Investigation of *in vitro* anti-leishmaniasis effect of quercetin

Reza Ranjbar1, Peyman Shayanfar2, Mahmoud Maniati2, Faham Khamesipour3*

The five-capacity antimony compounds, as the current treatment for cutaneous leishmaniasis, have disadvantages such as drug resistance and risk of relapse. Therefore, we studied the effect of quercetin on the growth of *Leishmania major* promastigotes and amastigotes as a new therapeutic approach. The effect of various concentrations of quercetin on *Leishmania major* promastigotes and amastigotes viability was evaluated after 24 hours of treatment. The percentage of parasite viability was determined by MTT colorimetric method. Thirty Wistar male rats were divided into five groups (n=6) including A1, A2, A3 and control which received 12.5, 25, 50 and 0 μg/ml of quercetin respectively after being infected by leishmaniasis through IP injection. The fifth group was SHAM which did not receive any compounds and were studied only for the confirmation of optimized housing conditions of rats. All rats were followed up every 5 days by measurement of lesion size and counting existing amastigotes in sample via Giemsa staining during the treatment period. After 24 hours of parasite culture, the promastigotes and amastigotes populations decreased in the presence of various concentrations of Quercetin, and at 1.61 and 50 μg/ml concentrations, the lethality rate was 21.65% and 73.29%, respectively. Data displayed that this compound efficiently relieved the lesions on the 30th day of treatment (1.8 compared to 9.1 mm in control) and reduced amastigotes population present in samples. Also, the mortality rate was not affected by Quercetin in rats 8 weeks after treatment. Our data indicated that Quercetin has in vivo and in vitro anti-leishmanial effects, triggering ideas to replace ordinance therapies with such new treatment which leads to patient improvements and reduced the side effects. Therefore, further studies on quercetin in human subjects are recommended.

**Key Words:** Quercetin; *Leishmania major*; Promastigote; Amastigotes

INTRODUCTION

*Leishmaniasis* is a parasitic zoonotic disease that is triggered by a protozoan from the *Trypanosomatida* family of *Trypanosomatophila* kingdom. Leishmaniasis presents itself in three forms of visceral, cutaneous, and mucocutaneous leishmaniasis. Its transmission often occurs from animal reservoirs to human by the *Phytocidae phlebotominae* sandfly bite [1,2]. Cutaneous leishmaniasis which causes skin lesions, can be seen in two dry and wet forms, which are caused by *Leishmania tropica* and *Leishmania major*, respectively [3,4]. The primary symptoms of infection in humans are the creation of a skin papule with a specific margin at the inoculation site, which is subsequently enlarged as a plaque or vascular node [5,6]. Cutaneous leishmaniasis as an endemic and hyper-endemic disease in some regions of Iran, is the most common type of leishmaniasis that has been expanding in recent years, and 20,000 cases diagnosed with cutaneous leishmaniasis, are reported annually from different countries [7].

Given the high prevalence of cutaneous leishmaniasis in Iran, the disease is now one of the most important priorities of the country's health care system. Unfortunately, no effective method has been devised for eradication of this disease, despite many suggestions for new therapeutic approaches. Common treatments of cutaneous leishmaniasis based on 5-capacity antimony compounds especially glucantime, bring about many side-effects such as anorexia, weight loss, indigestion, epigastric cramps, and hematological complications such as leukopenia and agranulocytosis [8]. In order to evaluate EC50, of quercetin on *Leishmania major* strains (MHOM/IR/75/ER), it was prepared from a laboratorial animal wound diagnosed with cutaneous leishmaniasis. The parasites were transferred to tubes containing two-phase NNN medium (Nony-MacNeal-Nicolle) and incubated for five days. Then, the *Leishmania major* parasites were passaged in a single-phase culture medium RPMI-1640 for proliferation [14].

**Preparation of different concentrations of Quercetin**

One mg of quercetin powder, purchased from Sigma Company, was dissolved in 200 μl methanol, and various concentrations of quercetin were prepared using RPMI-1640 culture medium and then stored in 4°C.

**Determination of promastigotes viability**

About 107 of parasites in logarithmic phase of growth were cultured in a 96-well plate containing RPMI-1640 medium with 10% bovine serum (purchased from National Cell Bank, Pasteur Institute of Iran, Tehran, Iran). The various concentrations of quercetin including 1.61, 3.12, 6.24, 12.5, 25, and 50 μg/ml were added to the wells in three times repeat. After 24 hours of incubation, 20 μl of MTT solution (with proportion 5 mg/ml) was added to each well and the samples ODs were analyzed by ELISA Reader at 650 nm [15].

**Determination of amastigotes viability**

In order to evaluate EC50 of quercetin on *Leishmania major* amastigotes, mouse macrophage J774 cells were used. Initially, these cells (J774) were infected with *Leishmania major* promastigote which were subsequently converted into amastigotes. For infection of macrophage by amastigotes, a 12-well plate was incubated in 5% CO2 for 4 hours at 37°C in order to allow the macrophages to adhere. To quantify the final concentration of quercetin EC50, various concentrations were added to the wells and analyzed by MTT assay.

1Molecular Biology Research Center, Systems Biology and Poisons Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran; 2Department of English, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; 3Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

Correspondence: Faham Khamesipour, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran; Email: Faham.Khamesipour@yahoo.com

Received: March 02, 2021, Accepted: March 16, 2021, Published: March 23, 2021

This open access article is distributed under the terms of the Creative Commons Attribution Non-Commercial License (CC BY-NC) (http://creativecommons.org/licenses/by-nc/4.0/), which permits reuse, distribution and reproduction of the article, provided that the original work is properly cited and the reuse is restricted to noncommercial purposes. For commercial reuse, contact reprints@pulsus.com
Rats and grouping

For animal studies, 6-8 weeks old Wistar male rats weighing 250-350 g (bought from Pasteur Institute of Tehran) were used. Ethical code of work involving the use of animals was obtained from Baqiyatallah University Research Ethics Committee (No. IRL.BMSU.REC.1397.368).

Thirty rats were examined in 5 groups of 6 each, including experimental groups A1, A2, A3, the control, and SHAM. All groups but SHAM underwent infection by leishmaniasis. The control group only received the non-quercetin solution. After getting infected, experimental groups A1, A2 and A3 received quercetin at different doses. The SHAM group only participated in the study to assess the suitability of animal housing conditions. At first, the parasites were counted after reaching the logarithmic phase and adjusted to 2×10⁶ per ml of parasitic solution.

Rat infecting

In order to infect the rats, 0.1 ml of the prepared parasitic solution was injected to the originating point of their tail. After skin lesions appeared in the injection site 2-3 weeks later, samples were taken from lesions, examined by Giemsa staining and microscopic observations, and were also inoculated in NNN culture to ensure the leishmaniasis presence.

Treatment of rats by quercetin

The starting day was considered as Day 0 and the rats of groups A1, A2 and A3 received doses at 12.5, 25 and 50 μg/ml per kilogram of quercetin by intraperitoneal injection for 30 days, twice daily (at morning and night). The size of the lesions was measured by caliper, and the presence of amastigotes in the lesion was investigated by Giemsa staining and sampling every 5 days in the treatment period. The mortality rate of the rats was followed up after treatment for up to 8 weeks.

Statistical analysis

The experiment was arranged in Completely Randomized Design (CRD) with three replications per treatment. All data were analyzed by SPSS (Statistical Package for the Social Sciences Statistical Package for the Social Sciences) version 20.0 (2016). Differences between treatment means in all experiments were analyzed based on Scheffe test using the probability of five percent.

RESULTS

Before running the analysis, the validity of assumptions was checked using graphical and statistical tests. The results indicated that the number of promastigotes in average clearly decreased with concentrations reaching 50 μg/ml, but the variation in the number of promastigotes was not stable in all groups (Figure 1). This result implied that the equality of variance may not hold for these data. However, the Levene statistic rejects the alternative hypothesis that the group variances are not equal (P=0.41).

![Figure 1](image)

The mean count of promastigotes exposed to different concentrations of quercetin including 1.61, 3.12, 6.24, 12.5, 25 and 50 μg/mL was measured to be 4.94, 4.61, 4.081, 3.83, 2.75 and 1.68, respectively after 24 hours (Table 1). According to the results, the mean number of promastigotes showed highly significant differences (P ≤ 0.0001) in different concentrations of quercetin. Indeed, quercetin showed a concentration- and time-dependent cytotoxic activity against Leishmania major promastigotes.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>The mean number of promastigotes and Quercetin’s inhibitory effect (%) on Leishmania major promastigotes growth at concentrations from 1.61 to 50 μg/mL are shown using MTT assay for EC₆₀ determination.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration (µg/ml)</strong></td>
<td><strong>No. of promastigotes</strong></td>
</tr>
<tr>
<td>1.61</td>
<td>6.31 ± 0.43</td>
</tr>
<tr>
<td>3.12</td>
<td>4.61 ± 0.4</td>
</tr>
<tr>
<td>6.24</td>
<td>4.08 ± 0.84</td>
</tr>
<tr>
<td>12.5</td>
<td>3.83 ± 0.73</td>
</tr>
<tr>
<td>25</td>
<td>2.75 ± 0.16</td>
</tr>
<tr>
<td>50</td>
<td>1.68 ± 1.2</td>
</tr>
</tbody>
</table>

Means followed by the same letter in the same column are not significantly different based on Scheffe test (P=0.05).

Calculate after 24 hours incubation

Higher concentrations of quercetin have more inhibitory effects on promastigotes

The number of promastigotes for the control group (with no quercetin) was 6.31 after 24 hours. In Figure 1, the fatality rate of various concentrations of quercetin (which is obtained by subtracting from 100) is shown. Quercetin EC₆₀ was measured 16 μg/ml after 24 hours. Also, the ODs of different concentrations from 1.61 to 50 μg/ml at 650 nm, indicated the gradual decrement of Leishmania major promastigotes count as a result of increased quercetin concentration. According to Table 1, quercetin shows a concentration-dependent inhibitory effect on Leishmania major promastigotes. The results also indicate that the growth inhibitory effect of various concentrations of quercetin on Leishmania major promastigotes standard strain increased significantly after incubation for 24 hours at 25°C. Based on these results, higher concentrations of quercetin improved the anti-parasitic activity (Table 1).

The measured optical density of different samples containing various concentrations of quercetin is depicted in Table 2. As displayed, contrary to quercetin concentration of 50 μg/ml which appropriates the lowest OD (0.244), the highest OD (0.633) belongs to the control group (with no quercetin). These data show the reverse correlation between quercetin concentrations and Leishmania major promastigotes counts and ODs.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>The OD and Growth Inhibition measured in different concentrations of quercetin.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration (µg/ml)</strong></td>
<td><strong>OD</strong></td>
</tr>
<tr>
<td>Control (0)</td>
<td>0.633 ± 0.018</td>
</tr>
<tr>
<td>1.61</td>
<td>0.437 ± 0.011</td>
</tr>
<tr>
<td>3.12</td>
<td>0.421 ± 0.04</td>
</tr>
<tr>
<td>6.25</td>
<td>0.409 ± 0.081</td>
</tr>
<tr>
<td>12.5</td>
<td>0.372 ± 0.029</td>
</tr>
<tr>
<td>25</td>
<td>0.261 ± 0.062</td>
</tr>
<tr>
<td>50</td>
<td>0.244 ± 0.08</td>
</tr>
</tbody>
</table>

Data are presented in Mean ± SD

Quercetin causes macrophages to react more vigorously against leishmaniasis

Although macrophages are the most important cells mediating leishmaniasis elimination, these cells are multifaceted elements. On the one hand, they swallow parasites and allow them to proliferate, and on the other hand, they present pathogen antigens to immune system cells making them the main cells in eliminating the infection. An interesting point we faced here, is that according to our data, these cells act more vigorously against pathogens in...
Investigation of in vitro anti-leishmaniasis effect of quercetin

The results showed that the mean size of the lesions in the control group increased from 2.9 mm on day 5 to 9.1 mm on day 30. Rats in group A3 which received quercetin at 50 μg/ml doses, showed significant changes (p=0.048) as the size of the lesions increased from 2.8 mm on day 5 to 3.7 mm on day 10, and to 4.5 mm on day 15, and then in a downward trend from day 20 onward, the mean size became 1.8 mm on day 30. Giemsa staining for group A3 showed an increase of amastigotes from days 0 to 15 with a smaller slope compared to that in controls, and then from day 15 to day 30, a considerable reduction was observed in the number of parasites in A3 group compared to control group such that few amastigotes were identified in the samples on day 30. On the other hand, according to the results of group A2 receiving the dose of 25 μg/ml, the size of the lesions was reported 2.7, 4.5, 5.5, 7, 8.6 and 8.8 on days 5, 10, 15, 20, 25 and 30, respectively. Although this group had a decrease in lesion size compared to the control group, these changes were not statistically significant (p=0.583). Also, the mean count of amastigotes was less than that of the control group from day 0 to 15, and on the day 30, fewer amastigotes were detected in the sample, but these differences were not significant in A3 on different days. Eventually, our results for group A1 showed no considerable alteration between groups A1 and A2 in terms of lesion size (p=0.376 in comparison to the control) and the number of amastigotes. Moreover, the mortality rate results indicated no remarkable finding with regard to the quercetin’s efficiency, though one of the rats in the control group died on the 24th day of treatment (Figure 2).

Table 2

<table>
<thead>
<tr>
<th>Amastigotes per 100 macrophages × 10^6</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>2.37</td>
</tr>
<tr>
<td>Quercetin-treated 1</td>
<td>1.62</td>
</tr>
<tr>
<td>Control 2</td>
<td>2.12</td>
</tr>
<tr>
<td>Quercetin-treated 2</td>
<td>1.5</td>
</tr>
<tr>
<td>Control 3</td>
<td>2.44</td>
</tr>
<tr>
<td>Quercetin-treated 3</td>
<td>1.54</td>
</tr>
</tbody>
</table>

The clinical symptoms of this disease are classified into three types of visceral, cutaneous and mucosal-leishmaniasis. Despite extensive advances in medical sciences, researchers have not yet succeeded in discovering effective vaccines in the treatment of leishmaniasis [16], and efforts to discover new drugs with least side effects continue to be pursued. Today, because of the side effects and drug resistance caused by common synthetic drugs such as antimony compounds, researchers are trying to introduce herbal compounds as a new and safe treatment for leishmaniasis. Plants have a large collection of secondary metabolites that show a wide range of medicinal activity. Quercetin, as a polyphenolic compound from flavonoids family, has been studied most of all to elucidate the biological effects of various flavonoids, and studies have demonstrated a vast span of its pharmacological and biological properties [17].

Quercetin’s anti-parasitic and specially anti-leishmaniasis effects have also been reported in many studies [18]. The response of the human and animal immune systems leading to leishmaniasis eradication is a process mediated by CD4+ T cells, Th1 and Th2, which play a reciprocal role in both host defense and parasite sensitization [19]. The main human immunity responding mechanisms against leishmaniasis consist of 1) Activation of macrophages by interferon gamma (IFN-γ) 2) Production of reactive oxygen products [20] such as anion superoxide and hydrogen peroxide, as well as nitric oxide by activated macrophages [21]. It has been shown that quercetin causes resistance to leishmanial infections, by differentiating T CD4+ cells to Th1. Quercetin also enhances the activity of eosinophil peroxide (EPO) by producing the active superoxide and superoxide anions in lymphocytes B, thereby reducing the host sensitization during immunity responses to leishmaniasis [22].

Quercetin also harnesses the GATA-3 transcription factor, but it stimulates the expression of Tbet factor in given cells by which the differentiation pathway of Th2 to Th1 cells is induced, and these finally eventuate to resistance against leishmaniasis infection [23]. Studies have also shown that quercetin enhances the activity of phagocytosis in macrophages by inhibiting the signaling of the transcription factor NF-kB in vitro. Quercetin directly decreases cell death in infections caused by parasites such as Leishmania donovani [24]. These could probably be the underlying mechanisms in our finding explaining how quercetin eventuated to decrement of mean lesion size and amastigotes count at high doses. Although one of the rats in group A3 died on the 24th day of treatment but it cannot be concluded that quercetin had any effect on mortality rate as the death seriously resulted from infection intensification, which could also be correlated to quercetin.

Sen et al. showed that Leishmania, as an intracellular parasite requires Fe (Fe^2+) ions for both growth and proliferation within phagosomes of host macrophages which are absorbed by its cell membrane called LIT1. Quercetin is highly lipophilic and it chelates iron ions and thus interferes with the iron absorption and metabolism by Leishmania. According to all these effective functions, quercetin can be used as a new candidate treatment of leishmaniasis [25]. Interestingly, it has been reported that quercetin inhibits the hexokinase enzyme in Trypanosoma brucei. This parasite uses exclusively produced ATP through glycolysis cycle, but quercetin may inhibit the hexokinase enzyme in Leishmania and thus interferes with the iron absorption and metabolism by Leishmania. According to all these effective functions, quercetin can be used as a new candidate treatment of leishmaniasis [25]. Interestingly, it has been reported that quercetin inhibits the hexokinase enzyme in Trypanosoma brucei. This parasite uses exclusively produced ATP through glycolysis cycle, but quercetin may inhibit the hexokinase enzyme in Leishmania and thus interferes with the iron absorption and metabolism by Leishmania. According to all these effective functions, quercetin can be used as a new candidate treatment of leishmaniasis [25]. Interestingly, it has been reported that quercetin inhibits the hexokinase enzyme in Trypanosoma brucei. This parasite uses exclusively produced ATP through glycolysis cycle, but quercetin may inhibit the hexokinase enzyme in Leishmania.

Regarding the endemic nature of leishmaniasis and properties of quercetin, we investigated the effects of quercetin on promastigote and amastigote of the Leishmania major (causative agent of cutaneous leishmaniasis) in vitro. Our findings show that all concentrations of quercetin have a significant effect on the parasite count in comparison to the control. This is, by increasing the concentration, a considerable reduction in the number of parasites was observed. Therefore, after 24 hours, the 16 μg/ml concentration of quercetin triggered 50% of the parasite population to disappear. Surprisingly, Quercetin, at the lowest concentration after 24 hours, showed 21.65% growth inhibition efficacy and higher concentrations were more effective such that at 50 and 25 μg/ml concentrations, the growth inhibition was reported to be 73.29% and 56.45%, respectively. We observed an increase in the death of Leishmania major promastigotes and amastigotes by increasing the concentration of quercetin. The effective concentration (EC_{50})
Investigation of in vitro anti-leishmaniasis effect of quercetin

of quercetin was measured to be 16 μg/ml for leishmania promastigotes after 24 hours and 32.54% for Leishmania major amastigotes after 72 hours [28].

CONCLUSION

The results of this study showed that quercetin’s in vitro inhibitory effect on the growth of Leishmania major promastigotes elevates by increasing its concentration. The optical absorption decrement in samples containing higher concentrations has proven the efficacy of quercetin’s anti-leishmaniasis effect.

The results of this study showed that quercetin has a lethal effect on Leishmania major promastigotes and the related infected macrophages. Hence, it is suggested that more in-vivo studies be done to investigate the efficacy of this herbal compound.

REFERENCES
