Thymoquinone Induced Leishmanicidal Effect via Programmed Cell Death in *Leishmania donovani*

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**ABSTRACT:** Visceral leishmaniasis (VL) or kala-azar is a vector-borne dreaded protozoal infection that is caused by the parasite *Leishmania donovani*. With increases in the dramatic infection rates, present drug toxicity, resistance, and the absence of an approved vaccine, the development of new antileishmanial compounds from plant sources remains the keystone for the control of visceral leishmaniasis. In this study, we evaluated the leishmanicidal effect of thymoquinone against *L. donovani* with an *in vitro* and *ex vivo* model. Thymoquinone exhibited potent antipromastigote activity with IC$_{50}$ and IC$_{90}$ concentrations achieved at 6.33 ± 1.21 and 20.71 ± 2.15 μM, respectively, whereas the IC$_{50}$ and IC$_{90}$ concentrations were found to be 7.83 ± 1.65 and 27.25 ± 2.20 μM against the intramacrophagic form of amastigotes, respectively. Morphological changes in promastigotes and growth reversibility study following treatment confirmed the leishmanicidal effect of thymoquinone. Further, thymoquinone exhibited leishmanicidal activities against *L. donovani* promastigote through cytoplasmic shrinkage, membrane blebbing, chromatin condensation, cellular and nuclear shrinkage, and DNA fragmentation, as observed under scanning and transmission electron microscopy analyses. The antileishmanial activity was exerted via programmed cell death as proved by exposure of phosphatidylserine, DNA nicking by TUNEL assay, and loss of mitochondrial membrane potential. Thymoquinone at a concentration of 200 μM was devoid of any cytotoxic effects against mammalian macrophage cells. Thymoquinone showed strong leishmanicidal activity against *L. donovani*, which is mediated via an apoptosis mode of parasitic cell death, and accordingly, thymoquinone may be the source of a new lead molecule for the cure of VL.

**INTRODUCTION**

Leishmaniasis is a vector-borne parasitic disease caused by obligate intracellular *Leishmania donovani* protozoan parasites, which are a member of Kinetoplastida. The disease manifests in many different ways, from a self-limiting cutaneous to a deadly visceralizing infection (bone marrow, spleen, and liver). The forms of leishmaniasis (cutaneous leishmaniasis and visceral leishmaniasis) depend on the type of *Leishmania* parasite and vary in geographical location, host, and vector involved. Visceral leishmaniasis, the most fatal form of leishmaniasis also known as kala-azar, is caused by *L. donovani*, which affects millions of people worldwide. Visceral leishmaniasis (VL) is endemic in 62 countries, with less than 1% of cases occurring in only five countries: Brazil, India, Bangladesh, Sudan, and Nepal. In regards to drug discovery, VL is considered a neglected tropical disease, and there are only a few drugs that are used to treat it. Owing to the present drug toxicity, high cost, and the emergence of new drug-resistant strains, an urgent need for a cheap, safe, and effective novel therapeutic agent is imperative. Plants and their product are very important sources of various bioactive agents, which include a varied group of essential oils and related compounds. Essential oils have well-known antifungal antibacterial and antiparasitic properties against many pathogenic microorganisms. Natural products from plant origin comprise a promising source of new antileishmanial drugs.

*Nigella sativa* seeds generally known as black cumin, black caraway, Roman coriander, and black seed are annual flowering herbs from the family Ranunculaceae and grow in southern Europe, the Middle East, North Africa, India, and Western Asia. *N. sativa* has traditionally been used as a natural remedy for the cure of many acute and chronic illnesses such as fever, cough, bronchitis, diabetes, skin diseases, jaundice, gastrointestinal problems, and hypertension.

Research shows that *N. sativa* exhibits anti-inflammatory, immunomodulatory, antioxidant, neuroprotective, antimicro-
bial, antitumor, and hepatoprotective effects.\textsuperscript{14} Thymoquinone, a yellow crystalline molecule, is one of the main bioactive constituents in \textit{N. sativa} oil (NSO) and extract.\textsuperscript{15−17} Thymoquinone IUPAC name and its chemical structure are presented in Figure 1. Approximately 35−50\% of thymoquinone is present in \textit{N. sativa} seeds,\textsuperscript{18} and because its isolation and characterization were done in 1963,\textsuperscript{19} it has been widely investigated by many scientists worldwide. Several investigations have reported antiviral, antibacterial, antifungal, and antiparasitic effects of NSO and thymoquinone.\textsuperscript{13,14,20−22} A few researchers showed \textit{in vitro} potent inhibitory effects of NSO nanoparticles against promastigotes and intracellular amastigote forms of \textit{Leishmania infantum}.\textsuperscript{23,24} A recent study showed that NSO and thymoquinone exhibited growth inhibition effects against parasites \textit{Leishmania tropica} and \textit{L. infantum}.\textsuperscript{25} Encouraged by the above studies and considering the potent antileishmanial activities of thymoquinone, the present study was aimed at assessing the antileishmanial potential of thymoquinone against \textit{L. donovani in vitro} and \textit{ex vivo}.

\section*{RESULTS}

\textbf{Thymoquinone Exhibits Death in \textit{L. donovani} Promastigotes.} The antileishmanial effect of thymoquinone against exponentially grown \textit{L. donovani} promastigotes was
investigated by an MTT degradation study. Treatment with thymoquinone (0–100 μM) showed a reduction in parasite growth in a dose-dependent manner, and after 72 h of incubation, all of the promastigotes seemed to be dead (Figure 2A). Pentamidine, taken as a reference drug control, exhibited a similar tendency in promastigote killing. Parasites without treatment propagate exponentially. Media with 0.1% DMSO exhibited inertness against parasite proliferation and viability (data not shown). The 50 and 90% inhibitory concentrations of thymoquinone were achieved at 6.33 ± 1.21 and 20.71 ± 2.15 μM, respectively, whereas pentamidine IC50 and IC90 values were found at 1.67 ± 0.4 and 6.11 ± 1.24 μM, respectively (Figure 2B).

**Leishmanicidal Effect of Thymoquinone.** To confirm the cytocidal effects of thymoquinone, untreated and treated promastigotes after 4 days of incubation were gently washed with incomplete media and resuspended in complete media.
with 10% FBS, and their growth was ascertained microscopically after an additional 96 h of culture. The number of survival parasites becomes zero in the case of earlier treatment with thymoquinone and the standard control drug (IC$_{90}$ concentration; 20.71 ± 2.15 and 6.11 ± 1.24 μM, respectively), confirming their cytocidal effect. While in the case of promastigotes without drugs, the residual live parasites expanded and restored their growth in the state of the exponential phase (Figure 2D).

**Thymoquinone Alters the Morphology of Promastigotes.** Bright-field microscopy examination of *L. donovani* promastigotes upon exposure at IC$_{90}$ concentration of thymoquinone (20.71 ± 2.15 μM) affirmed that the cellular morphology of promastigotes changes to an ovoid shape with cytoplasmic condensation, loss of flagella, and cell shrinkage, resulting in a substantial reduction in size and complete circularization of the promastigotes compared to the parasites without exposure to thymoquinone. A similar morphological alteration was detected in the case of pentamidine-treated promastigotes (Figure 2E). Overall, the microscopy study pointed out that the killing of the parasites was mediated through programmed cell death.

**Antileishmanial Effect of Thymoquinone against Intramacrophagic Amastigotes.** The antileishmanial activity of thymoquinone toward intramacrophagic amastigotes was evaluated using Giemsa-stained macrophages under an immersion oil microscope. Flagellated promastigotes transformed into nonmotile aflagellated amastigotes inside the parasitophorous vacuoles of macrophages. Parasite survival inside the parasitophorous vacuoles of the macrophages is an index of pathogenesis. Therefore, it is essential to test the activity of the test drug toward the macrophage-resident amastigotes. Treatment of amastigotes with the compound of interest at IC$_{90}$ concentration resulted in the reduction of the amastigote load within macrophages in a dose-dependent pattern (Figure 3A) with IC$_{50}$ and IC$_{90}$ achieved at 7.83 ± 1.65 and 27.25 ± 2.20 μM, respectively (Figure 3B). Giemsa-stained slides of infected and treated macrophages display almost complete clearance of the intramacrophagic amastigotes under a microscope at a higher dose (100 μM), and a similar result was detected with the standard drug, pentamidine.

**Cytotoxicity of Thymoquinone toward Mammalian Macrophages.** Cytotoxicity evaluation was performed on the mammalian macrophage cell line (RAW264.7) to study the toxic effects of the test compounds and the standard drug pentamidine. Exposure of RAW264.7 to thymoquinone (Figure 3C) up to 200 μM did not alter the morphology and viability of the cell line, unlike the standard drug.

**SEM Analysis of Thymoquinone-Treated L. donovani Promastigotes.** Alteration in the cellular morphological
Figure 5. (A) Phosphatidylserine externalization in thymoquinone-treated promastigotes using annexin V and PI staining. Promastigotes at a cell population of \(2 \times 10^6\) cells/mL are exposed to thymoquinone and pentamidine at IC\(_{90}\) concentration for 72 h, and PS exposure is analyzed by co-staining with annexin V and PI through flow cytometry as explained in the Methods section. The % of positive cells is marked in different quadrants of the dot plot. (B) Bar graph represents the percentage of apoptotic cells (annexin V positive). (C) DNA fragmentation in thymoquinone-treated parasites depicted by the TUNEL study. The flagellated promastigotes at a parasite density of \(2 \times 10^6\) cells/mL are incubated with IC\(_{90}\) concentration of thymoquinone for 72 h at 26 °C; treated parasites are then processed as per the manufacturer’s directions, and the sample is
acquired on a flow cytometer. Bar graph depicting the mean fluorescence intensity values of TUNEL positivity in test drug-treated and untreated samples. (D) Thymoquinone induced a reduction in the membrane potential of mitochondria in flagellated promastigotes. Exponentially growing phase parasites (2 × 10^6 cells/mL) are incubated in the presence of test drugs (thymoquinone and pentamidine) at IC_{90} concentration for 72 h and investigated with JC-1 dye; bar graph image presenting the ratio of red/green fluorescence intensity (MFI) obtained from histogram statistics. Each bar corresponds to the mean ± SE of triplicate samples and is representative of one of the three separate experiments. p < 0.05 is treated as statistically significant (**p < 0.001).

Thymoquinone Induces Ultrastructural Alterations in Promastigotes. Transmission electron microscopy (TEM) analysis of the ultrastructure of L. donovani promastigotes treated with IC_{90} dose of thymoquinone showed that the test compound induced ultrastructural morphological alterations, such as flagellated promastigotes becoming aflagellated and spherical accompanied by a gain in the size of lipid bodies and vacuoles and marked subcellular changes, particularly in the mitochondria—kinetoplast complex. Intense swelling of mitochondria, disorganization of the mitochondrial inner membrane, loss of electron density of the mitochondrial matrix, and decondensation of kinetoplast are detected, and pentamidine exhibited similar effects on parasites. Promastigotes without drugs presented cylindrical and elongated bodies, large flagellum and a regular subcellular structure with a single nucleus, and electron-condensed mitochondria containing well-organized cristae and dense kinetoplast (Figure 4A).

PS Externalization in Promastigotes by Thymoquinone. PS externalization as a characteristic feature of apoptosis. Apoptosis occurred in unicellular parasitic cells when phosphatidylserine (PS) was exposed from the inner leaflet of the plasma membrane to the outer side. To determine whether thymoquinone induced apoptosis or necrosis during cell death in promastigotes, parasites with and without drugs were stained using annexin V and PI (live cells, annexin V −ve and PI −ve; apoptosis, annexin V +ve and PI −ve; necrosis, annexin V −ve and PI +ve; late apoptosis, annexin V +ve and PI +ve). Following treatment with test drugs, 48.26 ± 5.17% of promastigotes underwent apoptotic mode of cell death (lower right quadrant). A small number of parasites was found to be late apoptotic (upper right quadrant), and apoptotic cells were also noticed. Whereas in the case of standard drug pentamidine, 61.65 ± 4.68% of promastigotes were found to be apoptotic (Figure 5A,B).

Thymoquinone Depolarized Mitochondrial Membrane Potential (ΔΨ_m). Changes in the membrane potential of mitochondria (ΔΨ_m) are an attribute of cellular apoptosis. The damage of membrane potential was ascertained using JC-1 dye. JC-1 dye is fluorescent and lipophilic in nature and freely enters the mitochondrial membrane of healthy cells and makes J-aggregates, emitting specific red fluorescence. An apoptotic drug causes a reduction in the membrane potential, and JC-1 dye is unable to permeate the mitochondrial membrane, as a result, they exist as monomers in the cytosol of cells and release green fluorescence. Therefore, the ratio of J-aggregates and monomers acts as an index of the energy state of the mitochondria, allowing for apoptotic cells to be easily differentiated from nonapoptotic cells. In parasites without any treatment, the J-aggregates/monomer fluorescence ratio was 2.84 ± 0.49, whereas treatment with IC_{90} dose of thymoquinone and pentamidine for 72 h exhibited a significant loss in the mitochondrial membrane potential, and the red/green fluorescent ratios were 0.65 ± 0.15 and 0.57 ± 0.14, respectively (Figure 5C).

Thymoquinone Treatment-Induced DNA Fragmentation in Promastigotes. Fragmentation of DNA to oligonucleosomal subunits is an indication of euerytropic apoptosis. DNA fragmentation resulting from treatment with thymoquinone was determined using a terminal deoxynucleotidyl transferase (TdT) δUTP nick-end labeling (TUNEL) study in which the relative amount of DNA fragments was measured by incorporation of δUTP-FLUOS via TdT to the fragmented ends of DNA. Accordingly, the number of DNA fragments was directly proportional to the intensity of fluorescence acquired from δUTP-FLUOS. Parasites exposed for 72 h at an IC_{90} concentration (20.71 ± 2.15 μM) of thymoquinone exhibited enhanced oligonucleosomal DNA fragmentation as proved by an increase in δUTP-FLUOS labeling. Promastigotes without drugs showed a mean fluorescence intensity (MPI) of 834 ± 52.32, which was increased to 3244.33 ± 143.23 and 3645.34 ± 138.85 after exposure to and being treated with thymoquinone and the standard drug, respectively (Figure 5D). Without any doubt, this finding demonstrated that thymoquinone induced DNA fragmentation in extracellular L. donovani parasites.

**DISCUSSION**

In immunocompromised patients, VL is studied as an opportunistic infection and reoccurrence of ostensibly cured VL infections as post-kala-azar dermal leishmaniasis and their co-infection in AIDS patients. Currently, there is no vaccine and the cure relies on antileishmanial synthetic drugs, mainly the first line of drugs such as antimonials (SbV), pentamidine, and amphoterin B, and the second-line drugs have several limitations, such as high toxicity, challenging treatment schedules, and development of resistance.26,27 Natural products may offer an inexhaustible source of chemical diversity with therapeutic potential.28 Plant-derived natural products have been central to drug discoveries for both antibiotics and antiparasitic drugs. Switching to alternate therapies, particularly plant and other natural product-derived drugs, may advance the search for better, more cost-effective, and safe antileishmanial agents.29,30 Numerous plant and herbal extracts and oils have been used as antiparasitic agents against liver flukes, tapeworm, and protozoan.31–34 N. sativa oil has been shown in several studies to have antibacterial, antifungal, and antiviral properties.35 The antibacterial activities
are due to the presence of the major component thymoquinone in the *N. sativa* oil. The antibacterial mechanism of thymoquinone has not been studied thoroughly yet. It was estimated that thymoquinone might prevent the synthesis of DNA and or block the ATP synthase enzyme synthesis of microorganisms. In the past, researchers have shown that thymoquinone induced apoptosis in leukemia cells by the mechanism of loss of mitochondrial membrane potential. In a recent study, thymoquinone exhibited strong leishmanicidal efficacy against promastigotes and amastigotes of *L. infantum* and *L. tropica*. Due to its antileishmanial activity toward other forms of leishmaniasis and species of *Leishmania* parasites, we preferred to use thymoquinone against *L. donovani*, which causes visceral leishmaniasis. In the present research work, we found and believed for the first time that thymoquinone exhibited a dose-dependent inhibition of growth in *L. donovani* promastigotes and amastigotes. Thymoquinone induced an intense antiparasitic effect against *L. donovani* promastigotes and intramacrophagic amastigotes, and the IC50 value was achieved at 6.33 ± 1.21 and 7.83 ± 1.65 μM, respectively, whereas the IC90 value was attained at 20.71 ± 2.15 and 27.25 ± 2.20 μM against flagellated promastigotes and intracellular amastigotes, respectively. The antileishmanial effect of the test drug was found to be cytotoxic rather than cytostatics as confirmed through the promastigote growth reversibility study. The antileishmanial activity of thymoquinone and *N. sativa* oil has also been affirmed by other researchers against *L. tropicana* and *L. infamatum*, and the IC50 was reported to be comparable with our findings. The leishmanicidal activities of plant metabolites such as curcumin, racemose A, artemisinin, aloe vera leaf exudates, and Piper betel leaf extract were reported, and the antileishmanial effect was mediated by apoptosis. *Leishmania* parasites undergo programmed cell death in response to natural secondary metabolites and essential oils. Apoptosis is a type of mode of programmed cell death, which involves alteration in cellular morphogenesis and homeostasis. Apoptosis is related to different clinical disorders, including playing an important role in preventing cancer. Apoptosis is characteristic of biochemical events that lead to membrane blebbing, chromatin condensation, cellular shrinkage, nuclear fragmentation, and mRNA decay. An earlier study on thymoquinone proved that it exhibited significant cytotoxic and apoptotic effects against human lung adenocarcinoma cells and the lymphocytic leukemia CEM cell line. In the present research, we illustrated that leishmanicidal activities of thymoquinone against *L. donovani* parasites are selective. Evidence of apoptosis in thymoquinone-treated promastigotes showed a typical alteration in morphological patterns of apoptosis with a decreased number of surviving parasites, cytoplasmic shrinkage, and membrane blebbing, all of which was observed using an inverted light microscope. Chromatin condensation, cellular shrinkage, membrane blebbing, and nuclear fragmentation were observed under scanning and transmission electron microscopy analyses. The antileishmanial effect of a few reported synthetic drugs and plant metabolites such as miltefosine, amphotericin B, CPT, aloe vera leaf exudates, artemisinin, racemose A, curcumin, and *Vernonia brasiliensis* essential oil was shown to be mediated by apoptosis. Along with identifying the morphological changes in drug-treated *Leishmania* parasites, we also define the mode of action of thymoquinone against promastigotes. We have shown that thymoquinone initiates cell death sharing many phenotypic characteristics observed with metazoan apoptosis, which includes phosphatidylserine externalization, DNA fragmentation, and depolarization of mitochondria. In addition to this, thymoquinone displays significant clearance of amastigote from the parasitic infected macrophages. Supposedly, we believe that this is the first report demonstrating that thymoquinone exhibits apoptotic mode of cell death in *L. donovani* promastigotes. Earlier studies reported that thymoquinone in a dose-dependent manner exerted apoptotic mode of cell death in As49 lung cancer cells. Thymoquinone-treated A549 cells underwent an increase in the fragmentation of nuclear DNA with an increase in drug concentration. In several studies, it was reported that thymoquinone showed anticancer activity by different mechanisms such as induction of apoptosis in cancerous cells by decreasing the mitochondrial membrane potential. Outer and inner membrane permeabilization of mitochondria may lead to the death of the cells by means of apoptosis and or necrosis. The fall in membrane potential of mitochondria is mainly an early alteration linked with apoptosis as the reduction of ΔΨm is followed by permeabilization of the inner mitochondrial membrane, activating the release of various apoptotic signals. Berberine chloride, artemisinin, Piper betel, racemose A, and oil from *Syzygium aromaticum* were reported to exhibit antileishmanial activity through the mechanism associated with a decrease in mitochondrial membrane potential. In our research, thymoquinone also caused a loss of mitochondrial membrane potential in *L. donovani* promastigotes.

In the present study, we demonstrated that thymoquinone levels up to 200 μM showed no adverse toxic effects on the mammalian macrophage cell lines. As per our findings, several other studies have shown that administration of *N. sativa* oil and its molecules either orally or intraperitoneally represents an insignificant level of toxicity in rodents. Accordingly, it is advised that thymoquinone is very safe for mammalian and host cells, taking into account that at very high concentrations, thymoquinone exerted significant cytotoxicity against host cells.

Thus, we conclusively showed that thymoquinone exhibited potent leishmanicidal activity against *L. donovani* promastigotes and macrophage-loaded amastigotes. The leishmanicidal impression interceded through the mechanism of programmed cell death as evidenced by means of phosphatidylserine exposure, nuclear DNA nicking, and reduction in the potential of the mitochondrial membrane without adverse effect on the murine macrophage cell line. Our study validates thymoquinone as a novel source for antileishmanial agents against visceral forms of this debilitating disease.

**METHODS**

**Materials.** Thymoquinone, pentamidine, M199 medium, RPMI 1640 medium, penicillin G sodium salt, streptomycin sulfate, and MTT from Sigma-Aldrich (St. Louis, MO). DMSO was from SRL, fetal bovine serum (FBS) procured from Himedia, methanol from Merck, and annexin V-FITC and Apo-Direct kits were from Roche Inc., Basel, Switzerland. All of the other chemicals were from Sigma-Aldrich unless otherwise stated.

**Leishmania Parasite Culture.** *L. donovani* flagellated promastigote parasites, strain MHOM/IN/AG/83, were maintained in M199 medium, which was completed with 10% heat-inactivated FBS, 2 mM glutamine, and penicillin/
The mean percentage of parasite viability was calculated by the comparative study, whereas 0.1% DMSO served as the solvent.

Dose-Dependent Antileishmanial Potential of Thymoquinone against Promastigote. The antileishmanial potential of thymoquinone on the promastigote stage of *L. donovani* was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] cell viability assay using the method described earlier. Briefly, promastigotes at a cell density of 2 × 10⁶ cells/mL (100 μL) were harvested from their exponential growth phase and incubated in a BOD incubator at 24 °C for 72 h in a 96-well tissue culture plate. Then, 100 μL of thymoquinone was added in two-fold serial dilutions, from 100 μM (100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, 0.195, and 0.1 μM). Pentamidine was taken as the reference drug (100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, 0.195 and 0.1 μM) in every experiment for the comparative study, whereas 0.1% DMSO served as the solvent control. Promastigotes without test drugs acted as the control. The mean percentage of parasite viability was calculated by the MTT assay. The inhibitory concentration of thymoquinone, which was induced for 50 and 90% in promastigote killing (IC₅₀ and IC₉₀), was evaluated through graphical extrapolation (GraphPad Prism 6). All experiments were performed in triplicate, and three independent experiments were conducted.

Time-Dependent Antileishmanial Effects against Promastigote. *L. donovani* promastigotes were treated with IC₉₀ concentration (20.71 ± 2.15 μM) of thymoquinone at different time intervals. The antileishmanial effect of the test drugs was evaluated in a time-dependent manner by a colorimetric cell viability MTT assay. Promastigotes at a cell number of 2 million/mL were incubated with IC₉₀ concentration (20.71 ± 2.15 μM) of test drugs for different time intervals (0, 12, 24, 36, 48, 60, and 72 h). At the end of each time interval, MTT was used to evaluate cell viability, and percentage viability was calculated through graphical extrapolation after plotting the mean % viability against the time interval. All experiments were performed in triplicate, and three independent experiments were conducted.

Analysis of Leishmanicidal Effect of Thymoquinone. To prove that the leishmanicidal effect of thymoquinone against *L. donovani* parasites is either cytostatics or cytocial, a reversion study was performed. Promastigotes (2 × 10⁶ cells/mL) were treated at a concentration of 20.71 ± 2.15 μM and incubated for 6 days in M199 medium at 24 °C. Pentamidine served as the standard drug (IC₉₀ concentration 6.11 ± 1.24). After proper incubation, treated and untreated promastigotes were thoroughly washed three times with sterile 1× PBS and resuspended in M199 supplemented with 10% FBS and further allowed to grow in a BOD incubator. The viable flagellated promastigotes were counted under a bright-field microscope after 96 h. All experiments were performed in triplicate, and three independent experiments were conducted.

Investigation of Parasite Morphology Treated with Thymoquinone. *L. donovani* promastigotes at a cellular population of 2 million/mL were treated at IC₉₀ concentration (20.71 ± 2.15 μM) of test drugs in a complete M199 medium. Pentamidine (6.11 ± 1.24)-treated parasites act as the positive control group while parasites without test drugs were taken as a negative control group. Following 72 h of incubation with test drugs, 10 μL of treated and untreated promastigotes were placed on a hemocytometer, and the cellular morphological structure was inspected with a 40× objective under a bright-field microscope. A minimum of 10 microscopic fields were examined for each group of samples, and a photomicrograph was captured and recorded. All experiments were performed in triplicate, and three independent experiments were conducted.

Scanning Electron Microscopy of Thymoquinone-Treated Promastigotes. Parasites were treated with thymoquinone and pentamidine at IC₉₀ dose (20.71 ± 2.15 and 6.11 ± 1.24 μM) for 72 h. Post-72 h of incubation, parasites were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde at 37 °C for 3 h and then finally incubated overnight at 4 °C. Thereafter, moisture was removed from the treated and untreated parasites by increasing the gradient of the ethanol washing solution, and imaging was performed utilizing a scanning electron microscope (ZEISS EVO LS 10). All experiments were performed in triplicate, and three independent experiments were conducted.

Ultrastructural Study of Leishmania parasite Using Transmission Electron Microscopy. Promastigotes in the exponential growth phase were treated with IC₉₀ concentration (20.71 ± 2.15 and 6.11 ± 1.24 μM) of thymoquinone and pentamidine for 72 h at 24 °C. Then, the treated and untreated parasites were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde with a fixing buffer (pH 7.2). Thereafter, the specimens were processed for conventional transmission electron microscopy (TEM), and using a transmission electron microscope (Tecnai G2 20 S-Twin), ultrastructural images were taken at SAIF, AIIMS, New Delhi. All experiments were performed in triplicate, and three independent experiments were conducted.

Adverse Toxicity Assay on Mammalian Macrophages. To determine the unfavorable toxicity effects of thymoquinone on the mammalian macrophage, RAW264.7 cells were cultured in 96-well flat-bottom cell culture plates in a complete medium (RPMI 1640). The cell line at a cell number of 2 × 10⁵ cells per well was seeded, and the cells were incubated with increasing concentrations (25, 50, 100, 150, and 200 μM) of thymoquinone and pentamidine for 48 h at 37 °C in a CO₂ incubator. The murine macrophage cell line in medium RPMI 1640 alone serves as a control. Post-48 h of incubation, each well was supplemented with MTT and incubated for 5 h, and the purple color formazan crystals formed were dissolved in DMSO. Finally, the reading of the culture plate was conducted on an ELISA plate reader machine at 570 nm wavelength. The % cell viability was determined as follows:

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\text{% of macrophages viability} = \frac{\text{mean OD of treated sample}}{\text{mean OD of untreated sample}} \times 100
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All experiments were performed in triplicate, and three independent experiments were conducted.

Macrophage-Loaded Amastigote Reduction Assay. To assess the efficacy of thymoquinone on intracellular amastigote forms of *L. donovani* parasites, RAW264.7 macrophage cells at a density of 1 × 10⁶ cells/mL (volume, 1 mL) were subcultured every 4–5 days in the fresh M199 medium at the mean density of the initial inocula being 1 × 10⁶ cells/mL.

Cell Culture. RAW264.7 macrophage cell line was grown at 37 °C in complete RPMI 1640 medium (FBS 10%) in an incubator with 5% CO₂ for 48–72 h. When the cells reached 90% confluence, the medium was replaced with a fresh RPMI 1640 medium and subcultured at a cell density of 2 × 10⁶ cells/mL.

To prove that the leishmanicidal effect of thymoquinone against *L. donovani* parasites is either cytostatics or cytocolidal, a reversion study was performed. Promastigotes (2 × 10⁶ cells/mL) were treated at a concentration of 20.71 ± 2.15 μM and incubated for 6 days in M199 medium at 24 °C. Pentamidine served as the standard drug (IC₉₀ concentration 6.11 ± 1.24). After proper incubation, treated and untreated promastigotes were thoroughly washed three times with sterile 1× PBS and resuspended in M199 supplemented with 10% FBS and further allowed to grow in a BOD incubator. The viable flagellated promastigotes were counted under a bright-field microscope after 96 h. All experiments were performed in triplicate, and three independent experiments were conducted.
Detection of Loss of Membrane Potential of Mitochondria ($\Delta \Psi_m$). Alteration in $\Delta \Psi_m$ was revealed using lipophilic cationic JC-1 dye. The dye releases fluorescence in a different manner inside apoptotic and live cells. JC-1 dye forms J-aggregates inside mitochondria and emits red fluorescence in living cells, whereas in apoptotic cells, the dye exists as a monomer in the cytoplasm and fluoresces green. Accordingly, the ratio of red and green fluorescence is an index of relative mitochondrial membrane potential.44 In determining the $\Delta \Psi_m$ of exponentially growing parasites treated with an IC_{90} concentration (20.71 ± 2.15 μM) of thymoquinone for 72 h at 24 °C, pentamidine (6.11 ± 1.24 μM) served as the reference drug. After treatment, promastigotes were harvested, pelleted, washed with 1× PBS, then finally stained with the dye (JC-1; 10 mM), and incubated at 37 °C for 15 min. The samples were then washed with 1× PBS, acquired on a BD FACS Aria flow cytometer, and analyzed with DEVA software. All experiments were performed in triplicate, and three independent experiments were conducted.

Statistical Analysis. All in vitro antileishmanial experiments were performed three times in triplicate, the results described are from one of the three separate experiments, and the values are reported as means ± SEM. Statistical analysis was done utilizing GraphPad Prism 6.0 software, and groups were compared using one-way ANOVA, followed by Tukey’s test. We considered $p$ values <0.05 to be statistically significant.

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Experiments conceived and designed: M.I. Experiments performed: M.I., Ab.A., O.A., I.A., and Am.A. Manuscript written: M.I. Data acquisition result interpretation and analysis: M.I., Ab.A., O.A., I.A., and Am.A. Reviewing and editing of the manuscript: K.K., A.S.A.A., M.A.A. and S.P. All authors have given approval to the final version of the manuscript.

Funding
M.I. is thankful to the University Grants Commission (UGC), Government of India, for providing financial assistance (F.4-2/2006 (BSR)/BL/18-19/0117) and the Japanese Society for the Promotion of Science (JSPS), Tokyo, Japan, for providing fellowship during the experimental design of the study (JSPS/OF322, ID No. P19108).

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
Ab.A. is thankful to Taif University Researchers Supporting Project Number (TURSP-2020/124), Taif University, Taif, Saudi Arabia. The authors are thankful to the Central Instrumental Facility, Jamia Millia Islamia, New Delhi (India), for providing flow cytometry facilities (BD FACSARIA).

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