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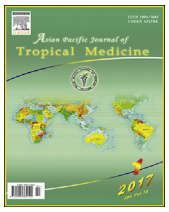

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Antileishmanial activities of caffeic acid phenethyl ester loaded PLGA nanoparticles against *Leishmania infantum* promastigotes and amastigotes *in vitro*

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ABSTRACT

Objective: To investigate and compare the antileishmanial effects of CAPE and $(CAPE)_{PLGA\ NPs}$ on *Leishmania infantum* (*L. infantum*) promastigotes and amastigotes *in vitro*.

Methods: Efficacies of CAPE, $(CAPE)_{PLGA\ NPs}$ and free PLGA nanoparticles (NPs) on promastigotes were evaluated using MTT and promastigote count assays, and their anti-amastigote effects were determined via infection index analysis. Griess reaction was also performed to calculate nitric oxide production of macrophages exposed to investigated molecules.

Results: It was determined that CAPE and $(CAPE)_{PLGA\ NPs}$ demonstrated significant inhibitory effects on *L. infantum* promastigotes and amastigotes, while free NPs did not exhibit any meaningful antileishmanial effectiveness. The IC_{50} values of CAPE for *L. infantum* promastigotes and amastigotes were assessed as (51.0 ± 0.8) and (19.0 ± 1.4) μg/mL, respectively ($P < 0.05$). On the other side, it was revealed that $(CAPE)_{PLGA\ NPs}$ had superior antileishmanial activity on both forms of parasites since its IC_{50} values for *L. infantum* promastigotes and amastigotes were (32.0 ± 1.3) and (8.0 ± 0.9) μg/mL, respectively ($P < 0.05$). It was also determined that both agents strongly stimulated nitric oxide production of macrophages.

Conclusions: The obtained results show that $(CAPE)_{PLGA\ NPs}$ have a great potential to be especially used in treatment of visceral leishmaniasis; however, *in vivo* antileishmanial screening of these molecules should be performed in the near future.

1. Introduction

Leishmaniasis, which is considered as one of the most important neglected diseases, is categorized as a Class I disease (uncontrolled and emerging) by the World Health Organization [1]. It has the second highest prevalence rate within endemic parasitic diseases behind that of malaria. The disease has widely distributed to five continents, and it is estimated that it is endemic in 98 countries [2]. It is also predicted that every year, 500 000 new cases of visceral leishmaniasis (VL) and 1 500 000 new cases of cutaneous leishmaniasis (CL) will occur globally which of the two is the main clinical form of the disease [3,4]. According to WHO records, 350 million people are living under the threat of leishmaniasis [5]. Due to the lack of an effective vaccine [6], the adverse impact of rapidly developed global warming [7], climate changes [8], wars and migration movements [9,10], more people may be affected from leishmaniasis in a near future, which is a frightening prospect.

Since there is no available effective and safe vaccine against leishmaniasis, treatment options are the only way to combat the disease. However, none of the current antileishmanial drugs are considered ideal medications because of their identified disadvantages, including high toxicity, expensiveness, long therapy duration and severe side effects [11–13]. On the other hand, recently evolved resistance of the *Leishmania* parasites to pentavalent antimonials are used as the gold standard in chemotherapy on the infection reveals that there is an immediate need, even a necessity, to develop new and effective antileishmanial formulations to cope with the infection [14,15].

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Drug delivery systems have recently taken the attention of researchers to develop new formulations against infectious diseases. These systems provide an opportunity to transport expected substances to targeted cells, tissues, or organs with high efficiency [16]. Due to the prevention of dispersion of drug molecules into undesired regions of the body and the cargo of desired dosages of the drugs into targeted areas, can make the therapy more effective and successful [17]. On the other hand, delivery systems may decrease the required dosages of the drugs in order to eliminate microorganisms residing within the tissues. In this way, drug toxicity may be diminished, and drug resistance may be prevented since there is no requirement to use high concentrations of drugs, as overdosing is one of the main reasons for evolved resistance [18–21]. Poly lactic-co-glycolic acid (PLGA) nanoparticles are among the most valuable and significant representatives of such drug delivery systems. These FDA-approved polymers are widely used in drug delivery systems thanks to their special features, such as biodegradability, biocompatibility, and large surface areas [22,23]. Further, due to their capacity to allow sustained drug release, and the possibility of surface modifications, PLGA nanoparticles are more convenient to use for drug delivery compared to bulk sized particles [24,25]. According to their specific properties, PLGA nanoparticles are determined to be promising agents for the treatment of infectious diseases and cancers [26,27]. In recent studies, it was further demonstrated that PLGA nanoparticles containing antileishmanial drugs, such as amphotericin B and artemisinin, were very efficacious in eliminating *Leishmania* parasites both *in vitro* and *in vivo* [28,29]. Since VL affects internal organs by infecting macrophages, blood, kidneys and spleen cells, it is thought that controlled and effective delivery of drugs by PLGA nanoparticles may contribute to developing new antileishmanial drug systems for the successful treatment of VL.

Caffeic acid phenethyl ester (CAPE) is known as one of the most active components of honeybee propolis extracts [30]. Their anticancerogenic, anti-inflammatory, antibacterial, and immunomodulatory effects have been demonstrated in various studies [31–34]. Possible antimicrobial action mechanisms of CAPE are predicted to relate to the disruption of DNA, RNA, and outer membranes of microorganisms [35,36]. There is also evidence available in the literature that exhibits their roles in impairing RNA polymerase enzymes and leading to oxidative stress within microbial cells [37–39]. It is thought that the presence of multiple attacking sites within microorganisms makes CAPE a strong antibacterial agent. Despite the fact that there are many studies in literature that demonstrate the antibacterial effects of CAPE on several bacteria, such as *Escherichia coli*, *Salmonella*, *Pseudomonas*, to our knowledge its antiparasitic, especially its antileishmanial effects have not been investigated until now.

On the other side, encapsulation of CAPE into PLGA nanoparticles was successfully achieved in previous studies [40,41]. In one of these researches, Arasoglu demonstrated that CAPE-encapsulated PLGA nanoparticles $(CAPE)_{PLGA\ NPs}$ provide high and sustained inhibitory efficacies on *Escherichia coli*, *Staphylococcus aureus*, and MRSA, while CAPE alone did not show any remarkable changes on the survival of these bacteria. These results displayed that PLGA nanoparticles lead to the time-dependent release of CAPE molecules, which increases their antibacterial effectiveness considerably [41]. Moreover, it was revealed that encapsulation into PLGA nanoparticles altered the size of the CAPE molecules and thereby improved the amounts of the accumulated substances within the bacterial cells. According to these features, systems based on CAPE-loaded PLGA nanoparticles may be also valuable for inhibiting intracellular infectious agents just like *Leishmania* parasites. By considering the high antimicrobial capacity of CAPE and the success of PLGA nanoparticles on the delivery of drug molecules into desired sites of body with high efficiency, we believe that $(CAPE)_{PLGA\ NPs}$ can be further used in treatment of VL. To support this hypothesis, we investigated the vitro antileishmanial performance of $(CAPE)_{PLGA\ NPs}$ on *Leishmania infantum* (*L. infantum*) parasites, the main causative agents of VL.

The main objective of the present study was to investigate the antileishmanial effects of CAPE and $(CAPE)_{PLGA\ NPs}$ on promastigote and amastigote-macrophage cultures of *L. infantum in vitro*, compare their efficacies, and develop a new approach based on PLGA nanoparticulate drug delivery systems in the treatment of VL.

2. Materials and methods

2.1. Leishmania parasite culture

Leishmania tropica (MHOM/TR/99/EP39) promastigotes were cultured in a Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with L-glutamine (Sigma Chemical Co., St. Louis, MO), 10% fetal bovine serum (FBS) (Sigma Chemical Co.), and Gentamicin (80 μg/mL) (Sigma Chemical Co.) at 27 °C. The passages of promastigote culture were maintained at fourth days of incubation. An inverted microscope (Olympus CKX 41) was used to monitor the daily growth of *L. tropica* promastigotes.

2.2. J774 macrophage cell culture

For cytotoxicity assays and to obtain a amastigote-macrophage culture, a murine macrophage cell line (J774) was used. J774 cells were cultured in a RPMI 1640 medium enriched by 10% fetal bovine serum (FBS), 2 mM L-glutamine, 80 μg/mL Gentamicine, and 1 M HEPES at 37 °C and 5% CO_2. The growth of the cells were observed every day by an inverted microscope, and the culture was passaged and added into fresh media when macrophages confluented 80%–90% culture flasks.

2.3. Preparation of the CAPE encapsulated PLGA nanoparticles

$(CAPE)_{PLGA\ NPs}$ used in the present study for screening antileishmanial effects were synthetized in a previous study prepared by Derman *et al*. Briefly, nanoparticles are synthetized by using an oil-in-water emulsion evaporation method. Characterization of synthetized nanoparticles was performed using the Dynamic light scattering technique, Scanning Electron Microscopy, and Fourier Transform Infrared Spectroscopy analysis. It was determined that $(CAPE)_{PLGA\ NPs}$ possessed 89% ± 3% encapsulation efficiency, (−34.4 ± 2.5) mV zeta potential, and (163 ± 2) nm particle size. Synthetized and characterized powder nanoparticles were solved with phosphate buffer solution (PBS), and a 1 mg/mL stock solution was prepared. Before its use, the solution was sonicated and filtered using 22 μm filters for sterilization.

2.4. Determination of antileishmanial effects on *L. infantum* promastigotes

2.4.1. Proliferation assay

L. infantum promastigotes (1×10^6/mL) at stationary phase were suspended in 5 mL RPMI 1640 media enriched with 10% FBS and then added into falcon tubes. Following one night of incubation at 27 °C, different concentrations of free NPsAPE and $(CAPE)_{PLGA\ NPs}$ ranging between 5 and 1000 μg/mL were added into test tubes. All the tubes were incubated at 27 °C for 120 h. Antileishmanial efficacies of all the analyzed concentrations were determined at the end of 120th h of exposure by counting the numbers of viable promastigotes in the test tubes with hemocytometer. For that purpose, a 100 μL *L. infantum* promastigote culture was obtained from all test tubes and fixed with 2% formalin at a ratio of 1:1. Then, 50 μL of suspensions were put into a hemocytometer, and the slide was investigated in an inverted microscope. IC_{50} values of all the samples were determined by finding the concentration that inhibited half of the promastigotes. Antileishmanial efficacies of samples were compared according to their IC_{50} values at the end of the 120th h of exposure.

2.4.2. Metabolic activity evaluation

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays were performed to consider the antileishmanial effects of free NPs, CAPE, and $(CAPE)_{PLGA\ NPs}$ on the metabolic activities of *L. infantum* promastigotes. For that purpose, 1×10^6 stationary-phased *L. infantum* promastigotes were seeded into each well of 96-well microplates. After overnight incubation at 27 °C, the promastigotes were exposed to different concentrations of analyzed test samples that ranged between 5 and 1 000 μg/mL. The group that was exposed to neither nanoparticles nor CAPE was considered the control. The microplate was incubated for 96 h. Following incubation, 10 μL MTT salt (10 μg/mL) were included in each well of the microplate, and the parasites were incubated for 4 h. Then, all of the wells were observed in an inverted microscope to detect the emergence of formazan crystals. After the detection, the crystals were dissolved by adding 100 μL of DMSO into each well and the plate was held in the dark at room conditions for 30 min. Following incubation, the absorbance values of each well were read by an ELISA reader at 570 nm.

2.5. Determination of antileishmanial effects on *L. infantum* amastigotes

2.5.1. Cytotoxicity assay

Prior to investigating the antileishmanial effects of samples on *L. infantum* amastigotes, an experiment was performed to evaluate the cytotoxicities of the specimens on J774 macrophage cells, since amastigote-infected macrophage culture is used to detect anti-amastigote efficacies. For that purpose, 1.5×10^4 J774 macrophages cells cultured in a RPMI 1640 medium supplemented by 10% FBS were seeded on 96 well-microplates. Cells were incubated in a humidified atmosphere at 37 °C and 5% CO_2 overnight. Afterwards, various concentrations of the free NPs, CAPE, and $(CAPE)_{PLGA\ NPs}$ (1 μg/mL to 1000 μg/mL) were individually added into each well. The microplate was incubated at 37 °C for 72 h. After that, cytotoxicity screening was conducted using MTT reaction, which was mentioned above in detail. IC_{50} and IC_{90} values were assessed by determining the concentration of the sample that led to death of the macrophage cells at a 50% and 90% ratio, respectively.

2.5.2. Infection index analysis

In order to screen the anti-amastigote effectivenesses of specimens, the infection indexes of macrophages were evaluated. Therefore, J774 macrophage cells were infected with *L. infantum* promastigotes at first to compose amastigote-macrophage culture systems. For that purpose, 3×10^5 J774 macrophage cells were seeded into 6-well plates including coverslips and incubated overnight at 37 °C. After detecting that all cells were totally confluent, 3×10^6 *L. infantum* promastigotes were included in each wells to provide infection of macrophages with parasites in a ratio of 1:10. The plate was held at 37 °C for 4 h, and during this period, the infection process was maintained. After 4 h of incubation, every well was washed with PBS, and fresh media was added to remove non-infective promastigotes from the medium. Afterwards, the infected macrophages were incubated at 37 °C for 48 h for the *L. infantum* amastigotes to proliferate within macrophages. After 48 h of incubation, the amastigote-macrophage culture was ready to investigate the anti-amastigote efficacies of the samples.

Therefore, J774 macrophages infected with amastigotes were treated with non-toxic concentrations of free NPs, CAPE, and $(CAPE)_{PLGA\ NPs}$ determined during the cytotoxicity analysis performed on the macrophages. Then, infected macrophages were again incubated at 37 °C for 96 h. Following this incubation, a medium including different concentrations of each sample was distracted and all of the wells were rinsed with 500 μL PBS three times. For fixation, the cells were respectively exposed to 500 μl PBS-methanol (1:1.5) solution and 250 μL methanol for 10 min. After that, all wells were again washed with methanol and macrophages were stained with 200 μL Giemsa for 3 min. Thereafter, each well was quickly washed with PBS to remove the stain. Later, coverslips on which macrophages were attached, were removed from the plate and placed onto slides to observe the propagates in an inverted microscope at 100 × dimensions.

During microscopic observation, the numbers of infected macrophages were counted, and % infectivity was assessed for each sample by dividing the numbers of infected macrophages by the uninfected macrophages. Afterwards, the *L. infantum* amastigotes that were determined within the vacuoles of infected macrophages were counted, and the average number of amastigotes per one macrophage was calculated. Infection index rates of macrophages were assessed by multiplying % infectivity values with the average amastigote numbers per a single macrophage. Therefore, the anti-amastigote efficacies of each sample at different concentrations were comparatively investigated in regard to their varieties in the infection index.

2.6. Nitric oxide screening

In the present study, we also investigated the effects of free NPs, CAPE, and $(CAPE)_{PLGA\ NPs}$ on the production of nitric oxide by J774 macrophage cells. For that reason, 3×10^4 J774 macrophage cells were seeded into each well of a 96-well microplate. Following overnight incubation, non-toxic concentrations of the analyzed samples that were established earlier were exposed onto macrophages, and the cells were incubated at

37 °C for 96 h. After that, supernatants of the macrophage cells were picked from all the wells and transferred into eppendorf tubes that were separated for each group. A Griess reaction was performed to assess the nitric oxide amounts of macrophages treated with different concentrations of the samples. Therefore, at first, the Griess reactant was prepared by solubilizing 0.1 g N-(1-Naphthyl)Ethylenediamine and 1 g Sulfanilamide within 20 mL distilled water, including 2.5 mL of phosphoric acid. On the other hand, standard solutions were prepared for determining the nitric oxide amounts in terms of ng/mL. Hence, 0.345 g Sodium Nitrite was dissolved within 10 mL distilled water to prepare a stock solution with final concentrations of 500 μM. Thereafter, the stock solution was diluted for preparing sub-concentrations of sodium nitrite varying from 100 μM to 0.5 μM.

After preparing the Griess reactant and sodium nitrite solutions, a 50 μL sample was taken from the standard and all test specimens and transferred into a 96-well microplate. Later, 50 μL Griess reactive was included in all wells, and the microplate was held in the dark at room temperature for 10 min. Finally, optical density in every well was read at 540 nm, and the generated nitric oxide amounts by macrophages were calculated in terms of nmol/mL based on the standard solutions.

2.7. Statistical analysis

All experiments were repeated at least three times. The results were expressed as mean ± SD. Statistical Packages of Social Sciences (SPSS 16.0 version for Windows) software with parametric tests (unpaired samples *t*-test, analysis of variance, and Mann–Whitney *U* test) was used fort the statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Proliferation of L. infantum promastigotes

Figure 1 represents the proliferation rates of the *L. infantum* promastigotes that were exposed to different concentrations of free NPs, CAPE, and $(CAPE)_{PLGA\ NPs}$, *in vitro*. It was demonstrated that in all the investigated concentrations, CAPE and $(CAPE)_{PLGA\ NPs}$ decreased the proliferation rates of *L. infantum* promastigotes when compared to the control that were exposed to neither nanoparticles nor CAPE. On the other hand, free PLGA nanoparticles did not lead to any statistically meaningful decrease in the proliferation values even if they were used at high concentrations, such as 500 and 1000 μg/mL. It was determined that $(CAPE)_{PLGA\ NPs}$ were more efficacious against promastigotes than was the use of CAPE alone ($P < 0.05$). The IC_{50} values of $(CAPE)_{PLGA\ NPs}$ and CAPE for *L. infantum* promastigotes were evaluated as (32.0 ± 1.3) μg/mL and (51.0 ± 0.8) μg/mL, respectively ($P < 0.05$). On the other side, the IC50 value of free PLGA nanoparticles was determined as >1000 μg/mL. Moreover we detected that exposure to 200 μg/mL $(CAPE)_{PLGA\ NPs}$ completely killed *L. infantum* promastigotes, while the concentration of CAPE molecules allowed for a total inhibition of promastigotes found as 500 μg/mL. These results demonstrated that $(CAPE)_{PLGA\ NPs}$ displayed a superior antileishmanial effect on the proliferations of *L. infantum* promastigotes than CAPE alone did ($P < 0.05$).

3.2. Determination of metabolic activity

Metabolic activity screening was performed in two ways, namely, microscopic examination and optical density assessment. In Figure 2, microscopic views of parasite clusters that were exposed to different concentrations of NPs, CAPE and $(CAPE)_{PLGA\ NPs}$ such as 5, 50 and 100 μg/mL. When parasites were exposed to investigate three samples at concentration of 5 μg/mL, it was observed that parasite clusters generated high amounts of formazan crystals which are the signs of viability and metabolic activity (Figure 2A, B and C). This demonstrates that free NPs, CAPE and $(CAPE)_{PLGA\ NPs}$ did not cause to any toxic effects on *L. infantum* promastigotes when they were applied at low concentrations. Similarly, *Leishmania* parasites that were exposed to 50 μg/mL and 100 μg/mL of free NPs generated purple colored formazan crystals indicating that parasites are alive (Figure 2D and G). On the other hand, 50 μg/mL of CAPE and $(CAPE)_{PLGA\ NPs}$ inhibited the production of formazan crystals and only dead parasite clusters were seen during the microscopic examination (Figure 2E and F). Likewise, parasites did not produce formazan crystals when they were

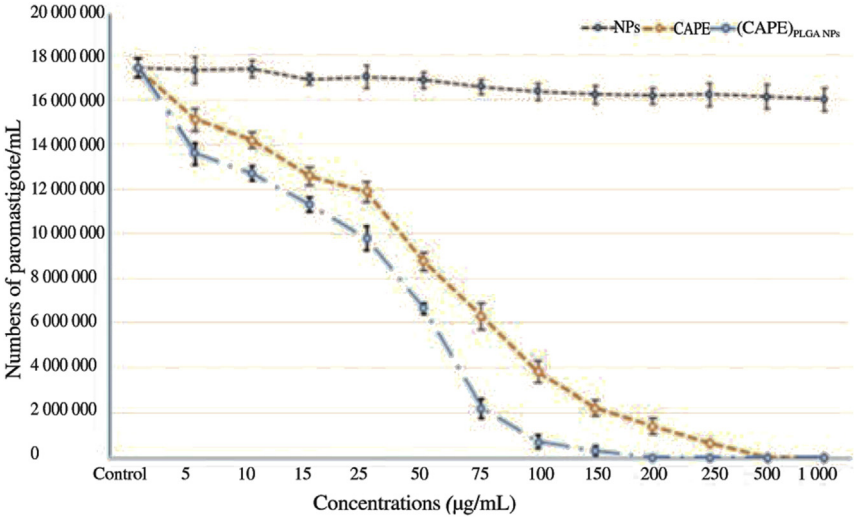

Figure 1. Antileishmanial effects of NPs, CAPE, and $(CAPE)_{PLGA\ NPs}$ on the proliferations of *L. infantum* promastigotes *in vitro*. The IC_{50} values were determined as 32.0 ± 1.3, 51.0 ± 0.8 and >1000 μg/mL for $(CAPE)_{PLGA\ NPs}$, CAPE and free NPs, respectively.

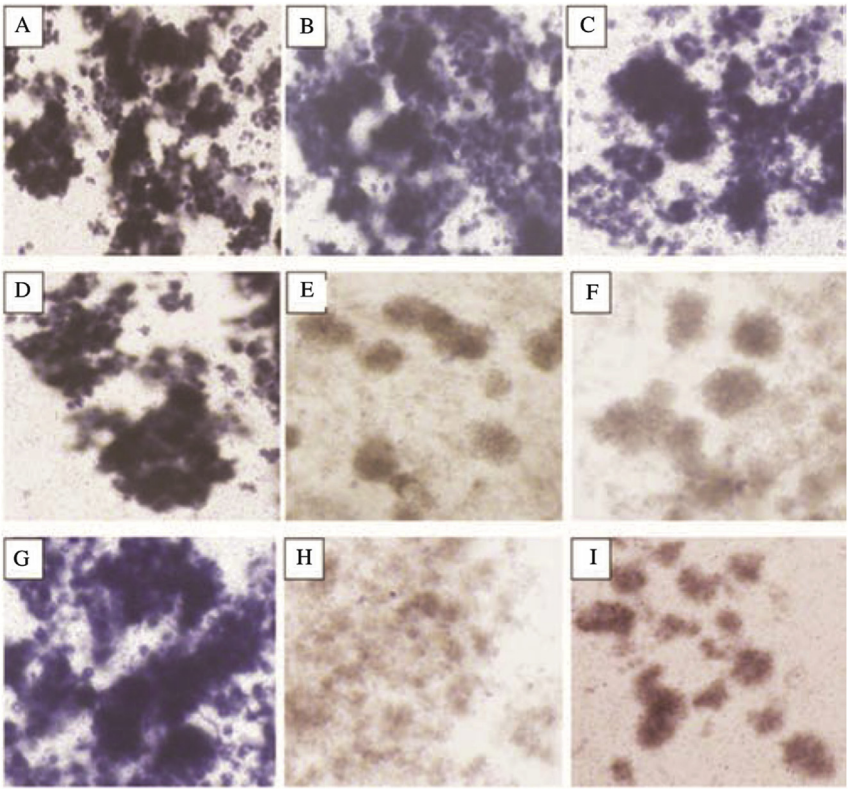

Figure 2. Microscopic examination of metabolic activities of *L. infantum* promasitgotes (×100).
(A) 5 μg/mL free NPs and the production of formazan crystals, (B) 5 μg/mL free CAPE and the production of formazan crystals, (C) 5 μg/mL $(CAPE)_{PLGA\ NPs}$ and the production of formazan crystals, (D) 50 μg/mL free NPs and the production of formazan crystals, (E) 50 μg/mL free CAPE and the lack of formazan crystals, (F) 50 μg/mL $(CAPE)_{PLGA\ NPs}$ and the lack of formazan crystals, (G) 100 μg/mL free NPs and the production of formazan crystals, (H) 100 μg/mL free CAPE and the lack of formazan crystals, (I) 100 μg/mL $(CAPE)_{PLGA\ NPs}$ and the lack of formazan crystals.

exposed to 100 μg/mL of CAPE and $(CAPE)_{PLGA\ NPs}$, respectively (Figure 2H and I). Microscopic examination reveal that application of CAPE and $(CAPE)_{PLGA\ NPs}$ at 50 and 100 μg/mL concentrations were efficacious in inhibiting metabolic activity of *L. infantum* promastigotes, while parasites that were treated with free nanoparticles at same concentrations were metabolically active.

When we evaluated the optical density values, it was determined that CAPE and $(CAPE)_{PLGA\ NPs}$ possessed inhibitory activities on the metabolic activities of *L. infantum* promastigotes, while free nanoparticle application did not cause any changes according to the investigated concentrations. Figure 3 shows that the effectivenesses of CAPE and $(CAPE)_{PLGA\ NPs}$ enhanced was due to the increase in concentrations. We considered that $(CAPE)_{PLGA\ NPs}$ were more efficacious than use of CAPE alone since $(CAPE)_{PLGA\ NPs}$ decreased the viability of parasites in a range between 1, 6 and 25 folds, while CAPE alone diminished metabolic activity values 9-fold at most ($P < 0.05$). At high concentrations, such as 500 and 1000 μg/mL, $(CAPE)_{PLGA\ NPs}$ totally inhibited the metabolic activities of the *L. infantum* promastigotes. These results verified the data obtained from the proliferation assay.

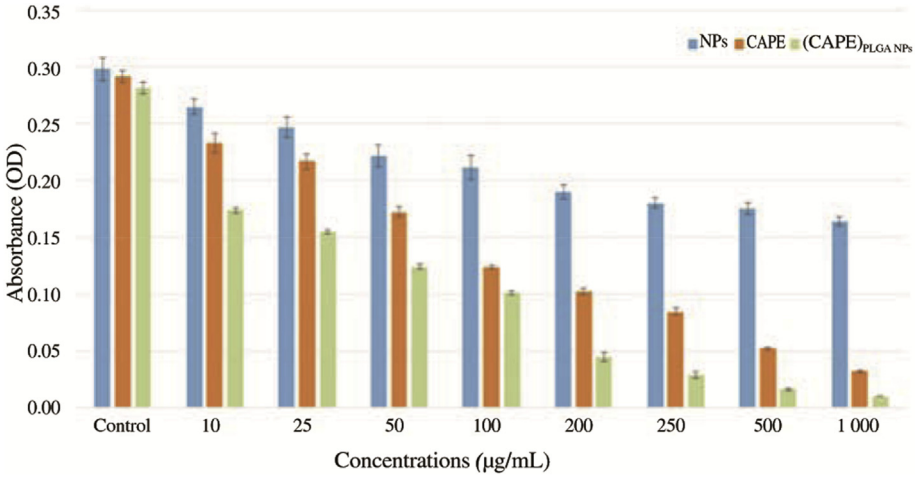

Figure 3. Evaluations of antileishmanial effects of NPs, CAPE, $(CAPE)_{PLGA\ NPs}$ on the metabolic activity rates of *L. infantum* promastigotes *in vitro*. The most powerful effect was determined in the group exposed to $(CAPE)_{PLGA\ NPs}$.

3.3. Cytotoxicity assay

In Table 1, the IC_{50} and IC_{90} values of NPs, CAPE, and $(CAPE)_{PLGA\ NPs}$ for J774 macrophage cells is shown. The IC_{50} values of CAPE and $(CAPE)_{PLGA\ NPs}$ were calculated as (42.0 ± 1.3) and (53.0 ± 2.7) μg/mL, respectively. The concentrations of CAPE and $(CAPE)_{PLGA\ NPs}$ up to 30 μg/mL were evaluated as non-toxic for macrophages, and these concentrations were also found to be convenient to use in the anti-amastigote screening assay. Exposure to concentrations higher than 30 μg/mL sharply declined the viability values of the macrophage cells. In concentrations higher than the IC_{50} values, macrophages were obviously detected to lose their compact structure and did not generate formazan crystals. On the other hand, the use of free PLGA nanoparticles did not cause any cytotoxic effect even when high concentrations like 500 and 1000 μg/mL were used. Microscopic investigation also verified the biocompatibility of PLGA nanoparticles since the macrophages in all test groups that were exposed to different concentrations of free nanoparticles were alive and generated high amounts of formazan crystals.

Table 1

Determination of IC_{50} and IC_{90} concentrations of free $(CAPE)_{PLGA\ NPs}$, CAPE, and $(CAPE)_{PLGA\ NPs}$ for J774 macrophage cells, *in vitro* (μg/mL).

Groups	NPs	CAPE	$(CAPE)_{PLGA\ NPs}$
IC_{50}	>500	42.0 ± 1.3	53.0 ± 2.7
IC_{90}	>1000	>1000	>500

3.4. Anti-amastigote effect screening

The *in vitro* antileishmanial efficacies of investigated samples at non-toxic concentrations on *L. infantum* amastigotes were determined by evaluating the infection index rates and a determination of IC_{50} values. Table 2 represents the infection index rates of amastigote containing macrophages that were treated with NPs, CAPE, and $(CAPE)_{PLGA\ NPs}$. In this experiment, the non-toxic concentrations of CAPE and $(CAPE)_{PLGA\ NPs}$, that were determined on J774 macrophage culture, varied between 5 and 30 μg/mL were used. Infection index rates of macrophages in the control group that were treated with neither nanoparticles nor CAPE were assessed as (407.0 ± 7.6). Furthermore, it was observed that free NPs demonstrated no efficacy on *L. infantum* amastigotes since the infection index values of macrophages that were exposed to nanoparticles alone were calculated in a range between 350 and 390. Since PLGA nanoparticles are biocompatible and non-toxic for macrophages, we explored their anti-amastigote activity at high concentrations like 500 μg/mL. However, it was also detected that free PLGA nanoparticles showed no antileishmanial activity on *L. infantum* amastigotes even if they were used in high concentrations. Accordingly, IC_{50} values of PLGA nanoparticles on *L. infantum* amastigotes were determined as >500 μg/mL. On the other hand, Table 2 demonstrates that CAPE and $(CAPE)_{PLGA\ NPs}$ had a superior impact on *L. infantum* amastigotes than the free nanoparticles. Infection index values of macrophages that were exposed to CAPE at non-toxic concentrations were evaluated in a range between (312.0 ± 5.1) and (142.0 ± 3.2) changing due to an increase in concentrations ($P < 0.05$). This result indicates that CAPE showed an anti-amastigote effect by decreasing the numbers of amastigotes within macrophages at 3-folds in contrast to the control ($P < 0.05$). IC50 value of CAPE on *L. infantum* amastigotes was calculated as (19.0 ± 1.4) μg/mL. On the other side, $(CAPE)_{PLGA\ NPs}$ exhibited nearly two times higher anti-amastigote efficacy than CAPE since its IC_{50} value was evaluated as (8.0 ± 0.9) μg/mL ($P < 0.05$). It was detected that the infection values of $(CAPE)_{PLGA\ NPs}$ were in a range between (240.0 ± 6.9) and (77.0 ± 4.1) and it was augmented in direct proportion to enhanced concentrations ($P < 0.05$). CAPE-encapsulated PLGA nanoparticles diminished the numbers of amastigotes within macrophages at approximately 6-fold in contrast to the control when it was applied at 30 μg/mL. This result means that $(CAPE)_{PLGA\ NPs}$ at 30 μg/mL inhibited more than 80% of the amastigotes hosted in macrophages.

3.5. Evaluation of nitric oxide production

Since superior concentrations of CAPE and $(CAPE)_{PLGA\ NPs}$ lead to remarkable toxicity on the macrophages, we evaluated the stimulatory effectivenesses of non-toxic concentrations of these molecules on nitric oxide production of macrophages that were treated with them. The amounts of generated nitric oxide by macrophages that were exposed to different concentrations of free NPs, CAPE and $(CAPE)_{PLGA\ NPs}$ are represented in Table 3. As it can be seen, investigating all samples significantly induce

Table 2

Determination of infection index rates of macrophages infected by *L. tropica* promastigotes and exposed to different concentrations of free NPs, CAPE, and $(CAPE)_{PLGA\ NPs}$.

Groups	Control	5 μg/mL	10 μg/mL	15 μg/mL	20 μg/mL	30 μg/mL
NPs	407.0 ± 7.6	392.0 ± 7.0	383.0 ± 7.2	376.0 ± 7.3	369.0 ± 7.4	364.0 ± 7.8
CAPE	410.0 ± 7.3	312.0 ± 7.1	275.0 ± 7.2	236.0 ± 7.1	195.0 ± 7.4	142.0 ± 7.2
$(CAPE)_{PLGA\ NPs}$	418.0 ± 2.7	240.0 ± 6.9	192.0 ± 4.0	155.0 ± 3.9	124.0 ± 3.7	77.0 ± 4.1

Table 3

Determination of nitric oxide amounts (ng/mL) produced by macrophages after their exposure to NPs, CAPE, and $(CAPE)_{PLGA\ NPs}$ at different concentrations ranging between 5 and 30 μg/mL.

Groups	Control	5 μg/mL	10 μg/mL	15 μg/mL	20 μg/mL	25 μg/mL	30 μg/mL
NP	3.80 ± 0.25	4.50 ± 0.24	5.70 ± 0.42	7.80 ± 0.51	10.10 ± 0.47	12.90 ± 0.51	15.30 ± 0.42
CAPE	3.50 ± 0.19	4.00 ± 0.24	4.90 ± 0.46	5.70 ± 0.37	7.30 ± 0.49	7.90 ± 0.43	9.90 ± 0.57
$(CAPE)_{PLGA\ NPs}$	3.70 ± 0.23	5.30 ± 0.22	8.20 ± 0.14	10.80 ± 0.19	14.70 ± 0.37	18.50 ± 0.27	21.90 ± 0.22

macrophages to produce high amounts of nitric oxide. Furthermore, the most effective stimulatory effect was observed when macrophages were exposed to 30 μg/mL $(CAPE)_{PLGA\ NPs}$. It was determined that the amounts of nitric oxide produced by macrophages exposed to $(CAPE)_{PLGA\ NPs}$ were 6.5 fold higher than for the control groups ($P < 0.05$). On the other hand, exposure to CAPE and free nanoparticles alone increased the produced amounts of nitric oxide 3 and 4 fold, respectively, in contrast to the control ($P < 0.05$).

4. Discussion

For the first time, in this study, the antileishmanial effects of CAPE and $(CAPE)_{PLGA\ NPs}$ were comparatively investigated on *L. infantum* promastigote and amastigote-macrophage culture systems using different parameters, such as proliferation screening, metabolic activity determination, infection index analysis, and stimulated nitric oxide evaluation, *in vitro*. It was determined that both of the investigated samples demonstrated significant inhibitory effects on *L. infantum* promastigotes and amastigotes, while free nanoparticles did not exhibit any meaningful antileishmanial effectiveness. The IC_{50} values of CAPE for *L. infantum* promastigotes and amastigotes were assessed as (51.0 ± 0.8) and (19.0 ± 1.4) μg/mL, respectively ($P < 0.05$). On the other side, $(CAPE)_{PLGA\ NPs}$ had superior antileishmanial activity on both forms of parasites since its IC_{50} values for *L. infantum* promastigotes and amastigotes were evaluated as being (32.0 ± 1.3) and (8.0 ± 0.9) μg/mL, respectively ($P < 0.05$). Furthermore, it was also determined that both agents strongly stimulated nitric oxide production of macrophages since $(CAPE)_{PLGA\ NPs}$ elevated nitric oxide production approximately 6.5 folds, while CAPE alone increased by 3 folds in contrast to the control.

The results obtained from the present study indicate that the CAPE molecule has high antileishmanial effects on both forms of *L. infantum* parasites and its efficacy is significantly enhanced by its encapsulation into PLGA nanoparticles. CAPE, which is isolated from honeybee propolis extracts, is a bioactive molecule, since its antibacterial, anticancerogenic, antiviral, anti-inflammatory, and immunomodulatory activities have recently been demonstrated [42–45]. On the other hand, its antileishmanial activities have been primarily shown in the current study. We think that this remarkable antileishmanial efficacy directly relates to the action mechanisms of the CAPE molecule.

In various studies that have investigated the antimicrobial efficacies of CAPE, it has been discussed that CAPE possesses different action mechanisms against microorganisms, such as impairing DNA, RNA or enzymes, producing high amounts of free radicals or arresting cellular cycles, even though its precise mode of action has not yet been discovered [35,38,39,46]. These action mechanisms are also effective against *Leishmania* parasites, and indeed, many antileishmanial drugs, such as miltefosine, pentamidine, paromomycine and sitamaquine use these pathways to eradicate parasites and show antileishmanial activity [47–50]. *Leishmania* parasites are also known to be very sensitive to the generation of large amounts of reactive oxygen species which lead to apoptosis following oxidative stress which is one of the predicted action mechanisms of CAPE against microorganisms [51,52]. Therefore, it is not surprising for us to have detected high antileishmanial activities of CAPE that have the potential to attack multiple targets that are also specific for antileishmanial drugs for eradicating *Leishmania* parasites.

In the present study, we also discovered that CAPE loaded PLGA nanoparticles were 1.6 times more efficacious against promastigotes and 2.4 times against amastigotes in contrast to the use of CAPE alone. This result may be explained by the specific features of PLGA nanoparticles when delivering drug molecules. It is known that PLGA nanoparticles are very successful in preventing drugs from enzymatic degradation, transporting them to desired sites, and also providing their controlled release [53–55]. In terms of the present study, it can be said that PLGA nanoparticles mediated to a sustained release of CAPE molecules, and this caused the long-term exposure of parasites to CAPE. Therefore, the amounts of CAPE released from PLGA nanoparticles gradually increased, and this release provided for more effective killing of *Leishmania* amastigotes and promastigotes than direct exposure to CAPE. Studies indicating the elevated antimicrobial effects of drug-entrapped PLGA nanoparticles involved with the sustained release of drug molecules are also present in this literature.

In a recent study, Kolate *et al.* synthetized PLGA nanoparticles containing netilmicin sulfate (NS), a highly water soluble antibiotic, and compared their antibacterial performance with free NS on *Pseudomonas aeruginosa* bacteria. It was determined that after an extended period of examination, NS-encapsulated PLGA nanoparticles strongly inhibited the growth of the bacteria, while the antibacterial efficacies of a free drug was significantly diminished at the end of this period. This result reveals that NS-encapsulated PLGA nanoparticles had superior antibacterial effects to a free drug [56]. In another study, Darvishi compared the antibacterial effectivenesses of 18-β-glycyrrhetinic acid (GLA) which is an effective antimicrobial substance isolated from the rhyzomes of *Glycyrrhiza glabra* plant and its formulations prepared by encapsulating into PLGA nanoparticles on *Pseudomonas aeuroginosa, Staphylococcus aureus and Staphylococcus epidermidis*. It was noted that GLA-encapsulated PLGA nanoparticles demonstrated much more inhibitory effects on each bacterial strain than did pure GLA, and this finding was explained by penetration and the accumulation of many more drug molecules within the microbial cells when NS were entrapped into PLGA nanoparticles [57]. In a similar way, we think that encapsulation of CAPE molecules into PLGA nanoparticles may increase the accumulated amounts of the molecule within *Leishmania* parasites, which may cause the death of high numbers of *Leishmania* parasites.

On the other side, the stronger anti-amastigote activity of CAPE-encapsulated PLGA nanoparticles than a free CAPE can be explained by high adsorption capacity of PLGA nanoparticles and their ability to penetrate cellular membranes of mammalian cells thanks to their special features that come from nano sizes [58]. In a similar study, Guedj *et al.*, investigated the interactions of bovine serum albumine (BSA)-loaded PLGA nanoparticles with THP-1 type macrophages. They found that the accumulated BSA amounts within macrophages significantly increased when they were encapsulated into nanoparticles than for the use of BSA alone. This result indicates the uptake of PLGA nanoparticles into macrophages has high efficiency. Furthermore, in some studies, it has been emphasized that decreasing the size of PLGA to nano levels sharply diminished negative charges of polymeric surface, which induces the interactions of polymeric nanoparticles with macrophage membranes that own low

polarity. In this way, the uptake of PLGA nanoparticles into macrophages is very easy when compared to microparticles, and therefore, PLGA nanoparticles are considered to be more effective and successful systems in the delivery of drugs into macrophages. Our results also verified the success of PLGA nanoparticles in the cargo of CAPE into macrophages. It is predicted that PLGA nanoparticles augment the amounts of CAPE molecules accumulated within macrophages and thus enhances their anti-amastigote activities more than the use of CAPE alone.

Other important data obtained from this study indicated that both CAPE and CAPE encapsulated PLGA nanoparticles demonstrated superior antileishmanial effects on amastigotes to promastigotes. In various studies, Leishmania amastigotes were found to be more susceptible to drug molecules than promastigotes were, and this situation can be further explained by the variations in structural, biochemical, and morphological features of each parasitic form [59–61]. It can also be thought that exposure to CAPE and PLGA nanoparticles stimulated macrophages to produce lytic enzymes, reactive oxygen species, or nitric oxide. Due to these facts, the investigated molecules may explore more inhibitory effects on amastigotes [62]. In order to verify this opinion, we assessed the nitric oxide production of macrophages after their exposure to free NPs, CAPE, and $(CAPE)_{PLGA\ NPs}$. We determined that all three samples increased the amount of nitric oxide, which is known as the most important antileishmanial agent generated by macrophages [63]. The capacity of PLGA nanoparticles to stimulate macrophages to produce nitric oxide was also demonstrated in a recent study prepared by Derman *et al* [58]. On the other hand, high stimulatory efficacies of the CAPE-encapsulated PLGA nanoparticle for nitric oxide production is primarily shown in the current study.

Consequently, for first time in this study, we demonstrated that the CAPE molecule has a high impact on the biological parameters of Leishmania parasites, such as proliferation, metabolic activity, infection index, and its efficacy significantly is enhanced by its encapsulation to PLGA nanoparticles due to the sustained release characteristics of nanoparticulate delivery systems. Especially, the emergence of the superior effects of CAPE-encapsulated PLGA nanoparticles on the intracellular stage forms of *L. infantum* parasites is promising for further studies targeting the development of new drug delivery systems for the treatment of visceral leishmaniasis. We think a conjugation of these nanoparticles with macrophage specific ligands may lead to more effective targeting of *Leishmania* amastigotes and may indeed increase the success of the treatment. Further still, *in vivo* antileishmanial screening of these molecules should be performed in the near future.

Conflict of interest statement

We declare that we have no conflict of interest.

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