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ORIGINAL ARTICLE

Evaluation of chalcone derivatives for their role as antiparasitic and neuroprotectant in experimentally induced cerebral malaria mouse model

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Abstract

Cerebral malaria is a severe complication of *Plasmodium falciparum* infection with a complex pathophysiology. The current course of treatment is ineffective in lowering mortality or post-treatment side effects such as neurological and cognitive abnormalities. Chalcones are enormously distributed in spices, fruits, vegetables, tea, and soy-based foodstuffs that are well known for their antimalarial activity, and in recent years they have been widely explored for brain diseases like Alzheimer's disease. Therefore, considering the previous background of chalcones serving as both antimalarial and neuroprotective, the present study aimed to study the effect of these chalcone derivatives on an experimental model of cerebral malaria (CM). CM-induced mice were tested behaviorally (elevated plus maze, rota rod test, and hanging wire test), biochemically (nitric oxide estimation, cytokines (IL-1, IL-6, IL-10, IL-12p70, TNF, IFN-y), histopathologically and immunohistochemically, and finally ultrastructural changes were examined using a transmission electron microscope. All three chalcones treated groups showed a significant ($p < 0.001$) decrease in percentage parasitemia at the 10th day post-infection. Mild anxiolytic activity of chalcones as compared to standard treatment with quinine has been observed during behavior tests. No pigment deposition was observed in the QNN-T group and other chalcone derivative treated groups. Rosette formation was seen in the derivative 1 treated group. The present derivatives may be pioneered by various research and science groups to design such a scaffold that will be a future antimalarial with therapeutic potential or, because of its immunomodulatory properties, it could be used as an adjunct therapy.

Keywords Chalcones · Cerebral malaria · In vivo · *P. falciparum* · Treatment

Abbreviations

ANOVA	One-way analysis of variance
BBB	Blood brain barrier
CM	Cerebral malaria
CMC	Carboxymethyl cellulose
COX-2	Cyclooxygenase-2
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
DPX	Dibutylphthalate polystyrene xylene
ECM	Experimental cerebral malaria
EPM	Elevated plus maze
ICAM-1	Intercellular adhesion molecule 1

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IFN -γ	Interferon-gamma
IL-1β	Interleukin-1 beta
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
NF-kB	Nuclear factor kappa B
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3)
NO	Nitric Oxide
OsO_4	Osmium tetroxide
pRBCs	Parasitized red blood cells
QNN-T	Quinine treated
RBCs	Red blood cells
SPSS	Statistical package for the social sciences
TLR 4	Toll-like receptor 4
TNF-α	Tumour necrosis factor alpha

Introduction

Cerebral malaria (CM) is a severe complication of *Plasmodium falciparum* infection that occurs mostly in non-immune adults and in young children. It results in a deep coma and may be accompanied by acidosis, respiratory distress, and/or severe anaemia. CM results in less than 10% of hospital admissions but has an extreme mortality rate of about 15% in children and 20% in adults (Wang et al. 2015). The pathogenesis of CM is a complex phenomenon and has been linked to both the adherence of infected red blood cells (RBCs) in the brain's endothelial microvasculature and the deregulation of the host's immunological response to the parasite (Medana and Turner 2006; van der Heyde et al. 2006). This complex pathophysiology offers a distinctive target which yet remains to be explored for better therapeutic outcome. The recommended treatment, i.e., quinine or artesunate, is mostly effective in managing *P. falciparum* parasitemia. However, even under ideal circumstances, the case–fatality rate is found to be unacceptably high in severe malaria treated with these medications (John et al. 2010). Moreover, the efficacy of these antimalarial drugs is mainly restricted by drug resistance, and the latest evidence elucidates the emerging capability of parasites to acquire resistance to even the newest agents (Ouji et al. 2018). These facts call for innovative treatments to improve CM therapy outcomes. Because of this, it is important to find new antimalarial drugs, drug combinations, or new approaches to treat CM (Jain et al. 2013).

From several decades, natural products continuously lead a noteworthy contribution to malaria chemotherapy. The discovery of quinine and artemisinin evinces that nature is an extravagant source of lead compounds and these compounds can be further modified synthetically to develop effective antimalarial agents (Kingston and Cassera 2022). In this context, chalcone (1,3-diaryl-2-propen-1-one) a vital intermediate of flavonoid synthetic pathway distributed in spices, fruits, vegetables, tea and soy-based foodstuff has diverse biological and pharmacological activities such as anti-hepatotoxic, anti-inflammatory, antimicrobial, anti-anginal, antioxidant, anti-allergic anticancer and antimalarial (Nowakowska 2007; Dixit et al. 2014; Sinha et al. 2013). The combination of ease structure and least expensive methods for synthesis of these chalcones have engaged the many scientists and researcher to find and expand unique analogues for numerous infectious diseases including malaria. Moreover, in recent years because of small molecular size, and flexibility for alterations to adjust lipophilicity ideal for blood brain barrier (BBB) permeability have make chalcones as a one of the preferred candidates for their therapeutic potential in brain diseases such as Alzheimer's (Thapa et al. 2021). Earlier, chalcones has been elucidated for their antimalarial activity by hindering enzymes either plasmodial aspartate proteases or cysteine proteases (Sriwilaijaroen et al. 2006; Domínguez et al. 2001), additionally they were also observed to restrict the parasite-induced channels (Go et al. 2004) and responsible for exacerbated membrane perturbations of the erythrocytes (Ziegler et al. 2004). Moreover, studies reflect chalcones and their different derivatives have neuroprotective effect (Chen et al. 2020). Isoliquiritigenin, a natural occurring flavonoid obtained from licorice root has been elucidated to revert the LPS stimulated cognitive impairment because of its anti-inflammatory and antioxidant properties (Zhu et al. 2019). Additionally, it was shown that isoliquiritigenin inhibited the NLRP3 inflammasome without affecting its inhibitory effectiveness on TLR4 (Honda et al. 2014). In BV-2 microglial cells, prenylated chalcones, bavachalcone, and isobavachalcone were also found to have anti-inflammatory action (Xu et al. 2018). Similarly, plant derived 2,2′,5′-trihydroxychalcone (225THC), a strong antioxidant, displays neuroprotective effect against the TLR4 driven inflammation in microglia. It reduced the LPS induced TNF-α and IL-6 production (Jiwrajka et al. 2016). Lee et al. elucidated anti-inflammatory effects of a synthetic chalcone derivative (2-hydroxy-3′,5,5′-trimethoxychalcone) in microglial cells (BV2) by hindering (TLR4)-stimulated inflammatory responses. The 2-hydroxy-3′,5,5′-trimethoxychalcone inhibits Akt through reducing IkB phosphorylation, which in turn downregulates the NF-ƙB. The downregulated NF-kB signaling pathway, excerates the generation of iNOS, COX-2, and the expression of pro-inflammatory cytokines such as, IL-1β and IL-6 (Lee et al. 2012). In other studies, carried out by Mateeva et al. shows that 2′-hydroxy-3,4,5,3′,4′-pentamethoxychalcone have most potent anti-inflammatory effect, evidenced by complete inhibition of NO, reduction of iNOS protein, and the reduction of various pro-inflammatory cytokines, IL-1α, IL-6 and IL-10 (Mateeva et al. 2015). Moreover, earlier studies stated that chalcones (synthetic or natural)

with capacity to reduce NO or attenuate iNOS expression are known to prevent neurological injury in experimental models (Gyengesi et al. 2020).

Additionally, due to proven efficacy of chalcones in preclinical studies, chalcone-based molecules such as hesperidin methyl chalcone (vascular protective), metochalcone (choleretic drug), and sofalcone (antiulcer and mucoprotective) have been approved for clinical applications (Gomes et al. 2017; Sahu et al. 2012; Higuchi et al. 2010).

The role of chalcone as an antimalarial has been studied with lots of chemical modifications in their primary structure, but none of them studied the effect of these chalcones on severe malaria such as CM, which is required to be explored, so the present study aimed to study the effect of these chalcone derivatives on an experimental model of CM.

Material and methods

Chemicals and reagents

The three chalcone derivatives namely, (E)-1-(2,5-Dimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one, (1); (E)-(3,4,5-Trimethoxyphenyl)-3-(4-methoxyphenyl) prop-2-en-1-one, (2); and. (*E*)-1-(3,4,5-Trimethoxyphenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one, (3), were synthesized and screened for potent antimalarial effect by our group (Sinha et al. 2019, 2020) The physio-chemical properties of these three derivatives are shown in Table 1, and detail NMR spectrum has been previously published (Sinha et al. 2021).

The other drugs and reagents such as Quinine hydrochloride and Griess reagent were purchased from Sigma Aldrich, USA. BD CBA Mouse Soluble Protein Flex Set System for IL-1, IL-6, TNF, IFN-γ, IL-10 and IL-12p70 were purchased from BD Biosciences, USA and ICAM-1 [Anti-ICAM1 antibody [YN1/1.7.4] from ABCAM, USA.

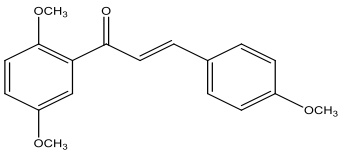
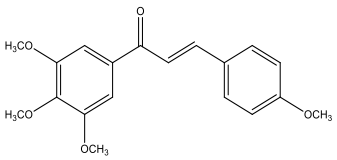
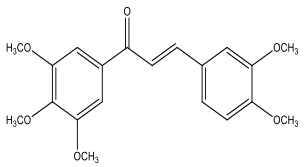
Experimental animals and ethics approval

The protocol was conducted after approval from the Institutional Animal Ethics Committee Ref. No. 69/IAEC/418 as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and the Institute Bio-Safety Committee Ref. No. 04/IBC/2013, at Postgraduate Institute of Medical Education and Research, Chandigarh. The study was reported in accordance with the ARRIVE guidelines. Inbred 6–8 weeks old C57BL/6 mice, weighing 18–24 g, were procured from the Advanced Facility for Small Animal Research, PGIMER, Chandigarh. The animals were kept in polypropylene cages under conventional laboratory settings with food and water ad libitum, controlled temperature (25 °C), and 12-h light/dark cycles for the specified period of the study. At the end of the experiment, each animal was sacrificed by giving anaesthesia followed by cervical dislocation. All procedures including euthanasia was done according to CPCSEA guidelines.

Plasmodium berghei ANKA and induced CM in C57BL/6 mice

The strain of *P. berghei* ANKA was procured from Department of Zoology, Punjab University, Chandigarh and was maintained in vivo by serial passage of parasitized erythrocytes from infected to naive mice. Thin blood smears were made from tail blood of mice every day to determine parasitemia.

Table 1 Physio-chemical properties of chalcone derivatives 1, 2 and 3 (Sinha et al 2019, 2020, 2021)

Compounds name	1. (*E*)-1-(2,5-Dimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one	(*E*)-(3,4,5-Trimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one	(*E*)-1-(3,4,5-Trimethoxyphenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one
Molecular formula	$C_{18}H_{18}O_4$	$C_{19}H_{20}O_5$	$C_{20}H_{22}O_6$
Chemical structure			
Molecular weight	298	328	359
Appearance	Pale to yellow solid crystal	Pale to yellow solid crystal	Pale to yellow solid crystal
Odour	Odourless	Odourless	Odourless
Solubility	$CHCl_3$, CH_2Cl_2, Ethyleacetate, MeOH, Acetone, DMSO	$CHCl_3$, CH_2Cl_2, Ethyleacetate, MeOH, Acetone, DMSO	$CHCl_3$, $CH2Cl_2$, Ethyleacetate, MeOH, Acetone, DMSO
Melting point	131–132 °C	121–122 °C	114–115 °C
Log *p* value	3.21	3.08	2.95

CM was induced in three C57BL/6 mice by intraperitoneal injection of 0.2 mL containing 10^6 *P. berghei* ANKA parasites. Starting on day 3 after infection, the mice were observed for clinical signs of ECM (ataxia, movement of the head, propensity to roll over in response to stimulation, and convulsions) (de Oca et al. 2013). The percentages of parasitemia calculated by examining Giemsa-stained thin blood films prepared every day beginning on day 3 post-infection and at the time of tissue collection. Finally, to confirm the presence of parasite invasion in vital organs such as, spleen, liver, lungs, brain, and kidney, histopathology was done (Supplementary Fig. 1).

Study design and treatment

Total of seventy two male C57BL/6 mice were recruited for the main experiment. These mice were randomly allocated to the following equally sized ($n=12$) groups as: Group1 (Non-infected): injected intraperitoneally with PBS; Group 2 (Infected): inoculated by intraperitoneal injection with 1×10^6 *P. berghei* ANKA infected erythrocytes; Group 3: infected mice treated with Quinine; Group 4: infected mice treated with Chalcone derivative 1; Group 5: infected mice treated with Chalcone derivative 2 and, Group 6: infected mice treated with Chalcone derivative 3. The schematic representation of study design is shown in Fig. 1.

Treatment procedure

Effective doses and route of administration of each chalcone derivative 1 (10 mg/kg), 2 (20 mg/kg), and 3 (10 mg/kg) were taken after extrapolation of in vitro data and pharmacokinetic study published previously (Sinha et al. 2019, 2021). Each Chalcone derivatives and the standard drugs groups were treated once daily for five days following Rane's test, in this case treatment begin at 5th day post infection (in this case onset of behaviour sign of CM) till 9th day. Thin blood smears were made from tail blood of mice every day. Levels of parasitemia in mice were assessed from Giemsa stained smears. For the intraperitoneal/oral administration, a suspension formulation of screened chalcones was prepared by triturating the weighed quantity of in a dry mortar with 0.5% carboxymethyl cellulose (CMC) and was prepared by drop wise addition of water and proper grinding.

Blood parasitemia

Parasitemia was monitored by light microscope examination under (1000 ×) magnification using Giemsa-stained blood smears on the 3rd, 5th, 7th day and 10th days after inoculation. The percentages of parasitemia were calculated by counting the number of parasite-infected erythrocytes per 2000 erythrocytes, according to the equations below (Attemene et al. 2018).

$$\text{Percent parasitemia} = (\text{No. infected RBCs/total no . RBCs counted}) \times 100$$

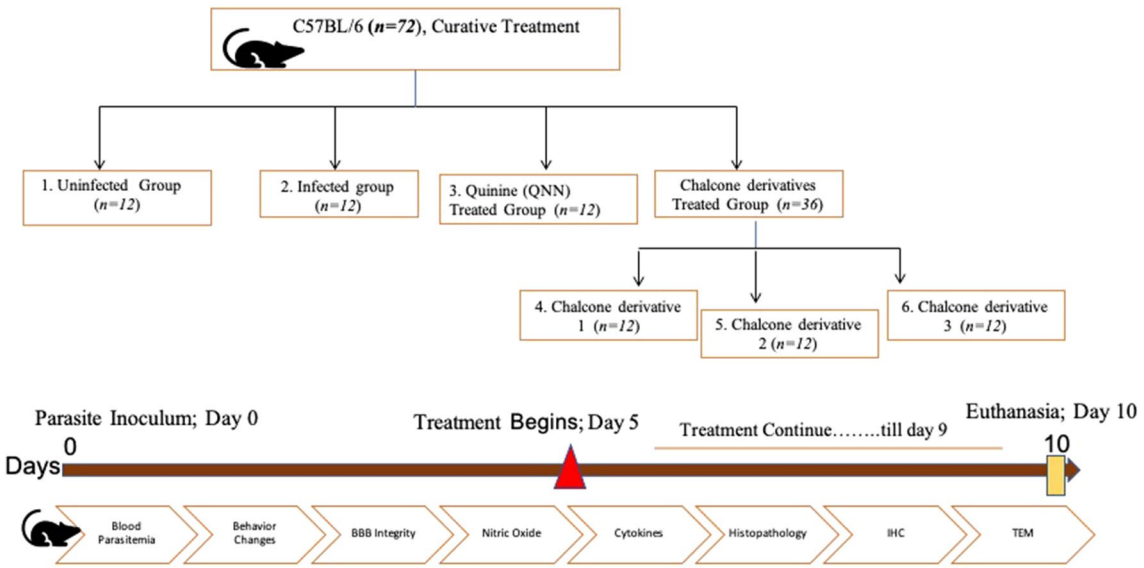


Fig. 1 Schematic diagram of study design

Behavior study

Elevated plus maze

Anxiety-related behavior was assessed using the Elevated Plus Maze (EPM) (Walf and Frye 2007). The apparatus had two opposing open arms (45 × 10 cm) and two closed arms (45 × 10 × 38 cm) that extend from a central platform (10 × 10 cm), elevated 50 cm above the floor. During testing, each mouse was positioned on the middle platform with their backs to the open arms of the maze and given five minutes to explore it. A computerized tracking system having video camera (Nodulus Ethovision XT 11.5 (EV115-06266) was used to observe and analyze the behavior. Time spent in the open and closed arms (and their edges) was recorded. The anxiety level was measured by the time spent in the open arm.

Rota rod

Mice were trained to stay on a scraped revolving rod (25 rpm) of the Rota rod assembly for at least three minutes prior to the experiment. Time of fall from revolving rod for animals was observed before and after treatment with standard drug and test compounds (Shiotsuki et al. 2010).

Hanging wire test

This test was performed as described by (Hattori et al. 2000) with slight modification. The mouse was placed on the center of the wire. Time to fall from the wire was recorded (Hattori et al. 2000).

Temperature and body weight

The rectal temperature and body weight of each mouse in all the groups was measured before infection (day 0) and on the 3rd, 5th, 7th and 10th days of post-infection.

Organ weight

The wet weight of two organs, i.e., the liver and spleen of each mouse in all the groups, was measured after sacrifices post-treatment.

Evans blue permeability

Permeability of the BBB was quantified before and after administration of chalcone derivatives using the modified Evans blue extravasation method as previously described (Radu & Chernoff 2013). Briefly, each mouse at day 10 post infection were intravenously given 200 µL of 0.5% Evans blue dye (E2129-10G, Sigma) in PBS. The mice were euthanized and perfused with PBS solution through the left ventricle. Brains were then extracted, weighted and dissolved in formamide. After extraction in formamide for 48 h at 55 °C, the absorption (at 610 nm) of the Evans blue extracted from the brain was measured using spectrophotometer and microgram Evans Blue extravasated per mg tissue was calculated against standard curve.

Nitric oxide estimation

For this, the blood samples were withdrawn from tail vein of each mouse and kept for clotting spontaneously at room temperature. Thereafter, serum was separated by centrifugation for 15 min at 3000 rpm at 4 °C and stored at – 80 °C. Nitrite level is estimated applying the Greiss reagent, served as an indicator of nitric oxide production. Briefly, serum (100 µL) was taken out in an empty 96-well plate, then 100 µL of Griess reagent (1:1, 0.1% -naphthylamine in water and 1% *p*-aminobenzene sulphamide in 5% phosphoric acid; Sigma Chemical, St. Louis, Mo., USA; Catalog Number. G4410) was added to each well and incubated at room temperature for 15 min. The absorbance was measured at 550 nm and thereafter nitric oxide concentration was computed using sodium nitrite standard curve and were expressed as µg/mL (Sun et al. 2003).

Cytokines estimation

Whole blood was collected in sterile tubes at the post-treatment stage and was allowed to coagulate for 2–3 h at 37 °C and then subjected to centrifugation. The sera thus obtained were stored at − 80 °C until cytokine measurement was performed using the BD CBA Mouse Soluble Protein Flex Set System for IL-1, IL-6, TNF, IFN-γ, IL-10, and IL-12p70 following the manufacturer's instructions.

Histopathology

Brain, liver and spleen from all experimental groups were removed aseptically after being fixed *in toto* for 48 h in a neutral buffered 10% formalin solution. The organs were cut into serial cross sections and fixed in formalin for another 12 h. Finally, tissue sections were fixed in paraffin and cut into 5 mm thick sections, which were stained with haematoxylin and eosin and examined for malaria parasites or pigments or any kind of inflammation (Sanni et al. 2002).

Immunohistochemistry

Tissue sections of the brain, liver and spleen were immunostained as described previously (Rudin et al. 1997). The Avidin–Biotin–peroxidase complex (ABC) technique

was followed using primary antibodies against ICAM-1 antigen (product name: Anti-ICAM1 antibody [YN1/1.7.4]): Rat monoclonal antibody to ICAM-1 with species reactivity to mouse. Briefly, 3 µm sections were incubated for 2 h at 37 °C with 1: 200 diluted rat monoclonal antibodies directed against murine ICAM-1. After washing, sections were incubated for 1 h at room temperature with biotin-conjugated secondary antibody (UltraTek HRP (Anti-Polyvalent), USA) ready to use. Peroxidase activity was developed in 0.5% 3, 3′-diaminobenzidine hydrochloride till the desired stain intensity was developed. Sections were washed in de-ionised H_2O for 5 min, and slides were dried and mounted with DPX.

Transmission electron microscopy

Ultrastructure changes after the treatment can be visualized by electron microscopy as described previously (Wisner-Gebhart et al. 1980). The specimens of liver, spleen and brain of approximately 0.5 mm^3 were fixed in 3% glutaraldehyde-0.1 M sodium phosphate buffer (pH 7.2), for 2 h at 4 °C. All samples were rinsed three times and kept overnight at 4 °C in a 0.2 M sucrose-0.1 M phosphate buffer (pH 7.2). After that, the samples were post-fixed in 2 percent OsO_4 in 0.1 M phosphate buffer, washed twice in 0.1 M phosphate buffer and twice in distilled water, dehydrated in a graded alcohol series and then in propylene oxide, and embedded in Epon. Using a Reichert-OmU-2 ultramicrotome and a glass or diamond knife, thin slices were cut and deposited on Formvar carbon-coated grids. The sections were poststained with 6% aqueous uranyl magnesium acetate and Reynold's lead citrate. The section was then examined with Phillip 200 or 400 transmission electron microscope at 60 or 80 kv, according to the thickness of the section and the required magnification.

Statistical analysis

Data variability was expressed as standard error (SE). A one-way analysis of variance (ANOVA) followed by *post-hoc* Bonferonni's test and Pearson Chi-square, contingency co-efficient, correlations test were used for data analysis using SPSS version 16 software. Values of $p < 0.05$ were considered significant.

Results

Sixty C57BL/6 mice were infected with *P. berghei* ANKA pRBCs by intraperitoneal injection on the first day (D0). On 5th day (D5), the mice were randomly divided into five groups of twelve mice each and one more additional groups of 12 mice without infection. Three groups of the mice were treated intraperitoneally or orally with derivative-1 (10 mg/kg), derivative-2 (20 mg/kg), and derivative 3 (10 mg/kg), respectively. The negative control group was treated with PBS while the positive control groups were treated with QNN (20 mg/kg), respectively.

Blood parasitemia level

There was daily increase in parasitemia level of the infected-control group. In case of QNN-T group there was significant ($p < 0.001$) reduction in percentage parasitemia

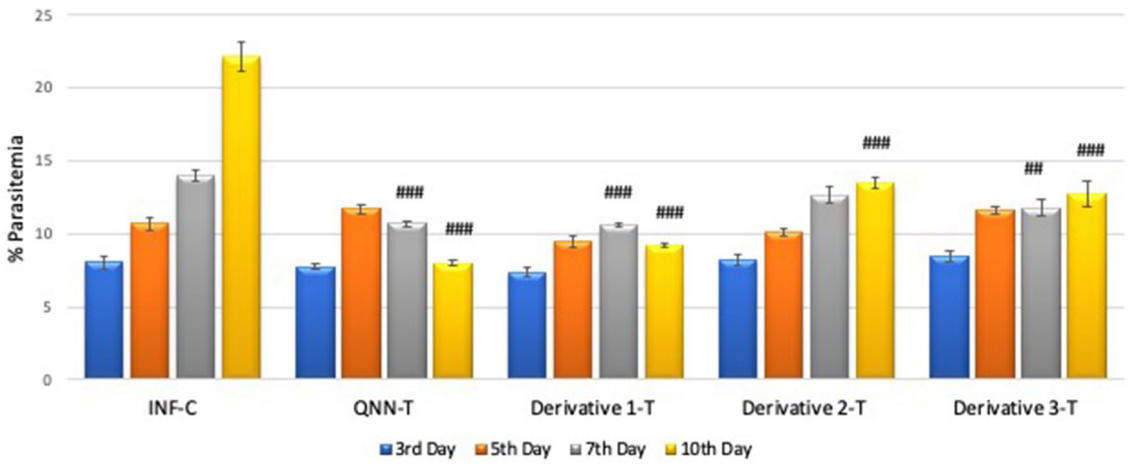


Fig. 2 Effect of Chalcone derivatives 1 (10 mg/kg), 2 (20 mg/kg) and 3 (10 mg/kg) and Quinine hydrochloride (20 mg/kg) on parasitemia (%) (parasitized RBC count) in Cerebral malaria-induced mice. Data are represented as mean ± SEM, $n = 12$ mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ ($^{\#}$Group represents QNN-T, chalcone derivatives 1, 2, and 3 compared to infected Control group)

on 7th (10.7 ± 0.16) and 10th (8 ± 0.18) day post-infection as compared to infected control (13.98 ± 0.39) and (22.17 ± 1.01). Also chalcone derivative 1 group showed significant ($p < 0.001$) reduction in parasitemia level on 7th day of post-infection. However, all three chalcones treated groups showed significant ($p < 0.001$) decrease in percentage parasitemia at 10th day (12.73 ± 0.87), derivative 1 (9.22 ± 0.1), 2 (13.47 ± 0.4) and 3 (12.73 ± 0.87), respectively (Fig. 2).

Performance in elevated plus maze task

The level of anxiety in non-infected, infected control and treated groups were evaluated by EPM. EPM experiment heat map presentation (Fig. 3A). CM-induced mice displayed high anxiety and fear by spending more time in the close arm of EPM. Infected control group spent more time in the close arm than the treated group, both before the treatment and after the treatment however no significant difference was observed (Fig. 3C). Significant difference ($p < 0.01$) was observed in time spent in open arm in infected

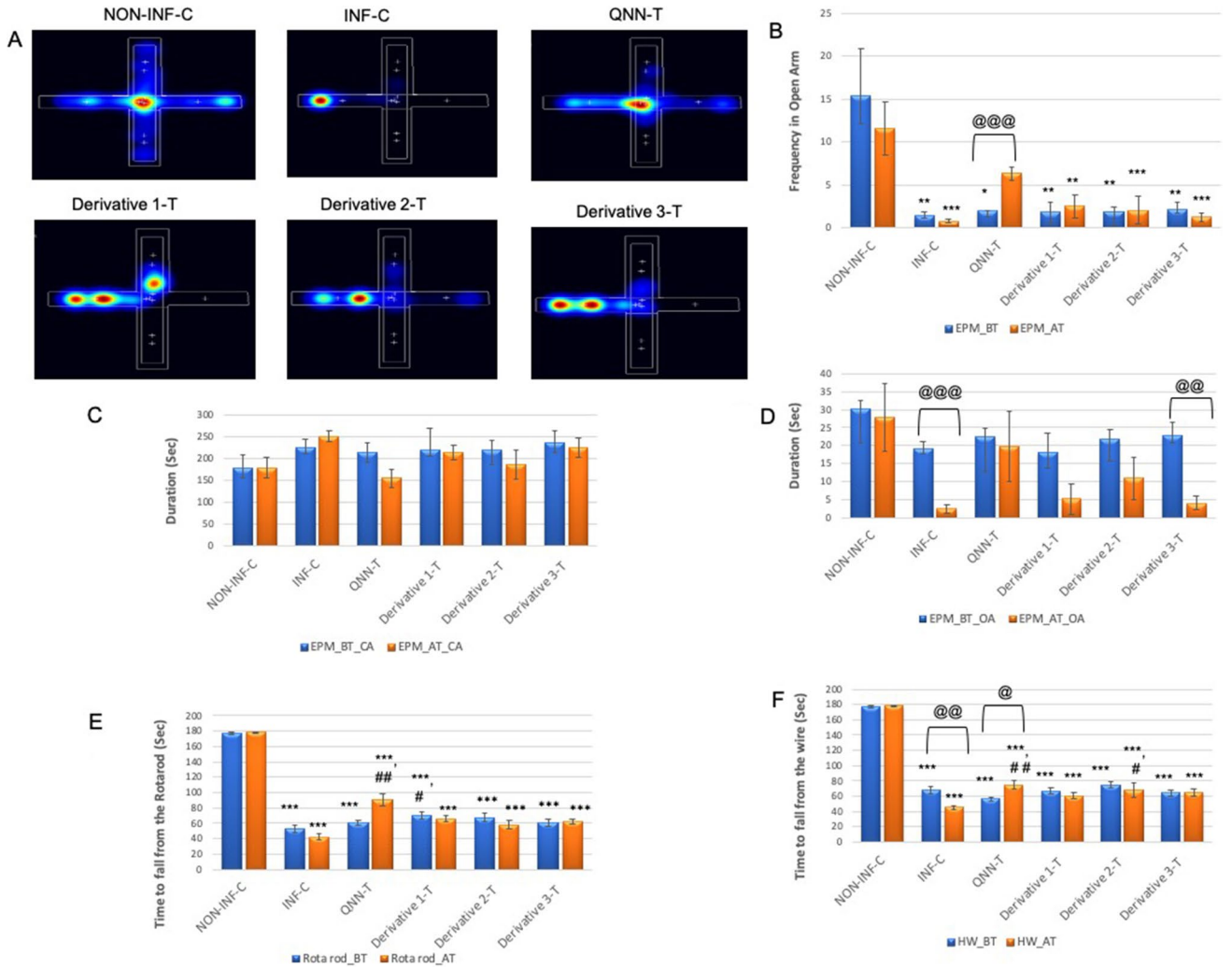


Fig. 3 **A** Elevated plus maze experiment heat map presentation. Cerebral malaria-induced mice displayed high anxiety and fear by spending more time in the close arm of EPM. Cerebral malaria-induced mice treated with Chalcone derivatives 1 (10 mg/kg), 2 (20 mg/kg), 3 (10 mg/kg) and Quinine hydrochloride (20 mg/kg) for five days. **B** EPM experiment. Number of entries in the open arm compared with non-infected mice before and after treatment. **C**, **D** EPM experiment. Cerebral malaria-induced mice displayed high anxiety and fear by spending more time in the close arm of EPM as compared to mice treated with Quinine hydrochloride (20 mg/kg). **E** Time (sec) to fall from rotarod. **F** Time to fall (sec) from wire. Data are represented as mean ± SEM, $n = 12$ mice per group. Analyzed by one-way ANOVA followed by Bonferroni multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001 (*Group represents INF-C, QNN-T, Chalcone derivatives 1, 2 and 3 compared to NON-INF-C Control group) and #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (#Group represents QNN-T, chalcone derivatives 1, 2, and 3 compared to infected Control group); Paired sample T test was performed for before and after treatment comparison. @$p < 0.05$, @@$p < 0.01$, @@@$p < 0.001$ (@ signifies for Paired sample T test)

control group and in derivative 3-T group ($p < 0.05$) before and after treatment (Fig. 3D). Also the frequency of entering in open arm was increased after treatment with quinine in the QNN-T group ($p < 0.001$) (Fig. 3B).

Motor co-ordination in rota-rod and hanging wire test

Motor co-ordination was evaluated by the rota-rod experiment. Significant ($p < 0.001$) reduction in motor co-ordination was observed in all infected and treated group. However, treatment with quinine and derivative 1 has improved the motor co-ordination ($p < 0.001$) and ($p < 0.05$) as compared to infected control (Fig. 3E). Grip strength was also significantly ($p < 0.05$) improved in quinine treated group (Fig. 3F).

Rectal temperature, body weight and organs weight

The rectal temperature of all infected control and treated group exhibited significant ($p < 0.001$) rise in temperature on 5th and 7th day compared to non-infected control. However significant ($p < 0.001$) decrease in temperature was observed at 10th day in infected control (94.74 ± 0.35 °F) and derivative 1-T group (95.49 ± 0.12 °F) compared to non-infected control (96.75 ± 0.08 °F). However, when compared to infected control there was significant increase in temperature at 10th day in QNN-T group ($p < 0.01$), derivative 1 ($p < 0.05$) and 2 ($p < 0.001$) shown in (Fig. 4 A).

There was no significant difference of increase or decrease in body weight between all six groups. However, gradual decline in weight was observed from 3rd day third to 10th day in infected control group and all treatment groups (Fig. 4B).

The wet weights of three organs, liver, spleen and brain were recorded at 10th day of post-infection after euthanesia. There was significant ($p < 0.001$) increase in liver weight of infected control (1.17 ± 0.067), derivatives 1 (1.05 ± 0.35), 2 (1.08 ± 0.08), and 3 (1.13 ± 0.04) and in QNN-T ($p < 0.05$; 0.92 ± 0.08) as compared to non-infected control (0.62 ± 0.02). Similarly, as compared to non-infected control there were significant ($p < 0.001$) increase in weight of spleen of infected control, QNN-T, derivative 1, 2 and 3 treated groups. However, no significant differences were observed in weight of brain in all infected and treated group as compared to non-infected group. Also, no significant differences were observed in the weight of liver, spleen and brain in all treated group as compared to infected group (Fig. 4C).

BBB permeability

The permeability of the BBB was measured using the Evans blue assay to further analyze the impact of chalcones derivatives on the integrity of the BBB and its contribution to neurological symptoms in *P. berghei* ANKA-infected mice. Between the infected and treated groups,

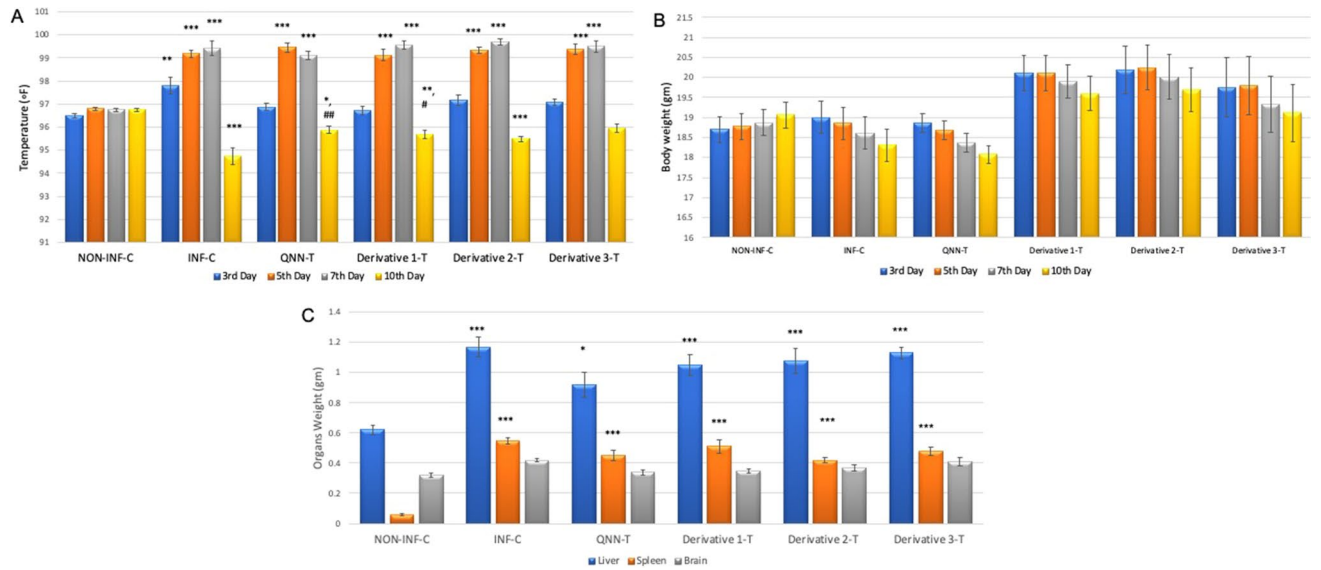


Fig. 4 Effect of Chalcone derivatives 1 (10 mg/kg), 2 (20 mg/kg) and 3 (10 mg/kg) and Quinine hydrochloride (20 mg/kg) on **A** temperature (°F), **B** body weight (g) and Organ weight (g) in cerebral malaria-induced mice. Data are represented as mean ± SEM, *n* = *12* mice per group. Analyzed by one-way ANOVA followed by Bonferroni multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (*Group represents INF-C, QNN-T, chalcone derivatives 1, 2 and 3 compared to NON-INF-C Control group) and #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (#Group represents QNN-T, chalcone derivatives 1, 2, and 3 compared to infected Control group)

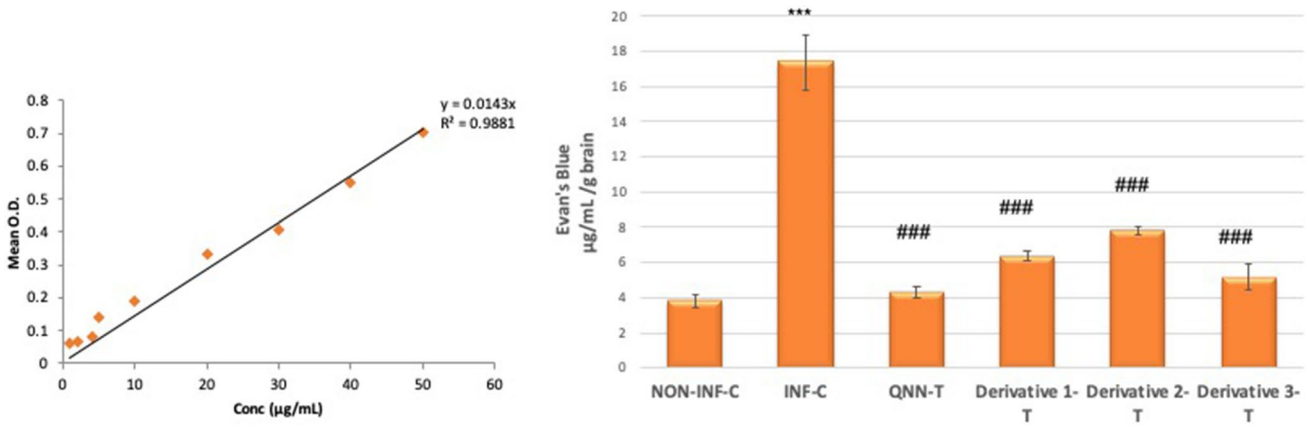


Fig. 5 Effect of Chalcone derivatives 1 (10 mg/kg), 2 (20 mg/kg) and 3 (10 mg/kg) and Quinine hydrochloride (20 mg/kg) on BBB Evans Blue Integrity (µg/mL) in Cerebral malaria-induced mice. Data are represented as mean ± SEM, $n = 6$ mice per group. Analyzed by one-way ANOVA followed by Bonferroni multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (*Group represents INF-C, QNN-T, Chalcone derivatives 1, 2 and 3 compared to NON-INF-C Control group) and $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ (#Group represents QNN-T, chalcone derivatives 1, 2, and 3 compared to infected Control group)

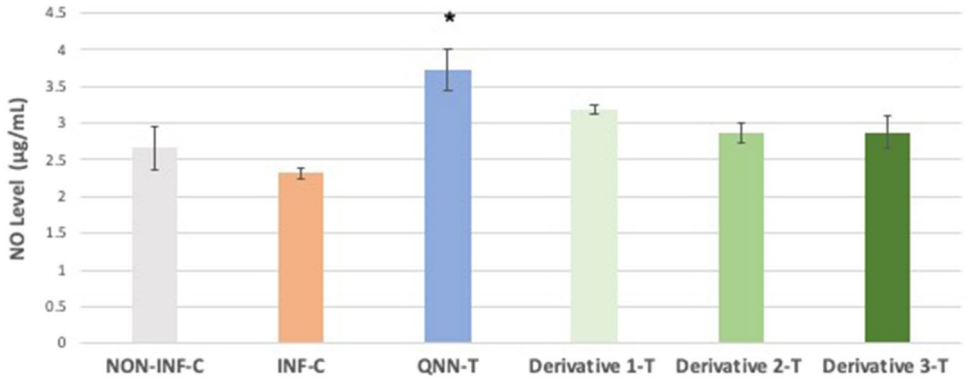


Fig. 6 Effect of Chalcone derivatives 1 (10 mg/kg), 2 (20 mg/kg) and 3 (10 mg/kg) and Quinine hydrochloride (20 mg/kg) on Nitric Oxide (µg/mL) in Cerebral malaria-induced mice. Data are represented as mean ± SEM, $n = 6$ mice per group. Analyzed by one-way ANOVA followed by Bonferroni multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (*Group represents INF-C, QNN-T, Chalcone derivatives 1, 2 and 3 compared to NON-INF-C Control group) and $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ (#Group represents QNN-T, chalcone derivatives 1, 2, and 3 compared to infected Control group)

there were statistically significant variations observed in the amounts of Evans blue dye that leaked from the mouse brains (Fig. 5).

Nitric oxide level

Nitric oxide (NO) production was estimated in serum samples of mice sacrificed on day 10th, post-infection using Griess reagent. The level of NO was found to be significant in QNN-T groups as compared to non-infected control group (Fig. 6).

Cytokines level

BD CBA Mouse Soluble Protein Flex Set (IL-6, IL-10, IL-12p70, IL-1β, TNF, IFN-γ) analysis revealed extremely significant ($p < 0.001$) increase in serum level of IL-6, IL-10, IL-1β, TNF and IFN-γ and significant ($p < 0.01$) increase in IL-12p70 level in infected group, as compared to non-infected control. There was extremely significant ($p < 0.001$) reduction in IL-6 level, IL-10 and TNF in all treated group as compared to infected control. The IL-1β expression was decreased extremely ($p < 0.001$) in QNN-T (4.05 ± 2.5 pg/mL) and derivative 1-T (1.25 ± 1.25 pg/mL) as compared to infected control (86.42 ± 22.8 pg/mL). However the level of IFN-γ was significantly ($p < 0.05$)

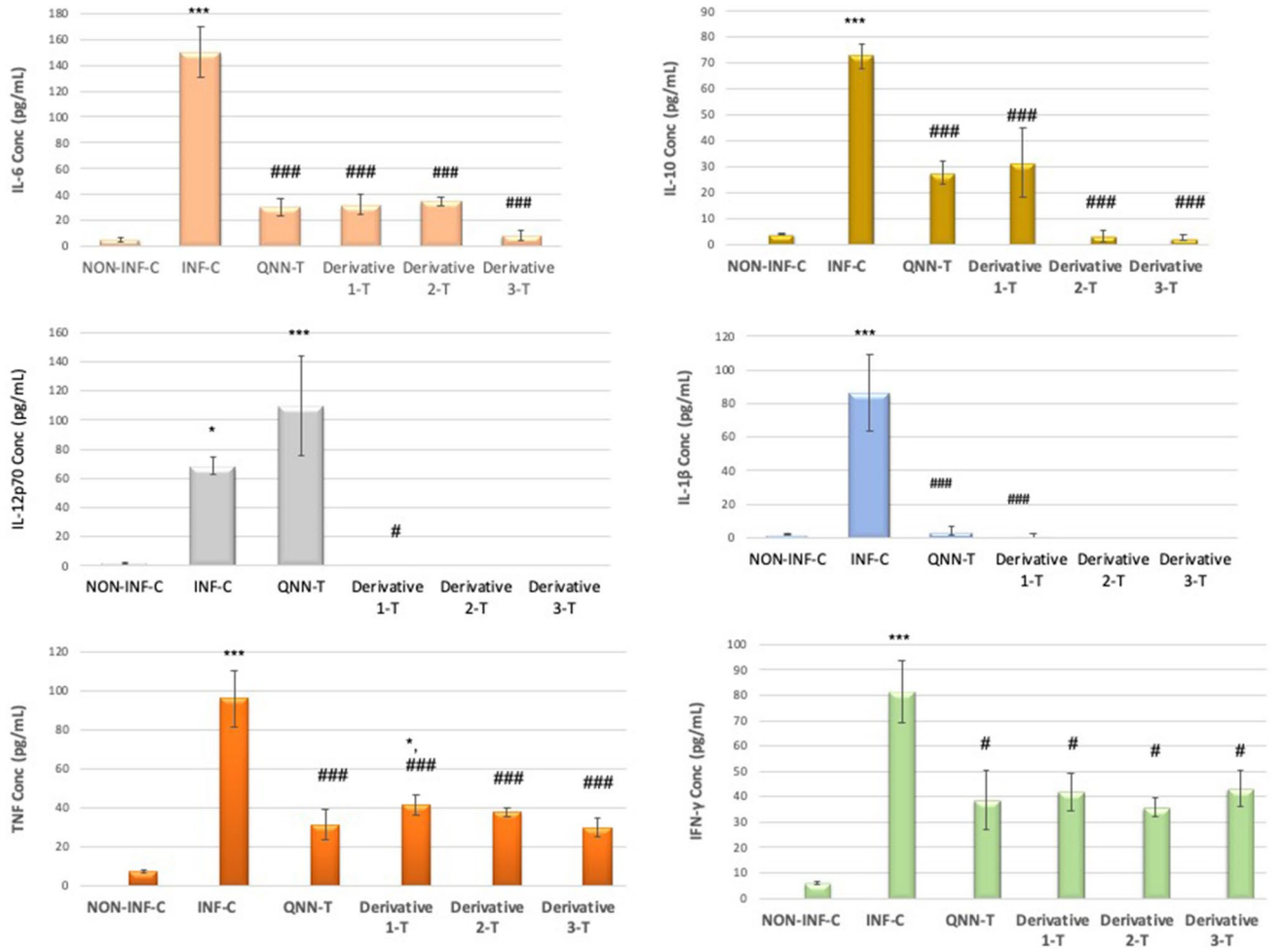


Fig. 7 Effect of Chalcone derivatives 1 (10 mg/kg), 2 (20 mg/kg) and 3 (10 mg/kg) and Quinine hydrochloride (20 mg/kg) on Cytokines expression (pg/mL) in Cerebral malaria-induced mice. Data are represented as mean ± SEM, $n = 5$ mice per group. Analyzed by one-way ANOVA followed by Bonferroni multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (*Group represents INF-C, QNN-T, Chalcone derivatives 1, 2 and 3 compared to NON-INF-C Control group) and $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ (#Group represents QNN-T, chalcone derivatives 1, 2, and 3 compared to infected Control group)

decreased in all treatment groups as compared to infected control (81.36 ± 12.26 pg/mL) (Fig. 7).

Histopathology

Histopathological sections of liver showed even more prominent accumulation of hemozoin pigment, in which kupffer cells were loaded with parasitized cells, congestion of the sinusoids by pRBCs was also observed in infected control group. The pigment accumulation is found to be very less in QNN-T group, but there was more pigment deposition in chalcone derivatives treated groups compared to QNN-T (Fig. 8A).

The spleen sections showed heavy deposition of hemozoin pigment in the pulp histiocytes and sinusoidal lining cells which was profoundly scattered all over the section and very few of these pigments were seen in all the treatment groups (Fig. 8B).

Brain histology showed pigment accumulation in the smaller vessels, microvascular occlusion caused by large numbers of sequestered iRBC only in infected control. No pigment deposition was observed in QNN-T group and other chalcone derivatives treated group (Fig. 8C).

Immunohistochemistry

Mild expression was seen in endothelial lining of sinusoids, was observed in non-infected control whereas, severe expression was observed in all infected control mice showing intense positivity on endothelial lining of

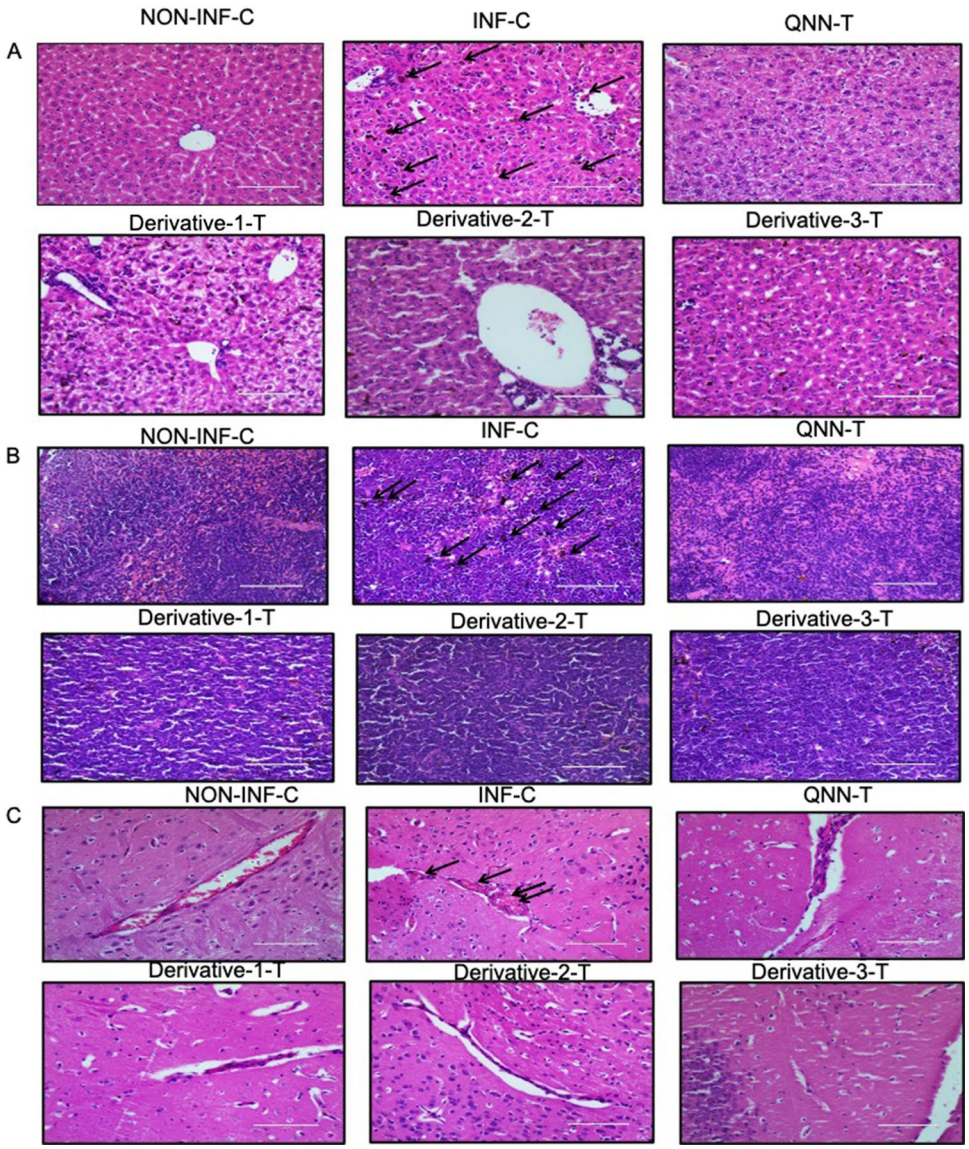


Fig. 8 Photomicrographs depicting Hematoxylin and Eosin stained Liver section at 400 X, spleen section 400 × and, brain 400 × magnifications of Cerebral malaria-induced mice treated with Chalcone derivatives 1 (10 mg/kg), 2 (20 mg/kg) and 3 (10 mg/kg) using Quinine hydrochloride (20 mg/kg) as standard antimalarial drug for 5 days **A** non-infected control, **B** infected control **C** quinine hydrochloride-treated (20 mg/kg) **D** chalcone derivative 1-treated (10 mg/kg), **E** derivative 2 Treated (20 mg/kg), and **F** derivative 3 Treated (10 mg/kg). Arrows indicates deposition of hemozoin pigment. Bar scale represents 100 µm

sinusoid as well as hepatocytes. Moderate expression was observed in sinusoidal endothelium and hepatocytes of QNN-T as well as in chalcone derivatives treated groups (Fig. 9A). Percentage ICAM-1 expression is shown in Table 2 of each six studied group ($n = 3$).

ICAM-1 expression in immunostained spleen section revealed 66.66% of mild expression and 33.33% moderate expression only in QNN-T, whereas 33.33% of severe and 66.66% of moderate expression was observed in derivative 1 and 3 and 100% of mild expression was observed in derivative 2 treated group when compared to non-infected control (100% mild expression) and infected control (100% severe expression) (Fig. 9B; Table 2). Similar observation was also observed in brain showing severe expression in all infected control mice (Fig. 9C; Table 2).

Electron microscopy

Electron Microscopy, elucidates several pRBC insides macrophages and endothelial cells via their surface knobs in liver and spleen in case of derivative 1 treated and QNN treated group (Fig. 10A, B).

In brain section, electron microscopy elucidates, cerebral vessel consisting of a number of loosely packed RBCs, marginated pRBCs and Ghosts RBCS. The two pRBCs appear to be surrounded by uninfected RBCs which represent rosette formation in derivative 1 treated group. In the small congested vessels, the majority of the RBCs were found infected (Fig. 10C), and in most of the cases, the parasites were distorted which may be because of drug treatment, and visualised as a small pyknotic or vacuolated (Fig. 10C, QNN-T) structures.

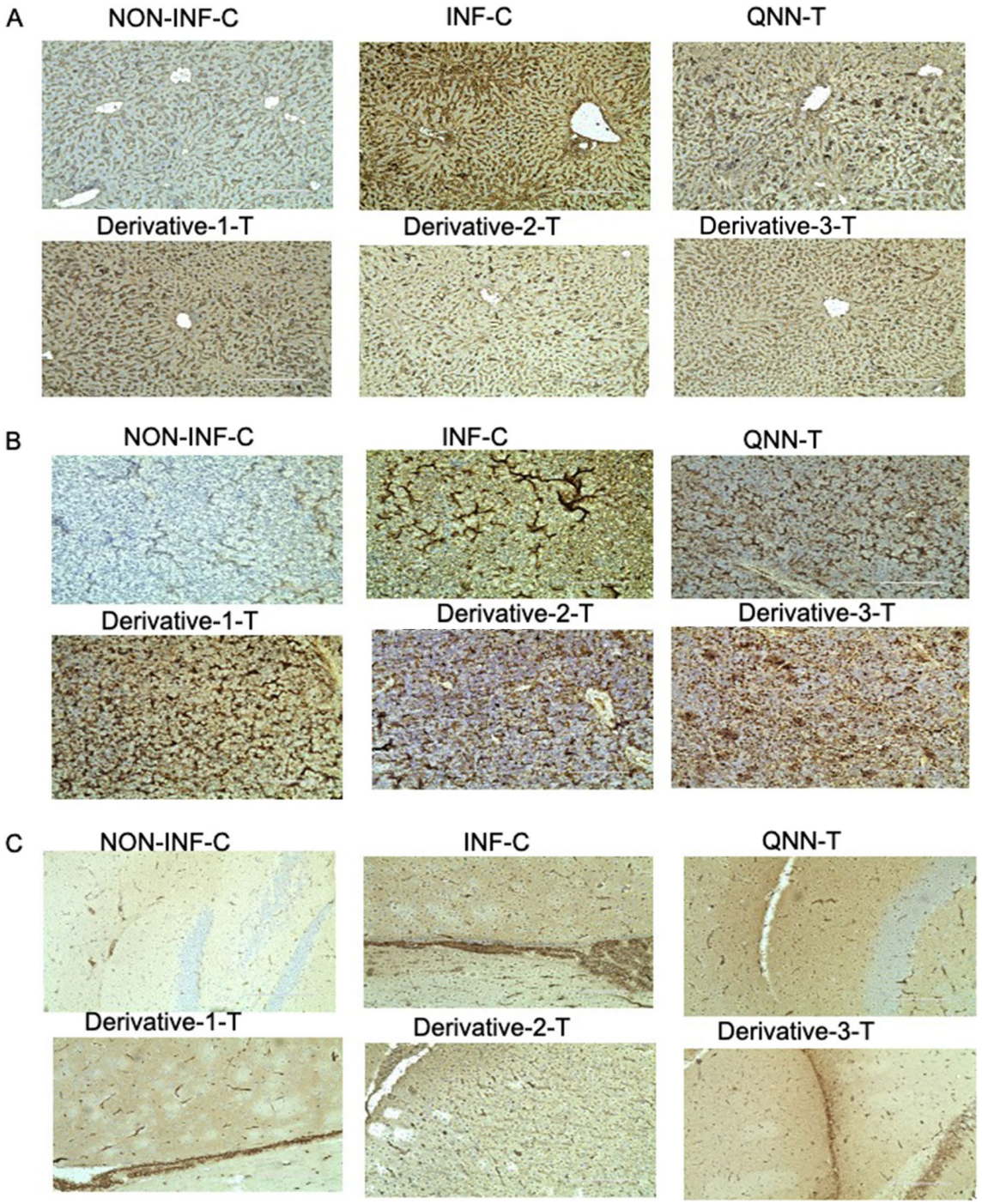


Fig. 9 Photomicrographs depicting ICAM-1 stained liver section at 200 ×, spleen 200 ×, and brain 200 × magnifications of Cerebral malaria-induced mice treated with Chalcone derivatives 1 (10 mg/kg), 2 (20 mg/kg) and 3 (10 mg/kg) using Qunine hydrochloride (20 mg/kg) as standard antimalarial drug for five days. Biotin-conjugated secondary antibody and streptavidin-conjugated horseradish peroxidase from DAB Substrate kit (Scy Tek) were applied to sections to amplify the antigen signal for subsequent 3,3-diaminobenzidine staining, which produces a permanent brown color

Discussion

Due to restricted range of therapeutic molecules for the CM treatment and fear of resistance, new drugs are always in demand to resolve future needs. Chalcones are natural precursors of flavonoids and isoflavonoids in higher plants and are related to other recognised antioxidants such as resveratrol, curcumin, and ubiquinone (Sahu et al. 2012; Lin et al.

Table 2 ICAM-1 Expression in tissue sections of *P. berghei* ANKA infected mice treated with chalcones derivatives and Quinine. Pearson Chi-square, contingency co-efficient, correlations test was applied for statistical significance (*p < 0.05)

Animal grouping ($n=3$)	ICAM-1 expression in tissues sections (liver, spleen and brain)			
	Mild (+)	Moderate (++)	Severe (+++)	p value
NON-INF-C	3 (100%); Liver 3 (100%); Spleen 3 (100%); Brain			
INF-C			3 (100%); liver 3 (100%); spleen 3 (100%); brain	*$p=0.014$; liver *$p=0.014$; spleen *$p=0.014$; brain
QNN-T	2 (66.6%); Spleen	3 (100%); liver 1 (33.33%); spleen 2 (66.6%); brain	1 (33.33%); brain	*$p=0.014$; liver *$p=0.083$; spleen *$p=0.083$; brain
Derivative 1-T		2 (66.6%); liver 1 (33.33%); spleen 2 (66.6%); Brain	1 (33.33%); liver 2 (66.6%); spleen 1 (33.33%); brain	*$p=0.05$; liver *$p=0.05$; spleen *$p=0.05$; brain
Derivative 2-T	1 (33.33%); Liver	2 (66.6%); liver 2 (100%); spleen 2 (100%); brain	1 (33.33%); brain	*$p=0.083$; Liver *$p=0.014$; spleen *$p=0.05$; brain
Derivative 3-T		2 (66.6%); liver 2 (66.6%); spleen	1 (33.33%); liver 1 (33.33%); spleen 3 (100%); brain	*$p=0.05$; liver *$p=0.05$; spleen *$p=0.014$; brain

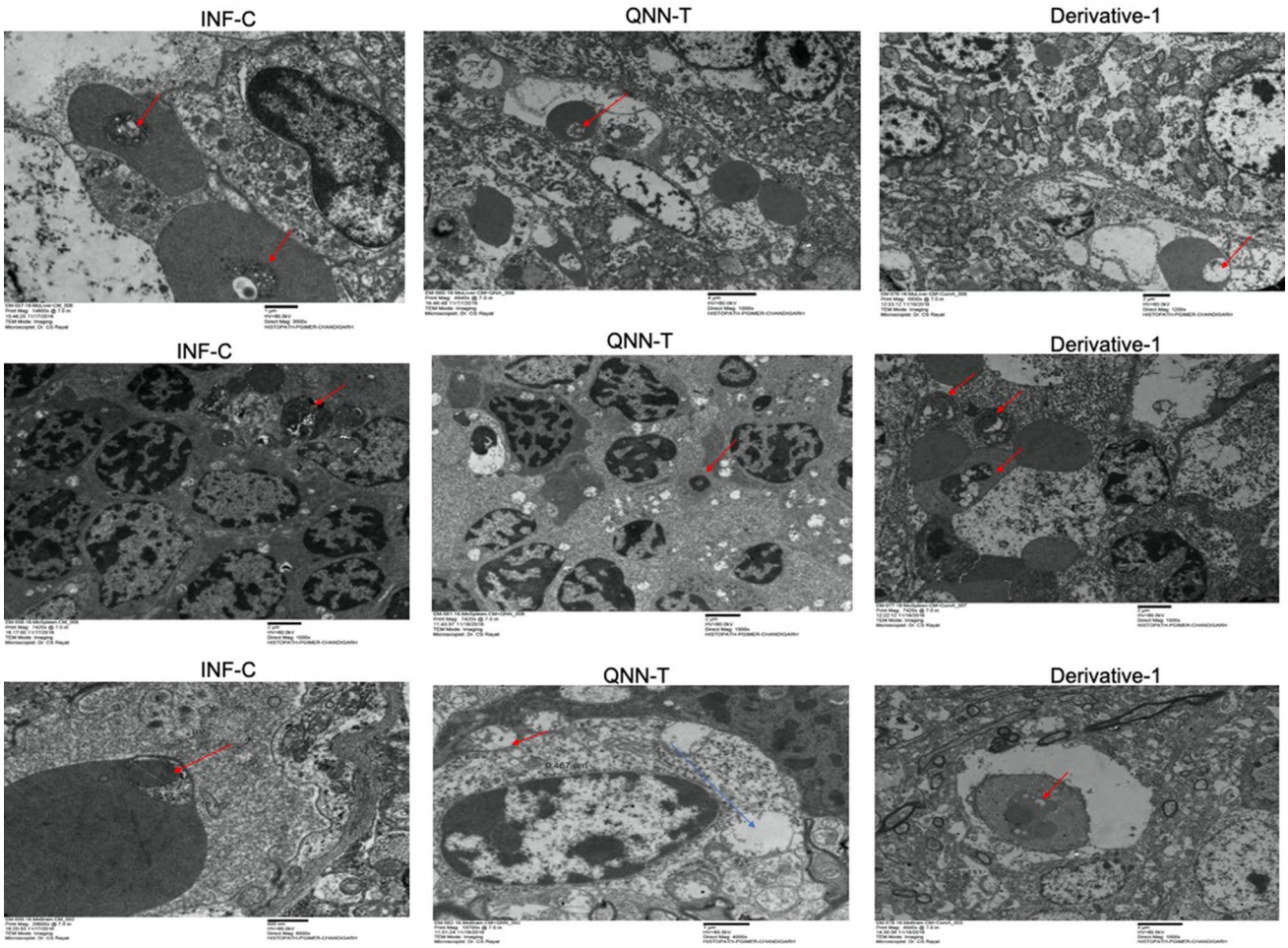


Fig. 10 Electron micrographs depicting effect Chalcone derivative on liver, spleen, and brain section at different magnifications of Cerebral malaria-induced mice treated with Chalcone derivatives (10 mg/kg), using Quinine hydrochloride (20 mg/kg) as standard antimalarial drug for five days

2002; Mojzis et al. 2008; Dal et al. 2010). Chalcones and their derivatives have previously been shown to have anti-cancer, anti-diabetic, anti-inflammatory, antioxidant, antibacterial, antiparasitic, psychotropic, and neuroprotective activities in preclinical studies (Salehi et al. 2021). In the present study, infected mice showed extremely significant decrease in percentage parasitemia at 10th day post-infection in all three chalcones treated groups compared to non-treated groups, which is concordant to previous studies which showed significant inhibition of parasite when chalcone derivatives was administrated intraperitoneally, or subcutaneously, or orally to *Plasmodium yoelii*–infected mice model. These results demonstrate potent antimalarial activity which can be exploited for development of new antimalarial drug (Chen et al. 1997; Tomar et al. 2010). Moreover, in CM, neurological signs are most important features for early diagnosis and treatment. Here, neurobehavior sign in the PbA infected mouse was observed and was tested for anxiety-like behavior in control and infected mice on day 5 post-infection and day 10th of infection and the resultant data elucidated very mild anxiolytic activity of chalcones as compared to standard treatment quinine. Interestingly, some studies have reported for anxiolytic effect of chalcones, such as 'isoliquiritigenin' (Cho et al. 2011), 5′-methyl-2′-hydroxychalcone (Higgs et al. 2019), and some like ^{1}H-1,2,3-triazole grafted tacrine-chalcone conjugates has potential to improve the motor-function and anxiety-like behavior, in mice pre-treated with scopolamine as compared to standard drug tacrine (Rani et al. 2021). Additionally, the study shows non-significant decrease in body weight, temperature of all treated and infected mice which indicates towards terminal illness and all these conditions may suggest extended therapy of chalcones.

Destruction of BBB integrity is the main mechanism that initiates complicated molecular processes and inflammatory responses. This further enhances neurodegeneration and delay the process of repair for neurologic function that can execrates neurological dysfunction and nerve cell injury (Obermeier et al. 2013). Next, BBB integrity was checked on 10th day post-infection in all groups and observed that chalcones are significantly effective in able to maintain the BBB integrity along with quinine compared to non-treated infected groups. Also, non-significant increase in NO in all treatment group including quinine treated compared to infected groups that suggest protective mechanism of Chalcones. Earlier Zhang et al. have shown that Isoliquiritigenin, maintain BBB integrity in vivo and prohibits the inflammatory responses as well as activation of microglia in mice after Traumatic brain injury (Zhang et al. 2018).

The chief pathophysiological phenomenon involved in the progression of CM are the activation of endothelial cell, a loss of BBB integrity and an imbalance of pro and anti-inflammatory responses (Dunst et al. 2017). Moreover, proinflammatory cytokines, such as TNF, lymphotoxin α (LTα), IFN-γ, interleukin-1α (IL-1α), and IL-1β, are elevated during CM and have been majorly contributed to the pathogenesis of the disease (Lyke et al. 2004). Chalcone derivatives in the present study, reduces the serum levels of the cytokines IL-10, TNF, IL-12(p70), IL-6 and IFN-γ, thereby improving the integrity of BBB during ECM. Additionally dysfunction of the endothelial cells may occur and this dysfunction in case of malaria is also linked with the lower levels of NO and its precursor, L-arginine (Yeo et al. 2007). NO is crucial for the optimum functioning of endothelial cells (Tousoulis et al. 2012). NO found to protects the endothelial cells by (i) diminishing the expression of cytokine-stimulated adhesion molecules like ICAM-1, VCAM-1, and E-selectin on endothelial cells (Yeo et al. 2007; Cooke 2004), which, results in poor proinflammatory cytokine responses by limiting the accumulation of leukocytes inside the brain, and (ii) diminishing the adherence of iRBCs to the microvascular endothelium (Serirom et al. 2003), the chief stimulator for CM-associated pathology. Further research is required in this direction where drug could obstruct the pathophysiological processes to control neurological complications and death (Mturi et al. 2003). Moreover, upregulated proinflammatory cytokines such as, TNF by the endothelial cells disturbs the BBB integrity by tailoring the tight junctions and this whole complex phenomenon is well reversed after treatment with chalcone derivative in this study. Also chalones down regulate the expression of ICAM-1 and hence may reduce the disease severity. Histology of vital organs showed sequestration of pRBCs in the microvessels of brain which revealed severity of the disease as compared to treated group which further illustrates the association of cytokine overproduction that may contribute to brain vascular pathology, as TNF and IFN-γ were shown to be crucial mediators in the pathogenesis of CM. Finally, in present study ultra-structure of brain, liver and spleen, all shows pRBCs and parasites in macrophages, in all infected and treated groups that suggest clearance of parasites from peripheral circulation which can be observed as low parasitemia in giemsa stained slides but those trapped in tissues vesicles are well observed through electron microscope and these trapped parasites can be gradually degraded with time, which can be also co-related with the persistence positive immunoreactivity in most of the treated group.

Earlier it has been suggested that, immunomodulators can be used for CM treatment in the form of an adjunct therapy which can regulate the production of pro-inflammatory cytokine and reduces the expression of adhesion molecules, thereby controlling the parasite sequestration which could finally improve the disease condition of the patient's (Cariaco et al. 2018; Mimche et al. 2011), which is found to be a similar perspective from our study as well.

Conclusion

CM, a severe malaria form that must be taken as a serious issues because of its left over treatment failure. The main mechanisms of CM include the sequestration of pRBCs in brain capillaries, the generation of cytokines, the accumulation of immune cells/platelets, and the release of microparticles, all of which contribute to various brain injuries (oedema, haemorrhages and ischemia) (Bruneel 2019). So, reaching to a drug molecule that can act on a multiple pathways along with directly killing the malarial parasite is a needed for future options for the treatment of severe form of malaria. The present derivatives can be pioneered by various research and science groups to design such scaffold that will be a future antimalarials with therapeutic potentials or because of its immunomodulatory properties, it can be used as an adjunct therapy.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13205-023-03676-y.

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Author contributions RS, BM, BDR and SS designed the study. SS carried out the experiment and wrote the initial draft of the manuscript. BDR examined the Histopathological and IHC Data. All authors SS, RS, BDR, BM, DIB and NM did the final editing of manuscript and agreed to the publication of this study.

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Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The ethical approval was taken from the Institutional Animal Ethics Committee, Postgraduate Institute of Medical Education and Research, Chandigarh, Reference No. 69/IAEC/418 as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

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