Rutin Ameliorates Malaria Pathogenesis by Modulating Inflammatory Mechanism: An In-Vitro and In-Vivo Study

Divya Bhatt
CSIR - Central Institute of Medicinal and Aromatic Plants
Saurabh Kumar
CIMAP: CSIR - Central Institute of Medicinal and Aromatic Plants
Parmanand Kumar
CSIR-CIMAP: Central Institute of Medicinal and Aromatic Plants CSIR
Surbhi Bisht
CIMAP: CSIR - Central Institute of Medicinal and Aromatic Plants
Anil Kumar Maurya
CSIR-CIMAP: CSIR - Central Institute of Medicinal and Aromatic Plants
Anirban Pal
CSIR-CIMAP: CSIR - Central Institute of Medicinal and Aromatic Plants
Dnyaneshwar Umrao Bawankule (bawankuledu@gmail.com)
Central Institue of Medicinal and Aromatic Plants https://orcid.org/0000-0003-0677-0981

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Abstract

Rutin (3,3',4' 5,7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonoid glycoside, found in many edible plants such as buckwheat and berries. Rutin as a food supplement is recommended for the treatment of various diseases, which directed us to investigate its valuable effects in malaria induced pathogenesis. In the present study, Rutin was tested for its anti-plasmodial activity against chloroquine sensitive and resistant strains (NF-54 and K1) of *Plasmodium falciparum* and studied for its anti-oxidative and anti-inflammatory potential against LPS stimulated macrophage cells. *In vitro* observations were further validated using an *in-vivo* physiological rodent model of *Plasmodium berghei*-induced malaria pathogenesis. Rutin was also tested for its effect in combination with chloroquine.

Rutin was found to have potent anti-plasmodial activity against both chloroquine sensitive and resistant strains of *P. falciparum* (NF-54 and K1). It was able to reduce the oxidative stress induced by LPS in macrophage cells with decreased production of pro-inflammatory cytokines (IL-6, TNF-α and IL-1β). Rutin was found to significantly suppress the parasitaemia, increase the mean survival time and restored the haemoglobin and glucose level in *in vivo* assays. This was corroborated by reduced production of malondialdehyde (MDA) and pro-inflammatory mediators in rutin treated mice in *P.berghei*-induced malaria pathogenesis. Interestingly, the combination of rutin with chloroquine had shown synergy in both *in vitro* and *in vivo* experiments. The findings of the present study thus highlighted the suitability of rutin for further study in the management of drug resistant malaria, alone or in combination with other compounds.

Introduction

Malaria is arguably the main public health concern worldwide due to its high rate of mortality and morbidity across the Tropical and Sub-tropical regions with an estimated 229 million cases in 2019 (WHO 2020). An increase in the malaria epidemic was witnessed owing to emerging parasite resistance to all the subsisting antimalarial drugs, turning up of the insecticidal resistance, dearth of vaccines countering malaria spread, and also due to alteration in the nature of Anopheles vectors (Menard and Dondorp 2017). During malaria infection, the host undergo severe oxidative stress (Franklin et al. 2011), which variously challenge the immune system of the infected host to activate macrophages, leading to the production of free radicals and pro-inflammatory cytokines such as TNF-α, IFN-γ and IL1-β ((De Souza et al. 2016). Any imbalance in this inflammatory response may result in immunopathology (Drewry et al. 2018). Thus, modulation of the host inflammatory response exerted by the malaria parasite could be a considerable therapeutic approach against malaria pathogenesis such as the signalling kinases activated in the host in response to the malaria infection manifested a promising target for the anti-malarial interference (Adderley et al. 2020). Similarly, antioxidant molecules such as vitamin C renders its protection against the outcome of malaria pathology by regulating inflammatory response (Kauffmann et al. 2021). The expansion of resistance developed by *P. falciparum* to the existing antimalarials has intensified the quest for drugs with a new mechanism of action (Rout and Mahapatra 2019). In that

regard, the plants or plant-derived bioactives by exerting immunomodulatory effects on the host immune system are considered as the desirable source (Wright et al. 2005).

Rutin, an important dietary flavonoid derived naturally from various plant sources such as buckwheat, tea, apple, apricots, cherries, oranges, grapefruit and plums. It is often referred to as rutoside or vitamin P and exhibits a various range of beneficial biological effects including cytoprotective, hepatoprotective, antioxidative, neuroprotective, antibacterial and vasoprotective activities (Ganeshpurkar and Saluja 2017; Prasad and Prasad 2019) and also known to exhibit enhanced cardio and renal protective activities by modulating the oxidative stress and inflammation (Nafees et al. 2015; Al-Harbi et al. 2019). Rutin showed evidence of parasiticidal activity by eliciting cell-mediated immune response through up-regulation of NF-kB and iNOS gene expression and reduction of the apoptosis induced by malaria infection (Chauhan et al. 2018; Oludele et al. 2020). Considering the pharmacological significance of rutin, the present study tested the effect of rutin on malaria pathogenesis with reference to the inflammatory response during *in vitro* and *in vivo* experimental models using the standard molecular pharmacological approach. The findings of this study exhibited that rutin treatment markedly diminish malaria pathogenesis with modulation of oxidative stress and inflammatory response. Interestingly, this study also showed that rutin, either alone or in combination with other compounds is suitable for further research on the management of drug-resistant malaria.

Materials And Methods

2.1. Chemicals

Rutin-trihydrate (Fig.1), chloroquine diphosphate, DMSO, D-sorbitol, gentamycin, hypoxanthine, LPS (Escherichia coli 055:B5), DMEM, penicillin, streptomycin, Fetal Bovine Serum, MTT, Phosphate Buffered Saline, Triton X-100, potassium chloride, Thiobarbituricacid, Trichloroacetic acid, TMB substrate, were bought from Sigma-Aldrich, USA. Albumax II, RPMI- 1640, Fetal Bovine Serum, and fungizone were purchased from Gibco (Grand Island, United States) while (CM-H_2DCFDA) chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate was procured from Invitrogen (Carlsbad, CA, United States). Mouse specific (IL-6, TNF-α, IFN-γ and IL-1β) ELISA Kits purchased from BD Biosciences.

2.2. *In vitro* Study

2.2.1. Maintainance of *in vitro Plasmodium falciparum* culture

The cultivation of *P. falciparum* sensitive and resistant (NF-54 and K1) strains was done in human red blood cells (B^+) added with RPMI-1640 medium containing the supplements like hypoxanthine (370μM), HEPES (25 mM), $NaHCO_3$ (0.2%), fungizone (25μg/mL), gentamycin (40μg/mL) and Alumax II (0.5%) at 37°C and 5% CO_2.Beside the change of growth medium every 24 hours, parasitaemia was routinely monitored through Giemsa stained thin smear of cultured parasite to achieve synchronized culture having initial stage parasite (ring stage).

2.2.2. Anti-plasmodial activity of rutin

For the *in vitro* study of the inhibition of *P. falciparum* growth, 200 µL of the ring stage *P. falciparum* culture having 1.2% parasitemia in 2% hematocrit was transferred to 96 well plate. The culture was then treated with rutin (0.1-100µM) and standard drugs (chloroquine and artesunate) and the culture without treatment served as negative control. After 48 hours of incubation at 37°C and 5% CO_2, thin smear of culture was prepared from each well and stained with Giemsa stain to count parasitaemia, Determination of parasitemia was done based on the parasitized RBCs counted in around total 1000 erythrocytes while the percentage suppression of parasitemia was enumerated as [(A-B)/A] 100, where A is the mean percent parasitaemia in the negative control and B refers to the mean parasitaemia of the treatment group. The IC_{50} (Mean±SEM) was determined using non linear regression analysis from concentration-mediated growth inhibition data.

2.2.3. Isolation of Primary Macrophages

The previously described method (Bawankule et al. 2008) was followed to isolate the primary cells from the peritoneal cavity. Briefly, 1.0 mL of intraperitoneal injection of 3% protease peptone was given to eight-week-old Swiss albino female mice three days earlier than harvesting macrophage cells. Just before isolating cells, mice were subjected to ether anaesthesia and sacrificed by cervical displacement. The macrophage cells from the peritoneum were collected by lavage of the peritoneal cavity using chilled PBS (pH-7.4). Then, cells were washed and filtered through sterile gauze and viable cells were adjusted to the required density (0.5×10^6 -1×10^6 cells/mL) in a DMEM medium containing 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). After seeding, cells were incubated overnight at 37°C with 5% CO_2.

2.2.4. The pro-inflammatory cytokine profile of rutin in primary macrophages

For the *in vitro* anti-inflammatory profile assessment of rutin, primary macrophages were seeded as described above. After 24 hours of seeding, cells were pre-treated with rutin at the concentration of 3, 10 and 30 µM along with dexamethasone (1 µM). The cells were then stimulated with LPS (1 µg/mL) and incubated for 24 hours at 37°C with 5% CO_2. After incubation, supernatants from each well were harvested and stored at −80ºC immediately until analysis. Supernatants were tested for estimating the levels of the pro-inflammatory cytokines (IL-6, TNF-α and IL-1β) using mouse-specific Enzyme Immuno Assay (EIA) Kits according to the instructions of the manufacturer.

2.2.5. Determination of cell viability

The effect of rutin on the viability of primary macrophages was studied using MTT assay. In short, primary macrophages (0.5 × 106 cells /well) were seeded in 96 well plate and incubated in CO_2 incubator at 37°C with 5% CO_2 for 24 hours. After incubation, 20 µL MTT (5 mg/mL in PBS) was added into each well and incubated for another 4 hours. Following incubation, media containing MTT was replaced with DMSO (100µL) to solubilize the formazan crystals formed. The absorbance was recorded at 550nm and viability of the cells has been represented in terms of percentage (%) of survival.

2.2.6. Quantification of Reactive Oxygen Species (ROS) generation

IntracellularROS level was determined as previously described (Becker et al., 2003). Briefly, RAW 264.7 cells were cultured using DMEM media containing 10% FBS. For the measurement of intracellularROS, 1×10^6 cells/well was seeded in a 6 well plate and incubated for 24 hours at 5% CO_2 and 37˚C. After incubation, cells were pretreated with rutin at the concentrations of 3, 10 and 30 µM along with dexamethasone (1 µM). After 30 minutes, cells were stimulated with LPS (1µg/mL) for intracellular ROS generation and again incubated for 24 hours. After 24 hours incubation, cells were incubated with fluorescent dye CM-H_2DCFDA (20µM) in culture medium and were further incubated in CO_2 incubator for 20 minutes. Following incubation, the cells were washed twice with PBS and also harvested in PBS after trypsinization. The fluorescence was measured using a flow cytometer (BD Biosciences) operational with a 488 nm argon laser as light source. Using FACS Diva software version 7.1 (BD Biosciences) percentage and mean fluorescence intensity of CMH_2DCFDA positive cells were calculated. Similarly, intracellular ROS level was also measured using spectro fluorometer (Spectramax i3x, Molecular Device) at 485 nm of excitation and 520 nm emission wavelength. Data was expressed as fluorescence unit which depicted the ROS level in rutin treated cells as compared to LPS-induced cells.

2.3. *In vivo* study

2.3.1. Animals and ethical approval

The protocol (CIMAP/IAEC/2020-23/01) followed for the anti-malarial experiment was approved by the Institutional Animal Ethics Committee (IAEC) along with the approval of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Government of India (Registration No: 400/01/AB/CPCSEA).

2.3.2. Mice and parasite infection

To evaluate the suppressive anti-malarial activity of rutin, adult Swiss albino male mice which were bred in-house and aged 6-8 weeks (20 ± 2 g) were chosen as experimental host. Random grouping of animals with 6 mice each in 5 groups was followed by seven days acclimatization in the experimental facility, kept under the standard environmental conditions of 23 ± 2°C, 12 hour light/dark cycle with the supply of food and water on an ad libitum basis. Chloroquine-sensitive rodent malaria strain *Plasmodium berghei* K-173 was maintained at institute by a continuous passage in mice and the blood-stage parasites were preserved in liquid nitrogen.

2.3.3. Anti-malarial study of rutin in *P. berghei* infected mouse model

According to the method of Knight and Peters and few modifications in an already published report (Mohanty et al. 2013), each experimental mice was inoculated intraperitoneally with 0.2 mL suspension of infected blood carrying approximately 1×10^6 *P. berghei* K-173 parasitized red blood cells diluted in sterile ACD. After an hour of infection, mice were administered with an oral dosage of rutin (25,

50, 100 mg/kg/day) prepared in 0.7% CMC while mice receiving vehicle (0.7% CMC) alone were considered as vehicle-treated group. The standard drug chloroquine at the dose of 10 mg/kg/day was orally given and considered as the positive control. The same dose regime was repeated once daily for four days.

2.3.4. Parasitaemia and survival determination

From 4th day post-infection, thin blood smears by tail snip were prepared on every alternate day until day 28th. After staining with Giemsa stain, percent parasitemia was examined by calculating total parasitized and normal RBCs over three optical fields with at least 300 RBCs per field. Determination of parasitemia was done based on the parasitized RBCs counted in around total 1000 erythrocytes, the percentage suppression of parasitemia was enumerated as [(A-B)/A] 100, where A is the mean percent parasitaemia in the negative control (vehicle-treated group) and B refers to the mean parasitaemia of the treatment group. Additionally, for assessing the mean survival time and percent survival, all the treated and non treated groups were followed up to 28 days to record and assess the mortality of individual mice in each group.

2.3.5. Quantification of Hemoglobin and glucose

On the peak day of parasitaemia (8th day), haemoglobin and blood glucose level were assessed in *P. berghei* infected mice. For estimating haemoglobin, the standard Drabkin's cyanmethemoglobin method was followed as per the manufacturer's guidelines. Glucose estimation was done using glucometer (Dr. Morepen, GlucoOne) according to the procedure described by (Mohanty et al. 2013).

2.3.6. Quantification of pro-inflammatory cytokines

Another set of experiment was performed to assess the role of rutin in modulating the profile of inflammatory mediators in the *P. berghei* infected mice. On the peak day (8th day), mice were bled through retro-orbital plexus and collected blood was processed to obtain serum. The animals were then sacrificed for the collection of the whole brain. The pro-inflammatory cytokines (IL-6, TNF-α and IFN-γ,) were then quantified from serum and brain homogenate by using the ELISA kits.

2.3.7. Quantification of malondialdehyde (MDA) content

The amount of MDA in liver, brain and spleen tissues was determined by following the method of Okhawa et.al. (1979) with the modifications described by Oakes & Van Der Kraak. (2003) . The quantification of LPO was done by using the standard curve of MDA and calculated as nM MDA / mL of tissue homogenate.

2.4. Combination study of chloroquine and rutin

2.4.1. Anti-plasmodial profile of chloroquine and rutin combination

The modified fixed ratio method was performed for the study of interaction between chloroquine and rutin (Fivelman et al., 2004). Firstly, the IC_{50} *i.e.*50% inhibitory concentration against the chloroquine-resistant strain K1 of *P. falciparum* was determined for chloroquine and rutin as described in section 2.2.2. The 5 times higher of the respective IC_{50} concentrations of chloroquine and rutin were diluted two-fold with culture medium to initial concentrations, then mixed volume by volume at 4:1 (1.016µM: 20.5µM), 3:2 (0.777µM: 41µM), 2:3 (0.518µM: 61.5µM), 1:4 (0.259µM: 82µM). All these ratios were further diluted two-fold. Then, IC_{50} values were determined for different combinations (chloroquine and rutin), the individual IC_{50} concentrations and the IC_{50} values of the combination were used for the calculation of fractional inhibitory concentrations (FIC) and ∑FIC using the following formula:

$$FIC = \frac{IC_{50} \text{ concentration in combination}}{IC_{50} \text{ concentration alone}}$$

∑FIC= FIC value of chloroquine + FIC value of rutin

2.4.2. Anti-malarial combination study of chloroquine and rutin in *P. berghei* infected mouse model

Another experiment was performed to evaluate the antimalarial potential of rutin in combination with chloroquine. The experiment was carried out as described in section 2.3.3 and mice were treated with an oral dose of rutin (25 mg/kg), CQ (2.5 mg/kg) and a combination of rutin (25mg/kg) and CQ (2.5 mg/kg) along with CQ (10 mg/kg) as the positive control. All the doses were prepared in 0.7% CMC (vehicle) and the group that received only CMC was called as vehicle-treated group. The parasitaemia,haemoglobin and survival were determined to evaluate the potential of rutin and chloroquine combination in *P. berghei* infected mice.

2.5. Statistical Analysis

The results in the present study were expressed in termsof Mean± SEM (*in vitro;* n=3, *in vivo*; n=6) and analysis was done using Graph Pad Prism 5. The comparison between vehicle-treated and rutin-treated groups was made using one-way analysis of variance (ANOVA) along with Turkey's multiple comparison tests. The P-value $P<0.05$ was taken as statistically significant.

Results

3.1. *In vitro* study

3.1.1. Rutin showed inhibition of *P. falciparum* growth

Rutin exhibited potent anti-plasmodial activity with an IC_{50} values of 20.5±0.59 and 26.4 ±1.31 µM against chloroquine sensitive (NF54) and resistant strains (K1) of *P. falciparum* respectively. The IC_{50} for chloroquine under similar conditions was 0.0021±0.0004 and 0.252±0.04 µM while artesunate showed

IC_{50} of 0.0067±0.0002μM and 0.010±0.0003μM against NF54 and K1 strains respectively. The data is represented in Table.1.

3.1.2. Rutin decreases the production of pro-inflammatory cytokines in primary macrophages

Rutin pre-treatment in LPS stimulated macrophages was able to diminish the pro-inflammatory cytokines production (IL-6, IL-1β and TNF-α) in concentration dependent manner and was found to be significant ($p<0.05$), when compared to the cells treated with LPS alone. At the highest concentration (30μM), rutin exhibited 43.82±1.08%, 57.00±7.50% and 35.04±1.60% chemo-suppression of IL-6, IL-1β and TNF-α (Fig.2).

3.1.3. Rutin did not exhibit cell cytotoxicity

MTT assay was done to ascertain the cytotoxic profile of rutin on primary peritoneal macrophage cells. The treatment of the rutin for 24 hours at the concentration of 10, 30 and 100 μM had posed no significant adverse effect on the viability of the cells ($P < 0.05$, (Table.2).

3.1.4. Protective role of rutin against intracellular ROS generation

The effect of rutin on ROS generation was analysed through spectrofluorometeric and flowcytometeric analysis. The spectroflurometeric analysis showed that after 24 hour of incubation, LPS (1 μg/mL) treated RAW 264.7 cells exhibited significant ($P<0.05$) increase in ROS generation (21.64±0.84 %) as compared with normal cells (untreated LPS). The concentration dependent decrease in ROS generation was observed as 11.03±0.31, 14.97±0.94 and 19.59±0.96% at 3, 10 and 30μM concentrations respectively as compared to the LPS-induced cells. In case of positive control (dexamethasone) at 1μM, decreased (18.14±0.93%) ROS level was observed as compared to LPS-induced cells. Similarly, in flowcytometric analysis, concentration dependent decrease in CM-H_2DCFDA positive cells was observed upon the treatment of rutin when compared to LPS treated cells (Fig.3).

3.2. In vivo study

3.2.1. Rutin decreases parasitaemia and increases survival in *P. berghei* infected mice

The antimalarial efficacy of rutin was undertaken in a Swiss albino mice model infected with *P. berghei*. The peak day of the parasitaemia was found to be on the 8^{th} day after infection and oral administration of rutin (25, 50 and 100 mg/kg) was found to decrease the parasitaemia significantly ($p<0.05$) in a dose-dependent manner as compared to the vehicle-treated infected group. Percentage suppression of parasitaemia on 8^{th} day post-infection is represented in the table along with the mean percentage of parasitaemia (Table.3). The vehicle-treated group succumbed to parasite infection in 6.7 days while the treatment of rutin increased the MST upto 12.38, 10.57, 8.3 and 8.36 days at doses of 25, 50 and 100 mg/kg respectively. The group treated with standard drug (chloroquine) have shown the MST of >28 days (Fig.4).

3.2.2. Rutin restores the haemoglobin and glucose level towards normal

The haemoglobin and glucose level in the blood of the vehicle-treated group was found to decrease significantly when compared to normal uninfected mice. When infected mice was treated with rutin and chloroquine, haemoglobin level as well as glucose level restored towards the normal uninfected mice (Fig.5 a and b).

3.2.3. Rutin shows reduction in pro-inflammatory cytokines production

The pro-inflammatory cytokines production(TNF-α, IL-6 and IFN-γ) in the serum and the brain homogenate of the vehicle-treated group was found to increase significantly when compared to the normal untreated mice. The treatment of the infected mice with rutin showed a significant ($p<0.05$) reduction in the secretion of pro-inflammatory cytokines when compared with the vehicle-treated group on the peak day of parasitaemia (Fig.6).

3.2.4. Rutin decreases the content of malondialdehyde in liver, brain and spleen tissues

The content of malondialdehyde in liver, brain and spleen tissues was found to increase significantly ($p<0.05$) in the vehicle-treated group while in the rutin treated group, a sharp decline in the malondialdehyde content was observed at the doses of 25, 50 and 100 mg/kg in liver, brain and spleen tissues in a significant manner ($p< 0.05$) (Table.4).

3.3. Combination study

3.3.1. Rutin in combination with chloroquine exhibit synergy

In the interaction study of chloroquine and rutin against the chloroquine-resistant K1 strain of *P. falciparum*, synergistic interaction was found at the ratio 1:4 (chloroquine : rutin) with Σ FIC 0.83 (≤1). In the presence of chloroquine and rutin combination at the ratio 1:4, the IC_{50} value of rutin decreasedfrom 20.5±0.59 to 11.22±0.03 μM and the IC_{50} of chloroquine was decreased from 0.252±0.04 to 0.07±0.00 μM. (Table.5, Fig.7).

3.3.2. Rutin and chloroquine combination reduces parasitaemia and increases survival

The oral administration of rutin in combination with chloroquine was found to significantly ($p<0.05$) inhibit the parasitaemia as compared to the vehicle-treated group (untreated) in *P. berghei* infected mice when compared to the groups which received chloroquine and rutin alone. The combination of rutin and chloroquine was able to increase the survival up to 18 days when compared to rutin (12.7 days) and chloroquine (14.1 days) alone. The increase in haemoglobin percentage was also observed in the group that received the combination of chloroquine and rutin up to 102.73 % as compared to rutin alone 65.65 % (Table.6, Fig.8).

Discussion

Excessive Inflammation and oxidative stress are critical pathological processes in malaria pathogenesis, which leads to severity like cerebral malaria, hypoglycaemia, hyperlactataemia and acidosis (Schofield and Grau 2005). In present research, we have studied the pharmacological profile of rutin against malaria pathogenesis with special reference to involvement of malaria parasite induced oxidative stress and inflammation. In *in vitro* study, rutin treatment showed the anti-plasmodial effect against NF-54 (chloroquine-sensitive) and K1 (chroroquine resistant) strains of *P. falciparum.* Rutin had also shown anti-inflammatory and anti-oxidative potential against LPS-stimulated macrophage cells. This finding is correlated with the previous observations that dietary flavonoids exhibits the anti-plasmodial and anti-inflammatory effects (Mohanty et al. 2013; Rudrapal and Chetia 2016; Gupta et al. 2018). To substantiate the beneficial role of rutin in *in vivo* condition, we have further evaluated its pharmacological profile alone and in combination with chloroquine in mice. The efficacy study performed *in vivo* in mice infected with *P. berghei* showed that peak parasiatemia in vehicle-treated group was found on day 8 and the oral treatment of rutin exhibited significant reduction in parasitaemia when compared to infected mice (vehicle-treated group) as well as found dose-dependent improvement in the mean survival time in rutin treated mice. Oral treatment of rutin and chloroquine had restored the blood glucose and haemoglobin level towards normal. Earlier published reports demonstrated that phyto-molecules were able to restore the glucose and haemoglobin level in experimental *P. berghei* infected animals (Mohanty et al. 2015; Saxena et al. 2016). Severe malaria is usually manifested in the form of anaemia and hypoglycaemia which is correlated to the increased rate of mortality specially of pregnant women and children in many parts of the world (Ivanova et al. 2016; Sharubutu and Usman 2019). This study also demonstrated that oral treatment of rutin is capable to provide protection from oxidative stress in vital organs during malaria pathogenesis. Oxidative stress due to malaria parasites if not checked by the host-antioxidant mechanism, can lead to oxidative damage in host tissues which contributes to the severe malaria pathogenesis (Fabbri et al. 2013). Naturally occurring flavonoids are known to possess anti-oxidant and significant chelating properties (Banjarnahor and Artanti 2014). These findings are in agreement with recent reports that mitochondrial dysfunction and oxidative stress induced by clozapine was reduced by the ellagic acid treatment in cardiomyocytes (Ahangari et al.,2020). The plant derived leads are able to reduce the extent of oxidative stress by improving the level of anti-oxidant enzymes in *P. berghei*-infected mice (Gupta et al. 2018). The pro-inflammatory cytokine production induced by malaria parasite was dose dependently inhibited in significant way by oral administration of rutin when compared to *P. berghei* infected vehicle-treated group. The most severe form of infection of *Plasmodium falciparum* is associated with overproduction of inflammatory response (IFN-γ, IL-1β, TNF-α, iNOS, IL-6) which contributes to severity of the disease (Schofield and Grau 2005). There are several reports which concluded that standard anti-malarial drugs (chloroquine, artemisnin) and other plant-derived leads which exert anti-malarial activity are also involved in the modulation of pro-inflammatory cytokines (Park et al. 2019; Adderley et al. 2020). Increased level of pro-inflammatory cytokines such as TNF-α are correlated with acute malaria episodes, such as severe malarial anaemia and cerebral malaria(L et al. 2020). Naturally occurring flavonoids are known to possess anti-inflammatory activities (Kumar and Pandey. 2013) by interfering with signalling pathways like nuclear factor kappa B transcription (Choy et al. 2019). The results of combination of rutin and chloroquine revealed the synergistic interaction in

experimental assays. It shows the possible reappearance of sensitivity of chloroquine against the malaria infection which could be suitable and safe combination. The extensive use of chloroquine for the treatment of malaria have intensified the extent of resistance to the malaria infection in tropical and sub-tropical countries resulting in a loss of efficacy of chloroquine. Chloroquine is an affordable treatment for the management of malaria infection in most of the under-developed and developing tropical and sub-tropical countries, especially the poor, rural families whose children are most likely to die from malaria (Arrow et al. 2004).

Conclusion

In conclusion, the current research findings demonstrate that rutin administration renders an anti-malarial activity evident by the reduction of parasitaemia and inflammatory mediators implicated in the malaria pathogenesis with an increase in mean survival time along with normalization of glucose and haemoglobin level. Targeting oxidative stress and inflammation could be a promising therapeutic strategy for the management of severe malaria pathogenesis. Therefore, dietary rutin postulated as a promising compound for further consideration towards the malaria pathogenesis management.

Abbreviations

P. falciparum, Plasmodium falciprum; MAPK, Mitogen activated protein kinase; NOS, Nitric oxide synthase; NF-κB, Nuclear factor kappa B: iNOS, Inducible nitric oxide synthase ; Roswell park memorial institute 1640 (RPMI-1640); CQ, Chloroquine; DMSO, Dimethylsulfoxide; DMEM, Dulbecco's modified eagle medium; FBS, Fetal bovine serum; PBS, Phosphate-buffered saline; LPS, Lipopolysaccharide; BSA, Bovine serum albumin; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium); ELISA, Enzyme-linked immuno-sorbent assay; ROS, Reactive oxygen species; CMH_2DCFDA, chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; TNF-α, Tumour necrosis factor-alpha; IL-6, Interleukin-6; IL-1β, Interleukin-1-beta; *P.berghei*, *Plasmodium berghei*; RBC, Red blood cell; ACD, Acid citrate dextrose saline; CMC, Carboxymethylcellulose; IFN-γ, Interferon-gamma; TMB, Tetramethylbenzidine; MDA, Malondialdehyde; TBA, Thiobarbituric acid; TCA, Trichoroacetic acid; SDS, sodium dodecyl sulphate; FIC, Fractional inhibitory concentrations; ∑FIC, Summation fractional inhibitory concentrations.

Declarations

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Conflicts of Interest

Authors declare no conflicts of interest.

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Tables

Table.1 The anti-plasmodial activity of rutin against chloroquine-sensitive (NF54) and resistant (K1) strain of *Plasmodium falciparum*

Group	IC_{50} (μM)	
	NF-54	K1
Rutin	26.4 ±1.31	20.5±0.59
Chloroquine	0.0021±0.0004	0.252±0.04
Artesunate	0.0067±0.0002	0.010±0.0003

Data are expressed as Mean ± SEM (n = 3)

Table.2 Cytotoxicity assessment in primary peritoneal macrophages after treatment with rutin at different concentrations

Test compound	Dose (µM)	% Cell viability
Rutin	10	98.67±0.05
	30	95.48±0.02
	100	94.18±0.03

Data are expressed as Mean ± SEM (n = 3)

Table.3 Effect of rutin against *P. berghei*-induced malaria in Swiss albino mice on the peak day of parasitaemia (day 8)

Group	Treatment (mg/kg)	Parasitaemia (%)	Suppression (%)	Mean survival time (MST)
Vehicle	-	41.26±0.86	NA	6.77
Rutin	25	16.31±1.61*	60.46±3.91	12.38
	50	20.41±3.53*	50.53±8.56	10.57
	100	27.71±2.51*	44.04±12.45	8.36
Chloroquine	10	-	100	28.00

Data are represented as mean percentage ±SEM (n =6), *p < 0.05 indicates a significant difference of rutin treated group compared with vehicle-treated on day 8; NA: not applicable.

Table.4 Effect of rutin on malondialdehyde content in brain, liver and spleen tissue of *P. berghei* infected mice

Group	Treatment (mg/kg)	MDA (nM/mL tissue homogenate)		
		Brain	Liver	Spleen
Normal	-	0.12±0.05	0.91±0.18	0.34±0.05
Vehicle	-	3.61±0.48#	22.15±0.85#	12.60±0.10#
Rutin	25	1.29±0.09*	8.71±0.51*	3.39±0.04*
	50	1.55±0.28*	9.36±1.02*	4.51±0.80*
	100	2.00±0.40*	14.16±1.75*	8.24±0.54*
Chloroquine	10	0.20±0.03*	0.91±0.18	0.77±0.19*

Data are expressed as Mean ± SEM.; # normal vs. vehicle-treated group; *P<0.05, vehicle-treated vs rutin-treated group; n=6.

Table.5 *In vitro* interaction of rutin with chloroquine at different concentrations against chloroquine-resistant strain (K1) of *P. falciparum*

Fix ratio combination	IC_{50}(µM)*		FIC			Interaction#
	Chloroquine	Rutin	Chloroquine	Rutin	FIC#	
5:0	0.254±0.04	-	-	-	-	-
4:1	0.39±0.02	7.71±0.42	1.53	0.38	1.90	Additive
3:2	0.28±0.00	22.52±0.06	1.11	1.10	2.21	Antagonism
2:3	0.20±0.07	24.40±6.78	0.78	1.19	1.97	Additive
1:4	0.07±0.00	11.22±0.03	0.28	0.55	0.83	Synergistic
0:5	-	20.5±0.59	-	-	-	-

*IC_{50} and FIC expressed as Mean ± SEM and #Σ FIC <1 synergism, Σ FIC ≥ 1 and < 2 additive, and Σ FIC ≥2 and < 4 antagonism

Table.6 Effect of rutin and chloroquine combination in *P. berghei* infected Swiss albino mice on the peak day of parasitaemia (day 8).

Group	Dose (mg/kg)	Parasitaemia (%)	Chemosuppression (%)	Mean Survival Time (MST)	Haemoglobin (Percent increase)
Vehicle	-	36.14±1.09	NA	7.7	-
Rutin	25	11.33±1.28*	54.06±5.21	12.7	65.65
Chloroquine	2.5	5.08±1.22*	79.41±4.96	14.1	79.10
Rutin+Chloroquine	25+2.5	2.21±0.62*	91.04±2.51	18	102.73
Chloroquine	10	0.00	100±00	28	147.56

Data are expressed as Mean±SEM (n=6); *P<0.05; vehicle-treated vs rutin-treated; NA: not applicable

Figures

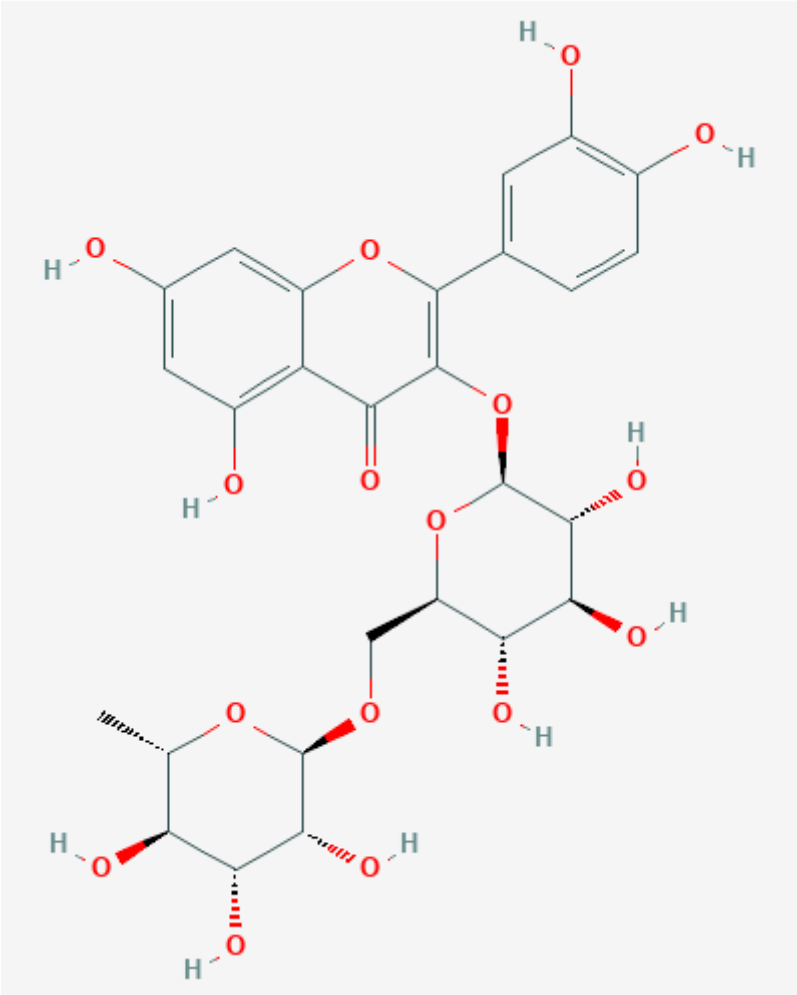

Figure 1

Chemical structure of quercitin-3-O-rutinoside.

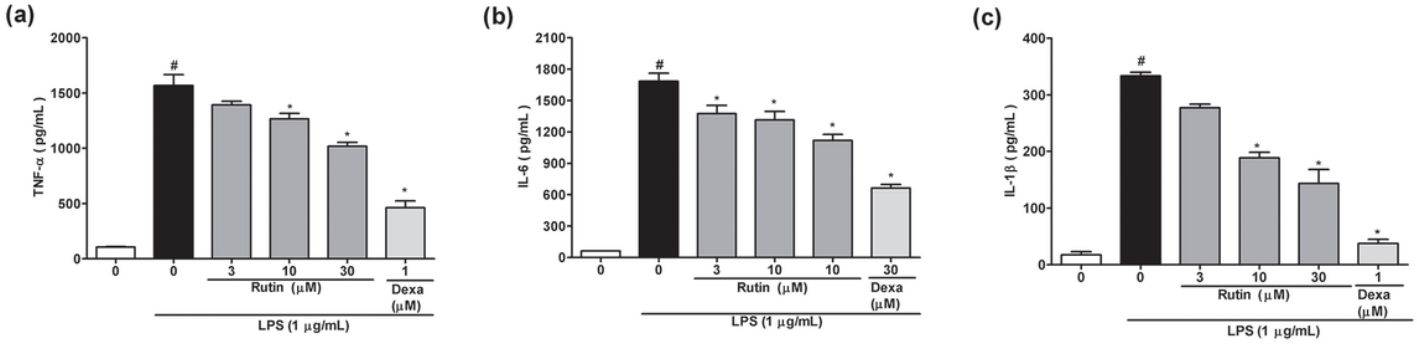

Figure 2

Effect of rutin on production of pro-inflammatory cytokines (a) TNF-α and (b) IL-6 and (c) IL-1β in primary macrophage cells. Data are expressed as Mean ±SEM: # Normal vs vehicle-treated group; *P<0.05; vehicle-treated vs rutin-treated; n=3

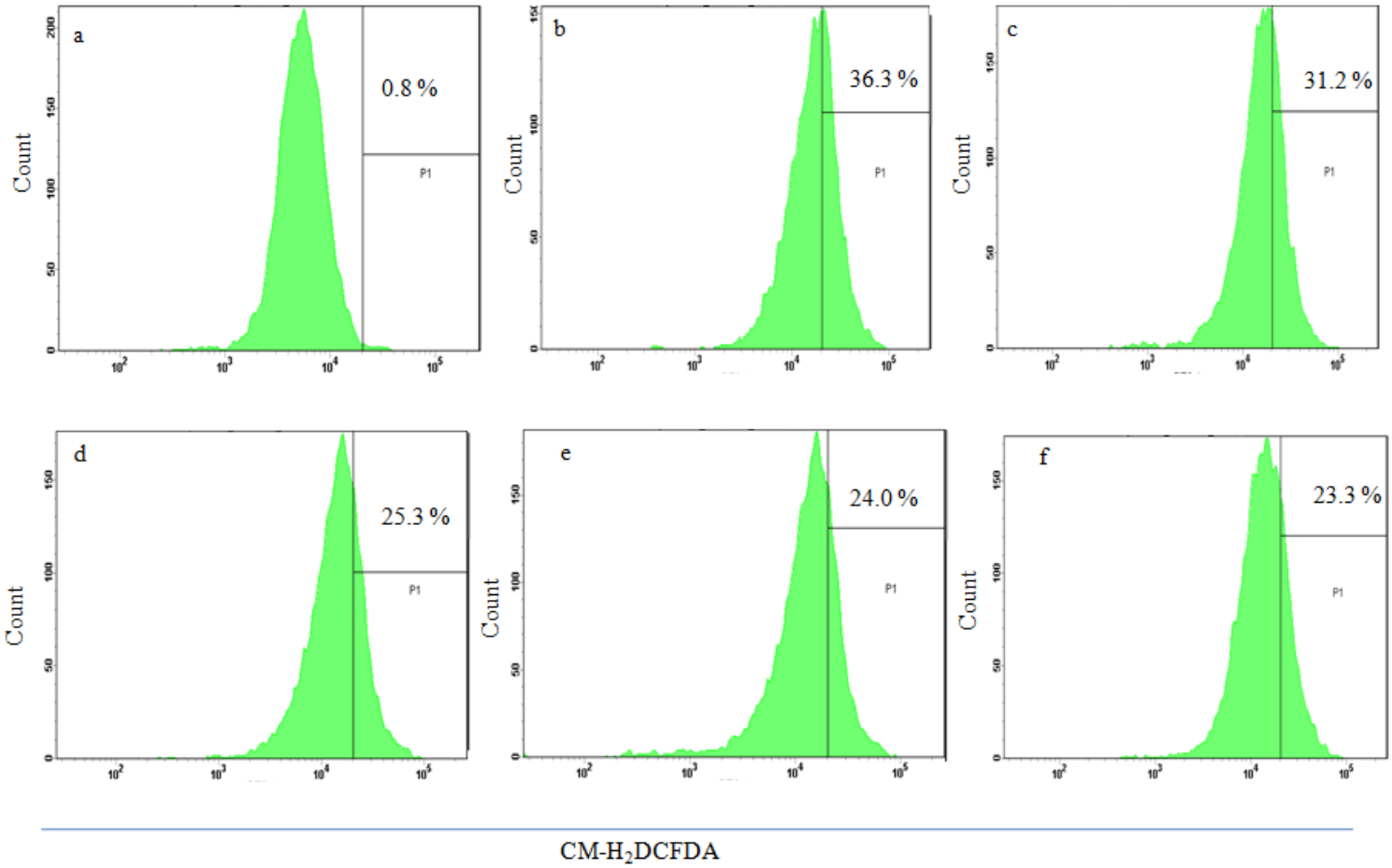

Figure 3

Effect of rutin on the generation of reactive oxygen species (ROS). Flow cytometry histograms showing change in DCF positive cells, (a) normal cells (negative control), (b) LPS (1μg/ml), (c) 3 μM, and (d) 10 μM, (e) 30 μM concentrations of rutin and (f) positive control (dexamethasone) at 1 μM. (f) The bar graph

indicates the fluorescence units. Data expressed as Mean ± SEM. # normal vs vehicle-treated group; *P<0.05; vehicle-treated vs rutin-treated; n=3

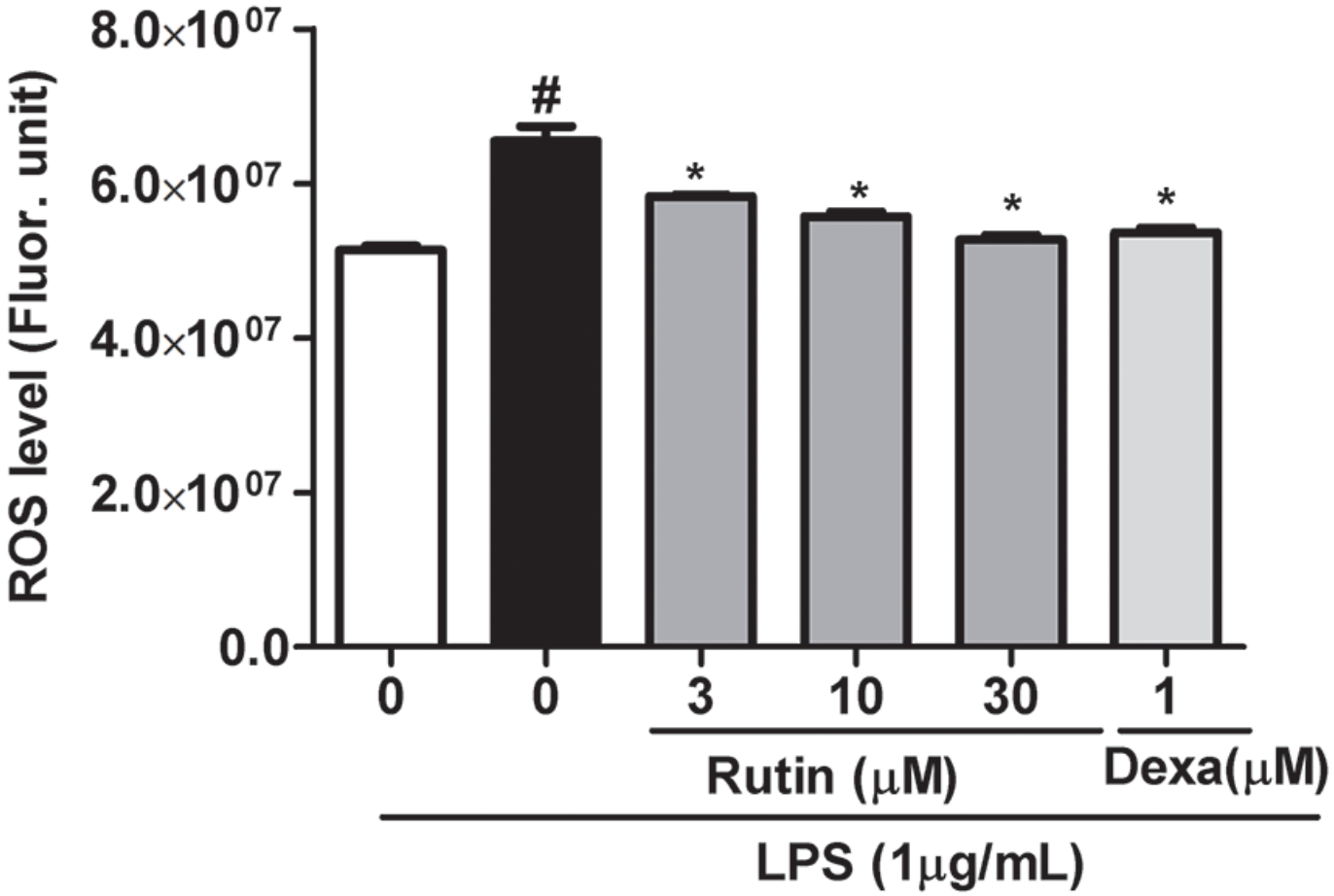

Figure 4

Survival curve of mice infected with P.berghei parasitized red blood cells for 28 days after infection. The percent of rutin treated and non treated mice that survived over time is represented in curve

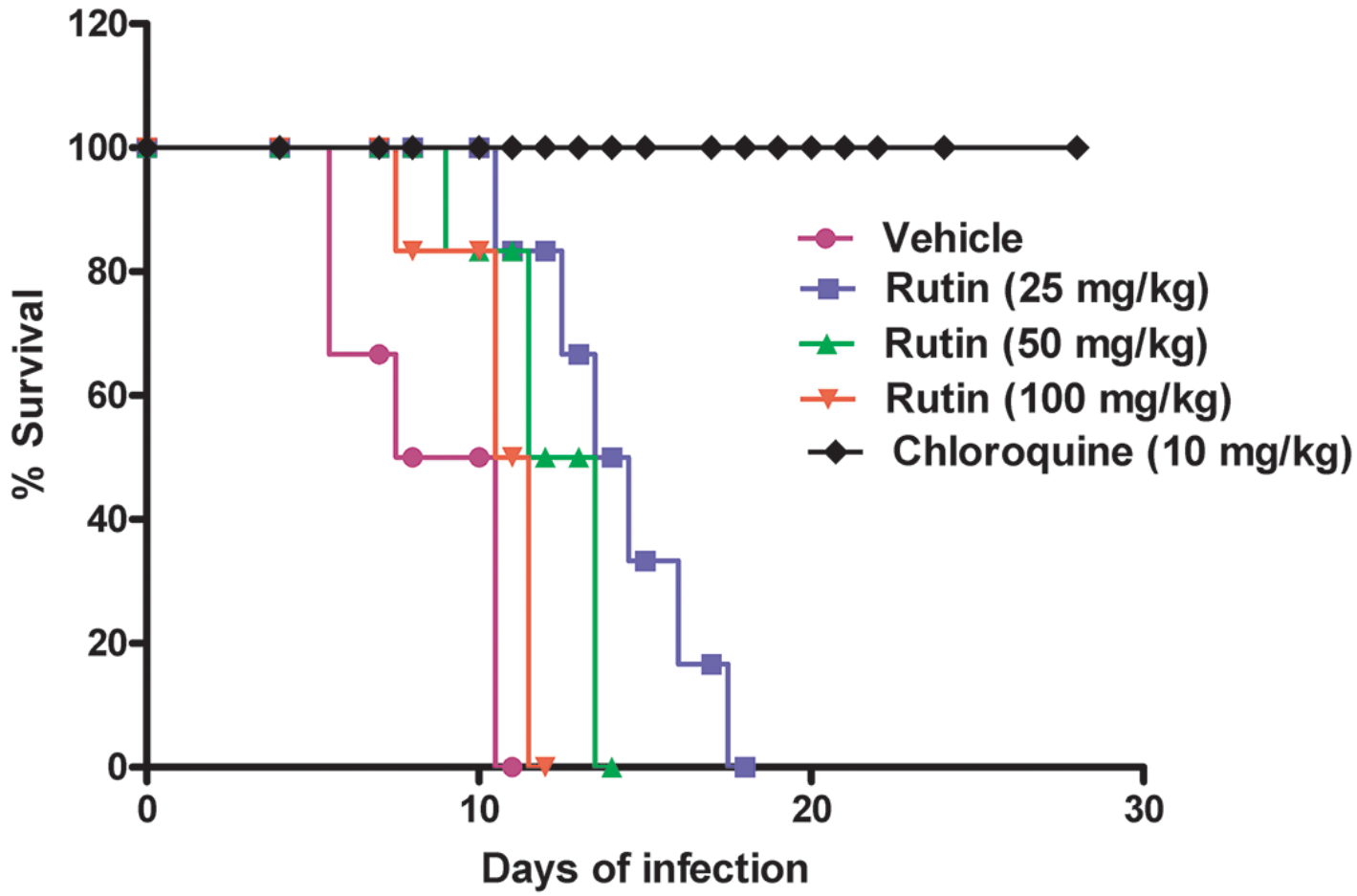

Figure 5

Effect of Rutin on haemoglobin and blood glucose level on the peak day of parasitaemia in P. berghei infected mice. (A) haemoglobin (B) Blood glucose. Data are expressed as Mean±SEM: #normal vs vehicle-treated group; *P<0.05; vehicle-treated vs rutin-treated; n=6

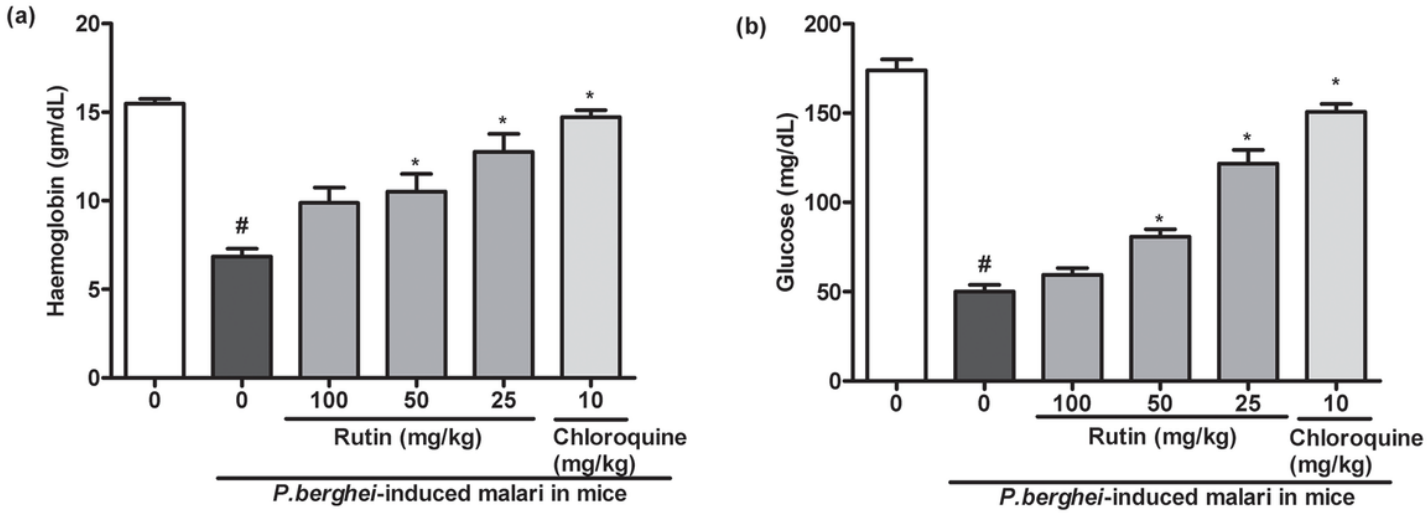

Figure 6

Effect of Rutin on production of pro-inflammatory cytokines TNF-α , IL-6 and IFN-Y on peak day of parasitaemia in malaria infected mice (a) Cytokines level in the serum (b) Cytokines level in the brain homogenate of the P. berghei infected mice. Data are expressed as Mean ± SEM; # normal vs vehicle-treated group; *P<0.05; vehicle-treated vs rutin-treated; n=6

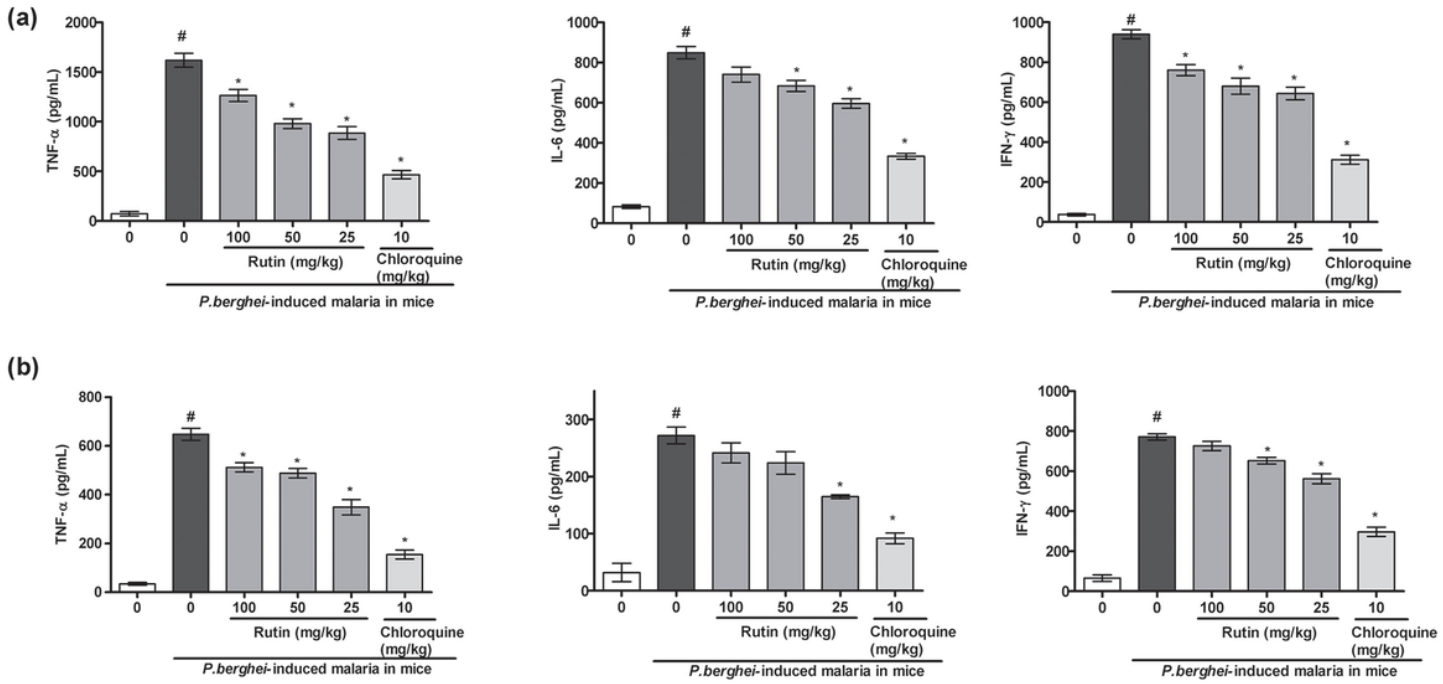

Figure 7

Isobologram representing interaction between rutin and chloroquine against drug-resistant strain (K1) of P. falciparum. Mean FIC of rutin and chloroquine represented on the y- and x-axes, respectively. Sum of

mean FIC values on the line shows additive, above the line shows antagonistic, and below the line shows synergistic interaction. Data expressed as Mean ± SEM. n=3

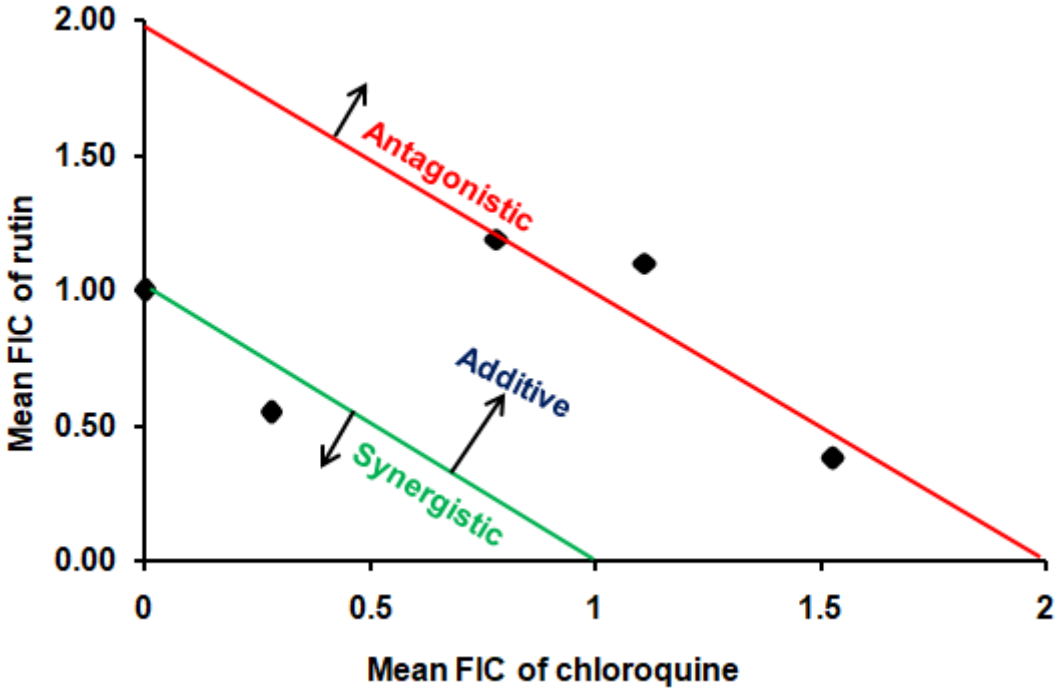

Figure 8

Survival curve of mice infected with P.berghei parasitized red blood cells for 28 days after infection. The percent of mice that survived over time in non treated group and the group treated with rutin and chloroquine alone and in combination is represented in curve

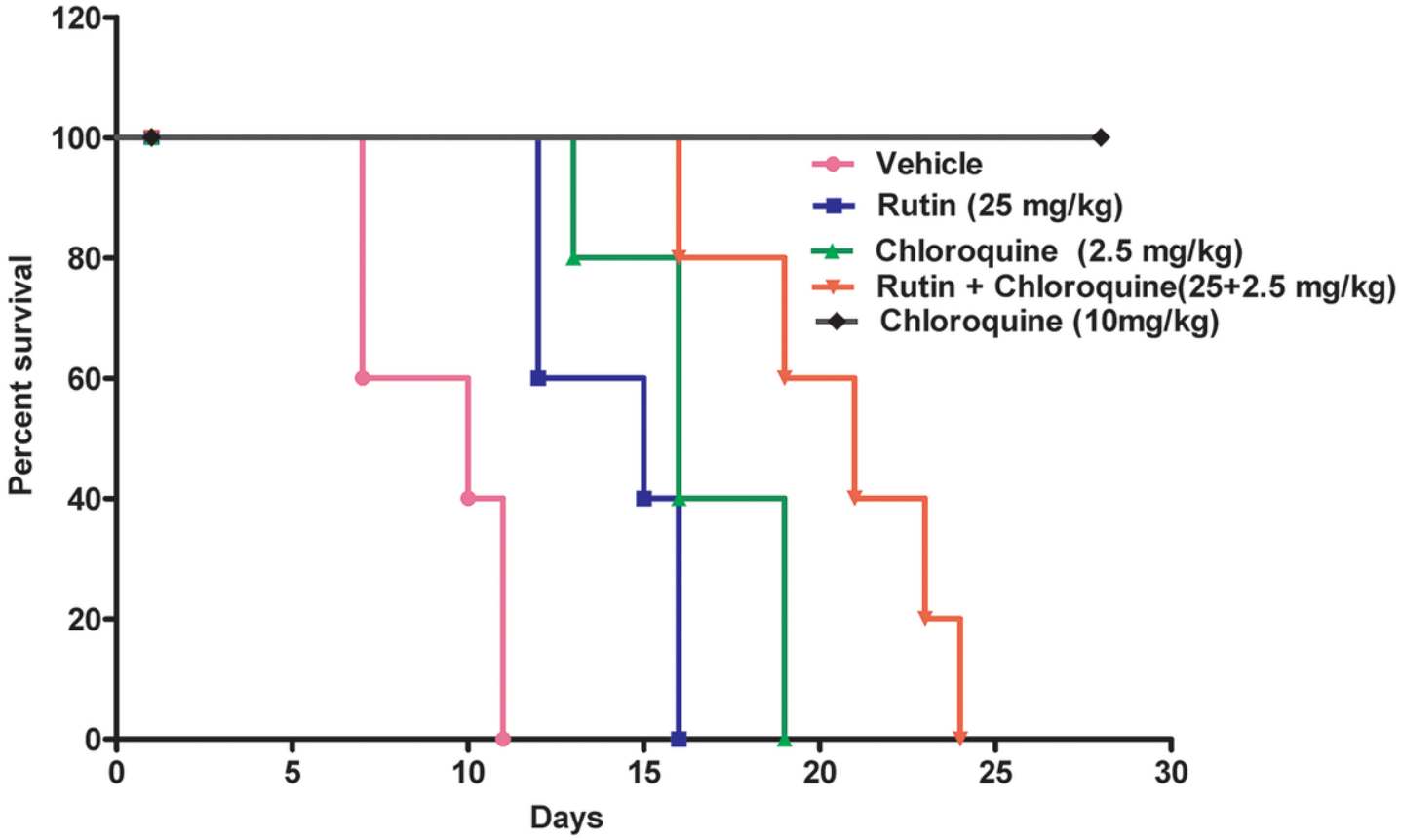

Figure 9

Caption not included with this version

Supplementary Files

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