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

Reza Ranjbar[1], Peyman Shayanfar[1], Mahmood Maniati[2], Faham Khamesipour[3*]

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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 5 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

SLeishmaniasis is a parasitic zoonotic disease that is triggered by a protozoan from the *Trypanosomatida* family of *Sarcomastigophora* kingdom. Leishmaniasis presents itself in three forms of visceral, cutaneous, and mucocutaneous leishmaniasis. Its transmission often occurs from animal reservoirs to human by the *Psychodidae 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 a 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 addition, drug-resistance and high production costs are other disadvantages of these chemical compounds used for cutaneous leishmaniasis therapy [9]. Therefore, researchers today are enthusiastic in treating acute leishmaniasis using herbal and traditional medicine compounds, and hence, the need for extensive research on herbal compounds is felt more than ever before [10].

As a polyphenolic compound, Quercetin is one of the most important flavonoid compounds in vegetables, fruits, leaves and seeds as a secondary metabolite [11]. Many studies have reported the various therapeutic properties of this herbal compound, particularly its antioxidant and anti-tumor features [12]. Matsuda et al., for instance, reviewed the effect of quercetin on African-American *Trypanosomiasis* (HAT) and concluded that quercetin directly induces apoptosis in *Trypanosoma brucei gambiense*, the main doer cell of this pathogen, without affecting human normal cells [13]. Along with these studies, the main objective of the current study is to investigate the *in vitro* effects of quercetin against *Leishmania major* strains (MHOM/IR /75/ER).

MATERIALS AND METHODS

Leishmania major culture

The *Leishmania major* standard strain of Iran (MHOM /IR /75 /ER) was prepared from a laboratorial animal wound diagnosed with cutaneous leishmaniasis. The parasites were transferred to tubes containing two-phase NNN medium (Novy-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 EC_{50} 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% CO_2 for 4 hours at 37°C in order to allow the macrophages to adhere. To quantify the final concentration of quercetin EC_{50}, various concentrations were added to the wells and analyzed by MTT assay.

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

Correspondence: *Faham Khamesipour, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran; E-mail: Faham.Khamesipour@yahoo.com*

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Rats and grouping

For animal studies, 6-8 weeks old Wistar male rats weighing 250-250 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. IR.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 2×10^6 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 Scheffea 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).

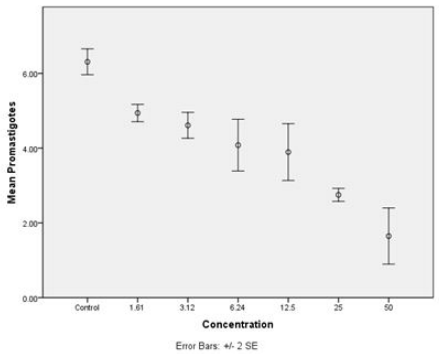

Figure 1) *The box plot of means and standard errors to check the equality of variances.*

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 ($P \leq 0.0001$) in different concentrations of quercetin. Indeed, quercetin showed a concentration- and time-dependent cytotoxic activity against *Leishmania major* promastigotes.

TABLE 1

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 EC_{50} determination.

Concentration (µg/ml)	No. of promastigotes*	Growth Inhibitory (%)
control	6.31 ± 0.43^{a}	0%
1.61	4.94 ± 0.14^{ab}	21.56%
3.12	4.61 ± 0.4^{b}	26.87%
6.24	4.08 ± 0.84^{bc}	35.33%
12.5	3.83 ± 0.73^{bc}	39.02%
25	2.75 ± 0.16^{cd}	56.45%
50	1.68 ± 1.2^{d}	73.29%

Means followed by the same letter in the same column are not significantly different based of Scheffea 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 EC_{50} 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 2

The OD and Growth Inhibition measured in different concentrations of quercetin.

Concentration (µg/ml)	OD*	Inhibition (%)
Control (0)	0.633 ± 0.018	0
1.61	0.437 ± 0.011	30.78
3.12	0.421 ± 0.04	33.5
6.25	0.409 ± 0.081	35.33
12.5	0.372 ± 0.029	41.16
25	0.261 ± 0.062	58.7
50	0.244 ± 0.08	61.37

Data are presented in Mean ± SD

Quercetin causes macrophages to react more vigorously against leishmania

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

presence of quercetin. We added quercetin at EC_{50} concentration to mouse macrophage cells J774 and compared the mean number of the *Leishmania major* amastigotes in 100 macrophages. The Table 3 below shows the mean number of amastigotes counted in 100 macrophages in the control and the quercetin-treated groups in the three treatments after 72 hours of incubation at 25°C. The mean inhibitory effect of this compound on *Leishmania major* amastigotes was 32.54%, which indicates the efficiency of quercetin.

TABLE 3

Quercetin EC_{50} inhibitory effect on *Leishmania major* amastigotes.

	Amastigotes per 100 macrophages $\times 10^6$	Inhibition (%)
Control 1	2.37	0
Quercetin-treated 1	1.62	31.8
Control 2	2.12	0
Quercetin-treated 2	1.5	29.15
Control 3	2.44	0
Quercetin-treated 3	1.54	36.69

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 lesions 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).

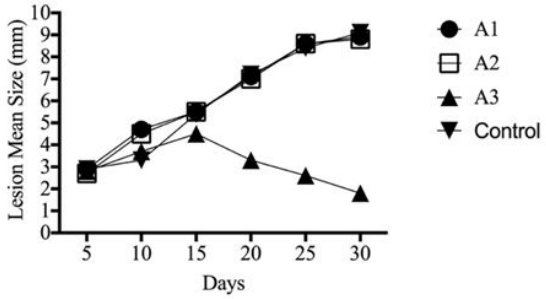

Figure 2) *The graph above, showing the mean size of lesions in different days, demonstrates that quercetin at high dose (50 µg/ml) is able to effectively reduce the lesion size. Although lower doses had ignorable effects, these changes could not be reported significant. According to these findings, anti-leishmaniasis activity of quercetin in animal models results from lethality of amastigotes in presence of this compound, which could be attributed to the more vigorous function of macrophage against parasites accompanying quercetin.*

DISCUSSION

Cutaneous leishmaniasis is a protozoan disease caused by *Leishmania major*. Cutaneous leishmaniasis has been considered as one of the six major diseases in the World Health Organization's Tropical Diseases Survey, and about 12 million people are suffering from this cutaneous disease worldwide [5]. The clinical symptoms of this disease are classified into three types of visceral, cutaneous and muco-cutaneous 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-leishmanial 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 reduces the activity of eosinophil peroxide (EPO) by preventing the activation of eosinophils and secretion of immunoglobulin IgE from 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 T-bet 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*. Quercetine directly induces 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 a protein on 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 growth and propagation of *Trypanosoma brucei* by inhibiting this enzyme, which catalyzes the first step of the glycolysis pathway [26]. Quercetin reduces the level of inflammatory factor TNF-α which is induced by lipopolysaccharide and nitric oxide (NO) released from active neutrophils and macrophages. In addition, it can lead to the production of reactive oxygen species [20], such as anion superoxide, hydrogen peroxide, which clean pathogens in bacterial and parasitic infections [27].

Regarding the endemic nature of leishmaniasis and properties of quercetin, we investigated the effects of quercetin on promastigote and amastigotes 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 group. That 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})

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.

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