Quassin alters the immunological patterns of murine macrophages through generation of nitric oxide to exert antileishmanial activity

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Objectives: The aim of this study was to characterize the in vitro antileishmanial activity of quassin, a traditional Chinese herbal medicine.

Methods: The cytotoxic effect of quassin was studied in murine peritoneal macrophages at various concentrations using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide method. The role of quassin as an antileishmanial agent was evaluated by microscopic counting of intracellular amastigotes in macrophages stained with Giemsa. To understand the effector mechanism of quassin-treated macrophages against leishmanial parasites, western blot and real-time PCR analysis of inducible nitric oxide (NO) synthase 2 (iNOS2) were done followed by measurement of NO generation by Griess reaction. The effect of quassin on the production of Th1 cytokines such as interleukin (IL)-12 and tumour necrosis factor (TNF)-α and Th2 cytokines such as IL-10 and transforming growth factor-β was measured by ELISA, and the mRNA expression of these cytokines was analysed by real-time PCR.

Results: Quassin at a dose of 25 μg/mL (64.36 μM) showed less cytotoxicity to the host murine peritoneal macrophages but at the same dose was effective enough to control the intracellular parasitic load compared with higher doses of quassin. *Leishmania donovani* is known to exert its pathogenic effects mainly by the suppression of NO generation and subversion of the cellular inflammatory responses in the macrophages. Quassin was found to induce a potent host-protective immune response by enhancing NO generation and iNOS2 expression both at a protein and mRNA level and by up-regulating pro-inflammatory cytokines such as TNF-α and IL-12 in *L. donovani*-infected macrophages with concurrent inhibition of anti-inflammatory responses.

Conclusions: These findings strongly support the effectiveness of quassin as a potent immunomodulatory tool for controlling the establishment of leishmanial parasite within the host macrophages.

Keywords: *Leishmania donovani*, pro-inflammatory cytokines, anti-inflammatory cytokines, NO

Introduction

The spectrum of the protozoan disease leishmaniasis encompasses ~350 million people in 88 countries and every year ~57,000 deaths are caused by this disease.¹,² Visceral leishmaniasis or kalazar, caused by the protozoan parasite *Leishmania donovani*, is the most severe form of leishmaniasis and is associated with irregular fever, weight loss, enlargement of the spleen and liver and anemia. The severity of this fatal disease is further magnified by the emergence of HIV/visceral leishmaniasis co-infection.¹,³ Currently, pentavalent antimony is the mainstay of the therapy of this infection,⁴,⁵ but treatment with antimonial agents has been unsuccessful due to high toxicity and resistance.⁴,⁶ Furthermore, improved formulations, such as liposome-encapsulated amphotericin B, in spite of their effectiveness have made general treatment unaffordable in many afflicted countries, revealing an urgent need for new, safer and cheaper drugs.⁷

Plants have been used traditionally for the treatment of protozoan diseases, and phytotherapy has recently filled the void in the search for alternative compounds with antiparasitic activity. Quassin, the first characterized member of the group quassinoids,⁸⁹ extracted from the bark of the Bitter tree, *Quassia*...
an antimalarial, antiviral, antifeedant, insecticidal, amoebicidal, parasitidal and herbicidal activities. Quassin itself has antitumorogenic and antiplasmodial activity.

In the present study, we demonstrate the antileishmanial role of quassin, where a dose of 25 μg/mL, post-infection, results in the induction of inducible nitric oxide (NO) synthase 2 (iNOS2) expression at the protein level leading to significantly enhanced generation of NO. In addition, it also induces Th1-type cytokines and suppresses Th2-type cytokines in L. donovani-infected BALB/c peritoneal macrophages. These observations suggest that quassin mediates the antileishmanial response of murine peritoneal macrophages not only via the generation of NO but also through the induction of the Th1 immune response.

Materials and methods

Chemicals and antibodies

RPMI-1640 medium, M-199 medium, penicillin and streptomycin were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fetal calf serum (FCS) was obtained from Gibco BRL (Grand Island, NY, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] (HiMedia, India) and an ELISA Assay Kit for tumour necrosis factor (TNF)-α, interleukin (IL)–12p70, IL–10 and transforming growth factor (TGF)-β (Quantikine M; R&D Systems, Minneapolis, MN, USA) were procured. For isolation of total RNA, TRI Reagent was purchased from Sigma. dNTPs, RevertAidH FMD M-MuLV Reverse Transcriptase, oligo dT, RNase inhibitor and other chemicals required for cDNA synthesis were purchased from Fermentas, USA. Power SYBR Green PCR Master Mix (×2) for quantitative real-time PCR was purchased from Applied Biosystems, UK. Oligos for real-time PCR were purchased from Sigma. All other chemicals were purchased from either Sigma or Merck. SDS was obtained from Qualigens, India. Authentic standard of quassin (Koch–Light Laboratories Ltd, UK) was obtained as a gift from Dr E. J. Allan (Department of Agriculture, University of Aberdeen, UK).

Animals and parasites

BALB/c mice (average weight, 25–30 g) were procured from the National Center for Laboratory Animal Sciences (NCLAS), Hyderabad, India. Animals were maintained at a standard temperature (25 ± 5°C), 12 h day/night cycle, fed a standard pellet diet and provided water ad libitum. For each experiment, 8–10 mice (6–8 weeks old) were used regardless of sex. All experimental protocols received prior approval from the Institutional Animal Ethical Committee.

L. donovani strain AG-83 (MHOM/IN/1983/AG83; a gift from Ananta Ghosh, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA) was maintained in vitro in M-199 medium containing 10% FCS. Amastigotes were prepared from the spleen of a Golden Hamster infected with L. donovani as described previously. For infection, hamsters were injected with 2 × 10⁷ amastigotes in 0.5 mL of normal saline via the intracardiac route. Amastigotes were then differentiated into promastigotes. The experiments were performed by infecting the cells with stationary-phase promastigotes.

Isolation of peritoneal macrophages

Mouse macrophages were isolated by peritoneal lavage with ice-cold PBS 48 h after intraperitoneal injection of 1.0 mL of sterile 4% thiglycolate broth (Difco). Cells were cultured as described previously. The adherent cell population was cultured for 48 h prior to any treatment, to achieve the resting state. More than 90% of the remaining adherent cells were found to be macrophages on the basis of morphological criteria and non-specific esterase staining.

Uptake and intracellular multiplication of L. donovani

To assess the activity of quassin against the amastigote stage of parasite, BALB/C peritoneal macrophages were cultured on glass coverslips (18 mm²; 5 × 10⁵ macrophages/coverslip) in 0.5 mL of RPMI-1640 supplemented with 10% FCS and were infected with L. donovani promastigotes at a 1:10 macrophage to parasite ratio for 4 h, after which the unbound parasites were washed off. The infected macrophages were incubated with 25 μg/mL quassin at 37°C for 24 and 48 h and then fixed with chilled methanol. Intracellular parasitic numbers were determined by staining with Giemsa. When indicated, the uptake and multiplication of L. donovani were studied in the presence and absence of 0.4 mM NO synthase inhibitor 1-N-monomethyl arginine (L-NMMA) (Sigma).

Cytotoxicity assay by MTT method

Monolayers of macrophages in 96-well tissue culture plates (Tarson) incubated with quassin at concentrations ranging from 1 ng/mL to 250 μg/mL were cultured in RPMI-1640 supplemented with 10% FCS for 48 h. Thereafter, the medium was replaced with fresh RPMI (without Phenol Red) containing 1 μg/mL MTT. Cells were incubated at 37°C for 3 h, the untransformed MTT was removed and 50 μL of 0.04 M HCl-isopropanolic solution was added to each well. After 15 min of incubation at room temperature, absorbance was measured using an automatic plate reader (Thermolab System Multiskan Ex), at a reference wavelength of 690 nm and test wavelength of 650 nm.

Nitrite generation assay

Nitrite accumulation in culture was measured colorimetrically by the Griess reaction using the Nitric Oxide Colorimetric Assay Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). For each assay, macrophages were cultured in 24-well tissue culture plates (Tarson) at a concentration of 1 × 10⁶ cells/mL. Cell-free supernatants were collected from different experimental sets after 24 and 48 h of treatment, and nitrite levels were estimated as per the manufacturer’s instructions. Data are expressed in terms of μM nitrite.

Measurement of reactive oxygen species (ROS)

To monitor the level of ROS, the cell permeant probe H₂DCFDA was used as described previously. Marine peritoneal macrophages from different treatment sets were incubated with H₂DCFDA (2 μg/mL) at room temperature for 20 min in the dark. Relative fluorescence was measured in a Perkin-Elmer LS50B Spectrofluorometer at an excitation wavelength of 510 nm and an emission wavelength of 525 nm. For each experiment, fluorometric measurements were performed in triplicate and data are expressed as mean fluorescence intensity units.
Measurement of cytokine release by sandwich ELISA
The level of murine TNF-α, IL-12p40, IL-10 and TGF-β in the conditioned medium of macrophage culture was measured using a sandwich ELISA Kit (Quantikine M; R&D Systems). The assay was performed as per the detailed instructions of the manufacturer. The detection limit of these assays was <5.1, <2.5, <4 and <3 pg/mL for TNF-α, IL-12p40, IL-10 and TGF-β, respectively.

Preparation of cell lysate and immunoblot analysis
Cells cultured overnight in RPMI 1640 (Sigma) containing 10% FCS (Gibco BRL) were subjected to respective treatment for 24 h after L. donovani infection. The cells were then pelleted by centrifugation at 400 g for 15 min at 4 °C and resuspended in ice-cold extraction buffer containing 10 mM Tris–HCl (pH 7.5), 4.5 mM EGTA, 50 mM 2-mercaptoethanol and an anti-protease mixture containing 10 mM Tris–HCl (pH 7.5), 4.5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM 2-mercaptoethanol and an anti-protease mixture containing 0.33 mM leupeptin, 0.2 mM phenylmethylsulphonyl fluoride, 4.8 trypsin inhibitor units of aprotinin/mL and 0.35 mM antipain. The cell lysate was sonicated and centrifuged at 4250 g for 10 min at 4 °C, and the supernatant was then used for western blotting.

Proteins in the supernatant were quantified with the Bio-Rad protein assay reagent using BSA as a standard. Equal amounts of protein (50 μg) in each lane were subjected to SDS–10% PAGE and transferred to a nitrocellulose membrane. The membrane was blocked overnight with 63% BSA in Tris-saline buffer (pH 7.5), and immunoblotting was done as described previously by Ghosh et al.

Isolation of RNA and real-time PCR
Total RNA was extracted from 3 × 10^6 murine peritoneal macrophages using TRI Reagent® (Sigma) according to the manufacturer’s protocol. Isolated total RNA (1 μg) was then reverse transcribed using Revert Aid™ M-MuLV Reverse Transcriptase (Fermentas, USA). The resulting cDNA was then used for real-time PCR for iNOS2 and cytokines (IL-12p40, TNF-α, IL-10 and TGF-β) using an ABI 7500 real-time PCR system (Applied Biosystems, UK) with the DNA binding SYBR green dye. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference. The forward and reverse specific primer sequences used were as follows. iNOS2, forward 5'-CCCTTCCAGGTCTGTCGAGC-3', and reverse 5'-GGCTGTCAGAGCCTCGTGG-3'; IL-12p40, forward 5'-CAACATCAAGAGCAGTGACGAC-3', and reverse 5'-TACTCCCAAGCTGACCTCC-3'; TNF-α, forward 5'-GGCAAGTTCTATTCCAGATGTCC-3' and reverse 5'-ACATTGAGGCTCAGTGAATTCCG-3'; TGF-β, forward 5'-GGATAACCACTATGCTCAGTTCC-3' and reverse 5'-GGCGTCCAATAATATAGGGGCAGGTC-3'; and GAPDH, forward 5'-CAAGGCTGTGGGGCAAGGTC-3' and reverse 5'-AGTGGAAGTGGGATGTCTG-3'. For real-time quantitative PCR, each reaction contained 1× SYBR Green PCR master mixture (Power SYBR Green PCR Master Mix; Applied Biosystems, UK), 10 pmol of each primer and 1.0 μL of cDNA in a final volume of 20 μL. The reaction conditions were as follows: initial activation step (5 min at 95 °C) and cycling step (denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C, and then extension for 1 min at 72 °C for 40 cycles) followed by melt curve analysis. Detection of the denatured probe, calculation of threshold cycles (Ct values) and further analysis of these data were performed by the Sequence Detector software. Relative changes in iNOS2 and cytokine (IL-12p40, TNF-α, IL-10 and TGF-β) mRNA expression were compared with an unstimulated control, normalized to GAPDH and were quantified by the 2^(-ΔΔCt) method. Thus, all the values for

Figure 1. Determination of the non-cytotoxic dose of quassin and its effect on the parasitic burden in L. donovani-infected murine peritoneal macrophages.
(a) Murine peritoneal macrophages cultured in complete RPMI 1640 medium were subjected to quassin treatment at specified doses (1, 10, 50, 100, 250 and 500 ng/mL) and (b) after 24 h of incubation, a cell viability assay was performed using the MTT method (spectrophotometric reading of the MTT-formazan formed was read at 650 nm and data are expressed as the percentage of viable cells). Single asterisks represent significant differences compared with the untreated control group at a level of P < 0.001. (b) Macrophages were cultured in complete RPMI medium overnight and infected with L. donovani promastigotes for 4 h at a macrophage to parasite ratio of 1:10. The cells were washed and 1, 5, 10, 25 and 50 μg/mL doses of quassin were added. The cells were incubated for another 24 and 48 h in coverslip culture, stained with Giemsa and the numbers of intracellular amastigotes per 100 macrophages were then counted. The bars show the number of parasites per 100 peritoneal macrophages. The experiment was repeated three times, yielding similar results, and data are expressed as means ± SD. Asterisks indicate statistically significant reductions (**P < 0.005; *P < 0.001) in the parasitic load compared with infected macrophages. Mφs, macrophages.
Figure 2. Quassin-mediated antileishmanial activity via nitrite generation and iNOS2 expression and ROS generation in infected macrophages. (a) Macrophages were challenged with *L. donovani* promastigotes (cell to parasite ratio of 1:10) for 4 h. Non-ingested promastigotes were removed and macrophages were cultured for another 20 h. Uninfected macrophages and macrophages infected with *L. donovani* were either kept untreated or treated with quassin for different durations. ROS generation was measured by H$_2$DCFDA staining of the peritoneal macrophages from different experimental sets. Data for ROS generation (mean fluorescence intensity in arbitrary units) are expressed as means $\pm$ SD from triplicate experiments yielding similar results. (b) In a separate experimental set, macrophages were incubated with LPS, *L. donovani* promastigotes (macrophage to parasite ratio of 1:10), quassin (25 $\mu$g/mL) alone or quassin (25 $\mu$g/mL) plus *L. donovani*. The cells were kept for 24 and 48 h for maximum nitrite generation, and the cell-free supernatants collected were subjected to the nitrite generation assay as mentioned in the Materials and methods section. Data for nitrite generation are expressed as means $\pm$ SD from triplicate experiments yielding similar results ($\mu$M nitrite). Asterisks indicate statistically significant induction (**$P < 0.005$; *$P < 0.001$) of nitrite generation compared with infected macrophages. (c) The nature of iNOS2 expression was determined by western blot analysis in macrophages treated with quassin (25 $\mu$g/mL) (lane 2), *L. donovani* (lane 3) or quassin (25 $\mu$g/mL) plus *L. donovani* (lane 4). Lane 1 represents untreated macrophages. The blot shown in (c) is representative of triplicate experiments.
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Densitometric analysis
Immunoblots were analysed using a model GS-700 imaging densitometer and Molecular Analyst version 1.5 software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis
The experiments were performed at least three times and the data are presented as means ± SD. One- or two-tailed Student’s t-test was employed to assess the significance of the differences between the mean values of the control and experimental groups. A P value of <0.05 was considered significant and <0.001 was considered highly significant.

Results
Determination of the non-cytotoxic dose of quassin and its effect on the intracellular parasitic load in L. donovani-infected macrophages
The cytotoxic effect of the naturally occurring plant product quassin was studied in murine peritoneal macrophages with increasing concentrations of quassin ranging from 1 ng/mL to 250 µg/mL using the MTT method. The results clearly indicated that treatment of peritoneal macrophages with 10, 25, 50 and 100 µg/mL quassin for 48 h led to 10%, 15%, 30% and 50% reduction in cell survivability, respectively (Figure 1a). Further, we examined the efficacy of quassin in reducing the intracellular parasitic load. It was found that quassin reduces the parasitic load in peritoneal macrophages in a dose-dependent manner after 24 and 48 h of L. donovani infection. Interestingly, at the 24 h timepoint, 10, 25 and 50 µg/mL quassin showed 36.36%, 70.45% and 82.95% reductions in the parasitic load of the macrophages, respectively, whereas at the 48 h timepoint, the same doses of quassin showed 58.45%, 86.47% and 91.93% reductions, respectively (Figure 1b). Based on these observations, we used 25 µg/mL (64.36 µM) quassin as the standard and effective dose for antileishmanial activity as it showed low cytotoxicity, corresponding to a 15% decrease in cell survivability, and high antileishmanial activity, corresponding to 70.45% and 86.47% decreases in the intracellular parasitic load at 24 and 48 h, respectively. Higher doses of quassin (50 µg/mL) showed 82.95% and 91.93% reductions in the intracellular parasitic load at 24 and 48 h but significantly reduced the host cell survivability.

Quassin-induced leishmanicidal activity by up-regulation of nitrite generation and iNOS2 expression
Probing into the probable mechanism of the quassin-mediated parasite clearance by the host macrophages, we examined the role of free radicals since NO and ROS are the two major microbicidal molecules.29,30 We have found that quassin-mediated protection against visceral leishmaniasis is not obtained through the release of ROS as it cannot induce the generation of this class of free radicals at early timepoints of infection (Figure 2a). This prompted us to study the release of NO at the early and late timepoints of Leishmania infection. There was no significant generation of NO after 1, 3, 6 and 12 h of infection, but, interestingly, after 24 and 48 h of infection, 25 µg/mL quassin could significantly (P < 0.001) induce the generation of NO (Figure 2b). As this generation of NO is dependent upon the activation of iNOS2, we studied its expression at the protein and mRNA levels after 24 and 4 h of quassin treatment, respectively, in infected macrophages. iNOS2 expression at the protein level showed a significant increase in L. donovani-infected macrophages treated with quassin (25 µg/mL) (64.36 µM) when compared with macrophages infected with L. donovani alone (Figure 2c and d). We also observed that there was a 9.8-fold increase in the expression level of iNOS2 mRNA in quassin-treated infected macrophages compared with only infected macrophages (Figure 2e). This result clearly suggests that quassin treatment induces the iNOS2 expression in the L. donovani-infected macrophages.

To further confirm the above data, the intracellular amastigote count was done in the presence or absence of L-NMMA, a competitive inhibitor of iNOS2 at the 24 and 48 h timepoints. It was observed that treatment of macrophages with quassin post-infection resulted in 70% and 88% reduction in the parasitic load, respectively, whereas treatment with L-NMMA pre-infection under similar experimental conditions could hardly reduce the parasitic load. Our results clearly indicated that in the presence of L-NMMA, the protective role of quassin is abrogated in L. donovani-infected macrophages (Figure 2f). Thus, quassin at a concentration of 25 µg/mL (64.36 µM) helps in the clearance of the intracellular parasitic burden by inducing an NO-mediated defence mechanism in the host macrophages.

Effect of quassin on cytokine production
It is well established that during leishmanial infection there is a critical skewing from pro-inflammatory to anti-inflammatory cytokine response that results in sustained intracellular survivability of the parasite. Hence, we planned to study whether quassin (25 µg/mL) treatment could modulate pro-inflammatory cytokine (TNF-α and IL-12p70) and anti-inflammatory cytokine (IL-10, TGF-β) release and expression at the mRNA level in...
Figure 3. Effect of quassin on cytokine profiles in *L. donovani*-infected murine peritoneal macrophages at protein and mRNA levels. Peritoneal macrophages cultured overnight in complete RPMI 1640 medium were infected with *L. donovani* promastigotes (1 macrophage to 10 parasites), washed after 4 h and treated with quassin at a dose of 25 μg/mL. These treated cells were kept for another 24 h. Then the levels of (a) TNF-α (pg/mL), (b) IL-12p70 (pg/mL), (c) TGF-β (pg/mL) and (d) IL-10 (pg/mL) in the culture supernatant were evaluated by sandwich ELISA, as described in the Materials and methods section. Results are expressed as means ± SD from three replicate experiments yielding similar results. Asterisks indicate a statistically significant increase or decrease (**P < 0.005; *P < 0.001) in cytokine release compared with infected macrophages. A separate set of cells, cultured similarly, was treated with quassin (25 μg/mL) for 4 h, after which the cells were collected in TRIZOL for mRNA extraction and analysed using real-time PCR (see the Materials and methods section) to study different pro-inflammatory and anti-inflammatory cytokine mRNA expression. Quantitative real-time PCR results show the expression of (e) IL-12p40, (f) TNF-α, (g) TGF-β and (h) IL-10 mRNA. Data are presented as fold changes compared with uninfected control cells. The data represent the means ± SD of data from three independent experiments that yielded similar results (**P < 0.005 and *P < 0.001).
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*L. donovani*-infected murine peritoneal macrophages to exert its NO-mediated intracellular parasite killing activity. We observed that following treatment with quassin (25 μg/mL), *L. donovani*-infected macrophages showed a 5-fold increase in the release of TNF-α and IL-12p70 in comparison with *L. donovani*-infected cells alone (Figure 3a and b). This significant increase in TNF-α release might play a pivotal role in triggering the signal for enhanced NO generation. On the other hand, the release of TGF-β and IL-10, the signature anti-inflammatory cytokines, was significantly abrogated upon treatment with quassin in *L. donovani*-infected murine peritoneal macrophages (Figure 3c and d).

To confirm our quantitative data as observed by the ELISA method, we further studied the mRNA expression of the above cytokines at the early timepoints of quassin treatment in infected macrophages by using real-time PCR analysis. The results revealed that there was a significant inhibition of IL-12p40 and TNF-α expression in the infected macrophages compared with uninfected macrophages (Figure 3e and f). In quassin-treated infected macrophages, there was a 9.5- and 12-fold increase in the expression levels of IL-12p40 and TNF-α, respectively, compared with infected macrophages (Figure 3e and f). On the other hand, in infected macrophages, there was a significant induction of IL-10 and TGF-β in comparison with uninfected macrophages at the mRNA level (Figure 3g and h). Interestingly, when infected macrophages were treated with quassin, there was a 3.62- and 3.45-fold reduction in the expression of TGF-β and IL-10, respectively, in comparison with infected macrophages (Figure 3g and h).

It is the balance between the Th1 and Th2 cytokines that determines the intracellular survival or clearance of a parasite. Our present findings showed that following treatment with quassin (25 μg/mL or 64.36 μM), *L. donovani*-infected macrophages induced Th1 cytokines and suppressed Th2 cytokines, thereby preventing intracellular survival of *Leishmania* amastigotes.

**Discussion**

The use of biologically active natural products is gaining increasing popularity day by day over traditional medicine as an attractive alternative approach for the treatment of various diseases. However, limited scientific evidence regarding the effectiveness of these natural derivatives and lack of mechanistic understanding have prevented their incorporation into mainstream medicine and their application in human therapy.

Quassin, a relatively non-toxic, naturally occurring triterpenoid compound, found in the bark of *Q. amara* has a number of medicinal properties.

In the present study we have demonstrated that *in vitro* treatment with quassin significantly reduced the intracellular parasitic load in *L. donovani*-infected murine peritoneal macrophages. We initially observed its cytotoxic profile in murine peritoneal macrophages by MTT assay (Figure 1) and determined its least comparative cytotoxic concentration to be 25 μg/mL (64.36 μM), whereas concentrations higher than 25 μg/mL showed increased cytotoxicity. So we used the 25 μg/mL concentration as the effective dose for further experiments.

To establish the antileishmanial activity of quassin, we have studied its effect on the intracellular parasitic load in *L. donovani*-infected murine peritoneal macrophages, and it has been shown that quassin dose-dependently reduced the parasitic load. Optimum reduction of the parasitic load was found to be 88% in the presence of 25 μg/mL quassin.

The killing of intracellular *Leishmania* parasites by quassin can be correlated with the induction of the iNOS pathway and concomitant production of reactive nitrogen derivatives. The major antileishmanial oxidant produced by activated macrophages is NO, which is responsible for the killing of established intracellular amastigotes. We demonstrated that quassin at a concentration of 25 μg/mL could not mediate NO generation at early timepoints of infection but could significantly induce the NO production at later timepoints, which paralleled maximal stimulation of leishmanicidal activity (Figure 2b). Quassin could effectively mediate NO generation as it significantly induced iNOS2 expression at both the protein and mRNA level in *L. donovani*-infected macrophages (Figure 2c–e). This iNOS2-mediated parasite killing by quassin was found to be abrogated when L-NMMA (Figure 2f), a competitive inhibitor of the iNOS enzyme, was used, confirming the involvement of NO in quassin-mediated protection during leishmaniasis. These observations clearly depicted the protective role of quassin-mediated generation of reactive nitrogen intermediates in the control of intracellular parasites by murine macrophages.

Quassin is a natural product that has been shown to possess antileishmanial activity. It acts through immunomodulation by altering the balance between Th1 and Th2 cytokines and inducing expression of iNOS, which is responsible for the killing of *Leishmania* amastigotes. The successful survival and establishment of intracellular parasites within host cells take place via the inhibition of NO generation and reduced pro-inflammatory cytokine response. The above observations clearly indicate the marked sensitivity of the macrophage effector responses of NO and pro-inflammatory cytokines to quassin and suggest that this natural product could be used as a potential immunomodulator to generate the required immunity not only for the treatment of leishmaniasis, but also for the treatment of other chronic infectious diseases.

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Transparency declarations

None to declare.

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