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Clotrimazole nanoemulsion for malaria chemotherapy. Part II: Stability assessment, *in vivo* pharmacodynamic evaluations and toxicological studies

Vivek Borhade[a,1], Sulabha Pathak[b,2], Shobhona Sharma[b,2], Vandana Patravale[a,*]

[a] Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, N.P. Marg, Matunga, Mumbai, 400019, Maharashtra, India
[b] Department of Biological Sciences, Tata Institute of Fundamental Research, Colaba, Mumbai, 400005, Maharashtra, India

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ABSTRACT

The aim of present investigation was to evaluate the potential of clotrimazole as antimalarial drug. Due to poor aqueous solubility and high lipophilicity, it was previously formulated in a nanoemulsion based system. The intrinsic effects of nanoemulsion on improvement of antimalarial activity of clotrimazole were assessed in mice infected with *Plasmodium berghei* and compared to its suspension formulation. In four-day suppressive test, mice treated with 10 mg/kg clotrimazole nanoemulsion showed the highest suppression of parasitemia and; parasitemia was significantly lower than that of 10 mg/kg clotrimazole suspension. In onset of activity and recrudescence test, percent reduction of parasitemia was significantly higher in 10 and 15 mg/kg clotrimazole nanoemulsion groups compared to 15 mg/kg suspension group. In both murine models, survival of mice treated with nanoemulsion was significantly prolonged compared to suspension at equivalent doses. The inhibition of parasite growth by clotrimazole in the nanoemulsion was dose dependent as determined by test for linear trend. In repeated dose oral toxicity, levels of serum liver enzymes and biomarkers of hepatotoxicity did not vary significantly from control. Six-month stability testing of the clotrimazole nanoemulsion exhibited no changes in various physiochemical attributes of drug product compared to initial analysis.

1. Introduction

Despite intensive research extending back to 1930s, when the first synthetic antimalarial drugs made their appearance, the repertoire of clinically licensed formulations remains very limited. The overreliance on single agent treatments progressively led to a situation in which parasites have developed resistance to most commonly used antimalarial drugs. Several of these are now greatly compromised by inexorable spread of multidrug–resistant strains (Gelb, 2007; Olliaro and Wells, 2009). In context of this, World Health Organization has recommended use of artemisinin combination therapy (ACT) as first line treatments of uncomplicated malaria (WHO, 2010). However, decline in the efficacy of such therapies have been reported in South Asia, and there is concern that this would spread to Africa, which would compromise the current ACT strategy (Bonnet et al., 2009; Dondorp et al., 2009). Further, the pharmaceutical industries have largely withdrawn from the area of parasitic diseases including malaria due to increased costs of developing and registering the drugs products, together with the prospect of inadequate commercial returns. Because of which, no new chemical class of antimalarials has been introduced into clinical practice since 1996 (Bangchang and Karbwang, 2009; Gamo et al., 2010). This current predicament accentuate for the incessant endeavors to develop new classes of antimalarials that are effective against multidrug resistant *Plasmodium* species. In this quest, the "piggyback approach", *i.e.* identification of antimalarial effects of classes of drug molecules that have already been evaluated as drug leads for other diseases represent a prospective strategy (Gelb, 2007; Bangchang and Karbwang, 2009).

Several investigators have demonstrated that extensively used antimycotic drug clotrimazole effectively and rapidly inhibit parasite growth in six different strains of *Plasmodium falciparum*, *in vitro*, irrespective of chloroquine sensitivity. The concentrations for 50% inhibition (IC_{50}) were between 0.2 and 1.1 μM. clotrimazole concentrations of 2 μM and above caused complete inhibition of parasite replication within single intraerythrocytic cycle (Saliba and Kirk, 1998; Tiffert et al., 2000). Pharmacokinetic studies on

Abbreviations: 5-NT, 5-nucleotidase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; CLT, Clotrimazole; DLS, dynamic light scattering measurements; GLDH, glutamate dehydrogenase; GST, αglutathione-S-transferase α; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; NE, nanoemulsion; SDH, sorbitol dehydrogenase; USP, United States Pharmacopeia.

* Corresponding author. Tel.: +91 22 3361 1111/2222/2217; fax: +91 22 3361 1020.
E-mail addresses: vivekborhade@gmail.com (V. Borhade), suepathak@tifr.res.in (S. Pathak), sharma@tifr.res.in (S. Sharma), vbp_muict@yahoo.co.in (V. Patravale).
[1] Tel.: +91 22 3361 1111/2222/2217; fax: +91 22 3361 1020.
[2] Tel.: +91 22 2278 2625/2865; fax: +91 22 2280 4610/4611.

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clotrimazole in healthy subjects indicate that, after single oral dose of 1 g clotrimazole (15 mg/kg), plasma levels reach mean peak concentrations of about 3.3 μM within 2–4 h of administration (Brugnara et al., 1995; Rifai et al., 1995). Infants and children have shown good absorption compared with adults (Weingartner et al., 1972). Further, a substantial concentration of clotrimazole is partioned into red blood cells (Brugnara et al., 1995). The total disappearance of parasites after 24 h exposure of ring stage to 2.5 μM clotrimazole concentrations (Tiffert et al., 2000) and its short elimination half life (~3 h) (Rifai et al., 1995) suggest that clotrimazole may be potentially effective in relatively short term treatments and may require multiple dosing over 3–4 parasite cycles for optimal *in vivo* antimalarial effects (White, 1997). Moreover, it has never been used for treatment of malaria and therefore can serve as a new molecule. These properties render it suitable for combination therapy as well. The development of fungal resistance to clotrimazole has not been observed *in vivo* and has been extremely difficult to elicit *in vitro* (Holt and Newman, 1972; Holt, 1974). Further, its short elimination half life and steep slope of concentration–response curve for parasite inhibition are additional features associated with a low probability of development of resistance (White, 1999).

Long term administration of high doses of clotrimazole (100 mg/kg) for few weeks resulted in gastrointestinal and hepatic adverse effects and; its interaction with hepatic microsomal enzymes with subsequent interference with its own metabolism (Burgess and Bodey, 1972; Bennett, 1974; Higgs, 1974). However, short term pediatric and adult administrations of high doses of clotrimazole (100 mg/kg) for treatment of mycotic infections have been well tolerated without significant adverse effects (Holt and Newman, 1972). Further in few of the clinical trials, it has been tested in humans for systemic treatment of severe Candida infections (Weuta, 1974), more recently, for systemic treatment of rheumatoid arthritis (Dennison et al., 1990) and sickle cell disease (Brugnara et al., 1996) and was well tolerated.

Taken together, this information has led our research group to evaluate the potential of clotrimazole as antimalarial drug. However, clotrimazole exhibited poor and erratic absorption from gastrointestinal tract when administered orally with marked intra and inter-individual variations (Burgess and Bodey, 1972; Brugnara et al., 1995; Rifai et al., 1995). This is attributed to its high lipophilicity ($\log P = 6.30$), poor aqueous solubility (0.49 μg/ml) and slow dissolution rate (Galichet, 2003; Budavari, 1989; Pedersen, 1993). In order to address aforementioned problem, in part I of this study, we formulated clotrimazole in nanoemulsion based drug delivery system. In this formulation, clotrimazole was stabilized and presented in solubilized form, which could eliminate absorptive barriers of low solubility and slow dissolution rate. The present paper is part II of the study, where we evaluated the antimalarial efficacy of clotrimazole in murine malaria model and assessed the intrinsic effects of nanoemulsion on the improvement of its antimalarial activity to establish the proof of concept. The study also investigated repeated dose toxicity of clotrimazole nanoemulsion with detailed assessment of hepatic adverse effects on oral administration. Furthermore, we also evaluated the stability of clotrimazole nanoemulsion as per ICH guidelines to ascertain its quality.

2. Materials and methods

2.1. Materials

Clotrimazole was obtained from Glenmark Generics Ltd., Mumbai, India. Solutol HS 15 (polyethylene glycol 660 hydroxystearate) was kindly provided by BASF India Ltd., Mumbai, India. Capryol 90 (propylene glycol monocaprylate) and Gelucire 44/14 (lauroyl polyoxylglycerides) were obtained as gift samples from Gattefosse, St-Priest, France. Methocel E5 Premium LV (hydroxypropyl methyl cellulose; HPMC 5 cP) was procured from Colorcon Asia Pvt. Ltd., Mumbai, India. HPLC grade acetonitrile and various buffer salts were purchased from s.d. Fine Chemicals, Mumbai, India. All excipients and chemicals were used as received. Freshly prepared double distilled water and buffers were filtered through 0.22 μm membrane filter (Pall India Pvt. Ltd., Ahmedabad, India) and used whenever required.

2.2. HPLC quantification of clotrimazole

A reversed phase HPLC method described in USP 30 NF 25 (USP, 2007) was modified suitably for in house analysis of clotrimazole. The HPLC system consisted of Jasco PU-2080 Plus Intelligent HPLC pump (Jasco, Tokyo, Japan) equipped with UV-2075 Intelligent UV–vis detector (Jasco, Tokyo, Japan), a Rheodyne 7725 injector (Rheodyne, Cotati, USA) and a Jasco ChromaPass Chromatography data system software (Version 1.8.6.1; Tokyo, Japan) was used. Chromatographic separation was performed on Hibar 250-4, 6, LiChrospher 60 RP-select B, 5 μm HPLC column (Merck KGaA, Darmstadt, Germany). The mobile phase consisted of acetonitrile: dibasic potassium phosphate buffer, pH 7.0 (75:25, v/v) was used. Freshly prepared mobile phase was filtered through 0.22 μm filter and degassed for 15 min before analysis. All samples were analyzed under isocratic elution at a flow rate of 1.0 ml/min, and effluent was monitored at 254 nm. A 100 μl of sample was injected onto the Rheodyne and analyzed at 25 °C. The retention time of ATQ was about 7.45 ± 0.31 min. The method was validated according to the ICH guidelines, Q2(R1) (2005). For identification of potential degradation products, a array of forced degradation tests were performed on bulk drug in accordance with the guidelines presented by International Conference on Harmonization Q3B(R2) (2006). Acidic, alkaline, oxidative, photo and thermal degradations were induced using 1 ml solution containing 1 mg/ml of clotrimazole in acetonitrile. Acid degradation was accomplished by adding 1 ml of 1 N HCl to drug solution and heated at 80 °C for 2 h. Thereafter solution was cooled and neutralized with 1 ml of 1 M NaOH. Similarly for base degradation, drug solution was mixed with 1 ml NaOH and heated at 80 °C for 8 h. The reaction was quenched with addition of 1 ml 1 N HCl. Oxidative degradation involved the addition of 1 ml 30% H_2O_2 to the drug solution and incubating it for 8 h at 80 °C. For thermal degradation, drug solution was heated at 80 °C for 8 h. All the samples were diluted to 10 ml with mobile phase. The samples were filtered through 0.22 μm syringe driven membrane filter unit before injecting into the chromatographic system. Degradation peaks were identified and quantified by the HPLC method described above. The developed method was used to analyze drug content over the period of stability study. To evaluate the drug content, 100 mg of nanoemulsion was accurately weighed and dissolved in 25 ml of acetonitrile. Clotrimazole content was analyzed after suitably diluting the sample.

2.3. Preparation of clotrimazole nanoemulsion

Clotrimazole was first dissolved into oily phase, Capryol 90 with gentle heating at 45–50 °C for 5 min. Required amount of surfactants, Solutol HS 15 and Gelucire 44/14 were weighed accurately, mixed and gently heated at 45–50 °C for 5 min. Both phases were mixed to form homogenous isotropic mixtures. Required amount of phosphate buffer, pH 7.5 was added to mixture to obtain nanoemulsion. The nanoemulsions were stored at room temperature until used.

2.4. In vitro evaluation of clotrimazole nanoemulsion

2.4.1. Dynamic light scattering (DLS) measurements

The average globule size and polydispersity index (PI) of nanoemulsions were measured in triplicate at 25 °C by DLS using Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The instrument utilizes a 4 mW He–Ne red laser at 633 nm. The light scattering is detected at 173° by noninvasive backscatter technology with a measuring range from approximately 0.6 nm to 6 μm. Disposable polystyrene cuvettes, 1 ml were used for measurements. Water or buffers were used to dilute the formulations.

2.4.2. In vitro dissolution profile

Dissolution studies were performed using USP Apparatus II; paddle (Electrolab, Mumbai, India) at 37 ± 0.5 °C. Accurately weighed samples (clotrimazole as plain drug and nanoemulsion) containing 300 mg equivalent of clotrimazole were placed in 900 ml of dissolution medium. USP buffers of pH 1.2, 4.5 and 6.8 were used as dissolution media. The paddle revolution speed was set to 50 rpm. At predefined time intervals (15, 30, 45, 60, and 120 min); 5 ml aliquots were withdrawn from each vessel and replaced with a similar volume of fresh media. Aliquots were centrifuged at 5000 rpm for 10 min and subsequently filtered through 0.22 μm syringe driven membrane filter unit. The filtrates were assayed for drug concentration. The dissolution profiles were characterized using percent dissolved at pre-selected time points, where the percent dissolved at 15, 60 and 120 min (*i.e.* Q15, Q60 and Q120%) was determined.

2.5. Stability studies of clotrimazole nanoemulsion

Clotrimazole nanoemulsion was subjected to various storage conditions of temperature and humidity to assess their stability as per ICH guidelines Q1A (R2) (2003). Clotrimazole nanoemulsion was subdivided into 10 ml glass vials, sealed with rubber stoppers and crimped with aluminum caps and were stored upright. Physical and chemical stability of nanoemulsion were evaluated for six months by storing them at 30 ± 2 °C/65 ± 5% RH and 40 ± 2 °C/75 ± 5% RH. Samples were withdrawn at specified time intervals (0, 3, 6 months of storage) and assessed for clotrimazole content and *in vitro* dissolution profile in buffer pH 6.8. Physical stability of formulations was determined by monitoring mean globule size and polydispersity index. Formulations were also evaluated for physical appearance, phase separation, pH, self-nanoemulsification efficiency and robustness to dilution. All parameters were evaluated as discussed in earlier section. The data obtained at various time points about the, globule size, pH, drug content and dissolution of developed nanoemulsions were analyzed by one way ANOVA followed by Bonferroni's test using GraphPad Prism 5 statistical software program (GraphPad Software Inc., La Jolla, USA). Differences were considered statistically significant at $p < 0.05$.

2.6. Preparation of clotrimazole suspension

The clotrimazole suspension was prepared by dispersing it in 0.5%, w/v Methocel E5 Premium LV solution with an over head stirrer (RQT 24A, Remi Motors, Mumbai, India) at 1000 rpm. The effective concentration of clotrimazole in suspension was 2.5%, w/v. The particle size of suspension was measured by laser diffractometry (Mastersizer 2000 Ver. 5.30.010 equipped with Hydro 2000MU sample dispersion unit, Malvern Instruments Ltd., Worcestershire, UK). The commercially available clotrimazole exhibited the particle size of: $d(v, 0.1) = 9.42 \pm 2.04$, $d(v, 0.5) = 35.17 \pm 6.83$, $d(v, 0.90) = 67.45 \pm 15.39$ and volume weighted mean = 49.84 ± 12.18 μm. The drug suspension was prepared freshly whenever required and diluted suitably with water to desired concentration for administration.

Table 1
Summary of different groups and their treatment regimen used for *in vivo* antimalarial efficacy studies.

Groups ($n = 8$)	Four-day suppressive test	Onset of activity and recrudescence test
Treatment (mg/kg)		
Control	Water	Water
Placebo nanoemulsion	Equivalent to 10.00 mg/kg	Equivalent to 15.00 mg/kg
CLT suspension	10.00	15.00
CLT nanoemulsion	1.00	2.50
CLT nanoemulsion	2.50	5.00
CLT nanoemulsion	5.00	10.00
CLT nanoemulsion	10.00	15.00

2.7. In vivo pharmacodynamic evaluation: antimalarial efficacy studies

2.7.1. Animal handling

Animal experiments were executed in compliance with the guidelines of Committee for the Purpose of Control and Supervision of Experimental Animals, India on the premises of Tata Institute of Fundamental Research (TIFR), Mumbai, India. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of TIFR (protocol number: TIFR/IAEC/2009-3). Healthy male Swiss albino mice aged 6–8 weeks and body weights of 30 ± 5 g were used for the study.

2.7.2. Parasites

In vivo antimalarial efficacy studies were performed on lethal rodent malaria parasite, *Plasmodium berghei* ANKA strain. This strain is highly infective in mice with life cycle that is essentially similar to human malaria parasite. It causes lethal infections in mice with high mortality rates and is sensitive to all currently used antimalarial drugs, providing a good model to estimate the efficacy and survival. The parasite was revived from frozen stocks and infection was initiated in the donor mice with intraperitoneal injection of 10^6 parasitized RBCs (Peters and Robinson, 1999).

2.7.3. Assessment of parasitemia in infected mice

Peripheral blood smears were prepared on glass slide by using blood obtained from tail veins of infected mice. The thin films were fixed in methanol for 5 min and stained with 5% Giemsa stain (Sigma Diagnostics, ST. Louis, USA). Blood smears were examined at a magnification of 100×/oil immersion lense with a light microscope (BX41TF, Olympus Optical Co. Ltd., Tokyo, Japan). Parasitemia was determined by counting 1000 erythrocytes covering 4–5 fields of view. For low parasitemias up to 2000 erythrocytes were counted.

2.7.4. Study protocol and drug treatment: four-day suppressive test

Blood taken from infected donor mice with approximately 10–15% parasitemia was diluted suitably in acid citrate dextrose buffer to contain approximately 10^6 parasitized erythrocytes. Experimental animals were infected intraperitoneally with this blood on day '0'. Mice were randomly divided into various groups ($n = 8$, Table 1). Two hours post infection, mice were treated orally with single dose of various formulations. Further, on days 1, 2, and 3, mice were treated again similarly as on day 0. Then, throughout the study, blood was withdrawn from tail vein from day 2 at regular time intervals for the assessment of parasitemia (Fidock et al., 2004).

2.7.5. Study protocol and drug treatment: onset of activity and recrudescence test

Mice were inoculated intraperitoneally with infected donor mice blood on day '0'. The mice were treated with a single oral dose (Table 1) 72 h after the infection and subsequently on days 4, 5, and 6 post infection in similar manner as on day 3. Parasitemia levels were determined on days 2, 3, 4 and later at regular time intervals throughout the study period (Osdene et al., 1967).

2.7.6. Evaluation parameters

The difference between mean values of parasitemia (infected erythrocytes) of the control group (taken as 100%) and those of experimental groups is calculated and expressed as percent reduction (activity) using the equation, activity = $100 - [(\text{mean parasitemia}_{\text{treatment}}/\text{mean parasitemia}_{\text{control}}) \times 100]$. Further, results are expressed as rapidity of onset of activity (disappearance of parasitaemia), time to onset of recrudescence, and an increase of parasitaemia (course of infection). For the treated mice, the survival time in days is recorded.

2.8. Repeated dose oral toxicity study

2.8.1. Animal handling

Animal experiments were executed in compliance with guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals, India on the premises of Haffkine Research Institute, Mumbai, India. The experimental protocol was approved by Institutional Animal Ethics Committee of Haffkine Research Institute (protocol number: 525/02/A/01/E). Six- to eight-week old, healthy adult Wistar rats ($n = 6$, each of either sex) were used for the study.

2.8.2. Study protocol and drug treatment

Clotrimazole nanoemulsion was evaluated at dose equivalent to 15 mg/kg. The formulations were diluted suitably with water. The animals, six males and six females, were assigned to three test groups namely Group I (control; water), Group II (Placebo nanoemulsion), and Group III (clotrimazole nanoemulsion). Animals in each group were administered respective doses for 14 days, once daily, by oral gavage.

2.8.3. Evaluation parameters

During the experimental period, animals were observed for any clinical signs of toxicity, mortality, changes in body weight or behavioral changes. At the end of treatment, animals were bled from orbital sinus for clinical pathology assessment which included analysis of various hematological and serum biochemical parameters. In addition, serum hepatic markers were evaluated in greater details. Hematology and serum biochemistry estimations were performed using standard kits (Spinreact, S.A. Ctra Santa Coloma, Spain). Consequently the animals were sacrificed by cervical dislocation and necropsied for gross evaluation of various organs. The necropsy also included careful and consistent dissection of various target organs like heart, liver, spleen, kidneys, intestine and stomach, determination of absolute organ weight and calculation of organ weight to body weight ratios. Dissected tissues were fixed in 10% neutral buffered formalin, processed and embedded in paraffin wax. Sections (5 μm) of these tissues taken on glass slides were stained using combination of hematoxylin–eosin for observing under a microscope for histopathological evaluations.

2.9. Statistical analysis

The results of percent parasitemia and activity were expressed as means ± standard deviations (SD), unless otherwise indicated. The data of different groups were compared and analyzed by one-way ANOVA followed by Tukey–Kramer multiple comparison post test. The dose–response relationship was evaluated by ANOVA followed by post test for linear trend. The survival data were analyzed by the Kaplan–Meier method and were compared among groups using log-rank test. All the statistical analysis was performed using GraphPad Prism 5 statistical software program (GraphPad Software Inc., La Jolla, USA). Differences were considered significant at $p < 0.05$. The results of repeated dose toxicity study are expressed as means ± SD, unless otherwise indicated. Statistical analysis of various parameters was performed using two tailed unpaired t test. All the statistical analysis considered as significant at $p < 0.05$.

3. Results and discussion

3.1. HPLC quantification of clotrimazole

The developed method was linear ($r^2 = 0.9997$) in the concentration range of 10–500 μg/ml. The method was accurate, precise and robust as percent relative standard deviation was consistently <2%. Forced degradation studies indicated that, clotrimazole was stable to alkaline, photo and thermal degradations. However, it was susceptible to acidic and oxidative degradation.

3.2. Preparation and characterization of clotrimazole nanoemulsion

Clotrimazole nanoemulsion was prepared by spontaneous emulsification process. As clotrimazole was liable to hydrolysis in acidic conditions, the pH of nanoemulsion was adjusted to 7.5. The nanoemulsion was dispersed effectively in various aqueous phases without any precipitation of the drug. It exhibited globule size < 25 nm with narrow distribution (PI < 0.2) when diluted with water and aqueous buffers in the pH range of 1.2–7.5. Furthermore, the nanoemulsion was robust to dilution as it did not show any phase separation, increase in globule size and drug precipitation even after 24 h storage at room temperature. The dissolution studies were performed in USP buffers of pH 1.2, 4.5 and 6.8. The summary of comparative dissolution profiles of clotrimazole as plain drug powder and clotrimazole nanoemulsion in various media is shown in Table 2. Clotrimazole as a plain drug was characterized by slow and poor dissolution. The dissolution of drug was strongly affected by pH, with significantly greater dissolution observed at pH 1.2 than at pH 4.5 and 6.8. Clotrimazole is a weak base with two ionizable nitrogen atoms. The pK_a value of 4.7 may result in protonation of nitrogens below pH 3 (Williams, 2002). This explains why the changes in pH profoundly influenced the dissolution of drug. Clotrimazole was characterized by less than 30 and 10% dissolution at the end of 2 h in pH 4.5 and 6.8, respectively. A 100% release of clotrimazole was obtained from nanoemulsion in 15 min in all dissolution media and was unaffected by the pH of dissolution medium. The dramatic increase in rate of release of clotrimazole from the nanoemulsion compared to clotrimazole as plain drug is attributed to its quick dispersability and ability to keep drug in solubilized state. The dissolution studies conducted for 2 h to observe the occurrence of precipitation. The amount of drug dissolved at the end of 2 h was close to 100% and was similar to value observed at 15 min. Visual observations also indicated no sign of drug precipitation.

3.3. Stability studies of clotrimazole nanoemulsion

Clotrimazole nanoemulsions stored at long term and accelerated conditions of stability were evaluated at the 3 and 6 months and; results are summarized in Table 3. In liquid state, there is a potential for phase separation or precipitation of drug substance

Table 2
Drug content and summary of *in vitro* release of CLT as plain drug and CLT nanoemulsion. Data expressed as mean ± SD ($n = 3$).

Formulation parameters	CLT as plain drug			CLT nanoemulsion		
	Buffer pH 1.2	Buffer pH 4.5	Buffer pH 6.8	Buffer pH 1.2	Buffer pH 4.5	Buffer pH 6.8
Drug content (mg)	299.70 ± 0.75	300.41 ± 1.03	300.21 ± 0.72	300.17 ± 0.64	299.56 ± 0.43	299.79 ± 0.47
Q15 (%)	8.87 ± 2.47	6.94 ± 2.31	3.48 ± 2.49	99.39 ± 2.01	98.89 ± 1.12	100.87 ± 2.74
Q60 (%)	50.82 ± 8.26	25.44 ± 5.47	7.54 ± 1.89	99.45 ± 1.12	100.47 ± 1.12	101.46 ± 1.85
Q120 (%)	51.15 ± 2.92	26.18 ± 3.85	7.01 ± 2.67	99.64 ± 3.42	100.09 ± 2.37	100.21 ± 3.52

Table 3
Stability evaluation of CLT nanoemulsion.

Period and condition	Globule size[a,b] (nm)	pH[c]	Drug content (%)[c]	Dissolution Q15 (%)[c]	Dissolution Q60 (%)[c]	Dissolution Q120 (%)[c]
Initial	20.14 (0.14)	7.62 ± 0.87	99.34 ± 1.25	98.47 ± 2.15	99.49 ± 2.81	99.15 ± 1.47
30 °C/65% RH						
3 months	19.17 (0.16)	7.48 ± 0.54	99.05 ± 0.89	99.14 ± 1.56	99.73 ± 2.36	99.08 ± 2.05
6 months	20.34 (0.17)	7.37 ± 0.73	99.24 ± 1.54	98.42 ± 2.45	99.31 ± 1.98	99.44 ± 2.43
40 °C/75% RH						
3 months	19.83 (0.14)	7.53 ± 0.84	98.67 ± 1.43	99.87 ± 2.89	99.16 ± 2.08	99.64 ± 2.46
6 months	20.78 (0.17)	7.61 ± 0.52	99.45 ± 1.07	99.08 ± 2.87	99.47 ± 1.47	99.13 ± 2.67

[a] Globule size expressed as mean ($n = 3$) where relative standard deviation was <10%.
[b] Values in parentheses represent polydispersity index ($n = 3$).
[c] Data expressed as mean ± SD ($n = 3$).

to occur with time. Therefore, the nanoemulsion was monitored closely for any occurrence phase separation or precipitation during the stability period. Clotrimazole nanoemulsion appeared as transparent, homogenous liquids without any signs of phase separation and drug precipitation. The pH of the nanoemulsion also did not change significantly at both the storage conditions during the study period. Clotrimazole nanoemulsions were diluted with 250 ml of pH 6.8 buffer and evaluated for self-nanoemulsification properties. During the entire period of the study, the nanoemulsion was found to disperse within a minute forming transparent, optically isotropic dispersions. After dilution, when observed for 24 h, none of the formulation exhibited any signs of precipitation of the drug. No significant changes in mean particle size and PI were observed. Chemical stability due to acid catalyzed degradation of drug in solution state was another concern. However, adjustment of pH of the nanoemulsion to 7.5 could successfully prevent degradation of clotrimazole. The chromatograms of clotrimazole nanoemulsion with and without pH adjustment are shown in Fig. 1, which elucidate the chemical stability of clotrimazole at accelerated storage condition. No significant changes were observed in drug content and *in vitro* release profiles of drug form the nanoemulsion. Formulations were found to release 100% of drug in 15 min in pH 6.8 buffer over a period of six months.

3.4. *In vivo* pharmacodynamic evaluation: antimalarial efficacy studies

3.4.1. Four-day suppressive test

The four-day suppressive test is useful for the assessment of suppressive activity where the infection is just initiated and was employed to evaluate the activity of clotrimazole nanoemulsion at different dose levels. The results pertaining to reduction in

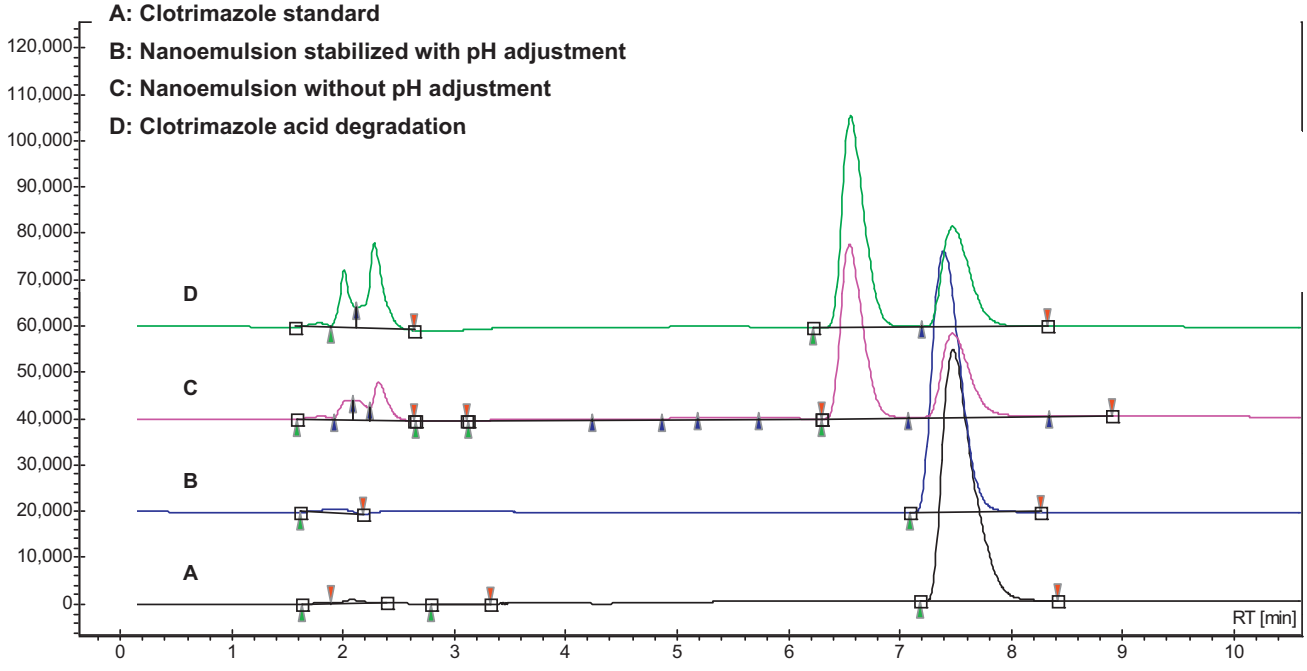

Fig. 1. Representative chromatograms of clotrimazole formulated in nanoemulsion with and without pH adjustment obtained in stability study.

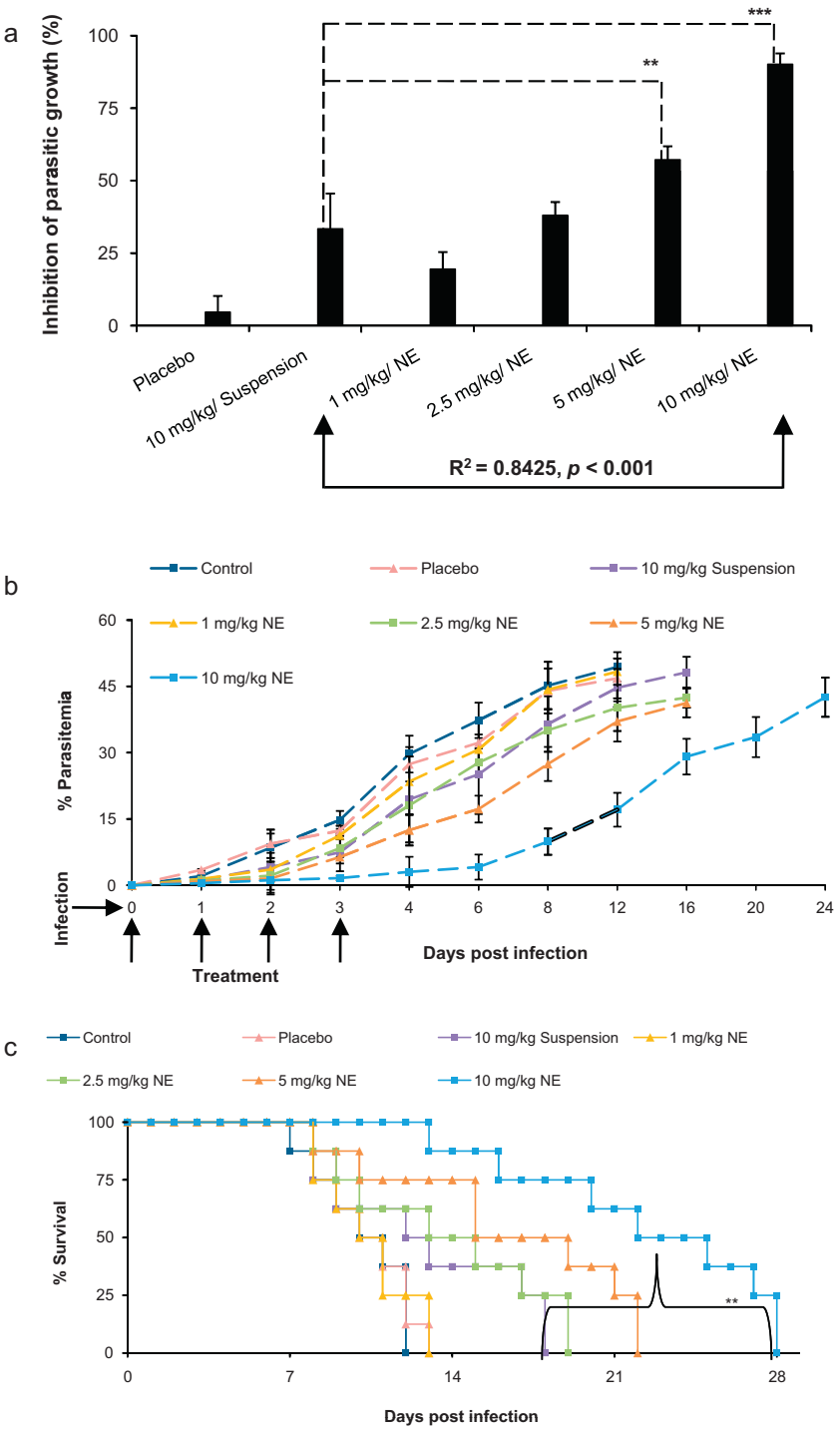

Fig. 2. The *in vivo* pharmacodynamic evaluation with four-day suppressive test. (A) Percent reduction in parasitemia in mice on day 4. The infected mice treated 2 h after the infection with a single dose of clotrimazole nanoemulsion and suspension and; subsequently on days 1, 2, and 3. Data expressed as mean ± SD, $n = 8$. Statistical analysis performed using one-way ANOVA followed by Tukey–Kramer multiple comparison post test where, $^{***}p < 0.001$ for 10 mg/kg clotrimazole nanoemulsion *versus* 10 mg/kg clotrimazole suspension and $^{**}p < 0.01$ for 5 mg/kg clotrimazole nanoemulsion *versus* 10 mg/kg clotrimazole suspension. Dose proportionality across 1–10 mg/kg of clotrimazole nanoemulsion analyzed by one way ANOVA followed by post test for linear trend. (B) Percent parasitemia–time profiles of infected mice. Data expressed as mean ± SD, $n = 8$. (C) Survival curves (Kaplan–Meier estimates) of infected mice treated with clotrimazole nanoemulsion and suspension. Statistical analysis performed using log-rank test where, $^{**}p < 0.01$ for 10 mg/kg clotrimazole nanoemulsion *versus* 10 mg/kg clotrimazole suspension.

percent parasitemia, parasitemia–time profiles and survival analysis are presented in Fig. 2A–C. Untreated control mice were highly parasitized by day 4 (29.70 ± 4.17). Infected mice that received placebo showed course of parasitemia similar to that of untreated control mice (27.41 ± 3.89). The parasite growth was considerably suppressed in treatment groups on day 4, *i.e.* 24 h after last treatment; with level of parasitemia in all treatment groups being significantly lower ($p < 0.05$) than that in control as determined by one-way ANOVA followed by Tukey–Kramer multiple comparison post test. The treatment of mice with clotrimazole nanoemulsion at different doses given at the start of infection had marked antimalarial activity (Fig. 2A). The animals treated with 10 mg/kg clotrimazole nanoemulsion showed highest reduction in parasitemia as compared to all other groups and; was

significantly higher ($p < 0.001$) than that of 10 mg/kg clotrimazole suspension. Further, mice treated with 5 mg/kg clotrimazole nanoemulsion exhibited significantly enhanced activity compared to 10 mg/kg clotrimazole suspension group ($p < 0.01$). However, no significant difference was observed for inhibition of parasitic growth between 10 mg/kg clotrimazole suspension and 2.5 mg/kg clotrimazole nanoemulsion treatment groups ($p > 0.05$); and exhibited similar parasitemia–time profile throughout the course of study (Fig. 2B). The percent inhibition of parasitic growth (day 4 post infection) of groups treated with various doses of clotrimazole nanoemulsion was analyzed by one-way ANOVA followed by post test for linear trend. The results exhibited significant linear trend ($p < 0.001$) with a R^2 of 0.8425 implying dose proportionality when animals were treated in escalating doses. The control and placebo group mice died between 7–12 and 7–13 days, respectively. Mice treated with clotrimazole suspension and nanoemulsion exhibited prolonged survival compared to control. Survival of mice treated with 10 mg/kg clotrimazole nanoemulsion was significantly prolonged compared to those treated with 10 mg/kg clotrimazole suspension ($p < 0.01$) as determined by log-rank test (Fig. 2C).

3.4.2. Onset of activity and recrudescence test

For this study, a different experimental protocol was chosen to distinguish further between clotrimazole nanoemulsion and suspension. An established infection murine model was employed where; severely infected mice were used as described by Osdene et al. (1967). Although the same strain of parasite was used, it is more severe model for efficacy testing as a more onerous 72 h treatment delay was studied compared to 2 h delay in four-day suppressive test. It also represents a better model for treatment of malaria in humans because the infection was already established when the treatment was initiated. The reduction in percent parasitemia, parasitemia–time profiles and survival analysis with respect to antimalarial activity are illustrated in Fig. 3A, B and C, respectively. Mice from untreated control and placebo groups were highly parasitized by day 3. Other treatment groups also developed comparable parasitemia on day of treatment, *i.e.* day 3. There was no significant difference among the groups in the mean parasitemia levels at the time of dosing ($p > 0.05$). Clotrimazole administered 3 days after the infection had marked schizonticidal activity against *P. berghei* with the level of parasitemia being significantly lower ($p < 0.05$) on fourth day, *i.e.* 24 h after administration of first dose. Further, parasitemia in the clotrimazole nanoemulsion treated groups (10 and 15 mg/kg) was significantly lower compared to 15 mg/kg clotrimazole suspension treatment group ($p < 0.05$). The results indicated rapidity of clotrimazole action in the nanoemulsion formulation. The continuation of treatment to total of four doses further reduced the parasitemia significantly ($p < 0.01$) compared to control. However, the percent reduction of parasitemia was significantly higher in 10 and 15 mg ($p < 0.001$) and 5 mg/kg ($p < 0.05$) treated clotrimazole nanoemulsion groups compared to 15 mg/kg clotrimazole suspension group as compared on 7th day of study, *i.e.* 24 h after administration of last dose. The clotrimazole nanoemulsion 2.5 mg/kg and suspension 15 mg/kg treated mice exhibited comparable reduction in the parasitemia ($p > 0.05$). The parasite growth was considerably suppressed up to day 16 of the study, where the parasitemia was <15% in 10 and 15 mg/kg clotrimazole nanoemulsion treated groups; as against the 45% in 15 mg/kg clotrimazole suspension treated group (Fig. 3B). Maximal inhibition of parasitemia was observed in the group receiving the highest dose of clotrimazole nanoemulsion (15 mg/kg), but even a relatively low dose of clotrimazole nanoemulsion (2.5 mg/kg) significantly suppressed the development of parasitemia compared with controls. The results showed that the inhibition of parasite growth by clotrimazole was dose dependent ($p < 0.001$, R^2 of 0.8032, as determined by one-way ANOVA followed by post test for linear trend). Due to shorter half life of the clotrimazole, none of the treated groups exhibited complete clearance of parasite, which resulted in steady increase in parasitemia in the follow up study period. However, the superior activity of clotrimazole nanoemulsion (15 and 10 mg/kg) resulted in significantly prolonged survival of mice compared to 15 mg/kg clotrimazole suspension group ($p < 0.01$ as determined by log-rank test, Fig. 3C).

A comprehensive evaluation of antimalarial efficacy in rodent malaria models with dose ranging studies is a standard part of malarial drug development pathway. Even though there are few reports in literature regarding *in vitro* activity of clotrimazole; there is a scarcity of studies elucidating its antimalarial effects in suitable animal models. The only *in vivo* study performed with clotrimazole was in female CD1 mice infected with *P. berghei* ANKA and *Plasmodium chabaudi* AS, where it exhibited less than 30% activity (Gemma et al., 2008). However it should be noted that, clotrimazole was administered as a suspension dispersed in tween 80. Therefore, in present study antimalarial effect of clotrimazole as a drug suspension was evaluated against its nanoemulsion. Clotrimazole suspension showed a moderate antimalarial activity, however at equivalent doses, clotrimazole nanoemulsion exhibited significantly potent activity. The improved therapeutic efficacy could be partly because of rapid and efficient dispersion of the nanoemulsion in gastrointestinal tract. This resulted in increased solubility and dissolution rate of clotrimazole which could keep the drug in solubilized state during gastrointestinal dilution and permeation process. In addition, nanoemulsion formed was sufficiently stable and had globule size in nanometer range. This could increase the accessibility and adhesion of clotrimazole to mucus and enterocyte surface and; could also provide larger interfacial surface area for drug diffusion (Borhade et al., 2008). The drug absorption behavior can further be facilitated by composition of nanoemulsion, *i.e.* the surfactants used such as Solutol HS 15 and Gelucire 44/14. These surfactants are reported to increase intestinal permeability and thereby have the absorption enhancing properties (Gao et al., 2009; Yin et al., 2009). Superior performance of clotrimazole nanoemulsion may also be attributed although not confirmed, to its intestinal lymphatic transport. This is based on observation that, clotrimazole is not only poorly water-soluble and hydrophobic but also highly lipophilic with log $P > 6$. It also exhibited good solubility in medium and long chain oils. These properties render clotrimazole a good candidate for lipid-based formulations and make it more amenable to access to intestinal lymphatic system (Porter et al., 2008; Pouton and Porter, 2008). Thus it was thought that, the combined effects of aforesaid aspects may result in an increased systemic exposure of clotrimazole on oral administration leading to its enhanced therapeutic efficacy in nanoemulsion formulation.

This murine model used in present investigation, with its all variations is valuable in studies of erythrocytic stage of malaria infection. In the four-day test, drug efficacy was evaluated in relation to suppression of infection, with drug administration commencing at the time of parasite inoculation. Whereas, the onset of activity and recrudescence test represented a better model for the treatment of severe malaria because the infection was already established when drug was administered. Thus, present study has demonstrated that murine *P. berghei* malaria treatment model can be an imperative tool for detailed preclinical investigation of pharmacodynamic effects of antimalarial drugs and their differentiating formulations.

3.5. Repeated dose oral toxicity study

Dose was selected based on what has been predicted to be efficacious in murine malaria models. No animal mortalities were observed for any of the treatment or control groups throughout the study period. The animals did not exhibit any treatment related

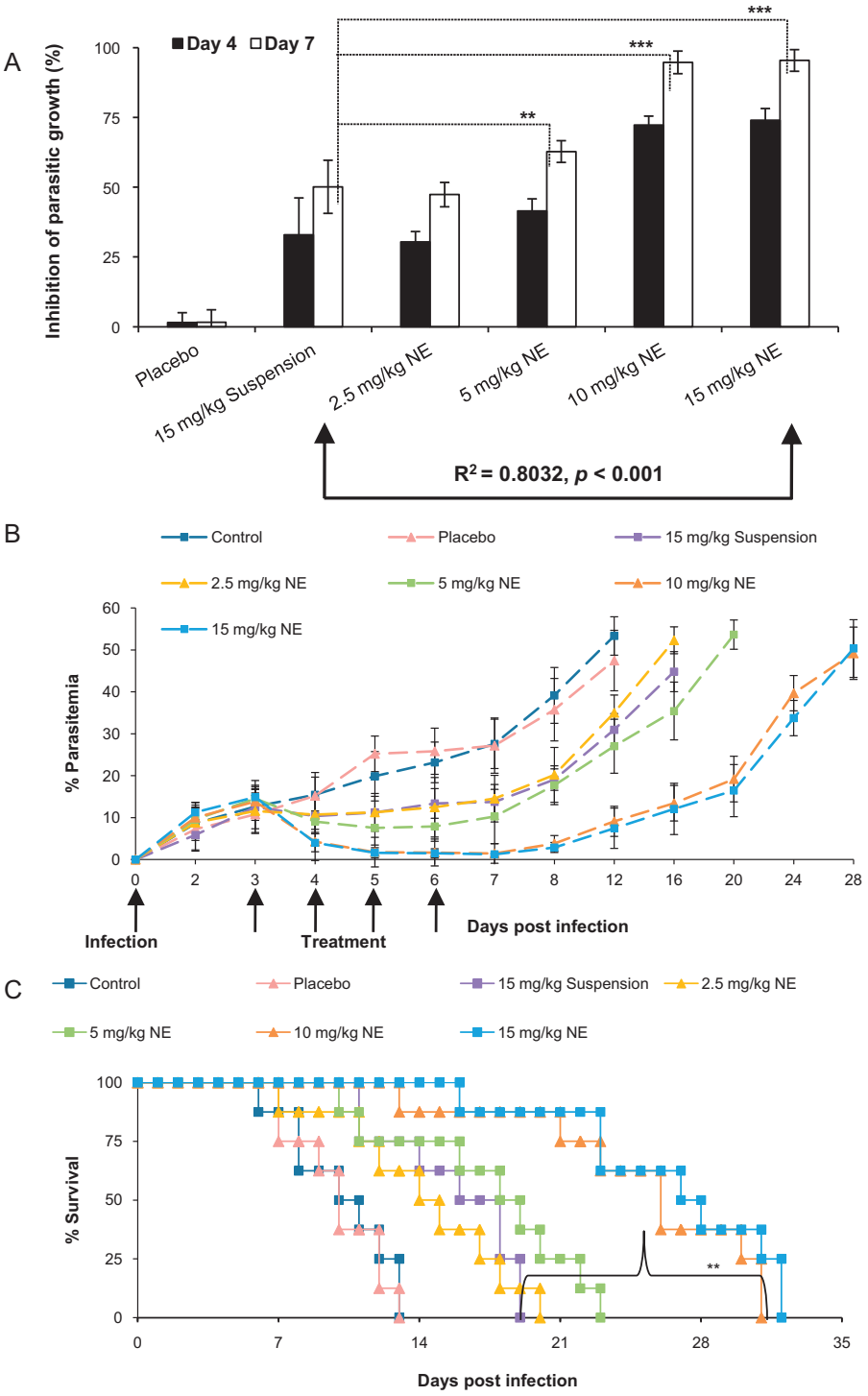

Fig. 3. The *in vivo* pharmacodynamic evaluation with onset of activity and recrudescence test. (A) Percent reduction in parasitemia on days 4 and 7. The infected mice treated 72 h after the infection with a single dose of clotrimazole nanoemulsion and suspension and; subsequently on days 4, 5, and 6. Data expressed as mean ± SD, $n = 8$. Statistical analysis performed using one-way ANOVA followed by Tukey–Kramer multiple comparison post test where, $^{***}p < 0.001$ for 10 and 15 mg/kg clotrimazole nanoemulsion *versus* 15 mg/kg clotrimazole suspension and $^{**}p < 0.01$ for 5 mg/kg clotrimazole nanoemulsion *versus* 15 mg/kg clotrimazole suspension. Dose proportionality across 2.5–15 mg/kg of clotrimazole nanoemulsion analyzed by one way ANOVA followed by post test for linear trend. (B) Percent parasitemia–time profiles of infected mice. Data expressed as mean ± SD, $n = 8$. (C) Survival curves (Kaplan–Meier estimates) of infected mice treated with clotrimazole nanoemulsion and suspension. Statistical analysis performed using log-rank test where, $^{**}p < 0.01$ for 10 and 15 mg/kg clotrimazole nanoemulsion *versus* 15 mg/kg clotrimazole suspension.

abnormal behavioral traits. The weekly feed consumption of animals of either sex in treatment groups did not vary significantly from that of the control group. The gain in body weight of animals in all treatment groups was comparable to that of the vehicle control group. The observations indicated that long term administration of the formulation had no adverse effects on the general health of the animals. The various hematological parameters of treatment groups did not vary significantly from control group (Fig. 4). These results also corroborated well with the serum biochemistry profiles of animals. The serum biochemical parameters of the treatment groups did not vary significantly from that of the control group (Fig. 5). It was in line with earlier reports that clotrimazole is not

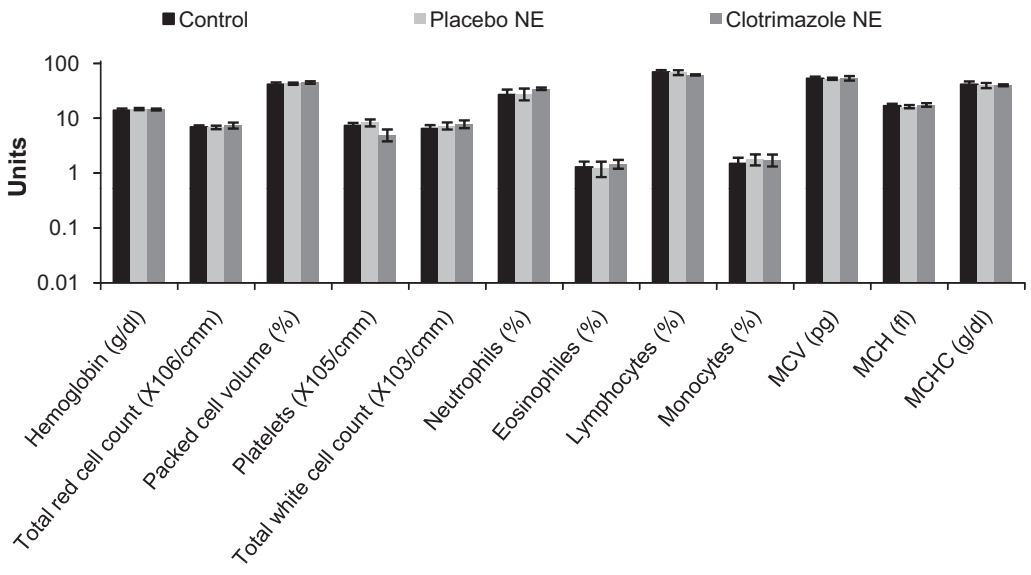

Fig. 4. Hematological parameters in Wistar rats. Data expressed as mean ± SD, $n = 6$.

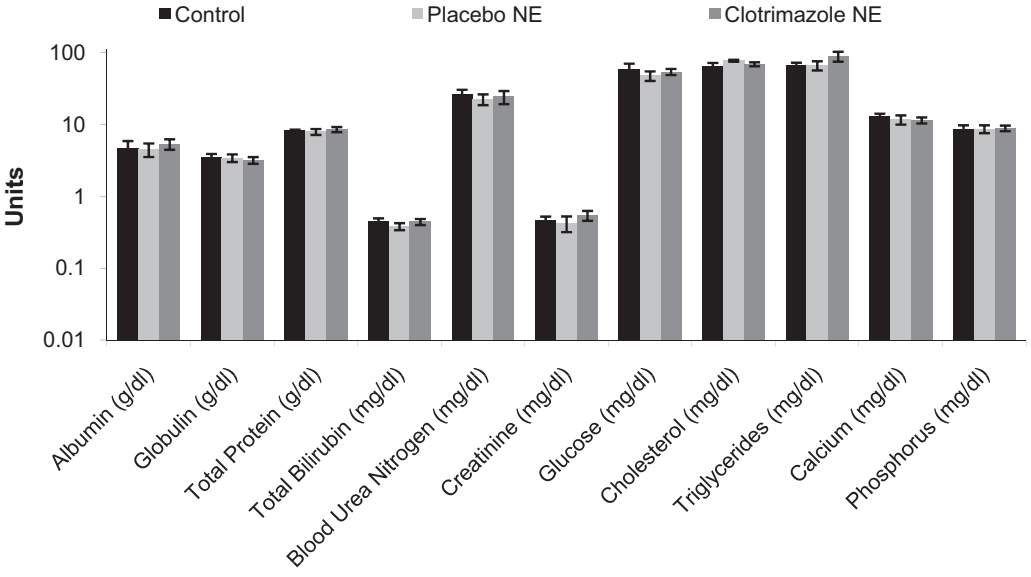

Fig. 5. Serum biochemistry parameters in Wistar rats. Data expressed as mean ± SD, $n = 6$.

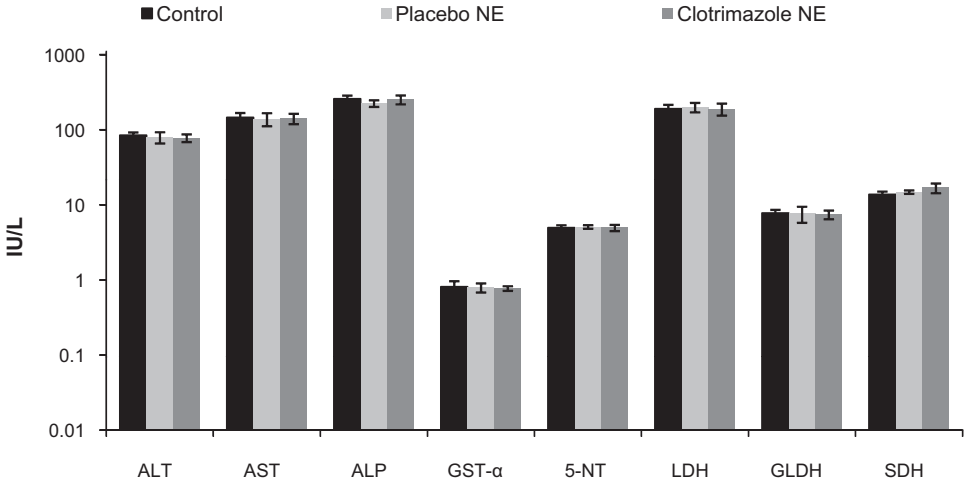

Fig. 6. Serum hepatic markers in Wistar rats. Data expressed as mean ± SD, $n = 6$.

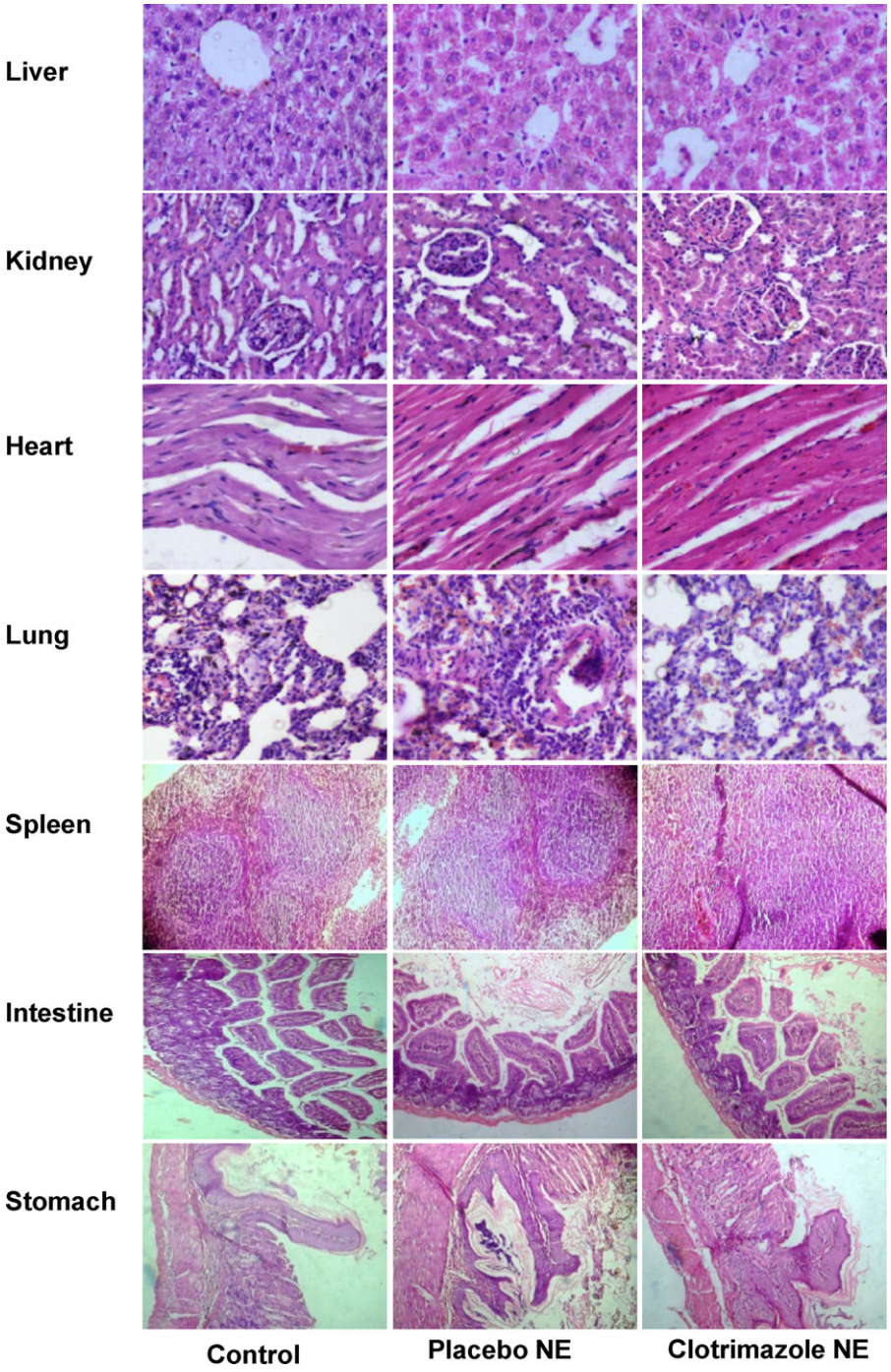

Fig. 7. Representative histological photomicrographs of different organs in control and treatment groups.

associated with hematological and renal adverse effects (Marget and Adam, 1971; Brugnara et al., 1996). However, few of the clinical studies performed with clotrimazole indicated that it was poorly tolerated when administered orally in high doses such as 100 mg/kg/day for few weeks and exhibited gastrointestinal symptoms such as nausea, abdominal pain, and diarrhea; and hepatic adverse effects such as elevation of the serum liver enzymes (AST and ALP) (Burgess and Bodey, 1972; Higgs, 1974; Tettenborn, 1974). Therefore in the present study various serum biomarkers of hepatotoxicity were investigated. The results are shown in Fig. 6. The serum ALT levels of treatment groups did not vary significantly from that of control group, in animals of either sex. Supplemental hepatotoxicity assays that support ALT measurements, for example, AST, ALP, GST α, total bilirubin were also investigated. Changes in serum hepatic markers of treatment groups were insignificant compared to control group. Histological examinations of liver showed no evidence of hepatotoxicity and were indistinguishable from controls. However, it should be noted that these clinical studies were performed in doses ranging from 60 to 200 mg/kg/day (for example; 1.5 g every 6 h) administered for several days for the treatment of systemic fungal infections (Burgess and Bodey, 1972; Sawyer et al., 1975). Higher doses were required to achieve clotrimazole serum concentrations in the range of MIC values of various pathogenic fungal species, when given orally (Burgess and Bodey, 1972; Holt and Newman, 1972; Shadomy, 1971; Waitz et al., 1971). Further, it was also reported that gastrointestinal and hepatic symptoms

associated with oral delivery of clotrimazole were dose dependent and subsided promptly in all patients when the drug was discontinued (Burgess and Bodey, 1972; Sawyer et al., 1975). This argument is also supported by few of earlier clinical studies of clotrimazole for systemic fungal infection; where it was administered orally at higher doses on intermittent basis and was found to be curative with minimum side effects (Ipp et al., 1977; Meade, 1977). Clinical studies performed in 1990s also indicated that, the administration of clotrimazole to humans in dose range of 10–20 mg/kg up to 19 days was well tolerated with minimal adverse effects (Brugnara et al., 1995, 1996).

In context of this, the *in vivo* antimalarial studies were performed, where clotrimazole was found to be effective, exhibiting >90% activity when administered at 10 and 15 mg/kg dose levels, formulated in nanoemulsion. One should also consider the acute nature of malaria as a disease compared to chronic nature and treatment of systemic or a mucocutaneous fungal infection, for which clotrimazole was investigated initially. The treatment of malaria is short term, as most of the currently designed regimens are of 3–4 days (Bangchang and Karbwang, 2009). Moreover, combination therapy can further minimize the frequent and high dosing of clotrimazole. Previous reports indicated that low IC_{50} value of clotrimazole even against chloroquine-resistant strains of malaria makes it a practical antimalarial drug because plasma clotrimazole concentration of 3 μM is achievable with an oral dose of 1 g (Huy et al., 2002; Saliba and Kirk, 1998; Tiffert et al., 2000). Therefore, higher doses of clotrimazole used for the antifungal treatment would not be required for its antimalarial activity.

Macroscopic and histologic evaluation of other target organs such as heart, kidney, spleen, intestine, and stomach tissues showed no evidence of inflammation, cell lysis, or lesions; the natural architecture of the organs remained unaffected (Fig. 7). The excipients used in formulation of nanoemulsion include Capryol 90, Solutol HS 15 and Gelucire 44/14, and are routinely used in variety of dosage forms for oral administration. These excipients are generally recognized as safe (GRAS), and therefore one would not expect considerable toxicity unless it was drug related. Thus, repeated dose toxicity study illustrated the safety of developed clotrimazole nanoemulsion on oral administration in the context of malaria infection.

4. Conclusion

Clotrimazole and its nanoemulsion formulations for the first time were systematically investigated in murine malaria model and the study evidently illustrated the effectiveness of lipid-based formulations in improving the performance of poorly-bioavailable molecules like clotrimazole. The repeated dose toxicity study indicated the oral safety of developed nanoemulsion without any significant hepatic adverse effects. The significantly enhanced antimalarial activity of clotrimazole in nanoemulsion at low doses than those required for its antifungal effects as demonstrated here suggest that clotrimazole holds much promise as an effective antimalarial agent. It may be suitable for a pilot clinical study in uncomplicated *P. falciparum* malaria and can serve as novel structural lead for designing new antimalarial molecules. In conclusion, the "piggyback approach" explored in present investigation may be an attractive and more economical strategy for malaria drug development.

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