Simultaneous Application of Thymoquinone and Hydroxychloroquine Suppresses Autophagy and Disrupts the Autophagosomal Trench Engulfed *Leishmania major*

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ABSTRACT

Background: Leishmaniasis is a vector-borne disease prevalent in 98 countries worldwide. The current treatment has shortcomings, including drug resistance and adverse effects, highlighting the need for novel medications and treatment strategies. This study aimed to investigate the anti-leishmanial effect of TQ during the regulation of autophagy in the macrophage cell line (RAW 264.7).

Methods: After culturing the macrophage cell line, an MTT assay was performed to assess the cytotoxicity effects of the agents at different concentrations of TQ, HCQ, MET, and GLU. The study groups included PBS, GLU, TQ, TQ + MET, GLU + MET, TQ + HCQ, GLU + HCQ, HCQ, and MET. The cells were then infected with *L. major* and treated with TQ, while autophagy was regulated using HCQ and MET. Subsequently, the infection index, the number of amastigote loads, and the fold change in the expression of specific autophagy-related genes (*LC3*, *P62*, and *Beclin*) in the treatment groups were evaluated.

Results: There was a significant decrease in the percentage of the infected macrophages treated with TQ and also the autophagy inhibitor HCQ compared to the control group. Macrophages treated with HCQ + TQ showed a significant reduction in the infection index and amastigote load compared to the TQ-treated group. Additionally, using HCQ as an autophagy inhibitor, along with TQ or GLU, enhanced the clearance of parasites and reduced the infection index of macrophages.

Conclusion: Downregulating autophagy could be a promising approach for Leishmania therapy, by which the leishmanicidal effect of TQ and GLU will be enhanced. ***DOI: 10.61186/ibj.4481***

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INTRODUCTION

The intracellular parasitic protozoan Leishmania is the causative agent of leishmaniasis, a vector-born zoonotic disease endemic to 98 countries worldwide[1]. The life cycle of Leishmania consists of two dominant stages: the amastigote, which resides in the vertebrate host, and the promastigote, which exists in the sand fly, the vector of the disease. The extracellular, flagellated promastigotes are motile and replicate within the midgut of the sand fly. Following multiplication and antigenic differentiation, the metacyclic promastigotes are transmitted to a specific mammalian host during a blood meal[2]. Inside the host,

List of Abbreviations:
DMEM: Dulbecco's Modified Eagle Medium; **GLU:** glucantime; **HCQ:** hydroxychloroquine; ***L. amazonensis*:** *Leishmania amazonensis*; ***L. major*:** *Leishmania major*; **MET:** metformin; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ***N. sativa*:** *Nigella sativa*; **PBS:** phosphate-buffered saline; **TQ:** thymoquinone

they invade macrophages and trigger the engulfment of them into the parasitophorous vacuoles. Inside these vacuoles, the parasites transform into the non-motile intracellular form known as the amastigote. This form of the parasite evades the host's immune system, multiplies, and ultimately causes the infected macrophage to burst, allowing the parasites to invade new macrophages. This process leads to the clinical manifestations of leishmaniasis and facilitates transmission to a new vector during the sand fly's biting and feeding[3].

Based on the species of Leishmania and the host's immune response, the clinical manifestations of leishmaniasis can present as visceral, cutaneous, and mucocutaneous leishmaniasis, the three main clinical forms of the disease[4,5]. Current treatments for leishmaniasis are primarily limited to pentavalent antimonial compounds as the first-line pharmacological option[6], along with paromomycin[7], amphotericin B[8], and miltefosine[9]. However, drug resistance and the adverse effects associated with these treatments have significant drawbacks, promoting researchers to develop new medications with fewer side effects[10-13].

TQ, the main bioactive constituent of black seed (*N. sativa*), has shown anti-leishmanial activity while exhibiting no toxicity to mammalian cells[14]. It has been demonstrated that autophagy plays a crucial role in the development of Leishmania within the host macrophage[15,16]. Moreover, 3-methyladenine, an autophagy inhibitor, decreased the infection index of *L. amazonensis* in macrophages, whereas autophagy inducers, such as rapamycin or fasting, exhibited no significant effects[15]. Macrophages infected with *L. amazonensis* or *L. major* showed a rise in the autophagic marker (LC3-II/Act) ratio after 24 h, although autophagy inhibitors had no noticeable impact on the infection ratio or parasite load[17]. In host macrophages, the downregulation of essential autophagy proteins (Atg5 or Atg9A) significantly declined the survival of Leishmania, suggesting that host autophagy is directly linked to Leishmania pathogenesis[18]. In this context, there are different reports on the effect of autophagy on leishmaniasis, and some evidence supports the leishmanicidal effect of TQ[14-18]. In this study, we investigated the effect of autophagy regulation during TQ treatment on the macrophage cell line RAW 264.7 infected with *L. major*.

MATERIALS AND METHODS

Preparation of drugs and reagents

TQ (Cayman, USA) was initially dissolved in DMSO (Merck, Germany), ensuring that the final concentration of DMSO did not exceed 2%. Different concentrations of TQ were then prepared by diluting in PBS or RPMI 1640 medium (Gibco). HCQ (Zahravi Pharmaceutical Company, Iran) was dissolved in PBS (pH 7.2) and subsequently diluted in RPMI 1640 medium (Gibco). MET (Merck) was prepared in deionized sterile water and used immediately. GLU from Sanofi Aventis (France) was provided as an injectable solution at a concentration of 1.5 g/5 ml; each 1.5 g of meglumine antimoniate corresponded to 0.405 g of antimony, which was diluted in sterile PBS or RPMI 1640 (Gibco). The dilution was performed based on the concentration of antimony.

Culture of promastigotes

Promastigotes of *L. major* were cultivated in RPMI 1640 (Gibco) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco), 10 mM of HEPES (Roth, Germany) buffer, and 5 mg/dl of gentamycin (Sigma-Aldrich, USA) at 24 °C. On days 4 to 5, during the stationary phase, the promastigotes were harvested, and the metacyclic promastigotes were isolated using the density gradient centrifugation method, as described previously[19]. The harvested promastigotes were washed with DMEM (Gibco) and centrifuged at 1,900 ×g. The pellet was then suspended in DMEM at a density of 2×10^8 cells/ml. Subsequently, 2 ml of this suspension was carefully transferred to a 15 ml Falcon tube containing two distinct phases: 2 ml of 40% Ficoll (Sigma-Aldrich) in sterile deionized water in the bottom and 2 ml of 10% Ficoll in M199 medium (Biowest, France) overlaid on top. The mixture was then centrifuged at 1300 ×g at room temperature for 10 min. The metacyclic promastigotes were collected from the interface, which is between the upper cell suspension layer and the 10% Ficoll layer[19]. Finally, the collected metacyclic promastigotes were washed with RPMI 1640 and used for subsequent tests.

Culture of macrophage cell line RAW 264.7

The macrophage cell line RAW 264.7 (Pasteur Institute of Iran, Tehran) was cultured in RPMI 1640 medium supplemented with 5 mg/dl of gentamycin and 10% (v/v) heat-inactivated fetal calf serum (Gibco) incubated in 95% humidity and 5% CO_2 at 37 °C[20].

MTT assay

Using the MTT (Sigma-Aldrich) assay, we assessed the effect of TQ, HCQ, MET, and GLU on the viability of RAW 264.7 cells. The cells were seeded in 96-well plates (SPL, Korea) at a density of 4×10^3 cells/well and incubated in 95% humidity and 5% CO_2 at 37 °C for 24 h. The MTT assay for each concentration of drugs and control was conducted in five replicates. The cells were exposed to different concentrations of TQ (2, 20, and 100 µM), HCQ (15 and 25 µM), MET (5 and 30 µM),

and GLU (20 µg/ml). These concentrations were selected based on previous reports of the noncytotoxic and therapeutic ranges of TQ[14, 21], HCQ[22,23], MET[24,25], and GLU[26] to ensure that the applied agents did not exert any cytotoxic effects on RAW 264.7 macrophages. After 24 h incubation, 20 µl of the MTT (0.5 mg/ml in PBS) solution was added to each well and incubated for another 3 h. Afterward, the formazan crystals were dissolved in DMSO (200 µl) and the absorbance was measured at 570 nm wavelength using a microplate reader (Hyperion, Germany).

Infection of RAW 264.7 cells with *L. major* and treatment of the infected cells

After seeding RAW 264.7 cells in 6 cm cell culture dishes (SPL) and incubating in 5% CO_2 and 95% humidity at 37 °C for 24 h, all the dishes were infected with metacyclic promastigotes at a ratio of 10:1, promastigotes to RAW 264.7 cells. Following 12 h of incubation, the dishes were carefully washed twice with fresh RPMI 1640, and the supernatant was removed to eliminate the unpenetrated promastigotes. The cells were subsequently treated with varying concentrations of TQ (2, 20, and 100 µM), along with either HCQ (15 and 25 µM; as an inhibitor) or Met (5 and 30 µM; as an inducer of autophagy). Treatment with GLU (20 µg/ml), the standard drug of leishmaniasis, was also conducted as a positive control group in the study. All dishes were incubated in 5% CO_2 and 95% humidity at 37 °C. After 48 h, Wright-Giemsa staining and microscopic observation were performed to assess the infection load in macrophages, RNA extraction, and measurement of autophagy-specific protein expression through real-time PCR.

Wright-Giemsa staining and microscopic examination

After 48 h treatment of the infected cells, smears of each treatment group were prepared and fixed with Methanol (Merck). Wright-Giemsa staining (Pakgene Yakhteh, Iran) was performed, and the stained smears were examined using a 100× objective microscope lens to estimate the percentage of the infected macrophages and the load of amastigotes within the infected macrophage. To accurately evaluate the infection rate, 100 macrophages were analyzed in each group, and the infection percentage was determined using the following formula: infection percentage = [(number of the infected macrophages × 100)/total number of macrophages]. For each group, the average number of amastigotes in the infected macrophages was assessed, and the infection index was calculated by the following formula: infection index = percentage of the infected macrophages × average number of the amastigotes per macrophage in the group.

RNA extraction and real-time PCR

After 48 h of treatment, the infected RAW 264.7 cells were harvested and centrifuged at 2000 ×g at 4 °C for 5 min. The pellet was then dissolved in RiboEx reagent (GeneAll, Korea), and RNA extraction was carried out according to the instructions recommended by the manufacturer. All the extracted RNAs were stored at -70 °C until examination. The Takara PrimeScript RT reagent Kit (Japan) was used to prepare complementary DNA. Real-time PCR was also performed using specific primers for the *LC3II*, *P62*, and *Beclin* genes, following the thermal program set as follows: denaturation at 95 °C for 10 s, annealing at optimized annealing temperature for 30 s for each pair primer (Table 1), and extension at 72 °C for 20 s. The PCR reaction included specific primers for each gene (0.3 µM of each), 20 ng of complementary DNA samples, and SYBR green DNA PCR Master Mix 1× (Ampliqon, Denmark). The calculation of gene fold change was conducted using the $2^{-\Delta\Delta CT}$ method normalized to β-actin as a reference gene.

Statistical analysis

The obtained data were displayed as mean ± SD and percentages. A one-way ANOVA test was used to compare quantitative variables among the tested groups, and Tukey's post hoc test was employed when there were significant differences. The distribution of data was assessed using the Kolmogorov-Smirnov test. All statistical analyses were conducted using SPSS version 24 software, and a *p* value of ≤0.05 was considered statistically significant.

Table 1. List of specific primers and their annealing temperature for each gene

Gene	Forward	Reverse	Annealing temperature (°C)
LC3	TCCGACCGGCCTTTCAAGCA	TCACCCTTGTAGCGCTCGAT	58.2
P62	GCTGCCCTATACCCACATCT	CGCCTTCATCCGAGAAAC	56.3
Beclin	CCAGGTGCGCTACGCCCAGA	TCCACTCCACAGGAACACTG	58.2
B-actin	TCCCTGGAGAAGAGCTATG	GTAGTTTCATGGATGCCACA	56.5

RESULTS

MTT assay results

The MTT assay showed no statistically significant difference in the viability rates among the groups treated with TQ (2, 20, and 100 µM), HCQ (15 and 25µM), MET (5 and 30 µM), and GLU (20 µg/ml) compared to the negative control (PBS) group, indicating the nontoxic effect of these agents at the mentioned concentrations on the macrophages. Hence, the selected concentrations for further tests were TQ (20 µM), HCQ (15 µM), MET (5 µM), and GLU (20 µg/ml), as shown in Figure 1.

Percentage of the infected macrophages

There was a significant decrease in the percentage of the infected macrophages treated with TQ (20 µM) and the HCQ (15 µM, autophagy inhibitor) compared to the control group ($p < 0.003$ for both comparisons). Additionally, macrophages treated with HCQ (15 µM) + TQ (20 µM) exhibited a significant reduction in infection rate ($p < 0.001$) compared to the untreated control group. This result indicates that TQ treatment along with autophagy inhibitor, prevents macrophages from being infected by Leishmania parasite. Furthermore, we found a significant decrease in the percentage of the infected macrophages in the groups treated with HCQ, TQ, HCQ + TQ, as well as HCQ + GLU and GLU as the positive control group. However, when comparing the protective effects of HCQ + TQ and TQ alone, the HCQ + TQ group demonstrated a significantly lower percentage of infected macrophages than the TQ group ($p < 0.001$; Fig. 2). A comparable outcome was observed when treating the cells with GLU (20 µg/ml) + HCQ (15 µM), compared to GLU (20

µg/ml) group, the standard treatment for leishmaniasis. This comparison revealed a decreasing effect of HCQ on the percentage of macrophage infection ($p < 0.015$). Also, there was a significant increase in the percentage of the infected macrophages in the group treated with TQ (20 µM) + MET (5 µM) compared to the group treated with TQ (20 µM; $p < 0.001$). There was no significant difference in the infection percentage between the TQ and GLU groups, in which GLU is the conventional drug for leishmaniasis, and also between the TQ + HCQ and GLU + HCQ groups ($p > 0.05$ for both comparisons). These results showed that both GLU and TQ similarly reduced the percentage of macrophage infection.

Amastigote load in macrophages

A significant difference was observed in the mean number of amastigotes in macrophages among the groups treated with HCQ ($p < 0.004$), TQ, HCQ + TQ, HCQ + GLU, and GLU as the positive control group compared to the untreated control group ($p < 0.001$). Based on the results, these compounds decreased the number of intramacrophage amastigotes. Additionally, comparing the average number of amastigotes in macrophages between the groups treated with TQ + HCQ and TQ alone revealed that concurrent application of HCQ and TQ enhanced the protective effect of TQ, leading to a significant reduction in the load of amastigotes in macrophages ($p < 0.006$). A similar effect was also observed when comparing the HCQ + GLU group with the GLU group alone ($p < 0.002$), suggesting that simultaneous application of HCQ with either TQ or GLU accelerated the clearance of Leishmania amastigotes in macrophages. The findings revealed that the inhibition of autophagy using HCQ,
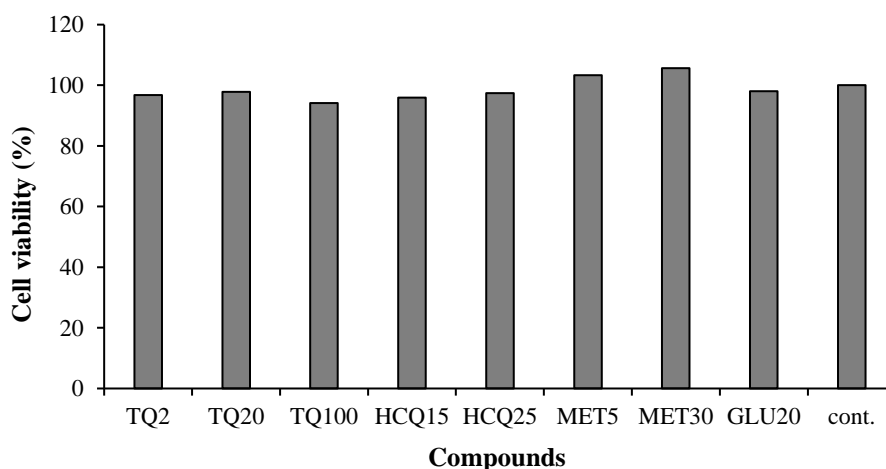
Fig. 1. Cell viability percentage. The MTT result revealed the nontoxic effect of TQ (2, 20, and 100 µM), HCQ (15 and 25 µM), MET (5 and 30 µM), and GLU (20 µg/ml) compared to the negative control (PBS) on RAW 264.7 cell line. There was no significant difference in the mean viability percentage among the compounds relative to the control group (PBS; $p > 0.05$).
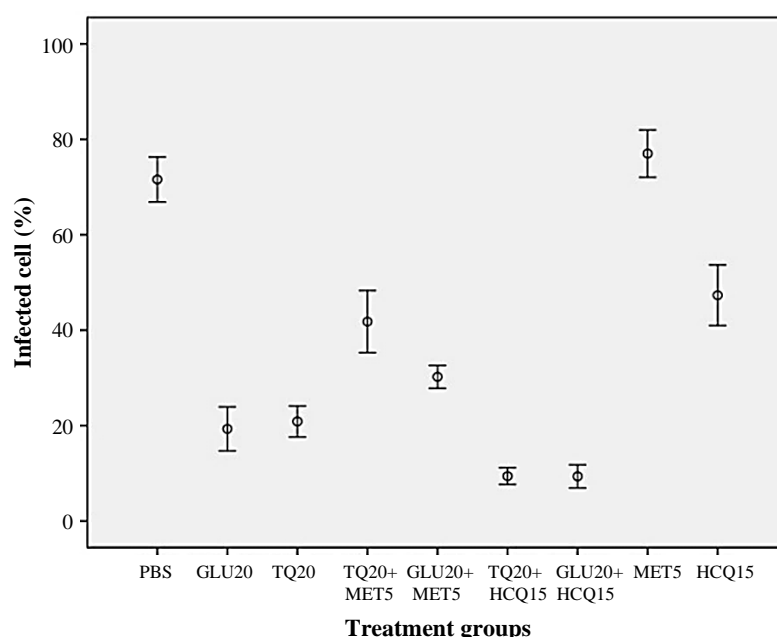
Fig. 2. Percentage of the infected cells in the treatment groups: PBS-treated group (negative control), GLU (20 µg/ml)-treated group, TQ (20 µM)-treated group, TQ (20 µM) + MET (5 µM)-treated group, GLU (20 µg/ml) + MET (5 µM)-treated group, TQ (20 µM) + HCQ (15 µM)-treated group, GLU (20 µg/ml) + HCQ (15 µM)-treated group, MET (5 µM)-treated group, and HCQ (15 µM)-treated group. There was a significant decrease in the percentage of infected RAW 264.7 macrophages in the groups treated with HCQ, TQ ($p < 0.003$), HCQ + TQ, as well as HCQ + GLU and GLU as the positive control group compared to the untreated control ($p < 0.001$). A significant decrease was also observed in the percentage of infected macrophages in HCQ + TQ group compared to the TQ ($p < 0.001$), as well as GLU (20 µg/ml) + HCQ (15 µM) group compared to the GLU (20 µg/ml) group ($p < 0.015$), indicating the role of HCQ in decreasing the percentage of macrophage infection. There was a significant increase in the percentage of the infected macrophages in the group treated with TQ (20 µM) + (MET 5 µM) relative to the group treated with TQ (20 µM), with a p value of 0.001.

together with TQ or GLU, decreased the infection load in macrophages and reduced the percentage of the infected macrophages. Conversely, MET, an inducer of autophagy, enhanced the infection of macrophages and increased the number of amastigotes in the treated cells ($p < 0.001$; Fig. 3).

Infection index

The infection index in all groups treated with TQ, HCQ + TQ, HCQ + GLU, and GLU decreased compared to the control group ($p < 0.001$). Remarkably, the reduction in the infection index was most pronounced in the group treated with HCQ + TQ, relative to the TQ group, and in the HCQ + GLU relative to the GLU group ($p < 0.016$ and $p < 0.05$, respectively). This outcome indicates that HCQ, as an autophagy inhibitor, reduces the infection index of RAW 264.7 macrophages and enhances the leishmanicidal effects of TQ and GLU (Fig. 4). There was no significant difference in the infection index between the TQ group and the GLU group, as well as between TQ + HCQ group and the GLU + HCQ group, denoting that these compounds have a similar effect on the infection index of macrophages ($p > 0.05$).

Real-time PCR

The real-time PCR results for *LC3II*, *P62*, and *Beclin* genes, specific markers of autophagy, demonstrated the suppression of autophagy in the TQ + HCQ, TQ, and GLU + HCQ-treated groups ($p < 0.001$; Fig. 5).

DISCUSSION

This study presents a novel approach that concurrently employs antileishmanial agents alongside autophagy inhibitors to enhance the efficacy of leishmaniasis treatment. Previous studies have illustrated the potential benefits of autophagy in combating pathogenic microorganisms, as well as the escape strategies employed by certain microorganisms, including *Streptococcus pyogenes*[27], *Salmonella typhimurium* enterica serovar[28], *Listeria monocytogenes*[29,30], and *Shigella flexneri*[31,32]. Studies have also explored the role of autophagy as an accelerator for the engulfment of Leishmania parasites within parasitophorous vacuoles, effectively acting as a "Trojan Horse"[33]. Autophagy plays a strategic role in supplying cells with essential nutritional resources. Leishmania parasites
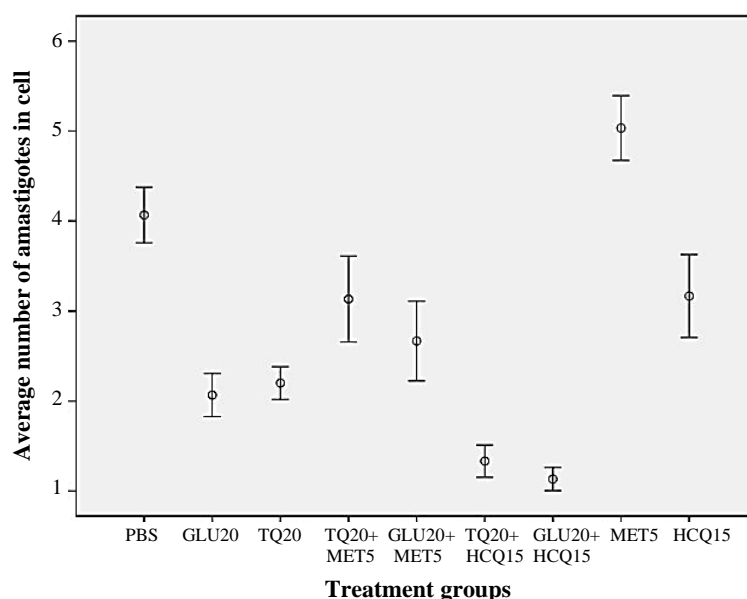
Fig. 3. Average number of amastigotes in macrophage cells in the treatment groups: PBS-treated group (negative control), GLU (20 µg/ml)-treated group, TQ (20 µM)-treated group, TQ (20 µM) + MET (5 µM)-treated group, GLU (20 µg/ml) + MET (5 µM)-treated group, TQ (20 µM) + HCQ (15 µM)-treated group, GLU (20 µg/ml) + HCQ (15 µM)-treated group, MET (5 µM)-treated group, and HCQ (15 µM)-treated group. There was a significant difference in the mean number of amastigotes in macrophages among the groups treated with HCQ ($p < 0.004$), TQ, HCQ + TQ, HCQ + GLU, and GLU as the positive control group ($p < 0.001$) in comparison to the control group, indicating that these compounds decreased the number of intramacrophage amastigotes. Comparing the group treated with TQ + HCQ and the group treated with TQ exhibited that concurrent application of HCQ with TQ, improves the protective effect of TQ, further reducing the load of amastigotes in macrophages ($p < 0.006$). A comparison between the HCQ + GLU group and the GLU-treated group revealed a similar enhancing effect for HCQ ($p < 0.002$). MET significantly increased the number of amastigotes in the treated cells ($p < 0.001$).
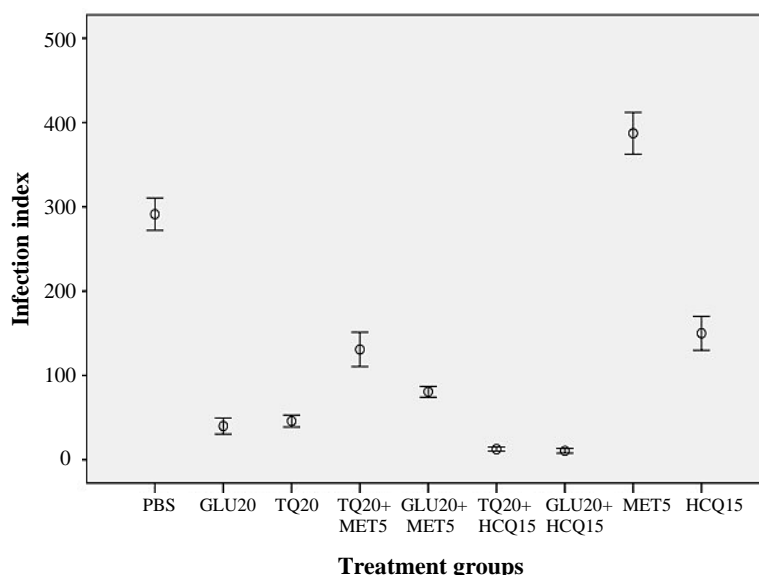
Fig. 4. Infection index in the treatment groups: PBS-treated group (negative control), GLU (20 µg/ml)-treated group, TQ (20 µM)-treated group, TQ (20 µM) + MET (5 µM)-treated group, GLU (20 µg/ml) + MET (5 µM)-treated group, TQ (20 µM) + HCQ (15 µM)-treated group, GLU (20 µg) + HCQ (15 µM)-treated group, MET (5 µM)-treated group, and HCQ (15 µM)-treated group. The infection index in all groups treated with TQ, HCQ + TQ, HCQ + GLU, and GLU significantly decreased compared to the control group ($p < 0.001$). Decreasing the infection index was significant in the group treated with HCQ + TQ compared to the TQ group, or HCQ + GLU compared to the GLU group ($p < 0.016$ and $p < 0.05$, respectively), indicating that HCQ as an inhibitor of autophagy reduces the infection index of RAW 264.7 macrophages and enhances the leishmanicidal effect of TQ and GLU.
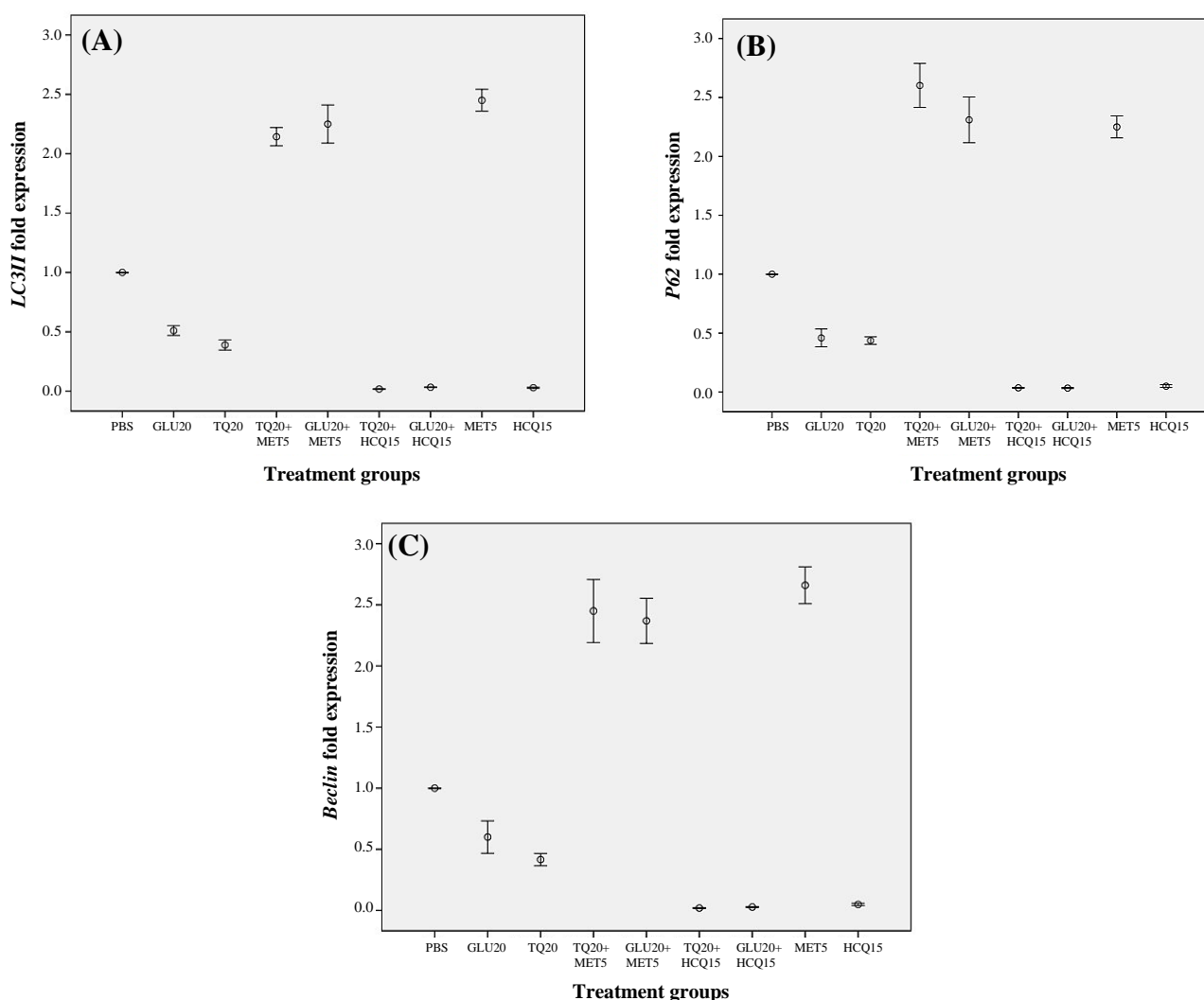
Fig. 5. The ratio of fold change expression of (A) *LC3II*, (B) *P62*, and (C) *Beclin* genes to *β-actin* in the treatment groups: PBS-treated group (negative control), GLU (20 µg/ml)-treated group, TQ (20 µM)-treated group, TQ (20 µM) + MET (5 µM)-treated group, GLU (20 µg/ml) + MET (5 µM)-treated group, TQ (20 µM) + HCQ (15 µM)-treated group, GLU (20 µg/ml) + HCQ (15 µM)-treated group, MET (5 µM)-treated group, and HCQ (15 µM)-treated group. In the treatment groups, the fold change expression ratio of *LC3II*, *P62*, *and Beclin* genes to *β-actin* decreased significantly, indicating that autophagy is suppressed in TQ + HCQ-, TQ-, and GLU + HCQ-treated groups ($p < 0.001$).

exploit a similar strategy to acquire nutritional elements from host cells. The parasitophorous vacuoles of *Leishmania mexicana* utilize two different mechanisms to obtain small anionic molecules or larger molecules from cytosolic materials within parasitophorous vacuoles[34].

Based on our results, MET, an inducer of autophagy, enhanced the percentage of the infected macrophages, increased the infection index of macrophages, and elevated the number of amastigotes in the treated cells compared to the control untreated group. Additionally, these parameters were amplified in the group treated with MET + TQ compared to the one treated with TQ alone. This observation signifies the enhancing effect of

MET in establishing the parasite within macrophages and providing an appropriate environment for macrophage infection, a finding that aligns with the results of Pinheiro et al.[16]. They found that inducing autophagy through starvation increased the parasite load of *L. amazonensis* in BALB/c macrophages. Moreover, starvation resulted in a rise in the number of amastigotes per macrophage, a higher proportion of the infected macrophage cells, and greater production of viable promastigotes. Of note, C57BL/6 mice, compared to BALB/c mice, were less susceptible to *L. amazonensis* infection. Thus, autophagy stimulation increased the parasite load in BALB/c mice, A/J macrophages, and the J774 cell line, whereas it did not show the same effects

on C57BL/6 macrophages, implying that host cell factors have a critical role in controlling the infection. Other autophagy inducers, such as rapamycin and glucagon, also increased the parasite load in BALB/c macrophages and yielded similar results[16]. These results, along with our findings, contradict the outcomes of the study conducted by Dias and colleagues[17]. They observed no significant changes in either the infection rate or the burden of parasites after treating CBA mouse macrophages infected with *L. major* and *L. amazonensis*. The treatment involved the use of rapamycin or physiological starvation as inducers of autophagy, as well as the inhibition of the autophagy using wortmannin, chloroquine, or VPS34-IN1 as autophagy inhibitors, compared to the untreated control groups. It is important to note that CBA mouse macrophages were resistant to *L. major* but susceptible to *L. amazonensis*. Dias et al. also observed that inducers of autophagy increased the viability of both *L. amazonensis* and *L. major* amastigotes. In contrast, pharmacological inhibitors of autophagy did not affect the viability of both species of Leishmania amastigotes[17]. Interestingly, several studies have revealed that Leishmania acts as an autophagy inducer in various hosts; however, the mechanisms by which autophagy enhances Leishmania survival within cells have remained contradictory[2]. For instance, the pattern recognition receptor, i.e. toll-like receptor, is believed to be responsible for inducing autophagy in *L. major*-infected macrophages. Additionally, the role of *ATG5* in preventing macrophage infection has been reported, as evidenced by the increased *L. major* parasite burden in *Atg5* knockdown BALB/c and C57BL/6 macrophages[35].

Our findings were in agreement with the results of the study conducted by Pinheiro et al. who demonstrated the inhibition of autophagy using 3-MA or wortmannin led to a decrease in the intracellular burden of *L. amazonensis*[16]. Treatment of RAW 264.7 macrophages with TQ significantly reduced the infection rate of *L. major* parasites compared to the untreated control macrophages. This protective effect was even more significant in the group treated with TQ + HCQ, as compared to the TQ alone. Similar results were obtained in the GLU group when compared to the untreated cells, as well as in the GLU + HCQ treated group relative to the GLU group. This outcome indicated that the GLU + HCQ treatment results in a lower infection rate in macrophages, a reduced load of amastigotes within the infected macrophages, and a decreased infection index compared to both the GLU-treated and untreated control groups. There was no significant difference in the infection index, percentage of the infected macrophages, and the burden of amastigotes between the GLU-treated and TQ-treated groups, indicating that TQ and GLU exhibit similar antileishmanial effects. The leishmanicidal properties of *N. sativa* and TQ against promastigotes and amastigotes of *Leishmania donovani* confirmed its leishmanicidal activity[14], as well as the significant antileishmanial effects of *N. sativa* on *L. major*[21]. Moreover, the encapsulated form of *N. sativa* in poly-ε caprolactone nanoparticles has also been reported to exhibit antileishmanial effects against *L. infantum*[36].

The current study demonstrated a leishmanicidal effect of TQ, which is consistent with previous research[14,21,36]. Our study also presents evidence that the simultaneous use of HCQ, as an autophagy inhibitor, with TQ or GLU, enhances the clearance of parasites and reduces the infection index in the macrophages. We observed that the induction of autophagy and increased levels of *LC3II* are associated with a higher infection index of RAW 264.7 cell line and a greater burden of *L. major* amastigotes in macrophages. This finding of our study aligns with a previous study that demonstrated the inhibition of autophagy with 3MA in RAW 264.7 cells eliminates both the infection index and parasite burden in macrophages[15]. Furthermore, inducing autophagy in RAW 264.7 cells through starvation or rapamycin did not affect the infection index compared to the non-starved cells[15], which contradicts our findings. Previous research has revealed that simultaneous treatment of human glioblastoma cells with TQ + chloroquine, or chloroquine alone as an autophagy flux inhibitor, reduces the clonogenicity of glioblastoma cells compared to normal astrocytes, indicating a new strategy for cancer treatment[37]. There are conflicting reports on the effect of TQ on autophagy, suggesting that this compound acts as an inhibitor[37,38] or inducer[39,40]. Our findings, however, revealed an inhibitory effect of TQ on autophagy.

CONCLUSION

The simultaneous use of TQ and HCQ enhanced the leishmanicidal effect of TQ due to the inhibitory activity of HCQ on autophagy. A similar effect was observed when using GLU in conjunction with HCQ, indicating the therapeutic potential of autophagy inhibition in treating leishmaniasis. These data suggest that greater attention should be given to HCQ-mediated autophagy inhibition alongside conventional or newly developed drugs. However, current treatments for leishmaniasis, particularly pentavalent antimony compounds, show side effects and drug resistance, which limit their effectiveness and highlight the need for alternative therapies. Based on the results, TQ a phytochemical

compound with numerous benefits, fewer side effects, and low cost, exhibits a significantly comparable antileishmanial effect to GLU, making it a promising candidate for substitution. Therefore, TQ and HCQ should further be investigated in a new approach as leishmanicidal drugs to achieve safe treatments with minimal side effects and high efficiency.

DECLARATIONS

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Ethical approval

This study was carried out with ethical principles and approved by the Research Ethics Committee of Ferdowsi University, Mashhad, Iran (ethical code: IR.UM.REC.1402.057).

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

AB: performed all experimental assays and prepared the draft; AM and GRR: supervised the study and revised the manuscript.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version does not contain supplementary material.

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