Rutin ameliorates malaria pathogenesis by modulating inflammatory mechanism: an in vitro and in vivo study

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Received: 13 October 2021 / Accepted: 25 December 2021 / Published online: 21 January 2022
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Abstract
Rutin (3, 3', 4', 5 and 7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonoid glycoside, found in many edible plants such as buckwheat and berries. Severe malaria is an inflammatory response triggered by oxidative stress that results in multi-organ pathologies and a high mortality rate in children and pregnant women worldwide. Rutin is recommended as a food supplement for the treatment of various diseases due to its anti-oxidative and anti-inflammatory properties, which prompted us to investigate its ameliorative effects in severe malaria pathogenesis against oxidative stress and inflammatory response using in vitro and in vivo bioassays. Rutin was examined in this work for its anti-plasmodial activity against chloroquine-sensitive and resistant Plasmodium falciparum strains, as well as its anti-oxidative and anti-inflammatory activity against LPS-stimulated macrophage cells. The in vitro data were subsequently verified in mice fed orally with rutin alone or in combination with chloroquine in Plasmodium berghei-induced malaria pathogenesis. The anti-plasmodial and anti-inflammatory properties of rutin were demonstrated in in vitro results. Apart from its anti-inflammatory and anti-oxidant effects in malaria pathogenesis, in vivo efficacy studies indicated that oral treatment with rutin reduced parasitaemia, increased mean survival time, and restored haemoglobin and glucose levels in mice at lower dose. Interestingly, both rutin and chloroquine demonstrated synergy in 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 in combination with standard anti-malarial drugs.

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Graphical abstract

**Keywords** Rutin · Anti-oxidative · Anti-inflammatory · *Plasmodium* · Malaria · Mice

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| *P. falciparum* | *Plasmodium falciparum* |
| MAPK | Mitogen-activated protein kinase |
| NOS | Nitric oxide synthase |
| NF-κB | Nuclear factor-kappa B |
| iNOS | Inducible nitric oxide synthase |
| RPMI-1640 | Roswell park memorial institute 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 immunosorbent assay |
| ROS | Reactive oxygen species |
| CMH₂DCFDA | 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 |
Rutin ameliorates malaria pathogenesis by modulating inflammatory mechanism: an in vitro…

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 anti-malarial drugs, turning up of the insecticidal resistance, dearth of vaccines countering malaria spread and alteration in the nature of Anopheles vectors (Menard and Dondorp 2017). During malaria infection, the host undergoes severe oxidative stress (Franklin et al. 2011), which variously challenges 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 and Harty 2020). 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, anti-oxidant 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 exerting immunomodulatory effects on the host immune system are considered the desirable source (Wright 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, anti-oxidative, neuroprotective, antibacterial and vasoprotective activities (Ganeshpurkar and Saluja 2017) and also known to exhibit enhanced cardio and renal protective activities by modulating the oxidative stress and inflammation (Nafees et al. 2015). Rutin showed 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 was to evaluate the pharmacological 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 diminished malaria pathogenesis with modulation of oxidative stress and inflammatory response and showed synergistic potential with chloroquine, a standard anti-malarial drug. The results of this study suggested the suitability of rutin for further detailed investigation in the management of drug-resistant malaria in combination with standard anti-malarial drugs.

Materials and methods

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, Thiobarbituric acid, 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-H₂DCFDA).

![Fig. 1 Chemical structure of rutin](image)
In vitro study

Maintenance 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 (370 μM), HEPES (25 mM), NaHCO₃ (0.2%), fungizone (25 μg/mL), gentamycin (40 μg/mL) and Albumax II (0.5%) at 37 ℃ and 5% CO₂. Besides the change of growth medium every 24 h, parasitaemia was routinely monitored through Giemsa stained thin smear of the cultured parasite to achieve synchronized culture having initial ring stage parasite (Trager and Jensen 1976).

Anti-plasmodial activity

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 (0.1–100 μM) and standard drugs (chloroquine and artesunate), and the culture without treatment served as a negative control. After 48 h of incubation at 37 ℃ and 5% CO₂, a 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

\[
\text{Percentage suppression of parasitemia} = \frac{[(A-B)/A] \times 100}{},
\]

where \(A\) is the mean percent parasitemia in the negative control and \(B\) refers to the mean parasitemia of the treatment group. The IC₅₀ (mean ± SEM) was determined using non-linear regression analysis from concentration-mediated growth inhibition data (Kumar et al. 2021).

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 8-week-old Swiss albino female mice, 3 days earlier than harvesting macrophage cells. Just before isolating cells, mice were subjected to ether anesthesia 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, adjusted viable cells to the required density (0.5 × 10⁶ – 1 × 10⁶ 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 ℃ with 5% CO₂.

Quantification of pro-inflammatory cytokine profile in primary macrophages

For the in vitro anti-inflammatory profile assessment of rutin, primary macrophages were seeded as described above. Cultured cells were treated with rutin at the concentration of 3, 10 and 30 μM for 30 min before LPS stimulation (1 μg/mL) to induce inflammatory cytokines. Culture supernatant was collected after 6 h of LPS incubation. The pro-inflammatory cytokine (IL-6, TNF-α and IL-1β) levels were quantified from culture supernatant using mouse-specific Enzyme Immuno Assay (EIA) Kits according to the instructions of the manufacturer.

Quantification of reactive oxygen species (ROS) generation in macrophages

Intracellular ROS level was determined as previously described (Kumar et al. 2021). Briefly, RAW 264.7 cells were cultured using DMEM medium containing 10% FBS. To measure intracellular ROS, 1 × 10⁶ cells/well were seeded in a 6-well plate and incubated for 24 h at 5% CO₂ and 37 ℃. After incubation, cells were treated with rutin at concentrations of 3, 10 and 30 μM with dexamethasone (1 μM) as a standard. After 30 min, cells were stimulated with LPS (1 μg/mL) for intracellular ROS generation and incubated for 6 h followed by incubation with fluorescent dye CM-H₂DCFDA (20 μM) in the culture medium for 20 min in CO₂ incubator. Following incubation, the cells were washed twice with PBS and also harvested after trypsinization. The fluorescence was quantified using a flow cytometer (BD Biosciences) operational with a 488 nm argon laser as a light source. Using FACS Diva software version 7.1 (BD Biosciences), percentage and mean fluorescence intensity of CMH₂DCFDA positive cells were calculated. Similarly, intracellular ROS level was also measured using a spectrofluorometer (Spectramax i3x, Molecular Device) at 485 nm of excitation and 520 nm emission wavelength. Data were expressed as fluorescence unit that depicted the ROS level in rutin treated cells compared to LPS-induced cells.

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 a CO₂ incubator at 37 ℃ with 5% CO₂ for 24 h. After incubation, cells were treated with rutin at
concentrations of 10, 30 and 100 μM for 24 h. After 24 h, 20 μL MTT (5 mg/mL in PBS) was added to each well and incubated for another 4 h. Following incubation, media containing MTT was replaced with DMSO (100 μL) to solubilize the formazan crystals formed. The absorbance was recorded at 550 nm, and viability of the cells has been represented in terms of percentage (%) of survival.

In vivo study

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).

Mice and parasite infection

To evaluate the suppressive anti-malarial activity of rutin, adult Swiss albino male mice bred in-house and aged 6–8 weeks (20 ± 2 g) were chosen as experimental hosts. Random grouping of animals with six mice each in five groups was followed by 7 days acclimatization in the experimental facility, kept under the standard environmental conditions of 23 ± 2 °C, 12 h 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 the institute by a continuous passage in mice and the blood-stage parasites were preserved in liquid nitrogen.

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

According to the method of Knight and Peters and a 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 × 10⁶ *P. berghei* K-173 parasitized red blood cells diluted in sterile ACD. After an hour of infection, mice were administered an oral rutin dosage (25, 50, 100 mg/kg/day) prepared in 0.7% CMC while mice receiving vehicle (0.7% CMC) alone were considered as a 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 regimen was repeated once daily for 4 days.

Parasitaemia and survival determination

From the 4th day post-infection, thin blood smears by tail snip were prepared every alternate days 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.

Quantification of haemoglobin and glucose

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

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, 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 using the ELISA kits.

Quantification of malondialdehyde (MDA) content

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

Anti-plasmodial profile of chloroquine and rutin combination

The modified fixed ratio method was performed for the study of the interaction between chloroquine and rutin (Fivelman et al. 2004; Kumar et al. 2021). First, the IC50, i.e., 50% inhibitory concentration against the chloroquine-resistant strain K1 of P. falciparum was determined for chloroquine and rutin as described in Sect. 2.2.2. The five times higher of the respective IC50 concentrations of chloroquine and rutin were diluted twofold with culture medium to initial concentrations, then mixed volume by volume at 4:1 (1.016 µM:20.5 µM), 3:2 (0.777 µM:41 µM), 2:3 (0.518 µM:61.5 µM) and 1:4 (0.259 µM:82 µM). All these ratios were further diluted twofold. Then, IC50 values were determined for different combinations (chloroquine and rutin), the individual IC50 concentrations and the IC50 values of the combination were used for the calculation of fractional inhibitory concentrations (FIC) and ΣFIC using the following formula:

\[ \text{FIC} = \frac{\text{IC}_{50} \text{ concentration in combination}}{\text{IC}_{50} \text{ concentration alone}} \]

\[ \Sigma \text{FIC} = \text{FIC value of chloroquine} + \text{FIC value of rutin}. \]

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

Another experiment was performed to evaluate the anti-malarial potential of rutin in combination with chloroquine. The experiment was carried out as described in Sect. 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 (25 mg/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 a vehicle-treated group. The parasitaemia, haemoglobin and survival were determined to evaluate the potential of rutin and chloroquine combination in P. berghei-infected mice.

Statistical analysis

The results in the present study were expressed in terms of 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

In vitro study

Anti-plasmodial effect of rutin

Rutin exhibited potent anti-plasmodial activity with IC50 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 IC50 for chloroquine under similar conditions was 0.0021 ± 0.0004 and 0.252 ± 0.04 µM while artesunate showed IC50 of 0.0067 ± 0.0002 µM and 0.010 ± 0.0003 µM against NF54 and K1 strains, respectively. The data are represented in Table 1.

Table 1 Anti-plasmodial activity of rutin against chloroquine-sensitive (NF54) and resistant (K1) strains of Plasmodium falciparum

| Group | IC50 (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)
Rutin ameliorates malaria pathogenesis by modulating inflammatory mechanism: an in vitro study.

Fig. 2 Effect of rutin on production of pro-inflammatory cytokines a TNF-α and b IL-6 in primary macrophage cells. Data are expressed as mean ± SEM. *normal vs vehicle-treated group; *p < 0.05; vehicle vs treatment; n = 3.

Fig. 3 Effect of rutin on the generation of reactive oxygen species (ROS). a Flow cytometry histograms showing change in Mean Fluorescence Intensity of CM-H₂DCFDA positive cells, (I) normal cells (negative control), (II) LPS (1 μg/mL), (III) 3 μM, and (IV) 10 μM, (V) 30 μM concentrations of rutin and (VI) positive control (dexamethasone) at 1 μM. b The bar graph indicates the Mean Fluorescence Intensity and the data expressed as mean ± SEM. *Normal vs vehicle-treated group; *p < 0.05; vehicle vs treatment; n = 3.

Table 2 Cytotoxicity assessment in primary peritoneal macrophages upon 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).
In-vivo study

Effect of rutin on parasitaemia, survival, blood glucose and haemoglobin level in *P. berghei*-induced malaria in mice

The anti-malarial 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 8th day after infection, and oral administration of rutin (25, 50 and 100 mg/kg) was found to decrease the parasitaemia significantly ($p < 0.05$) as compared to the vehicle-treated infected group. The lower oral dose (25 mg/kg) of rutin was the most effective dose as compared to the higher doses (50, 100 mg/kg) to ameliorate malaria pathogenesis in experimental mice. Percentage suppression of parasitaemia on 8th 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 had increased the MST up to 12.38, 10.57 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). In the vehicle treated infected mice the blood glucose and hemoglobin levels were significantly decreased on the day of peak parasitemia, compared to the un-infected normal mice. However, the oral treatment with rutin at lower doses (25 mg/kg and 50mg/kg) to the infected mice restored ($p < 0.05$) the blood glucose and the hemoglobin (Fig. 5).

Effect of rutin on pro-inflammatory markers and malondialdehyde contents of liver, brain and spleen tissues in malaria pathogenesis

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 (Fig. 6). The treatment of the infected mice with rutin at lower doses (25 and 50 mg/kg) showed a significant ($p < 0.05$) reduction in the secretion of pro-inflammatory cytokines when compared to the vehicle-treated infected mice.

### Table 3 Effect of rutin on *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                | 0 ± 0.00         | 100 ± 0.00      | > 28.00                 |

Data are represented as mean percentage ± SEM ($n = 6$)
NA not applicable

$^*$p < 0.05 indicates a significant difference of rutin treated groups compared with vehicle-treated group on day 8
Rutin ameliorates malaria pathogenesis by modulating inflammatory mechanism: an in vitro...

compared with the vehicle-treated group on the peak day of parasitaemia (Fig. 6). 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).

Fig. 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 $\pm$ SEM; #normal vs vehicle; vehicle vs treatment; $n=6$, *$p<0.05$

Fig. 6 Effect of Rutin on production of pro-inflammatory cytokines TNF-$\alpha$, IL-6 and IFN-$\gamma$ 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 $\pm$ SEM; #normal vs vehicle; vehicle vs treatment; $n=6$, *$p<0.05$
Combination study

Combinatorial anti-plasmodial effect of rutin and chloroquine

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 IC₅₀ value of rutin decreased from 20.5 ± 0.59 to 11.22 ± 0.03 μM and the IC₅₀ of chloroquine was decreased from 0.252 ± 0.04 to 0.07 ± 0.00 μM (Table 5; Fig. 7).

Effect of the combination of chloroquine and rutin on parasitaemia and survival in *P. berghei*-induced malaria in mice

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% 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, hyperlactateemia and acidosis (Schofield and Grau 2005). In the present research, we have studied the pharmacological profile of rutin against malaria pathogenesis with special reference to the involvement of malaria parasite-induced oxidative stress and inflammation. In an in vitro study, rutin treatment showed the anti-plasmodial effect against chloroquine-sensitive (NF-54) and chloroquine resistant (K1) strains of *P. falciparum*. Rutin has also shown anti-inflammatory and anti-oxidative potential against LPS-stimulated macrophage

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**Table 4** Effect of rutin on Lipid peroxidation 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*

**Combination study**

Combinatorial anti-plasmodial effect of rutin and chloroquine

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

| Fix ratio combination | IC₅₀ (μM)* | FIC | ΣFIC* | Interaction |
|-----------------------|------------|-----|-------|------------|
|                       | Chloroquine | Rutin | Chloroquine | Rutin |
| 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 | – | – | – | |

*IC₅₀ and FIC expressed as mean and *Σ FIC < 1 synergism, Σ FIC ≥ 1 and < 2 additive and Σ FIC ≥ 2 and < 4 antagonism*
Rutin ameliorates malaria pathogenesis by modulating inflammatory mechanism: an in vitro…

This finding is correlated with the previous observations that dietary flavonoids exhibit 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 conditions, 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 parasitaemia in vehicle-treated group was found on day 8 and the oral treatment of rutin exhibited significant reduction in parasitaemia, improvement in the mean survival time, haemoglobin and blood glucose level when compared to infected mice (vehicle-treated group). 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 (Sharubutu and Usman 2019). This study also demonstrated that oral treatment of rutin is capable of providing protection from oxidative stress in vital organs during malaria pathogenesis. Oxidative stress due to malaria parasites, if not checked by the host-anti-oxidant mechanism, can lead to oxidative damage in host tissues which contributes to severe malaria pathogenesis (Fabbri et al. 2013). The plant-derived leads can reduce the extent of oxidative stress by improving the level of anti-oxidant enzymes in \(P. berghei\)-infected mice. The pro-inflammatory cytokine production induced by malaria parasite was also significantly inhibited 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-\(\gamma\), IL-1\(\beta\), TNF-\(\alpha\), iNOS, IL-6), which contributes to the severity of the disease (Schofield and Grau 2005). Several reports concluded that standard anti-malarial drugs (chloroquine, artemisinin) and other plant-derived leads that exert anti-malarial activity are also involved in the modulation of pro-inflammatory cytokines (Park et al. 2019; Adderley

### Table 6

| 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 ± 0.00         | 100 ± 0.00           | > 28                     | 147.56                         |

Data are expressed as mean ± SEM (\(n = 6\))

NA not applicable

*p < 0.05; vehicle-treated vs rutin-treated

![Fig. 8](image-url) Percent survival of mice treated with rutin alone and in combination with chloroquine in \(P. berghei\)-infected mice. \(n = 06\)
et al. 2020). Increased levels of pro-inflammatory cytokines such as TNF-α are correlated with acute malaria episodes, such as severe malarial anaemia and cerebral malaria (Leao 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 lower oral dose of rutin (25 mg/kg) was found to be more effective at ameliorating malaria pathogenesis in experimental mice than the higher doses (50, 100 mg/kg). This finding could be explained by rutin’s pro-oxidant activity at higher doses during malaria pathogenesis. This observation is supported with previous findings that flavonoids operate as a pro-oxidant at high concentrations and as an anti-oxidant at low concentrations (Kessler et al. 2003; Chobot et al. 2013). This discussion is further substantiated based on the previous report that the vitamin C, a standard anti-oxidant has a switch over role from being an anti-oxidant in physiologic conditions to a pro-oxidant under pathological conditions (Chakraborty et al. 2014). The results of a combination of rutin and chloroquine revealed the synergistic interaction in experimental assays. It shows the possible reappearance of chloroquine sensitivity against the malaria infection which could be a suitable and safe combination. The extensive use of chloroquine for the treatment of malaria has 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 managing 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 (Gelband et al. 2004). Our studies hypothesize the utility of rutin in the combination therapy for the treatment of malaria. However, the ongoing pharmacokinetic studies can better conclude our postulate.

**Conclusion**

Malaria is an oxidative stress-driven inflammatory response that leads to multi-organ pathologies with high mortality in children and pregnant women globally. Targeting oxidative stress and inflammation could be a promising therapeutic strategy for the management of severe malaria pathogenesis. Results of this study demonstrated the ameliorative potential of rutin against malaria pathogenesis by modulating inflammatory and oxidative stress mechanism at lower dose and showed synergistic potential with chloroquine. Therefore, this study suggested the suitability of rutin for further detailed investigation in the management of drug-resistant malaria in combination with standard anti-malarial drugs.

**Acknowledgements** We are thankful to Director, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India for rendering essential research facilities and support. The study was financially supported by CSIR under projects HCP-0007 and HCP-00352. The first author also acknowledges the fellowship provided by the Department of Science and Technology (DST), New Delhi, India.

**Declarations**

**Conflict of interest** Authors declare no conflicts of interest.

**References**

Adderley JD, von Freyend SJ, Jackson SA et al (2020) Analysis of erythrocyte signalling pathways during *Plasmodium falciparum* infection identifies targets for host-directed antimalarial intervention. Nat Commun 11:1–13. https://doi.org/10.1038/s41467-020-17829-7

Bawankule DU, Chattopadhyay SK, Pal A et al (2008) Modulation of inflammatory mediators by coumarinflavonoids from *Cleome viscosa* in female swiss albino mice. Inflammopharmacology 16:272–277. https://doi.org/10.1007/S10787-008-8012-0

Chakraborty A, Ramani P, Sherlin HJ, Premkumar P, Natesan A (2014) Anti-oxidant and pro-oxidant activity of vitamin C in oral environment. Indian J Dent Res 25:499–504. https://doi.org/10.4103/0970-9290.142547

Chauhan K, Kaur G, Kaur S (2018) Activity of rutin, a potent flavonoid against SSG-sensitive and -resistant *Leishmania donovani* parasites in experimental leishmaniasis. Int Immunopharmacol 64:372–385. https://doi.org/10.1016/J.INTIMP.2018.09.026

Chobot V, Kubicova L, Bachmann G, Hadacek F (2013) Versatile redox chemistry complicates anti-oxidant capacity assessment: flavonoids as milieu-dependent anti- and pro-oxidants. Int J Mol Sci 14:11830–11841. https://doi.org/10.3390/ijms140611830

Choy KW, Murugan D, Leong XF et al (2019) Flavonoids as natural anti-inflammatory agents targeting nuclear factor-kappa B (NFκB) signaling in cardiovascular diseases: a mini review. Front Pharmacol 10:1–8. https://doi.org/10.3389/fphar.2019.01295

De Souza MC, Padua TA, das Graças HM (2016) Multiple organ dysfunction during severe malaria: the role of the inflammatory response. In: Current topics in malaria, p 85. IntechOpen

Drewry LL, Harty JT (2020) Balancing in a black box: potential immunomodulatory roles for TGF-β signaling during blood-stage malaria. Virulence 11:159–169. https://doi.org/10.1080/2150594.2020.1726569

Fabbri C, De Cássia MNR, Lalwani P et al (2013) Lipid peroxidation and anti-oxidant enzymes activity in *Plasmodium vivax* malaria patients evolving with cholestatic jaundice. Malar J 12:1–7. https://doi.org/10.1186/1475-2875-12-315

Fivelman QL, Adagu IS, Warhurst DC (2004) Modified fixed-ratio isobologram method for studying in vitro interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of *Plasmodium falciparum*. Antimicrob Agents Chemother 48:4097–4102. https://doi.org/10.1128/AAC.48.11.4097-4102.2004

Franklin BS, Ishizaka ST, Lamphier M, Gusovsky F et al (2011) Therapeutical targeting of nucleic acid-sensing Toll-like receptors prevents experimental cerebral malaria. Proc Natl Acad Sci 108:3689–3694. https://doi.org/10.1073/pnas.1015406108

Ganeshpurkar A, Saluja AK (2017) The pharmacological potential of rutin. Saudi Pharm J 25:149. https://doi.org/10.1016/J.JSPS.2016.04.025
Rutin ameliorates malaria pathogenesis by modulating inflammatory mechanism: an in vitro…

Gelband H, Panosian CB, Arrow KJ (eds) (2004S) Saving lives, buying time: economics of malaria drugs in an age of resistance. The National Academies Press, Washington, DC. https://doi.org/10.17226/11017

Gupta AC, Mohanty S, Saxena A et al (2018) Plumbagin, a vitamin K3 analogue ameliorate malaria pathogenesis by inhibiting oxidative stress and inflammation. Inflammopharmacology 26:983–991. https://doi.org/10.1007/s10787-018-0465-1

Kauffmann N, da Penha LKRL, Braga DV et al (2021) Differential effect of anti-oxidants glutathione and vitamin C on the hepatic injuries induced by Plasmodium berghei ANKA infection. Biomed Res Int 2021:1–8. https://doi.org/10.1155/2021/9694508

Kessler M, Ubeaud G, Jung L (2003) Anti- and pro-oxidant activity of rutin and quercetin derivatives. J Pharm Pharmacol 55:131–142. https://doi.org/10.1211/002235702559

Kumar S, Pandey AK (2013) Chemistry and biological activities of flavonoids: an overview. Sci World J 4:1–16. https://doi.org/10.1155/2013/162750

Kumar S, Mina P, Kumar R, Pal A, Ahmad A, Tandon S, Darokar MP (2021) 4-Chlorothymol exerts anti-plasmodial activity impeding redox defence system in Plasmodium falciparum. Front Pharmacol 12:93. https://doi.org/10.3389/fphar.2021.628970

Leao L, Puty B, Dolabela MF, Povoa MM, Ne YG, Eiro LG, Fagundes NC, Maia LC, Lima RR (2020) Association of cerebral malaria and TNF-α levels: a systematic review. BMC Infect Dis 20:1–7. https://doi.org/10.1186/s12879-020-05107-2

Menard D, Dondorp A (2017) Antimalarial drug resistance: a threat to malaria elimination. Cold Spring Harb Perspect Med 7:1–24. https://doi.org/10.1101/CSHPERSPECT.A025619

Mohanty S, Srivastava P, Maurya AK et al (2013) Antimalarial and safety evaluation of Pluchea lanceolata (DC.) Oliv. & Hiern: In vitro and in vivo study. J Ethnopharmacol 149:797–802. https://doi.org/10.1016/j.jep.2013.08.003

Mohanty S, Maurya AK, Jyotshna et al (2015) Flavonoids rich fraction of Citrus limetta fruit peels reduces proinflammatory cytokine production and attenuates malaria pathogenesis. Curr Pharm Biotechnol 16:544–552. https://doi.org/10.2174/138920101660150407114023

Nafees S, Rashid S, Ali N et al (2015) Rutin ameliorates cyclophosphamide induced oxidative stress and inflammation in Wistar rats: role of NFκB/MAPK pathway. Chem Biol Interact 231:98–107. https://doi.org/10.1016/j.cbi.2015.02.021

Oakes KD, Van Der Kraak GJ (2003) Utility of the TBARS assay in detecting oxidative stress in white sucker (Catostomus commersoni) populations exposed to pulp mill effluent. Aquat Toxicol 63:447–463. https://doi.org/10.1016/s0166-445x(02)00204-7

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358. https://doi.org/10.1016/0003-2697(79)90738-3

Oludele OJ, Adisa BA, Olufunso OO (2020) Regulated rutin co-administration reverses mitochondrial-mediated apoptosis in Plasmodium berghei-infected mice. Biochem Biophys Res Commun 522:328–334. https://doi.org/10.1016/j.bbrc.2019.11.067

Park TY, Jang Y, Kim W et al (2019) Chloroquine modulates inflammatory autoimmune responses through Nurr1 in autoimmune diseases. Sci Rep 9:1–11. https://doi.org/10.1038/s41598-019-52085-w

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