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Original Article

Quercetin and Nano Quercetin: Cytotoxicity, Antileishmanial and Antimicrobial Activities against Resistance Strains

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Abstract

Background: Quercetin, a natural polyphenolic flavonoid compound, showed high anti-cancer, anti-bacterial, and anti-viral activities. Given the importance of microbial diseases, the lack of definitive treatment for many of them, and the emergence of drug resistance, it is essential to use various natural compounds to investigate their antimicrobial effects. We aimed to assess the anti-bacterial and anti-leishmanial activity *in vitro* and *in vivo* study.

Methods: Anti-leishmanial effects of quercetin and nano-quercetin were evaluated on promastigote and amastigote stages *in vitro*. The minimal inhibitory concentrations (MICs) were determined by the broth dilution method using six species of clinical pathogenic bacteria strains, including *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Campylobacter jejuni*, and *Pseudomonas aeruginosa*. Furthermore, the cytotoxicity effects of the drugs were evaluated using MTT assay.

Results: All tested compounds presented anti-leishmanial and anti-microbial properties. Gram-negative bacteria were more resistant than gram-positive bacteria. Quercetin and nano-quercetin in concentrations of 200 and 400 µg/ml showed similar effectiveness on promastigote and amastigote of *L. infantum* in comparison to positive controls. In all experiments, nano-quercetin was more effective than quercetin. Moreover, no cytotoxicity activity was observed on Normal mouse fibroblast cell line (L929) *in vitro*.

Conclusion: Nano-quercetin and even quercetin had excellent anti-microbial and anti-parasitic effects, and given that no toxicity was observed from these compounds even at higher concentrations, these compounds can be used as a suitable alternative for the treatment of parasitic and microbial diseases.

Introduction

Infection is one of the most important factors that endanger human life and cause great economic, social, and psychological damages (1). With the advancement of pharmaceutical science, many anti-microbial drugs and compounds have been produced or discovered (2). Most of the drugs have excellent effects (3, 4) but, many drugs have lost their beneficial effects over time, and resistance to these drugs has now emerged. Due to these defects, searching for a new, effective, accessible and cost effective anti-microbial drugs is crucially essential (5). Leishmaniasis is one of the most important protozoan parasitic diseases which cause a wide spectrum of manifestations including cutaneous, mucocutaneous, and visceral forms in humans and in some reservoir hosts (6). Up to now, there is not a proven vaccine for leishmaniasis, and chemotherapy by pentavalent antimonials is the last choice of treatment (7). *Escherichia coli*, *Salmonella typhi* are the most important bacterial infection causing prolonged diarrhea in human and animals. Among bacterial infections, the highest drug resistance was reported in *E. coli* and *S. typhi*, and many mechanisms of resistance were predicted (8, 9). Given the emergence of drug resistance, which can be very important and can lead to occur major epidemics, pharmaceutical researchers should consider an alternative drug for many infectious diseases (10, 11).

Natural products are branded as the single foundation of medications by traditional people for decades and are used as the most important remedies in Africa, Asia and even in developed countries (12, 13). Several products presented anti-microbial effectiveness but, approving a medicinal plant and/or natural products as an anti-microbial treatment is puzzling and necessitates more calculation comprising efficacy and safety prior to clinical study (13, 14).

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a flavonoid found in fruits and vegetables as a natural product in the human diet, which is useful in improving mental and physical health and diminishing the rate of infection(15). Of more than 4000 available natural phenolics in plants, quercetin is a unique and most plentiful flavonol, with an extensive spreading as quercetin glycosides (16). Berries, apples, Brassica vegetables, onions, grapes, capers, tea, shallots, and tomatoes, as well as many nuts, seeds, barks, flowers, and leaves are the main source of quercetin and quercetin glycosides (17, 18). Many unique biological properties such as anti-cancer, platelet aggregation, ability to inhibit lipid peroxidation, capillary permeability, antioxidant, anti-inflammatory, and anti-microbial were reported by this brilliant compound (19). Quercetin showed anti-inflammation activity mainly by maintaining the stability of mast cells, decreasing the production of COX and LOX, and inhibiting the release of cytokines. It also plays anti-cancer roles by arresting the cell cycle of cancer cells with a decrease in Cyclin D and E and an increase in Cyclin B and cause apoptosis by decreasing Bcl-$_{XL}$, Bcl-2, and Mcl-1 and increasing Bax and BAD (20). Nevertheless, no accurate pharmacological evaluation does not exist on the characterized seeds oil.

Thus, the primary objective of conducting this study was the evolution of the anti-microbial, anti-parasite and cytotoxic activities of quercetin.

Materials and Methods

The Research and Ethics Committee of the Shahid Beheshti University of Medical Sciences, Laser Application in Medical Sciences Research Center has approved this study (IR.SBMU.LASER.REC.1403.001).

Preparation, Charatrization of quercetin and nano-quercetin

Quercetin was purchased from Xi'an Senmu Biological Technology Co Ltd (Xi'an, China), and nano-niosomes were prepared by

the thin-film hydration technique. The hydrophobic phase was dissolved in a mixture of ethanol and chloroform, and a uniform layer was prepared by drying in a rotary evaporator, and was hydrated by a hydration medium contained hyaluronan, Tween 80, and quercetin using a rotary evaporator (60 °C at 270 rpm). Then the prepared dispersion probe sonicator was used for 8 minutes (60s on, 60 s off). Purification was performed by centrifugation and purified modified nano-niosomes were used for further analysis (21).. The shape of modified nano-niosomes was analyzed by TEM and SEM (ZEISS, Germany).

Parasites

L. infantum (MCAN/IR/07/Moheb-gh) was provided by the Department of Parasitology, Iran University of Medical Sciences, Iran. Briefly, promastigote forms at stationary phase were cultured in RPMI medium supplemented with 10% fetal bovine serum (Gibco-USA), 100U/mL of penicillin (Gibco, USA), and 100 µg/mL of streptomycin (Sigma, USA) at 26°C and sub-cultured to increase the number of parasites in the stationary growth phase (22).

Anti-promastigote Assay

L. infantum promastigote (100µl, 1×10^5 parasite) were placed in 96-well plates with different concentrations of quercetin and nano-quercetin (100– 400 µg/mL) glucantime (300 µg/mL) and Miltefosine (10 mg/mL), at a final volume of 200 µL per well for 72 h. The colorimetric method with tetrazolium-dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were employed to assess the viability of the parasites. After incubation time, 10 µL of MTT (5 mg/mL) was added to each well, the culture medium was removed after five hours and 150 µL of DMSO was added to dissolve the formazan crystals. Absorbance was read using a spectrophotometer at a wavelength of 570 nm. The data was normalized, with the absorbance value of control used as 100%, and the results were used to calculate the 50% inhibition of parasite growth (IC_{50})(23).

Anti-leishmanial activity against intracellular amastigotes

J774 cell line were incubated in DMEM complemented with 10% heat-inactivated FBS in 5 % CO2 at 37 °C without phenol red. 10^5/ml of stationary phase of promastigotes were added to the J774 cells in eight-well chamber slides, incubated at 37 °C in CO2 5% for 24 hours. The infection of monolayers with promastigotes were performed at a range of infection of 5:1 (parasite/macrophage) after 24 h of adhesion and growth on the chamber slides, and they were incubated in 5% CO2 for overnight (at 37 °C). After incubation time, the wells were washed using PBS until removal of non-phagocytosed promastigotes. The quercetin in different concentrations were added, after 48h the chamber slides were fixed by absolute methanol and stained with Giemsa 4%. The percentage of infected macrophages were counted and the calculation of inhibition percentage was calculated (23). The IC_{50} was calculated from the total intracellular amastigote from 200 cells. Amphotericin B was used as the positive control. The selectivity index (SI) was obtained from the ratio of BALB/c peritoneal macrophages CC_{50} and intracellular amastigote IC_{50} (23).

Bacterial Culture

Bacterial reference strains including *B. cereus* (ATCC 13640), Staphylococcus *aureus* (ATCC 12600), *Listeria monocytogenes* (ATCC 7644), *Escherichia coli* (ATCC 25922), *Campylobacter jejuni* (ATCC 49943), and *Pseudomonas aeruginosa* (ATCC 27853) were cultured in Brain Heart Infusion (BHI) broth for 24h at 37 °C and diluted to 10^8 CFU/mL following the MacFarland scale (24).

Antimicrobial Assays

One mL of each bacterial suspension was homogeneously spread on a solid growth medium in a Petri dish. Impregnated filter paper

with 50 µL of each concentration of quercetin and nano-quercetin were placed on the surface of each agar plate, and the plates were incubated at 35 °C, and after 24 hours, the inhibition halo was measured with an electronic digital caliper. Disks impregnated with sterile distilled water and ethanol served as negative controls, and the disk with glucantime, and Miltefosine antibiotic served as a positive control. Broth dilution methodology was used to measure the minimum inhibitory concentration (MIC). Serial dilutions of quercetin and nano-quercetin were prepared from the concentrations of 2000 to 5 µg/mL. $1.5x10^8$ CFU/mL of the Microbial suspensions added at each well incubated at 35°C for 24 h. Tubes without treatment are used as a control for bacterial growth, and Tubes without bacteria were used as a control of broth sterility. BHI broth was used to confirm growth inhibition (25).

Cytotoxicity assay

Normal mouse fibroblast cell line (L929) was purchased from Pasteur Institute of Iran and was cultured in RPMI-1640 medium with 10% bovine fetal serum (FBS) and L-glutamine at the rate of 100 units/mL at 37°C, 5% CO2, and humidity of 95%. To assess cell cytotoxicity, 1×10^4 cells (200 µl) were added to every 96 well plates and incubated for 24h. 100µl of the different concentrations (100, 200, and 400 mg/ml) of quercetin and nano-quercetin were then added to each well and stored at 37 ºC for 24h. Wells without treatment and Cis-Platine were considered as negative and positive controls, respectively. After incubation time, the culture media was removed, 10 µL of MTT (5 mg/mL) was added to each well, and after five hours, 150 µL of DMSO was added to dissolve the formazan crystals. Absorbance was read using a spectrophotometer at a wavelength of 570 nm (26-28) .

Anti-leishmania activity on promastigote and amastigote

Fig. 2 shows the leishmanicidal activity of quercetin, nano-quercetin, glucantime, and Miltefosine against promastigote of *L. infantum* at concentrations of 100, 200, and 400 µg/ml after 3, 12, 24, and 48 hours. The effects of these treatments were dose and time-

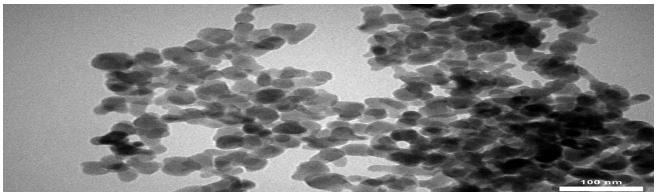
Statistical Analysis

The numerical outcomes were showed as mean ± standard deviation and were organized into tables and/or graphs. The IC_{50} was achieved from a nonlinear regression curve of concentration log versus normalized response. One-way ANOVA and Tukey's multiple comparisons test and GraphPad Prism 7.00 software were applied to comparison the IC_{50} values. All differences less than $p<0.05$ were considered significant.

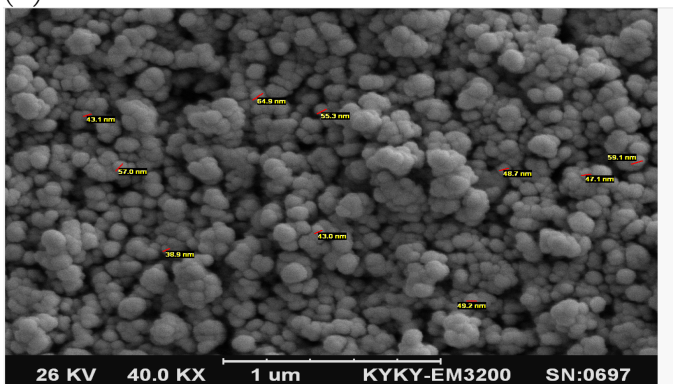
Results

Characterization of Quercetin Nanoparticles

According to the representative TEM and SEM micrograph, Quercetin Nanoparticles appeared spherical with sizes of about 70-150 nm (Fig. 1).



(A)



(B)

Fig. 1: A representative TEM (A) and SEM (B) micrograph to assess the size and morphology of Quercetin Nanoparticles. (magnification: ×100,000)

dependent. Anti-leishmanial efficacy was significantly different in the treated groups compared to the PBS as negative controls ($P < 0.001$). Positive controls in this study (glucantime, and Miltefosine) showed better leishmanicidal activity than quercetin, nano-quercetin, and nano-quercetin was better than quercetin, but the differences were not significant ($P = 0.072$ and $P = 0.063$).

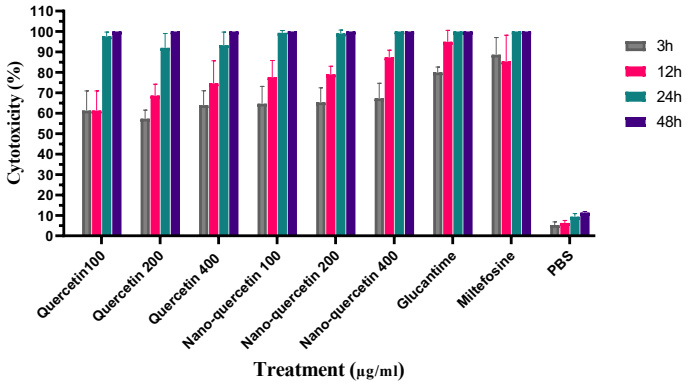


Fig. 2: Anti-promastigote activity of quercetin and nano-quercetin against *L. infantum* at concentrations of 100, 200 and 400 µg/ml in comparison to the glucantime, and Miltefosine as positive controls and PBS as negative control after 3, 12, 24 and 48 hours

According to Fig. 3, the IC50 value of the quercetin, nano-quercetin, glucantime, and Miltefosine were evaluated against *L. infantum* amastigotes, and our results indicated that nano-quercetin was the most effective treatment in all concentration even better than positive controls, since its IC50 values were obtained to be 31.5, 27.3, and 20.4 µg/ml after 12, 24, and 48 hours, respectively.

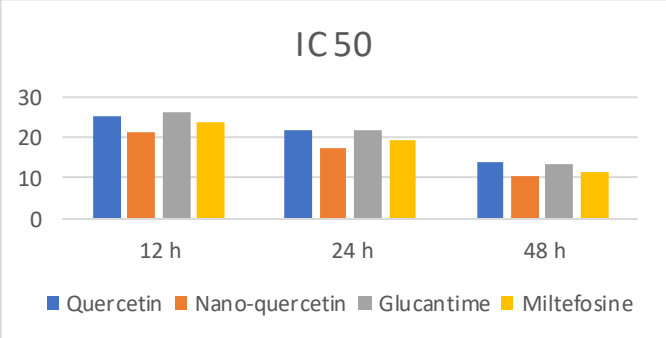


Fig. 3: The IC50 values of quercetin and nano-quercetin against *L. infantum* in comparison to the glucantime, and Miltefosine as positive controls and PBS as negative control after 12, 24 and 48 hours

Evaluation of MIC values determined by different methods

The anti-microbial activity (MIC values) of quercetin and nano-quercetin were determined against *S. aureus*, *E. coli*, and *P. aeruginosa* bacteria, and the broth dilution method was applied to find the ability of bacteria to produce visible growth during incubation with quercetin and nano-quercetin treatments. MIC values as determined by the broth dilution method

ranged from 0.1 to 12.8 mg/ml for quercetin, nano-quercetin, oxytetracycline, and nalidixic acid and our results revealed that MIC values for Quercetin were from 0.8 to 1.6 against gram-negative bacteria, including *E. coli*, *P. aeruginosa* and *C. jejuni*. Gram-positive bacteria were more susceptible than gram-negative bacteria with the MIC value of 0.2- 0.4 mg/ml. But nano-quercetin had better antibacterial effects than quercetin alone. The MIC in gram-negative bacteria was between 0.4 and 0.8 mg/ml, and in contrast, it was between 0.2 and 0.4 mg/ml for gram-positive bacteria (Fig. 4). In all experiments, positive controls showed better efficacy but were not statically significant (0.001).

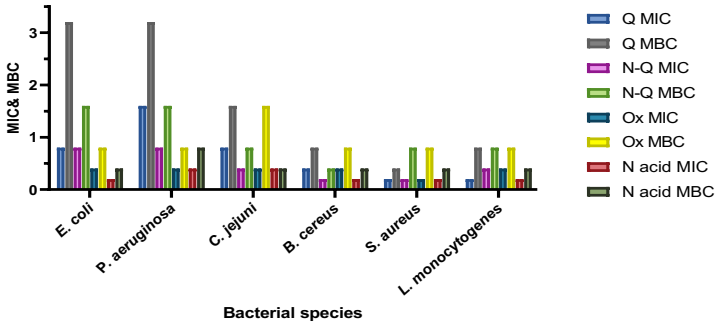


Fig. 4: Antimicrobial activity of quercetin and nano-quercetin expressed as MIC (mg/mL) determined by the broth macrodilution methods for gram-positive and gram- negative bacteria

Cell cytotoxicity efficacy

The cytotoxic effect of quercetin and nano-quercetin on normal mouse fibroblast cell line (L929) was determined using MTT assay at concentrations of 800 µg/ml after 48 h of treatment in comparison to the Cis-Platine as positive control and non-treated groups. The results indicated that quercetin and nano-quercetin have 93.59 and 94.37% cytotoxicity on L929 Cell Line whereas Cis-Platine showed 23.46% cytotoxicity effects (Fig. 5).

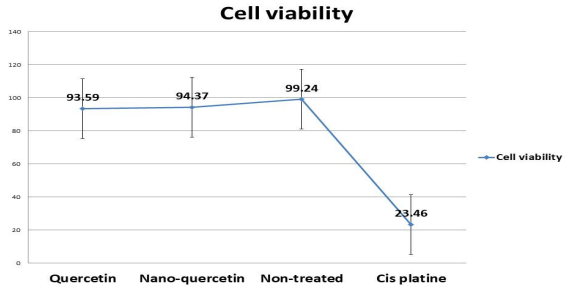


Fig. 5: Effects of quercetin, nano-quercetin on viability of L929 Cell Line at concentration of 800 µg/ml

Discussion

In the present study, the leishmanicidal and bactericidal effects of quercetin and nanoquercetin at concentrations of 100, 200, and 400 µg/ml were investigated in vitro and the brilliant effects were obtained. Moreover, it showed that quercetin and nanoquercetin exhibited a selective action against parasites without any cytotoxicity effects on host cell viability. In the last decades, there has been particular attention to the consumption of plentiful naturally arising anti-microbials such as plants, spices, and herbs (29). There are numerous natural products that have biological functions for clinical uses, and some of them are now approved and using as treatments for several diseases (2, 30). Quercetin is from the flavonoid group of polyphenols and is one of the most important plant molecules with a wide range of activities by several mo-

lecular pathways and mechanisms effects which we too experienced during our work (31). Hyun Kim et al. determined the anti-viral effectiveness of quercetin by a plaque-reduction assay and reported that the quercetin displayed potent anti-viral activities against both HCMV and VZV, with estimated IC50 values of 5.931 ± 1.195 and 3.835 ± 0.56 μg/mL, respectively. Quercetin has been reported to inhibit HSV-1 infection in the early stages of the viral life cycle by suppressing NF-κB activation in infected cells and reduce HSV-1-induced interferon (IFN) regulatory factor 3 (IRF3) activation (32).

Quercetin is a potential anti-bacterial agent for periodontitis such as *Aggregatibacter actinomycetemcomitans*, *Actinomyces viscosus*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Actinomyces naeslundii* with IC50 of 625, 1250, 1250, 625, and 2500 μg/mL, respectively (33). Recently, several kinds of literature proved that quercetin has anti-bacterial and anti-viral activity, and researchers of the current study were encouraged to explore other effects of this natural compound (34, 35). The anti-microbial activities of quercetin and nano-quercetin were determined and were similar to the described works in current study; the MIC values were ranged from 0.8 to 1.6 mg/ml for gram-negative and 0.4 and 0.8 mg/ml for gram-positive bacteria with no statistical significance to positive controls (P=0.071).

Based on the many published studies, anti-cancer drugs are also being effective against trypanosomatid parasites, and it has been hypothesized that cancer cells and trypanosomatid parasites share various biochemical similarities which affect protein kinases pathways, DNA metabolism, polyamines metabolism, and glucose catabolism enzymes (36). Suramin, Melarsoprol, and eflornithine are the most famous anti-cancer drugs that are also used for trypanosomatid parasites. On the other hand, pentamidine as an approved treatment for several diseases caused by protozoan parasites such as babesiosis, pneumocystosis, leishmaniasis, and trypanosomiasis has been recently reported as a worthwhile agent in cancer chemotherapy (36).

This has prompted researchers to assess the anti-*leishmania* effect of quercetin and nano-quercetin on promastigote and amastigote stages. Our results indicated that quercetin and nano-quercetin at concentrations of 200 and 400 μg/ml had leishmanicidal activity similar to oxytetracycline and nalidixic acid as standard positive controls. Non-toxicity, inexpensiveness, eco-friendliness, and suitability for pharmaceutical and biomedical applications are the vital aspects of drugs, and quercetin and nano-quercetin showed no toxicity on normal mouse fibroblast cell line, and our results were consistent with the earlier reports. Researchers found that quercetin has excellent anti-oxidant effects *in vitro*, which can reduce the expression of TGF-β1 and α-SMA through the construction of the BDL model, prevent oxidative stress, and have neuroprotective effects (37). Moreover, Salehi et al. indicated no toxicity or side effects of quercetin in a clinical trial on the human population (38). Our *in vitro* studies showed that quercetin and nano-quercetin could significantly inhibit bacterial growth and kill *Leishmania* promastigotes and amastigotes. These drugs are effective against gram-positive and gram-negative bacteria, and gram-positive bacteria were more susceptible than gram-negative.

Conclusion

The anti-leishmanial activity of quercetin and nano-quercetin were similar against promastigotes but amastigotes were more susceptible to nano-quercetin during incubation. Finally, our results indicated that quercetin and nano-quercetin could be useful for bacteria and *Leishmania* with minimum toxicity.

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Conflicts of interest

The authors declared that there is not any conflict of interest.

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