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In vitro Investigation of Rutin-Loaded PLGA Nanoparticles on *Leishmania infantum* Promastigotes

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PLGA

Abstract: Leishmaniasis is a group of illnesses occasioned *Leishmania* (*L.*) parasites transmitted by the bites of infected female *Phlebotominae* class flies and it is endemic in 102 countries. It is seen worldwide, particularly in developing countries. In the present study, the antileishmanial efficacy of free rutin and nanoparticles formed by encapsulating flavonoid rutin in a polymer nanoparticle system on *Leishmania infantum* promastigotes were contrasted. The efficacy of rutin-loaded PLGA nanoparticles $(RT)_{NPs}$ on the proliferation of promastigote form of *L. infantum* parasites was examined for the first time by counting the *in vitro* antileishmanial activities of $(RT)_{NPs}$ using the MTT assay and counting on the thoma slide. It has been observed that $(RT)_{NPs}$ significant affect the proliferation of parasites at concentrations of 1000, 750, and 500 µg/ml at 72nd and 96th hours. The viability% value decreased 10-fold at 1000 µg/ml concentration of $(RT)_{NPs}$. While the IC_{50} value of promastigote form of *L. infantum* parasites was 29.2 ± 4.5 µg/ml in the specimens treated with RT at varied concentrations, the IC_{50} value of promastigote form of *L. infantum* parasites was found to be 23.0 ± 2.7 µg/ml in the specimens treated with $(RT)_{NPs}$. It was observed that the absorbance measurements of $(RT)_{NPs}$ were lower compared to RT at concentrations of 1000, 750, and 500 µg/ml at 48th hour.

Rutin Yüklü PLGA Nanopartiküllerinin *Leishmania infantum* Promastigotları Üzerinde *In vitro* İncelenmesi

Anahtar Kelimeler
Leishmaniasis,
Antileishmanial etkinlik,
Flavonoid,
Rutin,
PLGA

Öz: Leishmaniasis, enfekte dişi *Phlebotominae* sınıfı sineklerin ısırmasıyla bulaşan *Leishmania (L.)* parazitinin neden olduğu bir hastalık grubudur ve 102 ülkede endemiktir. Dünya genelinde bilhassa gelişmekte olan ülkelerde görülmektedir. Bu çalışmada serbest rutinin ve bir polimer nanopartikül sistemi içerisine bir flavonoid olan rutinin enkapsüle edilerek üretilen nanopartiküllerin *Leishmania infantum* promastigotları üzerindeki antileishmanial etkinliğini karşılaştırıldı. Rutin yüklü PLGA nanopartiküllerinin ($(RT)_{NP}$) *in vitro* antileishmanial etkililiklerinin MTT analiziyle ve thoma lamında sayım yapılmasıyla, ilk sefer olarak $(RT)_{NP}$'lerinin promastigot formdaki *L. infantum* parazitlerinin çoğalmasına etkisi incelenmiştir. $(RT)_{NP}$'lerinin parazitlerin çoğalmasına, 72. ve 96. saatteki 1000, 750 ve 500 µg/ml'lik konsantrasyonlarında büyük oranda etki ettiği görülmüştür. %Canlılık değeri, $(RT)_{NP}$'lerinin 1000 µg/ml'lik konsantrasyonunda 10 kat azalmıştır. Değişik konsantrasyonlarda RT uygulanan örneklerde, promastigot formdaki *L. infantum* parazitlerinin IC_{50} değeri 29,2 ± 4,5 µg/ml iken $(RT)_{NP}$'leri uygulanan örneklerde promastigot formdaki *L. infantum* parazitlerinin IC_{50} ölçümünün 23,0 ± 2,7 µg/ml olduğu belirlenmiştir. 48. saatteki 1000, 750 ve 500 µg/ml'lik konsantrasyonlarda $(RT)_{NP}$'lerinin, RT'e kıyasla absorbans ölçümlerinin daha alçak olduğu görülmüştür.

1. Introduction

Leishmaniasis is a group of illnesses occasioned more than 20 *Leishmania* parasites in the protozoa group transmitted by the bites of infected female *Phlebotominae* class flies [1]. Visceral leishmaniasis (VL) is a systemic disorder that causes high fever, malaise, weight loss, swelling of the spleen, liver and lymph nodes, pancytopenia and anemia. VL, the strongest form of leishmaniasis, which is endemic in 102 countries, is found in many tropical and subtropical regions and war zones, particularly

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affected by poverty [1-3]. The annual worldwide prevalence of leishmaniasis is approximately 12 million. In addition, 350 million people are in danger of being infected. Approximately 2 million cutaneous leishmaniasis and 500,000 VL cases are registered each year, and 60,000 deaths from the disease are reported worldwide [4, 5].

Recently, there is concern that the number of people who will get leishmaniasis is increasing due to the lack of an effective enough vaccine, the negative effects of global warming, wars and migrations. Although *Leishmania* parasites have developed resistance to pentavalent antimonials in recent years, chemotherapy is used as the gold standard in infection [1, 3, 5]. It is important to develop new antileishmanial compounds due to the inadequacy of current therapies and severe toxic reactions to conventional drugs [6]. There is immediate essential to discover new natural remedies from herbs such as herbal derivatives (flavonoids) or herbal extracts that are efficient, non-toxic, safe, and cheaper to combat such diseases [6]. Natural compounds are the source of new, potential and discriminating agents for the treatment of neglected tropical diseases, particularly protozoan parasites. Since humans have continued to use plant-derived materials for centuries, it is not surprising that herbal products are used in drug development [7]. Many compounds obtained from plant sources to date have shown antileishmanial activity potential [8].

Flavonoids are a huge group of phenolic secondary metabolites belonging to the family of natural polyphenols [9]. Rutin (RT), a flavonoid, is found mainly in different parts of plants such as fruit pods, leaves, flowers, and roots. Among the plant species, the highest rutin concentrations were found in grapes and buckwheat [10]. RT (3,3',4',5,7-pentahydroxyflavone-3-rutinoside) has been found to have many biological effects such as antimicrobial, antifungal, antioxidant, antidepressant, antiviral, antiprotozoal, antiallergic, antiulcer, anti-diabetes, and anticarcinogenic [3, 11, 12]. RT is a non-oxidizable molecule that is significantly less toxic than other bioflavonoids [13]. Therefore, RT is a promising drug candidate for the therapy of all forms of leishmaniasis with potent antileishmanial activity [7].

Poly Lactic-co-Glycolic Acid (PLGA) nanoparticles (NPs) are one of the most commonly used compounds in nanotechnology and are considered to be the best-known carriers among drug delivery systems. The PLGA macromolecule is preferred because it is biodegradable, biocompatible, and non-toxic. Furthermore, it has the approval of the American Food and Drug Administration as drug delivery systems [14-16]. One of the most important features of PLGA NPs is that it increases the bioavailability and biocompatibility of biologically active molecules such as drugs, peptides, proteins [17].

In order to exhibit their therapeutic effects, drug delivery systems are used to deliver pharmaceutical and bioactive compounds to exact localizations in a continuous and enhanced manner [12, 14]. Polymeric nanoparticles have lately been evaluated as possible carriers for encapsulation of flavonoids. Because of the colloidal nature of NPs, they can cross various barriers in the body, with the inclusion of the gastrointestinal mucosa and blood-brain barrier [9]. PLGA has been widely used for drug release models. PLGA is one of the maximum effective structures for encapsulation of antimicrobial compounds, particularly given its high hydrophobicity, strong mechanical strength, controlled drug release, biodegradability, biocompatibility, large surface area, and non-toxicity. It also does not require surgical intervention after drug delivery [18].

Current antileishmanial drugs are cause serious side effects and very expensive. It is also known that several *Leishmania* species develop resistance to existing antileishmanial treatments. Hence, there is a serious need to improve new therapeutic targets that are less toxic, more effective, and accessible to the poorest affected population [19]. New oral therapies should be investigated for potential toxicity and emerging drug resistance [20]. It is known that RT limits clinical applications due to its low solubility in water [21]. Therefore, the main objective of the present study, to increase the antileishmanial activity by encapsulating the RT molecule, which has low solubility in water such as quercetin, with drug delivery systems.

The purpose of the present study is to investigate the antileishmanial efficacy of RT and $(RT)_{NPs}$ on promastigote cultures of *L. infantum in vitro*, compare their efficacies, and improve a new model based on PLGA nanoparticulate drug delivery systems in the treatment of VL.

2. Material and Method

2.1. Promastigote cultures of *L. infantum*

The culture of promastigote form of *L. infantum* parasites, which is the VL agent, was carried out in Roswell Park Memorial Institute-1640 (RPMI-1640) media containing 10% FBS in a 27 °C refrigerated oven. Promastigote form of *L. infantum* parasites were cultured within RPMI-1640 medium, which was added with Gentamycin (80 mg/mL), 10% fetal bovine serum, and L-glutamine at 27 °C. An inverted microscope was used to watch the daily growing of promastigote form of *L. infantum* parasites.

2.2. Properties of $(RT)_{NPs}$

$(RT)_{NPs}$ obtained from Kızılbey [21] (shown as NP10) were made by single emulsion-solvent evaporation technique. As it descripted in that article; reaction yield (%), Encapsulation Efficiency (%), Average Particle Size (nm), polydispersityindexes (PDI) and ζ-

Potential Values of NP are respectively 67 ± 3; 87 ± 6; 570.3±66.13; 0.524±0.046; −1.80±0.912 [21]. Drug Loading (DL%) value of the nanoparticles used was calculated with the formula given below [22].

$$\%DL = \frac{\text{Amount of Material Encapsulated (mg)}}{\text{The Amount of Dry Nanoparticles Obtained (mg)}} \text{ x } 100$$

Powder nanoparticles were dissolved in purified water and a 5 mg/mL solution was made ready for stock. Prior to use, the solution sterilized by sonication and afterwards by filtration with 22 µm filters. Subsequently, drug concentrations were prepared at 10, 25, 50, 100, 250, 500, 750 and 1000 µg/mL.

2.3. Investigation of antileishmanial activities of promastigote form of *L. infantum* parasites

Parasites taken from promastigote form of *L. infantum* culture incubated at 27 °C were calculated as $1x10^6$/mL and prepared using RPMI-1640 medium enriched with 10% FBS for a minimum of three specimens for each experiment. After 24 hours, RT and $(RT)_{NPs}$ with concentrations of 10-1000 µg/mL were added. Also, specimens containing only parasites were prepared as control groups. The effect of RT and $(RT)_{NPs}$ on proliferation and antileishmanial activity at the 24th, 48th, 72nd, and 96th hours after the application of the determined concentrations were evaluated. Accordingly, the proliferation of promastigote form of *L. infantum* parasites was obtained by determining the parasite numbers in eppendorfs using Thoma slide. In addition, the viability % of parasites were calculated by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay to determine antileishmanial activity.

2.3.1. Proliferation assay

Proliferation was determined by counting viable promastigotes in test tubes with hemocytometry at 24th, 48th, 72nd, and 96th hours. Briefly, 100 µl of promastigote form of *L. infantum* parasites was taken from all test tubes, immobilized with 10% formalin, and counted with a hemocytometer by examining under an inverted microscope. IC_{50} numbers of all specimens were determined by finding the concentration that inhibited semi of the promastigotes. The antileishmanial effects of the specimens were contrasted considering the IC_{50} numbers at 24th, 48th, 72nd, and 96th hours of exposure.

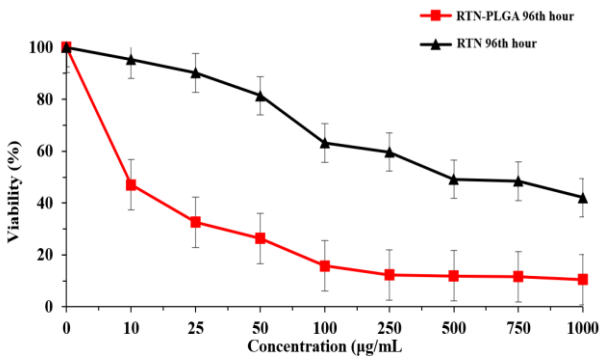
2.3.2. Metabolic activity assessment

Antileishmanial activities of promastigote form of *L. infantum* parasites of RT and $(RT)_{NPs}$ in different concentrations were also investigated with MTT assay at the same hours used in proliferation analysis. Accordingly, promastigote form of *L. infantum* parasites were taken from the tubes at the relevant hours and transferred to a 96-well plate. Then, after adding 10 µl of sterile MTT solution (5 mg/mL) to each of, it was left to incubate at 27 °C for 4 hours. In conclusion, 100 µL of DMSO was put inside each of the microplate wells to dissolve the formazan crystals. After waiting in the dark for 30 minutes, the absorbance values of promastigotes in the wells were determined by reading them on an ELISA reader (ThermoLabsystem Multiskan Ascent) at a wavelength of 540 nm.

3. Results

3.1 Proliferation of promastigote form of *L. infantum* parasites

The effects of RT and $(RT)_{NPs}$ on parasite count were calculated at 24th, 48th, 72nd, and 96th hours. As the concentration of RT and $(RT)_{NPs}$ increased, the decrease in viability% values showed its effect on the proliferation of promastigote form of *L. infantum* parasites. In Graph 1, it is seen that concentrations of $(RT)_{NPs}$ are more effective on viability% than concentrations containing RT alone. In addition, it has been determined that in specimens exposed to $(RT)_{NPs}$, $(RT)_{NPs}$ reduce the number of parasites in all hours compared to the control. While $(RT)_{NPs}$ decreased the metabolic activity values to 1/10th at a concentration of 1000 µg/mL, RT alone reduced it to about half. Thus, $(RT)_{NPs}$ have been observed more effective than RT alone.

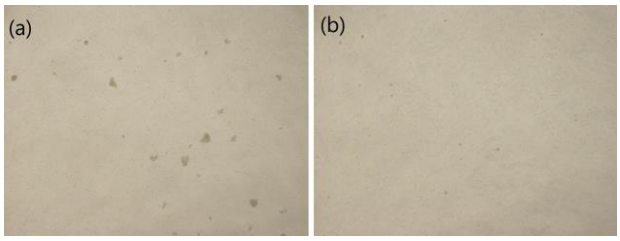


Graph 1. Antileishmanial effects of RT and $(RT)_{NPs}$ on the proliferation of promastigote form of *L. infantum* parasites by calculating viability% values *in vitro* at the 96th hour by counting.

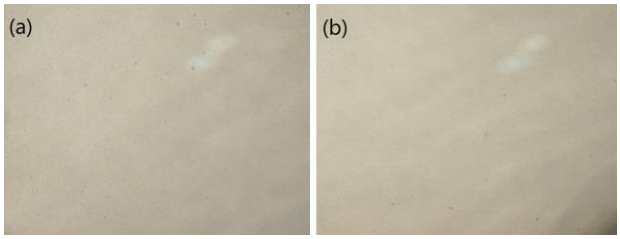
Parasite count decreased as $(RT)_{NPs}$ concentration increased in promastigote form of *L. infantum* cultures examined at the same magnification under an inverted microscope. In addition, parasite clusters decreased (Picture 1).

Promastigote form of *L. infantum* clusters were less in specimens exposed to $(RT)_{NPs}$ compared to RT at the same concentration (Picture 2).

Accordingly, $(RT)_{NPs}$ exhibited a greater antileishmanial efficacy on the proliferation of promastigote form of *L. infantum* parasites than RT alone.



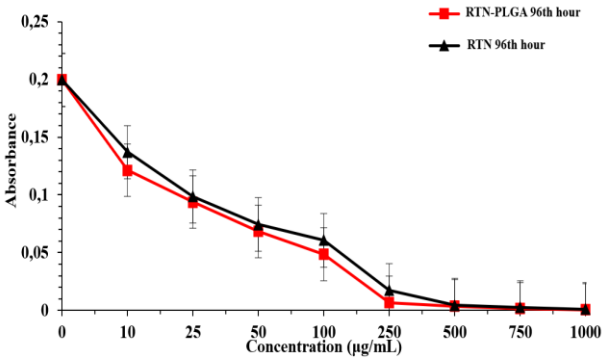
Picture 1. Microscopic images of promastigote form of *L. infantum* parasites exposed to $(RT)_{NPs}$ at (a) 10 µg/mL, (b) 1000 µg/mL concentration at 48th hour (10x)



Picture 2. Microscopic images of promastigote form of *L. infantum* parasites exposed to (a) RT and (b) $(RT)_{NPs}$ concentrations of 1000 µg/mL at 96th hour (10x)

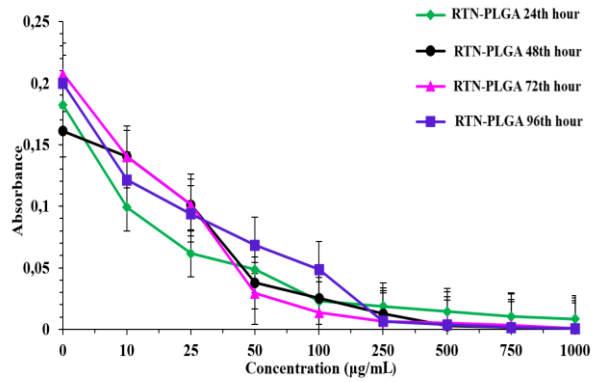
3.2. Determination of Metabolic Activity

Metabolic activity monitoring was achieved in two methods: optical density and evaluation microscopic examination at 24th, 48th, 72nd, and 96th hours. In specimens exposed to RT and $(RT)_{NPs}$, clusters of parasites were observed to produce high formazan crystals with signs of viability and metabolic activity below 250 µg/mL and 100 µg/mL, respectively. This shows that RT and $(RT)_{NPs}$ do not reason toxic effects on promastigote form of *L. infantum* parasites when administered at lower concentrations. Likewise, *Leishmania* parasites exposed to the same concentrations of free NPs formed purple colored formazan crystals, demonstrating that the parasites survived. On the other hand, RT and $(RT)_{NPs}$ inhibited the production of formazan crystals at concentrations above 250 µg/mL and 100 µg/mL, respectively, and clumps of dead parasites were seen during microscopic examination. Microscopic examination showed that RT and $(RT)_{NPs}$ were effective in inhibiting the metabolic activity of promastigote form of *L. infantum* parasites at both 750 and 1000 µg/mL concentrations, while parasites healing with free nanoparticles at the exact concentrations were metabolically active. While evaluating the optical density measurements, it was defined that RT and $(RT)_{NPs}$ have inhibitory activities on the metabolic activities of promastigote form of *L. infantum* parasites, and free nanoparticle application didn't lead to any change according to the concentrations examined. It shows that the efficacy of RT and $(RT)_{NPs}$ is because of the increase in concentrations. As seen in Graph 2, the absorbance values decreased at all concentrations at the 96th hour. It has been determined that the absorbance values of RT and $(RT)_{NPs}$ are significantly reduced compared to the control and are even more effective at high concentrations. The strongest effect was detected in the group exposed to $(RT)_{NPs}$ [23].



Graph 2. Evaluations of antileishmanial effects of RT and $(RT)_{NPs}$ on the metabolic activity rates of promastigote form of *L. infantum* parasites *in vitro* at the 96th hour by MTT

As seen in Graph 3, it was found that the absorbance values of promastigote form of *L. infantum* parasites exposed to $(RT)_{NPs}$ at all concentrations gradually decreased in all hours. This proved to be an increasingly powerful effect in time until the 96th hour. As the concentration increased and the absorbance values decreased more at the 96th hour compared to the 24th hour.



Graph 3. Evaluations of antileishmanial effects of $(RT)_{NPs}$ on the metabolic activity rates of promastigote form of *L. infantum* parasites *in vitro* at the 24th, 48th, 72nd, and 96th hours by MTT

The antileishmanial activities of promastigote form of *L. infantum* parasites in the specimens were compared considering their IC_{50} numbers after exposure. In specimens with different concentrations of RT, it was determined that the IC_{50} value of promastigote form of *L. infantum* parasites was 29.2 ± 4.5 µg/mL. In the specimens applied with different concentrations of $(RT)_{NPs}$, it was defined that the IC_{50} value of promastigote form of *L. infantum* parasites was 23.0 ± 2.7 µg/mL. Thus, the lower IC_{50} value of $(RT)_{NPs}$ on promastigote form of *L. infantum* parasites compared to RT proved higher antileishmanial activity.

4. Discussion and Conclusion

The RT molecule, a flavonol, is frequently studied and used for its ability to treat many diseases. In the experiments, the effect of $(RT)_{NPs}$ on the proliferation of promastigote form of *L. infantum* parasites was examined by enumeration in thoma slide and the *in vitro* antileishmanial activities of $(RT)_{NPs}$ by the MTT assay. In line with the results obtained in both methods, as the concentration increased, both viability% values and absorbance values decreased. Thus, it was determined that the results obtained using different methods are compatible with each other.

Natural products of plant origin containing various flavonoids have been studied as antileishmanial candidates [24]. Various flavonoids, such as quercitrin, quercetin, and luteolin, which are abundant dietary flavones, are effective against some *Leishmania* species [25]. Nanocapsulation of RT, an important plant flavonoid, can further increase its effectiveness [26].

The consequences show that for the first time in the world, a new approach to leishmaniasis treatment can be developed based on the use of RT flavonoid loaded nanoparticles. The proliferation effect of $(RT)_{NPs}$ was established at a concentration of 1000 µg/mL, reducing the viability% value 10 times. In the specimens applied with different concentrations of RT and $(RT)_{NPs}$, the IC_{50} numbers of promastigote form of *L. infantum* parasites were determined to be 29.2 ± 4.5 µg/mL and 23.0 ± 2.7 µg/mL, respectively. At 48th hour, the absorbance measurements of RT and $(RT)_{NPs}$ at concentrations of 500, 750, and 1000 µg/mL decreased significantly in comparison with lower concentrations. Compared to lower concentrations of RT and $(RT)_{NPs}$, the absorbance measurements at concentrations of 500, 750, and 1000 µg/mL decreased significantly, at 48th hour. In addition, it has been observed that the absorbance values of $(RT)_{NPs}$ are lower compared to RT. $(RT)_{NPs}$ compared to RT, the increase in the concentration at all hours decreased the viability% of promastigote form of *L. infantum* parasites.

The low IC_{50} value of RT proves that it is effective against resistant parasites at concentrations that are not poisonous to the host. RT could potentially be used to treat leishmanial infections resistant to commercially available drugs such as antimony [3]. IC_{50} numbers of RT were found to be 12.64 ± 0.86 µg/mL on susceptible promastigotes of *L. donovani* and 13.07 ± 1.42 µg/mL on resistant promastigotes [3]. In our study, the IC_{50} value of RT was found as 29.2 ± 4.5 µg/mL on promastigote form of *L. infantum* parasites. Approximately 2 times the RT concentration used on *L. donovani* promastigotes appears to have the same antileishmanial activity when used on promastigote form of *L. infantum* parasites.

In a study by de Medeiros et al. (2019), RT-loaded microparticles showed a faster analgesic effect compared to non-microencapsulated RT [12]. Studies by Mauludin et al. (2009) have shown that the absorption of orally administered RT can be developed using a nanocrystal formulation that can be incorporated into solid dosage forms such as capsules or tablets [27, 28]. RT nanocrystals showed more effective properties than the raw drug in the case of oral administration with prolonged and increased dissolution rate [27, 28].

PLGA NPs have been studied on a large scale, particularly against cancer and infectious diseases, due to their biocompatibility and biodegradable properties that lead to controlled releases for a long time [17, 29, 30]. Active ingredients are bound to the surface or are trapped inside the nanoparticles [29, 31]. In several studies, encapsulation of antigenic molecules into PLGA nanoparticles has been shown to increase their bioavailability while reducing their toxicity [17]. In studies conducted by Abamor (2018), the fact that quercetin loaded NPs are larger compared to free NPs can be accepted as an indicator of efficient and effective encapsulation by quercetin loaded PCL NPs. Abamor (2017) in addition, the biocompatibility of PLGA NPs loaded with caffeic acid phenethyl ester has been verified [32, 33].

Pandey et al. (2018) stated that there are several articles reporting that the encapsulation of various anticancer drugs with PLGA NPs was successfully transmitted *in vitro* and *in vivo*. Treatment with $(RT)_{NPs}$ has been shown to play a serious role in preventing the development of cancer by preventing oxidative stress [34]. In our study, the IC_{50} value of $(RT)_{NPs}$ on promastigote form of *L. infantum* parasites was determined as 23.0 ± 2.7 µg/mL. Thus, it is seen that the concentrations of $(RT)_{NPs}$ used on promastigote form of *L. infantum* parasites are almost 1/4 compared to quercetin loaded PCL NPs which shows the same antileishmanial activity [35]. In addition, the IC_{50} value of RT was found to be 29.2 ± 4.5 µg/mL on promastigote form of *L. infantum* parasites. Thus, using RT concentrations of approximately 1/5 compared to quercetin on promastigote form of *L. infantum* parasites shows the same antileishmanial activity. As a result, based on the specimens used at the same concentrations; it was found that both $(RT)_{NPs}$ and RT showed more antileishmanial activity compared to quercetin loaded PCL NPs and quercetin, respectively [35]. In a study by Allahverdiyev et al. (2013), it was shown that titanium dioxide silver (TiO_2@Ag) nanoparticles have antileishmanial efficacy on *L. infantum* and *L. tropica* parasites by inhibiting their biological properties such as viability, metabolic activity, and survival in host cells [36]. From the articles examined, the active molecules were encapsulated in the nanoparticular drug carrier system, showing more antileishmanial activity and thus, better results were obtained. In our

study, it was determined that $(RT)_{NPs}$ showed more antileishmanial activity compared to free RT.

As a result, this study has shown for the first time that the antileishmanial activities of RT and $(RT)_{NPs}$ are effective against promastigote form of *L. infantum* parasites, and it is believed that it will shed light on other studies on this subject. In addition, it has been shown that the antileishmanial activity of $(RT)_{NPs}$ on promastigote form of *L. infantum* parasites *in vitro* is higher compared to RT alone. It is thought that the results found will guide future *in vivo* studies in investigating the utility of $(RT)_{NPs}$ as drugs.

In conclusion, in order to be used as an alternative to the current treatment of leishmaniasis, *in vivo* antileishmanial efficacy tests of $(RT)_{NPs}$ are recommended on promastigote form of *L. infantum* parasites, which are the VL agent. Due to the antileishmanial activity of the RT and $(RT)_{NPs}$ we used in our study, it is believed that the results of this study can be developed with *in vivo* models and may be useful in terms of being a step for clinical studies.

Acknowledgment

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Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

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