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Anti-leishmanial, immunomodulatory and anti-oxidative activity of quercetin against cutaneous leishmaniasis caused by *Leishmania major*

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ABSTRACT

Objective: To evaluate the *in vitro* and *in vivo* efficacy of quercetin and its immunomodulatory and anti-oxidative activity against *Leishmania major* (*L. major*).

Methods: *L. major* promastigotes and amastigotes were incubated with different concentrations of quercetin to estimate EC_{50}. For *in vivo* study, the base of tails of mice was infected with *L. major*. After developing ulcers in the inoculation site, mice were treated with 50 mg/kg quercetin orally for 28 consecutive days. The wound-healing potential of quercetin was evaluated by histopathological analysis of tissue sections stained by hematoxylin and eosin as well as Masson's trichrome. In addition, the levels of tumor necrosis factor-α, interleukin-6, malondialdehyde, and adiponectin, the ferric reducing ability of plasma, as well as superoxide dismutase and glutathione peroxidase activities were measured.

Results: The EC_{50} values of quercetin against *L. major* promastigotes and intracellular amastigotes were 0.27 and 0.85 μM, respectively. Histopathological analysis showed that fewer inflammatory cells, more fibroblasts, and more collagen deposition were observed in tissue sections of quercetin-treated mice. In addition, treatment with quercetin markedly increased glutathione peroxidase activity, the ferric reducing ability of plasma and adiponectin levels while decreasing malondialdehyde, interleukin-6, and tumor necrosis factor-α levels.

Conclusions: Quercetin shows anti-leishmanial activity, immunomodulatory, anti-oxidative, and anti-inflammatory effects. Therefore, it may be further explored as an effective drug in treating leishmaniasis.

KEYWORDS: *Leishmania major*; Cutaneous leishmaniosis; Wound healing; Quercetin; Immunomodulator

1. Introduction

Leishmaniosis is a serious public health problem throughout the world, mostly affecting poor populations in developing countries[1]. Cutaneous leishmaniosis (CL) is the most prevalent form of leishmaniosis, with an incidence of about 600 000 to 1 million new cases annually. Despite extensive attempts to develop effective treatments and vaccines, the incidence of the disease is not decreasing[2]. There are different clinical manifestations of CL,

Significance

Medicinal plants are a source of molecules with therapeutic potential. Quercetin is the most abundant polyphenolic flavonoid present in plants. However, there is limited available investigation on the effects of quercetin on immunological and histopathological responses in mice with cutaneous lesions caused by *Leishmania major*. This study shows that quercetin accelerated the healing of cutaneous leishmaniosis-caused skin lesions by immunomodulatory, anti-oxidative, and anti-*Leishmania* activity. However, further research needs to be conducted on combination therapy and finding therapeutic drugs that act synergistically with quercetin.

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including localized CL, diffuse CL, mucocutaneous leishmaniosis, and post-kala-azar dermal leishmaniosis[3,4]. According to the Ministry of Health and Medical Education of Iran, annually, about 30 000 cases of CL occur in Iran. However, the number of actual cases is estimated to be four to five times. Numerous kinds of research have shown an association between environmental factors and CL occurrence[5]. Pentavalent antimonials, miltefosine, amphotericin B, liposomal amphotericin B, paromomycin, and azoles are the current drugs for treating CL[6]. Several drawbacks, such as high costs, toxic side effects, low efficacy, increased drug resistance, long-duration treatment, and hospitalization, limit these drugs' application[2]. Therefore, treatment of CL is still challenging, and developing new drugs with high efficiency, low cost, easy administration, and minimum adverse side effects is urgent[2,7].

Several *in vitro* and *in vivo* studies have indicated that many plant extracts possess significant anti-leishmanial properties[5]. The anti-leishmanial activity of some plant extracts has been attributed to flavonoids[8]. Quercetin (3,3',4',5,7-pentahydroxyflavone) is the most abundant polyphenolic flavonoid in fruits and vegetables, such as onions, apples, and tea[1,2]. It exhibits anti-oxidative, anti-inflammatory, anti-hypertension, anticancer, and antimicrobial effects[6,9–13]. It has been indicated that quercetin has activity against some species of *Leishmania in vitro* and animal models[14]. Based on the different *in vitro* studies, induction of protease-independent programmed cell death, the increment of ROS generation and the collapse of mitochondrial membrane potential, inhibition of topoisomerase Ⅱ, ribonucleotide reductase, and arginase enzymes are the main mechanisms of actions of quercetin against *Leishmania* spp.[1,6,14,15].

To the best of our knowledge, there is limited available investigation on the effects of quercetin on intracellular amastigotes of *Leishmania major* (*L. major*) and clinical forms of the parasite, and there are few *in vivo* studies regarding the effects of quercetin on immunological and histopathological responses of mice with cutaneous lesions caused by *L. major*.

Therefore, the present work aimed to investigate quercetin's *in vitro* and *in vivo* efficacy against *L. major*. The wound-healing potential of quercetin was also evaluated in an animal model of CL by histopathological analysis of tissue sections stained with hematoxylin and eosin (H & E) and Masson's trichrome. TNF-alpha (TNF-α), interleukin-6 (IL-6), malondialdehyde (MDA) and adiponectin levels, the ferric reducing ability of plasma (FRAP), superoxide dismutase (SOD), and glutathione peroxidase (GPX) were all measured.

2. Materials and methods

2.1. Culture of *L. major*

L. major (MRHO/IR/75/ER) promastigotes were purchased from the Department of Parasitology of Razi Institute (Karaj, Iran) and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% streptomycin solution (50 μg/mL) and penicillin (50 U/mL) (Sigma, St. Louis, Mo., USA) at 26 ℃. Constant maintenance and re-isolation of infected BALB/c mice ensured the parasites' virulence during the experiments. Promastigotes were used after three successive *in vitro* passages.

2.2. Cell culture

Raw 264.7 (murine macrophage adherent cell line) was purchased from the Iranian Biological Resource Center. The cell line was maintained in RPMI-1640 supplemented with 10% FCS, 1% penicillin (50 U/mL), and streptomycin (50 μg/mL) solution at 37 ℃ with 5% CO_2 in a humidified atmosphere.

2.3. Cytotoxicity assay

The cytotoxicity effect of 0-50 μM quercetin (Sigma, St. Louis, Mo., USA) against raw 264.7 macrophage cell lines was evaluated by staining with trypan blue. The final concentration of trypan blue was considered 0.2% in phosphate buffer saline. The macrophages (1×10^6 cells/well) were maintained in a 200 μL culture medium in 96-well culture plates at 37 ℃ with 5% CO_2. After 24 h of incubation, cell cultures were submitted to different concentrations of quercetin (0-50 μM) (Sigma ≥95%) and incubated for an additional 24 h. Macrophage viability was verified by microscopic quantification of the number of viable macrophages among 100 macrophages[16].

The following equation was employed for the selectivity index (S.I.): S.I. = CC_{50} RAW 264.7 cells/EC_{50} amastigotes.

2.4. *In vitro* assessment of quercetin against *L. major*

The promastigotes were cultured in a 200 μL culture medium in 96-well plates (1×10^6 parasites/well) in various concentrations of quercetin (0.01-0.64 μM) for 48 h. Glucantime [Aventis, Paris, France, containing 81 mg pentavalent antimony (SbV)/mL] was used as a control. To estimate the half-maximal effective concentration (EC_{50}) of SbV, different concentration (4-64 μM) was used. 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay was used for the estimation of EC_{50} of quercetin against the *L. major* promastigotes[17]. Forty-eight hours later, the plates were centrifuged at 3 000 rpm for 15 min, and the aqueous phases were discarded. Then, 100 μL MTT (2 mg/mL) was added to the wells in the culture, and the plates were incubated at 37 ℃ for 4 h. Finally, to dissolve the formazan dye, 200 μL dimethyl sulfoxide was added. The wells with medium and promastigotes served as the negative control, while the medium with no organism was the blank control. The absorbance was measured at 570 nm. The viability

percentage was calculated by: $[(A_T–A_B)/(A_C–A_B)]×100$, where A_B is the OD of the blank well, A_C is the OD of the negative control, and A_T is the OD of the treated cells. For each condition, at least three independent experiments were conducted.

To estimate EC_{50} of quercetin against *L. major* amastigotes, raw 264.7 macrophages (Iranian Biological Resource Center, Tehran, Iran) were maintained at 37 ℃, and 5% CO_2 in RPMI 1640 medium (Gibco, UK) supplemented with 10% FCS. Promastigote suspensions (10 parasites per adherent cell) were dispensed into the wells, and infection was allowed to occur for 4 h in a humid atmosphere with 5% CO_2 at 37 ℃. After incubation, the noninternalized parasites were washed away. The cultures were then treated with various concentrations of quercetin (0.01-1.28 μM) and SbV (4-64 μM) and incubated at 37 ℃ with 5% CO_2 for 48 h. The *L. major*-infected macrophages and the uninfected ones were considered positive and negative controls, respectively. Phosphate-buffered saline (PBS) was flashed onto the adhered macrophages after the medium of the culture chambers was removed. A Giemsa staining dilution of 1∶10 was used to fix macrophages, and the infection index was determined based on the following formula: infection index = (mean number of amastigotes per 100 treated macrophages/mean number of amastigotes per 100 control macrophages)×100[18]. At least three independent experiments were conducted for each condition.

2.5. *In vivo* study

Female inbred BALB/c mice aged 5-7-week-old were obtained from the Pasteur Institute (Tehran, Iran). The mice were kept in the faculty's animal house and had free access to food and water. Infectivity of a standard strain of *L. major* (MRHO/IR/75/ER) was maintained through regular passages in susceptible BALB/c mice at the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. For each mouse, 100 μL of PBS containing $1×10^6$ amastigote was obtained from the donor BALB/c mice employed for the infection. The mice weighing (19.81±1.78) g were inoculated subcutaneously into the base of tails. Five weeks after inoculation, when ulcers appeared in the injection site, the mice were randomly divided into four groups (six mice each), and treatment started daily for 28 consecutive days. The group that had no treatment was used as a negative control group. The vehicle group received Tween 80 (1%) orally. The glucantime group was treated with 60 mg/kg drug intraperitoneally, and the quercetin group was treated orally with 50 mg/kg quercetin suspended in Tween 80 (1%) (based on the pilot study). Oral drugs were administered through a stomach tube.

After 28 days of treatment, the mice were euthanized. A sterile vacuum tube containing EDTA was used to collect blood samples to determine antioxidant enzyme activity. After centrifugation, the plasma was stored at −70 ℃ until it was used to measure MDA, FRAP, and adiponectin levels. Two replications of the *in vivo* experiments were conducted.

2.6. Measurement of the lesions' size

From the beginning of treatment, photographs were taken every four days. The images were analyzed by Digimizer 4.2.6.0 to calculate the surface area of the wound.

2.7. Evaluation of the parasite load in margins of the lesions

Two Giemsa-stained smears were prepared from the margins of lesions from each animal. The parasite load was calculated by determining the mean number of parasites per 100 macrophages. The infection intensity was estimated by scoring the infection according to the defined criteria, *i.e.*, weak (<200 parasites/100 macrophages), moderate (200-2 000 parasites/100 macrophages), and severe infection (>2 000 parasites/100 macrophages)[19].

2.8. Histological analysis

Tissue sections from the skin lesions were fixed in formalin, paraffin-embedded, and stained with Masson's trichrome as well as H & E.

2.9. Determination of SOD and GPX activity

Spectrophotometric methods were used to evaluate SOD and GPX activities using diagnostic kits produced by Randox laboratories (Randox Laboratories Ltd., Crumlin, County Antrim, UK). The level of the enzymes is expressed as units per gram of hemoglobin.

2.10. Measurement of the FRAP

The plasma total antioxidant capacity was determined using the FRAP assay[20]. The antioxidants donate electrons to the Fe^{2+} 2,4,6-tripyridyl-S-triazine-reduced form from the colorless oxidized Fe^{3+} form of iron in this assay. The blue color can be monitored at 593 nm.

2.11. Measurement of adiponectin and MDA levels

A commercially-available ELISA kit was used to measure adiponectin levels in plasma (Bosterbio, China). The level of MDA in plasma was measured using the method described by Placer *et al.*[21]. This method determined MDA by the thiobarbituric acid reaction. The MDA level in plasma was analyzed against bi-distilled water as a blank at 548 nm.

2.12. Preparation of formalin–fixed Leishmania antigens

Promastigotes of *L. major* were collected and rinsed three times in sterile PBS before being used in *in vitro* lymphocyte proliferation and secretion assays. The parasites were fixed in 1% formol saline for 1

h and then washed three times in PBS. The parasites were counted in the hemocytometer counting chamber, adjusted in a concentration of 5×10^8/mL in sterile PBS, and stored at −70 ℃ until required.

2.13. Culture of lymph node cells and measurement of cytokines

After four weeks, single-cell suspensions were obtained from inguinal lymph nodes. The cells were seeded into RPMI-1640 medium (Shellmax, China) containing 2 mM *L*-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% heat-inactivated FCS (Shellmax, China), and 0.05 mM β-mercaptoethanol at a density of 1×10^6 cells/well in 12-well plates and cultured at 37 ℃ in 5% CO_2 in medium alone or medium with *L. major* formalin-fixed antigens. The cell culture supernatants were collected after 24 h for TNF-α and 72 h for IL-6. Levels of TNF-α and IL-6 were measured by ELISA using commercial kits (Bosterbio, China).

2.14. Statistical analysis

The EC_{50} values were determined using the nonlinear regression analysis of the dose-response curve [log(inhibitor) *vs.* normalized response-variable slope] by GraphPad Prism 6 software. The data were expressed as mean±SE, and the statistical difference was analyzed by one-way analysis of variance (ANOVA) followed by the LSD test using SPSS version 16.0. *P* values less than 0.05 were considered significantly different.

2.15. Ethical statement

Animal care and all experiments were performed as recommended by the Institutional Animal Care and Use Committee of the School of Veterinary Medicine, Shiraz University. All procedures followed the standard approved protocols for animal research (Ethical approval number 26-4-1395).

3. Results

3.1. In vitro studies

The cytotoxic concentration of quercetin to reduce 50% of viable macrophages was 27.3 μM. The effects of quercetin on the viability of *L. major* promastigotes and amastigotes were evaluated. Concentration-dependent inhibition against *L. major* promastigotes and amastigotes was seen after quercetin treatment with EC_{50} values of (0.27±0.08) and (0.85±0.30) μM, respectively. The EC_{50} values of glucantime against *L. major* promastigotes and amastigotes were (16.8±1.8) and (5.30±0.08) μM, respectively. The selectivity index in relation to the intracellular form demonstrated that the antiparasitic activity of quercetin was approximately ten times lower than its toxicity to mammalian cells.

3.2. In vivo studies

3.2.1. Area of the lesions

As shown in Figure 1, on the 12th day after the onset of treatment, the lesion area significantly decreased in the quercetin-treated group compared to the untreated group ($P<0.05$). On other days, there was a reduction in the average size of the lesions in the quercetin group compared with the untreated groups with no significant difference ($P>0.05$).

3.2.2. Parasite load in skin lesions

The infection intensity was weak in the quercetin-treated group (156.00±62.65), while it was severe in the untreated (2 214.2±230.6) and vehicle (2 363.2±337.2) groups.

3.2.3. Histological results

A few fibroblasts, a large necrotic area, and many neutrophils, macrophages, and amastigotes were seen in the vehicle and untreated groups. Granulation tissue formation was also found in the depth of ulcers in the glucantime and quercetin-treated groups (Figure 2). The number of inflammatory cells (macrophages and neutrophils) was also reduced significantly in the quercetin-treated group in comparison to the vehicle and untreated groups ($P<0.05$). On the other hand, neovascularization, fibroblasts, and fibrocytes were increased significantly in the quercetin-treated group ($P<0.05$) (Table 1). Moreover, the sections stained with Masson's trichrome showed that quercetin treatment increased collagen density compared to the untreated mice (Figure 3).

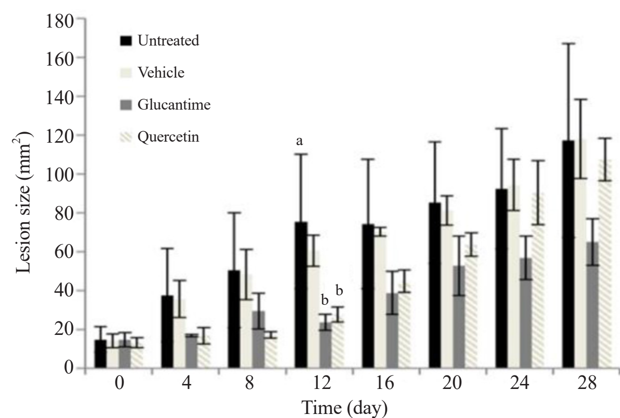

Figure 1. Changes in the lesion surface area after 28 days of quercetin treatment. The data are expressed as mean±SE and analyzed by ANOVA and LSD test. Means with different letters represent significant differences at $P<0.05$.

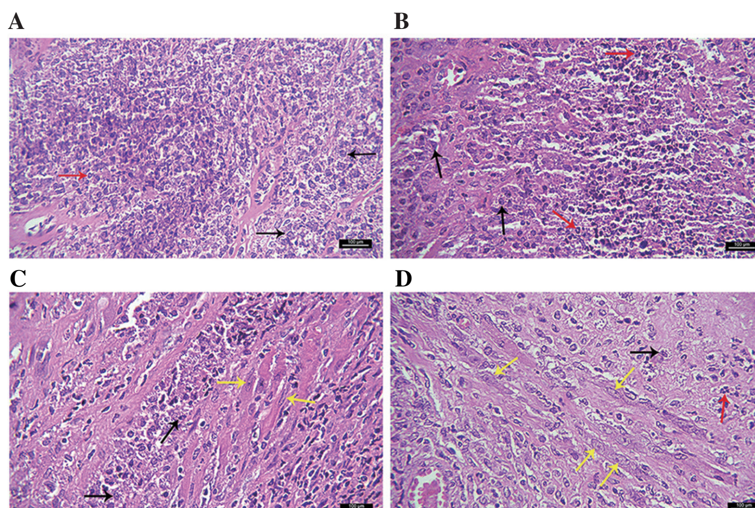

Figure 2. Histological sections of the lesions from *Leishmania major* infected mice treated with quercetin (H & E, magnification: ×400, scale bar: 100 μm). Compared with the untreated (A) and vehicle (B) groups, the glucantime (C) and quercetin (D) groups show decreased inflammatory cells (macrophages, lymphocytes, and neutrophils) and increased fibroblasts. Macrophages (black arrow), neutrophils (red arrow), and fibroblasts (yellow arrow).

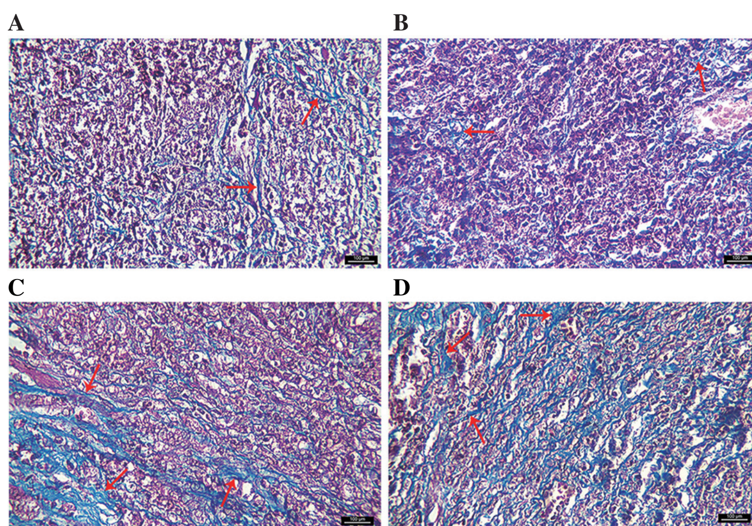

Figure 3. Masson's trichrome stained sections of *Leishmania major* infected mice treated with quercetin (magnification: ×400, scale bar: 100 μm). Compared with the untreated (A) and vehicle (B) groups, increased collagen deposition (arrow) is found in the glucantime (C) and quercetin (D) groups.

Table 1. Histopathological results after quercetin treatment in mice infected with *Leishmania major*.

Group	Macrophage	Neutrophil	Plasma cell & Lymphocyte	Fibroblast & Fibrocyte	Blood vessel
Untreated	234.22±21.80[a]	436.55±108.50[a]	6.55±2.20[a]	7.11±1.10[a]	0.77±0.40[a]
Vehicle	279.11±15.70[a]	379.88±51.90[a]	8.88±2.80[a]	6.11±0.94[a]	0.33±0.30[a]
Glucantime	85.22±10.90[b]	75.55±22.00[b]	87.44±13.60[b]	94.44±6.40[b]	2.21±0.60[b]
Quercetin	80.33±26.00[b]	51.66±8.00[b]	10.66±2.00[a]	51.33±5.67[c]	2.83±0.20[b]

The data are expressed as mean±SE and analyzed by ANOVA and LSD test. Means with different letters represent significant differences at $P<0.05$.

Table 2. Effect of quercetin treatment on biochemical parameters in mice infected with *Leishmania major*.

Group	SOD (U/mL)	GPX (U/mL)	MDA (nmol/nL)	FRAP (nmol/L)	Adiponectin (μg/mL)	TNF-α (pg/mL)	IL-6 (pg/mL)
Untreated	5.48±0.18[a]	60.49±10.30[b]	2330.0±366.5[a]	700.38±35.20[a]	18.02±3.14[a]	482.11±61.32[b]	68.32±9.18[b]
Vehicle	6.15±1.41[a]	89.72±21.82[b]	2370.0±826.5[a]	819.30±244.40[a]	15.14±2.47[a]	491.01±68.17[b]	61.17±11.30[b]
Glucantime	5.72±0.17[a]	77.91±13.60[b]	1010.1±521.4[b]	977.40±80.20[a]	17.30±3.32[a]	161.41±23.50[a]	18.64±3.20[a]
Quercetin	5.35±0.18[a]	127.83±11.60[a]	570.7±296.5[b]	1017.20±36.40[b]	31.07±1.69[b]	221.71±77.30[a]	29.66±10.61[a]

The data are expressed as mean±SE and analyzed by ANOVA and LSD test. Means with different letters represent significant differences at $P<0.05$. SOD: superoxide dismutase, GPX: glutathione peroxidase, MDA: malondialdehyde, FRAP: ferric reducing ability of plasma, TNF-α: tumor necrosis factor-alpha, IL-6: interleukin-6.

3.2.4. Effect of quercetin treatment on antioxidant enzyme activities and the levels of FRAP, adiponectin, MDA, TNF–α, and IL–6 in mice infected with L. major

As shown in Table 2, GPX activity was significantly increased in the quercetin-treated group compared to other groups ($P<0.05$), while SOD activity was not significantly different among the four groups ($P>0.05$). In addition, quercetin markedly lowered MDA concentration while increasing the levels of FRAP and adiponectin compared to other groups ($P<0.05$). Furthermore, quercetin significantly decreased TNF-α and IL-6 compared with the vehicle and untreated groups ($P<0.05$).

4. Discussion

There are many clinical manifestations of CL as one of the most important infectious diseases causing skin injury[22–24]. In most cases, CL is self-healing, but the resolution of the disease is slow and can take several months to years[24]. Some cases of CL may evolve into other forms of leishmaniosis, such as diffuse CL and mucocutaneous leishmaniosis; these forms never heal spontaneously, making the treatment complicated[3]. Thus, in treating CL, both limitation of tissue damage and control of parasite replication should be the target to improve cure, promote clinical healing of the lesions, reduce scar formation, and prevent parasite dissemination or relapse[4,16].

Our *in vitro* study indicated that quercetin has potential activity against the *L. major* promastigotes and intracellular amastigotes. The EC_{50} of quercetin (0.27 and 0.85 μM against the *L. major* promastigotes and amastigotes, respectively) is comparable to that of glucantime (16.8 and 5.3 μM against the *L. major* promastigotes and amastigotes, respectively), one of the conventional first-choice drugs in the treatment of leishmaniosis[25]. Consistent with our result, it has been shown that quercetin can inhibit the growth of *Leishmania donovani* and *Leishmania amazonensis* promastigotes and amastigotes[1,6,7].

Additionally, oral administration of quercetin (50 mg/kg/day for 28 consecutive days) reduced the parasite load in the margin of cutaneous lesions compared to the untreated group. Oral drugs are convenient because they reduce hospitalization and related costs, allow home treatment, are easier to administer, and have better coverage and accessibility[26]. Similar to our study, in some studies with different treatment regimens, it has been shown that quercetin is active by oral administration in experimental cutaneous and visceral leishmaniosis and can reduce the parasite burden in animal models[1]. For example, Mittra *et al*. demonstrated that oral administration of quercetin (14 mg/kg twice a week for one month) to golden hamsters significantly diminished splenic *Leishmania donovani* burden four days post-infection, compared to the infected untreated control hamsters[1]. Also, oral administration of quercetin at 20 mg/kg three times a week for four weeks reduced the parasite burden in the spleen cells of the *Leishmania donovani*-infected hamsters[13].

The average area of the lesion in the quercetin group was less than the vehicle and untreated groups during the treatment, but this reduction was not significant except on the 12th day of treatment. Like our study, oral administration of quercetin reduced the lesion size in *Leishmania amazonensis*-infected BALB/c mice[27]. Cutaneous wound healing is one of the most complex processes with different molecular and cellular pathways[22]. Sakthianandeswaren *et al*. demonstrated that resistance to CL is closely linked to the host's ability to heal a skin wound, and the wound repair response is critically important for the rapid cure of murine CL caused by *L. major*[28]. In our histopathological sections stained with H & E and Masson's trichrome, there were fewer inflammatory cells, more fibroblast proliferation, and more collagen deposition in the quercetin-treated group compared to the untreated group. On the other hand, TNF-α and IL-6 were prominently reduced, while the adiponectin level was increased after quercetin treatment. These findings suggest that quercetin can reduce inflammation and accelerate granulation tissue formation, positively promoting wound healing.

The excessive inflammatory response is one of the main reasons for delayed healing[29]. It has been shown that controlling the immune response and developing a balance between the pro-and anti-inflammatory factors in CL eliminates the parasites with minimal tissue damage, decreases inflammatory reactions, and promotes wound healing[6,30–32]. In mice, Th1 responses involving nitric oxide and the cytokines, including IL-12, IFN-γ, and TNF-α, result in parasite killing. In contrast, Th2 responses are characterized by producing IL-4, IL-13, and IL-10, resulting in susceptibility to infection[33]. The reduced IL-6 level in quercetin-treated mice with CL was associated with decreased inflammatory cells. It has been indicated that quercetin has an inhibitory effect on IL-6 production by neutrophils[34]. A broad spectrum of cells produces IL-6 in response to stimuli leading to tissue damage or stress[34]. IL-6 is an essential cytokine in the early stages of wound healing because of its crucial roles in acute inflammation and the transition from neutrophil to monocyte recruitment at the inflammatory site[11,12,35]. But the excess amount of IL-6 causes persistent inflammation and impaired healing resulting in chronic wounds[35].

TNF-α is another central mediator of inflammatory responses, and its small quantity is essential for host defense against infection[11]. Local and systemic TNF-α levels increase in impaired healing and chronic wounds[11,29]. In CL, the lesions' size positively correlates with *Leishmania* antigen-specific TNF-α production from peripheral blood mononuclear cells[36]. The synergistic action of TNF-α and interferon-gamma is necessary to activate macrophages to kill *Leishmania*[37]. However, a robust inflammatory response at the site

of infection leads to ulceration and tissue destruction in CL[4,22]. It has been shown that some TNF-α inhibitors, such as pentoxifylline and infliximab, alone or in combination with antimonial compounds, can cure CL patients[38]. Additionally, Ashcroft *et al*. demonstrated that applying TNF-α antagonists in impaired wound healing blunts leukocyte recruitment, enhances extracellular matrix synthesis, and accelerates wound healing[29]. Therefore, it seems the administration of immunomodulators in treating CL combined with current antileishmanial drugs can be appropriate to relieve the pathologic lesions of the disease and shorten the course of healing. Topical application of quercetin on cutaneous wounds increased wound closure along with downregulating the expression of TNF-α[39]. Similarly, in our investigation, accelerating wound healing of CL was associated with a reduction in TNF-α levels in quercetin-treated mice. The present study suggests that quercetin might modulate the immune response in CL.

It has been shown that there is a bi-directional inverse relationship between TNF-α and adiponectin[40]. In our study, the adiponectin level was increased in quercetin-treated mice. However, whether quercetin directly or indirectly increased the adiponectin level by inhibiting the *TNF–α* gene expression needs further investigation. Adiponectin is a protein secreted by adipocytes with anti-inflammatory effects[41]. It has been demonstrated that adiponectin has a critical role at different stages of cutaneous wound healing by inducing the proliferation of dermal fibroblasts, increasing collagen production, stimulating angiogenesis, and promoting keratinocyte proliferation and migration[42]. Some of these changes were seen in our histopathological sections, including more fibroblast and fibrocyte counts and more collagen deposition along the increment of adiponectin level in the quercetin-treated group compared to the untreated group. Therefore, we propose combining anti-leishmanial drugs with compounds that increase adiponectin levels may accelerate CL wound healing. Wijnant *et al*. revealed that combination therapy is likely to increase the effectiveness of treatment as well as reduce adverse outcomes[43].

In response to phagocytosis of *Leishmania* and inflammatory cytokines (TNF-α, and interferon-gamma), macrophages produce reactive oxygen species (ROS) and reactive nitrogen species to kill the parasite[44]. Although ROS are essential for the eradication of parasites and control of infection, excessive production or insufficient detoxification of these molecules causes oxidative stress and damage to the surrounding tissues[44]. It is widely believed that oxidative stress plays a significant role in the pathogenesis of chronic non-healing wounds. Several studies have demonstrated that infection with *L. major* induces ROS production and oxidative stress over time, associated with increased MDA level, a secondary marker of tissue damage, in mouse skin. The non-enzymatic and enzymatic antioxidants are involved in scavenging and detoxifying ROS and reactive nitrogen species[45]. Anti-oxidative enzymes, including SOD, GPX, and catalase, exist abundantly in the skin[19]. In our study, quercetin increased GPX activity but did not affect SOD activity in an animal model of CL. Biological compounds with antioxidant properties protect cells and tissues from ROS and other free radical damage. Quercetin is a biological compound with the highest antioxidant activity among flavonoids and is characterized by multiple mechanisms, including inhibition of lipid peroxidation and production of pro-inflammatory cytokines, as well as scavenging of oxygen radicals[11]. In the present study, FRAP activity was increased while the MDA level decreased in the quercetin-treated group compared to other groups. Therefore, quercetin could reduce tissue damage in CL.

In conclusion, histopathological findings supported the immunological results. These results indicated that quercetin, in addition to possessing anti-*Leishmania* activity, could accelerate the healing of CL lesions through its immunomodulatory and antioxidant effects. Although there was a reduction in the average size of the lesions after quercetin treatment, the lesion was not cured completely. Therefore, quercetin combined with current anti-leishmanial drugs is recommended to improve the effects. However, further research needs to be conducted on combination therapy and finding therapeutic drugs that act synergistically with quercetin.

Conflict of interest statement

The authors declare there is no conflict of interest.

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Authors' contributions

SB and AO conceived and designed the study. EB and SB analyzed the data. EB and SB interpreted the data. SB and EB drafted the manuscript. SB, EB, and AO critically revised the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

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