA-NLCs.

Table 3: Percentage of Parasitemia and Growth Inhibition in the Studied Mice with Different Concentrations of KA Nanoformulations and KA Solution Compared with the Chloroquine Treatment Control Group and the Placebo Control Group on D7 and D10.

Group	Treatment	Concentration Used	Parasitemia on D7 (%)	Growth Inhibition (%) on D7	Parasitemia on D10 (%)	Growth Inhibition (%) on D10
1	mg/kg NLC	100	0.04 ± 0.02	66.7	13.75±1.49	42.71
2		200	0.035 ± 0.03	70.9	10± 2.1	58.34
3		400	0.04 ± 0.042	66.7	10±3.09	58.34
4	mg/kg SLN	100	0.06 ± 0.023	50	11±1.58	54.2
5		200	0.061 ± 0.036	49.17	11.2±4.12	53.34
6		400	0.06 ± 0.029	50	12±2.14	50
7	mg/kg KA solution	400	0.07 ± 0.023	41.7	14±3.46	41.67
8	mg/kg Chloroquine	20	0	100	0	100
9	without treatment		0.12 ± 0.062	0	24±1.41	0

and other groups (P>0.05). It can be concluded that the drug did not have any toxic effect on the liver. According to the Post hoc test in Figure 3 about spleen evaluation, there is no significant relationship between the negative control group and most of the groups treated with drug concentrations (P>0.05).

The evaluation of KA nanoformulation, KA solution and KA-NLCs on RBCs showed that the KA-NLCs had less lysis effect than the KA on RBCs. This test also showed that the lysis of RBCs increased with increasing drug concentration in KA nanoformulations. At a concentration of 800 mg/kg of the drug, 100 % lysis of

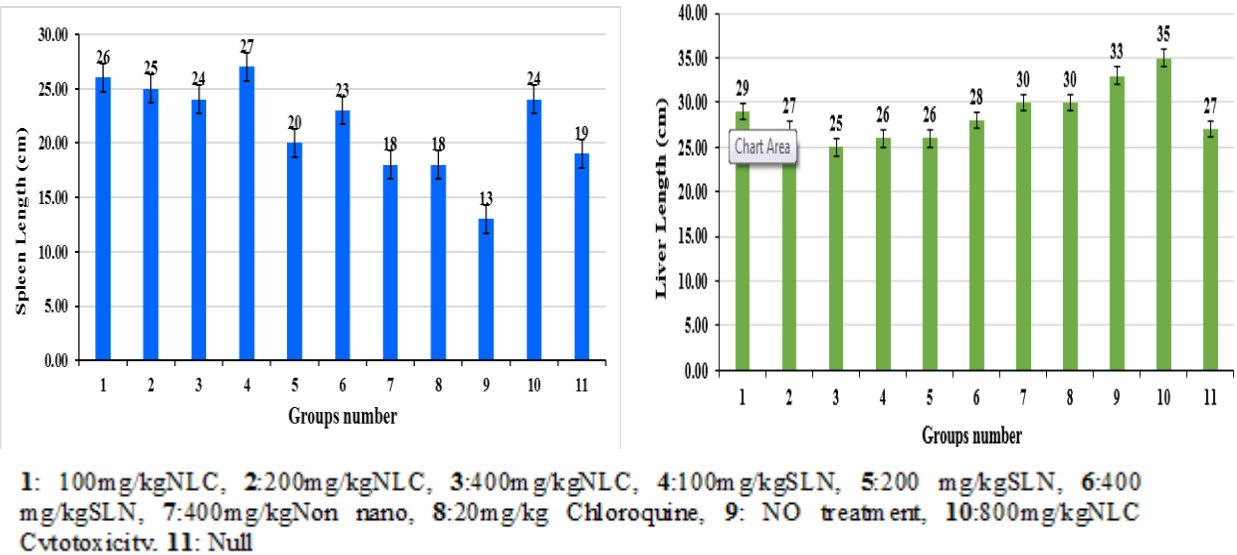

Figure 3: Graphs of Mean Liver and Spleen Length (in millimeters) Measured from the Mice Treated with KA Nanoformulations and KA Solution Comparison with the Groups Treated with Chloroquine, Placebo, and the Cytotoxic Substance.

Table 4: Assessment of the Hemolysis Effect (%) of KA Nanoformulations and KA Solution Compared with the Negative Control (Placebo Hemolysis) and Positive Control (Distilled Water Hemolysis) Using *in vitro* Method.

Group	Concentrations	Erythrocyte Hemolysis%
1	100 mg/kg NLC	10
2	200 mg/kg NLC	15
3	400 mg/kg NLC	29
4	100 mg/kg SLN	32
5	200 mg/kg SLN	47
6	400 mg/kg SLN	58
7	400 mg/kg KA	100
8	Negative control (hemolysis with placebo)	0
9	Positive control (hemolysis with distilled water)	100

RBCs in KA-SLNs was observed, probably because of the presence of solid particles in the KA-SLNs (Table 4).

The Kruskal-Wallis test was used to statistically evaluate the results of liver enzyme tests (AST, ALT, and ALP). The studied groups had no significant differences (P-value> 0.005). It can be concluded that the drug did not affect the change of AST, ALT, and ALP enzymes compared with the untreated group. Furthermore, by comparing the cytotoxic group with the treated groups, it could be concluded that the drug did not have any toxic effect on AST, ALT, and ALP enzymes. Moreover, in cytotoxicity test, no suspicious or negative cases were observed after careful evaluation of the mice in terms of diarrhea, weight loss, clarity and opacity, necrosis at the injection site, and mortality. Measurements of liver size, spleen, and liver enzymes showed that the drugs had no toxic effect on the spleen, liver, and liver enzymes but had a small negative effect on size (**Figure 4**).

Due to the prevalence of drug resistance in the treatment of malaria, the discovery and synthesis of new drugs with high anti-parasitic properties and minimal side effects has been carried out, and some efforts have resulted in the development of novel drugs (4). Nevertheless, it seems that it is still necessary to develop more new drugs. Emergence of drug resistance against the treatment of malaria refers to the ability of different strains of the parasite to survive and multiply in the presence of various concentrations of the drug. In the case of non-drug resistance, these drug concentrations can kill or inhibit the growth of parasites. Increased drug-resistant parasite lines have increased malaria mortality, particularly in children. *Plasmodium falciparum* causes the most acute form of malaria and bears the highest parasitemia rate. *Plasmodium falciparum* resistance to chloroquine and other antimalarial drugs has become common in most malaria-prone areas.

A literature review on herbal and natural medicines and their therapeutic activities showed that natural antiparasitic drugs have noticeable therapeutic potential against different parasites such as the parasites that cause malaria. Due to the unique anti-parasitic properties of natural agents, fewer side effects, cheapness, availability, and low cytotoxicity, the World Health Organization has recommended finding the therapeutic potential of plants in any region with anti-parasitic properties (5).

Low solubility of some drugs in water, low bioavailability, chemical instability in biological fluids, and short biological half-life in the plasma of new drug molecules are among the most challenging issues in this field. To resolve such problems, developing new drug delivery systems facilitates overcoming these difficulties. Recently, the development of new drug formulations using nanotechnology has made it possible to achieve intelligent treatment targeted by drug nanoparticles with unique properties such as greater effectiveness and variety in drug forms. Precise formulation of these nanoparticles leads to greater drug stability. It increases the dissolution rate to reach the appropriate biological levels, thus accelerating the

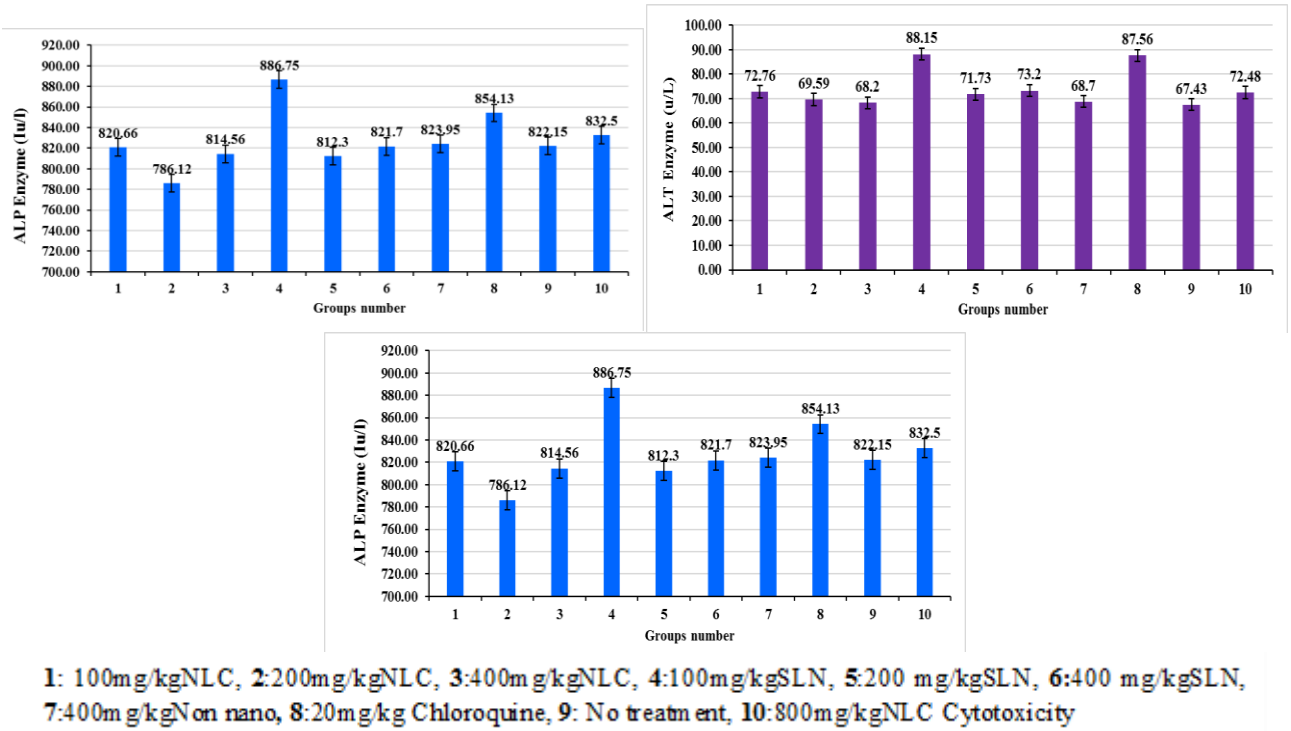

Figure 4: Diagrams of Liver Enzymes Measured from the Mice Treated with KA Nanoformulations and KA Solution and the Groups Treated with Chloroquine, Placebo, and the Cytotoxic Substance.

therapeutic effects of drugs and improving their bioavailability.

This study aimed to investigate the impact of KA-SLNs and KA-NLCs nanoformulations on the inhibition of *Plasmodium berghei* growth in Balb/c mice and to compare it with chloroquine. This research enhances knowledge about the use of nano-natural formulations as an eco-friendly and green strategy in parasitology. It allows us to use KA-SLNs and KA-NLCs nanoformulations as a potential therapeutic candidate for managing and inhibiting *Plasmodium berghei* growth in Balb/c mice. In this investigation, we have reported the findings of an in vivo study on the efficiency of KA-SLNs and KA-NLCs nanoformulations in inhibiting *Plasmodium berghei* growth in Balb/c mice.

According to the graphs, all the concentrations of KA reduced the number of parasites compared with the control group injected with a placebo. The concentration of 200 mg/kg had the highest effect inhibiting the parasite's growth (70.9% on the seventh day and 58.34% on the tenth day). The 400 mg/kg concentration had the highest half-life among drug concentrations, with the lowest mortality in the mice up to the fourteenth day. It also showed the least difference in growth inhibition on the seventh and tenth days (66.7% and 58.34%, respectively) and had less hepatitis than other drug concentrations. The KA-SLNs and KA-NLCs nanoformulations had more beneficial effects than the KA solution and also the KA-NLCs was more effective than the KA-SLNs on the growth of the parasite.

In the post-hack statistical study, there was a significant relationship between the untreated group and those groups that were treated with KA nanoformulations in terms of liver length (p-value <0.05). It was found that KA nanoformulations at all concentrations effectively reduced liver inflammation compared with the untreated group.

In a study, Motevalli *et al*. evaluated the efficiency of the ethanol extract of *Peganum harmala* L. seeds on *Plasmodium berghei* growth inhibition in saurian mice and compared it with chloroquine. This study showed that a concentration of 100 mg/kg of this extract could be introduced as the most effective concentration on the parasite. Although this extract at concentrations above 100 mg/kg had a high lethal effect on the parasite, but it also had many toxic effects on the host. Thus, 100

mg/kg concentration was considered the best. In that study, the ethanol extract of *Peganum harmala* L. seeds on the fourth and seventh days prevented 70% and 48% of parasite growth, respectively (23). However, in the present study, the concentration of 400 mg/kg KA-NLCs inhibited the growth of the parasite 66.7% and 58.34% on the seventh and tenth days, respectively.

In another study that was conducted by Karbalaei Pazoki *et al*., using the ethanolic extract of *Artemisia annua* on *Plasmodium* berghei in white mice indicated that the concentration of 1100 mg/kg could inhibit 67.7% and 14% of the growth of the parasite on the fourth and seventh days of treatment, respectively (24).

In a study conducted by Khodadadi *et al*., using the ethanolic extract of *Artemisia aucheri* against chloroquine sensitive strain of *Plasmodium berghei* in Sourian mice showed that the concentration of 1000 mg/kg was the best concentration to reduce parasitemia in mice. At higher concentrations, although the rate of growth inhibition was higher, toxic effects have been evident at these concentrations. Hence, the treatment concentration in the study was 1000 mg/kg of the extract (25).

Rodrigues *et al*. employed KA against *Leishmania (Leishmania) amazonensi* using *in vitro* and *in vivo* methods. The results of their study showed that KA (50 mg/mL) had 62% (IC50 34 mg/mL) and 79% (IC50 27.84 mg/mL) inhibitory effects on the parasite burden of promastigotes and amastigotes *in vitro*, respectively (19). However, in the present study, ED50 was obtained at 150 mg/kg for KA-NLCs and 400 mg/kg for KA-SLNs on D10.

Pestechian *et al*. evaluated the effect of the alcoholic extract of saffron fractions against *Plasmodium berghei* on small white mice in an *in vivo* study. The results of their study showed that the concentration of 700 mg/kg of ethinyl acetate extract and concentrations of 350 and 700 mg/kg of aqueous extract of the plant on the seventh day reduced the parasitemia compared with the untreated group. According to the research, although the effect of the saffron extract was less than chloroquine, this extract could be used in combination with chloroquine for more synergism (26).

Montazeri *et al*. reported that KA (100 mg/kg/day) could decrease the infection index of *Toxoplasma gondii* in experimental models of acute toxoplasmosis compared with untreated infected cells. Moreover, the survival time of the infected mice was higher by KA (100 mg/kg/day) treatment compared with the negative control groups ($P < 0.05$) (17).

Conclusion

The results of the present study revealed that KA solution was safe and had no side effects in the range of evaluated concentrations. Furthermore, our results showed that KA-SLN & KA-NLC nanoformulations had better therapeutic effects on *Plasmodium berghei* in Balb/c mice, particularly KA-NLC in the concentration of 200 mg/kg on the seventh day compared with the KA solution.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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