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Evaluation of the *in vitro* and *in vivo* inhibitory effects of quercetin on the growth of *Babesia* and *Theileria* parasites

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A B S T R A C T

Quercetin has antioxidant, anti-inflammatory, antitrypanosomal, and antimalarial activities. The hindering properties of quercetin were appraised for three *Babesia* species and *Theileria equi in vitro* and against *Babesia microti* in mice. Quercetin exhibited substantial growth restraint for *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi* with IC_{50} values of 8, 7, 5, and 4 nM, respectively. *T. equi* growth was suppressed at 50 µM concentration. Parasite regrowth was repressed in the consequent viability test at 100 µM for *Babesia bovis* and *Babesia bigemina* and 50 µM for *Babesia caballi* and *Theileria equi*. Quercetin at a dose rate of 14.5 mg/kg raised a 77.5 % reticence of *Babesia microti* progression in BALB/c mice. Quercetin might be of value as a treatment in babesiosis and theileriosis.

Keywords: Quercetin; *Babesia; Theileria equi; In vitro; In vivo*

1. Introduction

Piroplasmosis affected bovine and equines. Hard tick is the spreader of the infection. *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi* are the most important cause of piroplasmosis in bovine and equine hosts all over the world. The infection results in heavy fiscal damages in the global cattle industry. The clinical signs include fever, malaise, hemolytic anemia, hemoglobinuria, and jaundice (Kuttler, 1988). *Babesia microti* is a rodent pathogen with zoonotic importance to humans in Europe and the USA (Genchi 2007). The control of infection centered on parasite identification and drug treatment. The offered drugs have some disadvantages such as venomousness to the host (Vial and Gorenflot, 2006). Therefore, the invention of novel effective chemotherapeutic drugs against babesiosis and theileriosis without harm to the hosts is highly desired.

Quercetin, a plant flavonoid, originates in various plants. Its abundance is high in, oats (*Avena sativa*), tea (in the form of tannins), cabbages (*Brassica* spp.), garlic (*Allium sativum*), onion (*Allium cepa*), pear, and spinach (Havsteen 2002). Quercetin, has antioxidant (Yu et al., 2016), neuroprotective (Dajas et al. 2015), anti-inflammatory (Rogerio et al. 2007), antitumor (Nair et al. 2004), bactericidal (Amin et al. 2015), anti-virus (Xu et al. 2000), antitrypanosomal (Tasdemir et al. 2006), anti-amoebic (Cimanga et al. 2006), antileishmanial (Tasdemir et al. 2006), and antimalarial (Lehane and Saliba 2008) activities. *Plasmodium*, *Babesia*, and *Theileria* have similarities; therefore, quercetin might have inhibitory effects on these blood parasites. The reason for this investigation was to appraise the inhibitory impacts of quercetin on the *in vitro* progression of four *Babesia* species and *T. equi* and on the *in vivo* development of *B. microti*.

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2. Material and methods

2.1. Chemical reagents

Quercetin was bought from Sigma-Aldrich (USA). A 100 mM stock solution in dimethyl sulfoxide (DMSO) was prepared and stored at −30 °C. Diminazene aceturate (Ganaseg) was bought from Ciba-Geigy Japan Ltd., Tokyo, Japan and used as an affirmative control drug. A functioning stock solution of 10 mM in double-distilled water (DDW) was prepared and stored at –30 °C until required for use.

2.2. Rodent Babesia and mice

B. microti (Munich strain) was retained by a successive passage in the blood of BALB/c mice (AbouLaila et al. 2010b). For the *in vivo* studies, 30 female BALB/c mice (CLEA Japan, Tokyo, Japan) of eight weeks old were used.

2.3. In vitro culture of Babesia parasites

Quercetin was valued for its chemotherapeutic effect against *T. equi* (U.S. Department of Agriculture) (Mehlhorn and Schein 1998), *B. caballi* (Aboulaila et al. 2012), *B. bovis* (Texas strain) (Hines et al. 1995), and *B. bigemina* (Argentina strain) (Bork et al., 2004). Parasites were cultured in erythrocytes of cattle or horses using a continuous micro-aerophilous stationary phase culture system (Bork et al., 2004). *B. bovis*, *B. bigemina*, and *T. equi* were cultured in medium M199 (Sigma-Aldrich, Tokyo, Japan) that was completed with 40 % equine or bovine serum and 0.15 µg/ml of amphotericin B (Sigma-Aldrich), 60 µg/ml of streptomycin, and 60 U/ml of penicillin G. Hypoxanthine, vital supplement to the *T. equi*, was added at 13.6 mg/ml (ICN Biomedicals, Inc., Aurora, OH). Medium RPMI 1640 was enriched with 40 % horse serum, amphotericin B, and antibiotics for *B. caballi* culture (Aboulaila et al. 2010c).

2.4.In vitro growth inhibition assay

The *in vitro* growth inhibition assay was done as formerly designated (Bork et al., 2004). Cultures of *B. bigemina*, *B. caballi*, *B. bovis*, and *T. equi* were adjusted to 1 % parasitemia by mingling infected RBCs with 5 % parasitemia with uninfected RBCs for the assays. 96-well plates were utilized for the growth inhibition assay. Each plate is containing 20 µl of red blood cells and 200 µL of a fitting medium containing 0.01, 0.1, 1, 5, 25, 50, and 100 µM for *B. bovis* and *B. bigemina* and 0.005, 0.05, 0.1, 1, 5, 25, and 50 µM for *B. caballi* and *T. equi* of quercetin. Diminazene aceturate at 5, 10, 50, 100, 1000 or 2000 nM was used as the positive control (Aboulaila et al. 2010a). Cultures containing no drug and with only the solvent (0.01 % DMSO, for quercetin and 0.02 % DDW, for diminazene aceturate) were prepared. The experiments were completed for three times in triplicate. Cultures were raised at 37 ° C in an atmosphere of 5 % CO_2, 5 % O_2, and 90 % N_2. For four days, the medium was relieved daily with 200 µl of fresh medium containing a suitable drug concentration. Giemsa-stained smears were completed every day to

Table 1: IC_{50} values of quercetin and diminazene aceturate for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*

	IC_{50} (nM) [a]	
	Quercetin	**Diminazene**
B. bovis	8 ± 2	300 ± 30
B. bigemina	7 ± 1	190 ± 20
B. caballi	5 ± 1	10 ± 2
T. equi	4 ± 0.5	710 ± 15
P. falciparum[b]	15000 ± 5000	ND
Cells		ND
MT-4[c]	47000	
L6 cells[d]	37100	

[a] IC_{50} values expressed as drug concentration are in nanomolar of the growth medium and were determined on day 4 of *in vitro* culture using a curve fitting technique. IC_{50} values represent the mean and standard deviation of 3 separate experiments. [b] Lehane and Saliba, 2008 [c]Cimanga et al. 2006 [d] Tasdemir et al., 2006 ND not determined

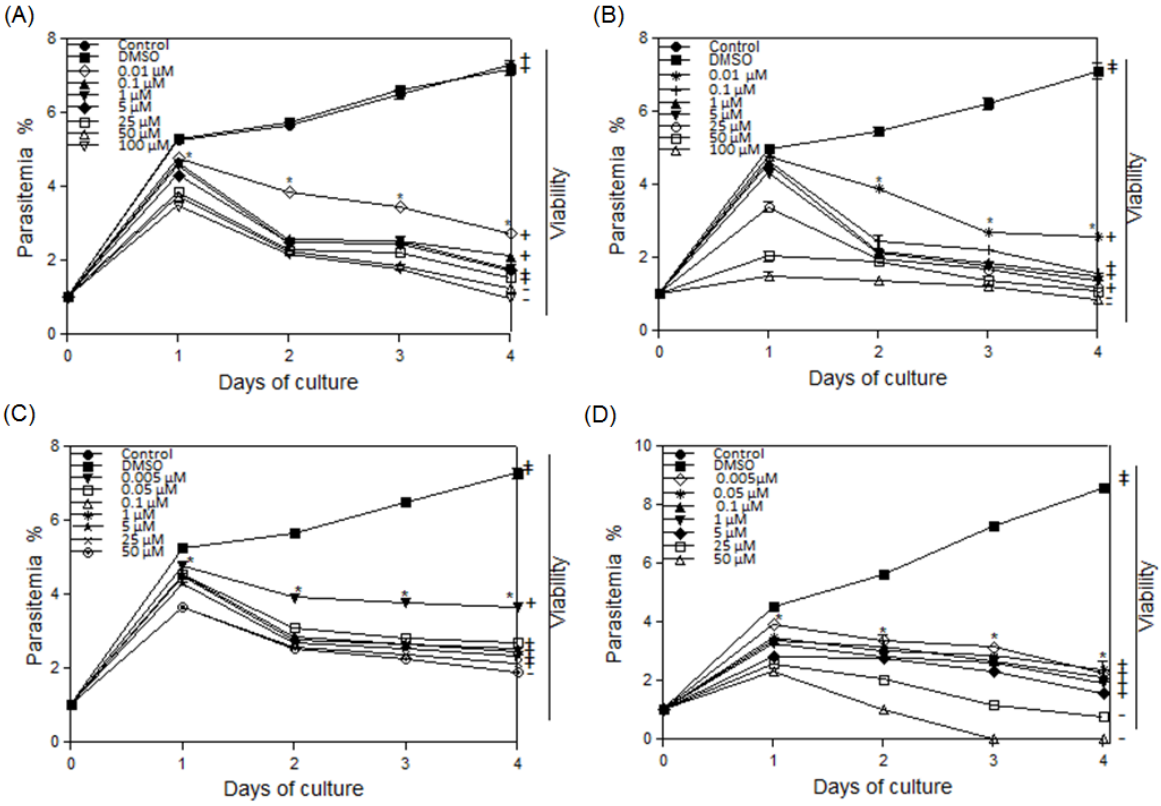

Figure (1): Inhibitory effects of different concentrations of quercetin on the *in vitro* growth. (A) *B. bovis*, (B) *B. bigemina*, (C) *B. caballi*, and (D) *T. equi*. Each value represents the mean ± standard deviation in triplicate. These curves represent the results of three experiments carried out in triplicate. Asterisks indicate a significant difference (* $P < 0.05$) between quercetin-treated and control cultures. Regrowth after 10 days was indicated as viable (+) and dead (-)

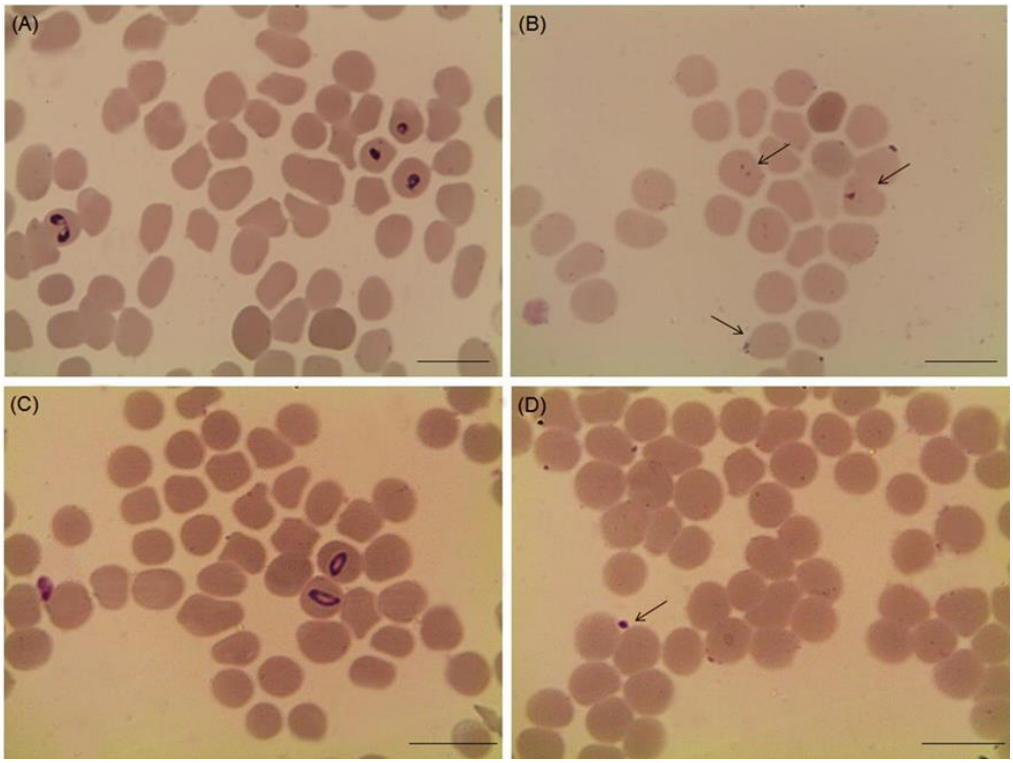

Figure 2: Light micrographs of *Babesia bovis* and *Babesia bigemina* treated with 25 µM quercetin in the *in vitro* cultures. (A) *Babesia bovis* control, (B) quercetin-treated cultures, (C) *Babesia bigemina* control, (D) quercetin-treated cultures. The drug-treated cultures showed higher numbers of degenerated parasites indicated by arrows than the control cultures. Micrographs were taken on day 4 of treatment. Scale bars, 10 µm

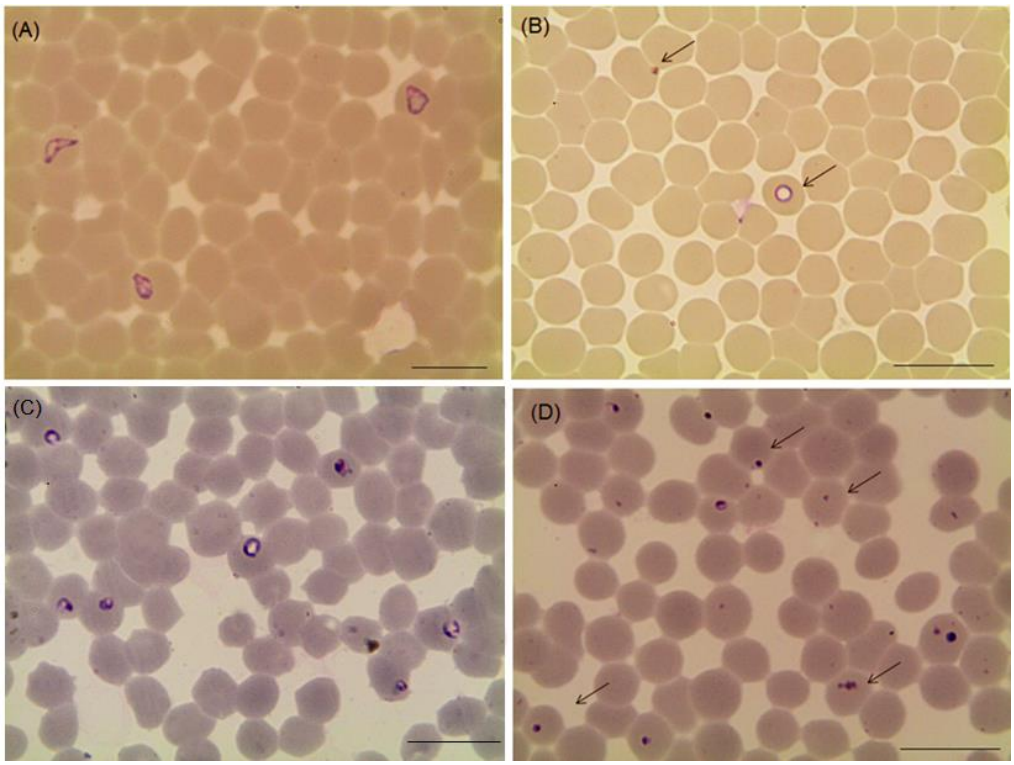

Figure 3: Light micrographs of *Babesia caballi* and *Theileria equi* treated with 25 µM quercetin in the *in vitro* cultures. (A) *Babesia caballi* control, (B) quercetin-treated cultures, (C) *Theileria equi* control, (D) quercetin-treated cultures. The drug-treated cultures showed higher numbers of degenerated parasites indicated by arrows than the control cultures. Micrographs were taken on day 4 of treatment. Scale bars, 10 µm

determine the parasitemia in 1,000 erythrocytes. Light microscopy was utilized to match the changes in the morphology of the treated parasites with that of the control. The 50 % inhibitory concentration (IC_{50}) values were valued by interpolation using the curve-fitting technique on the third day of *in vitro* culture (AbouLaila et al. 2010b).

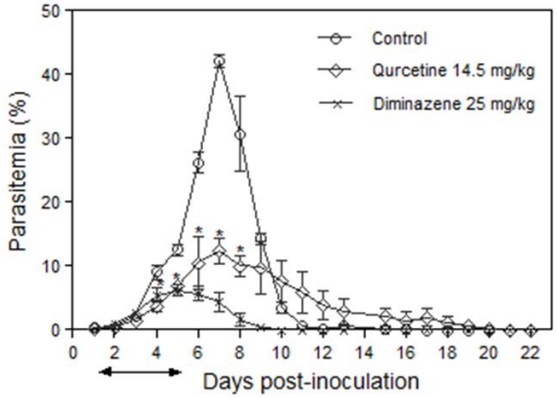

Figure 4: Inhibitory effects of I.P. quercetin 14.5 mg/kg and S.C. diminazene aceturate 25 mg/kg on the *in vivo* growth of *Babesia microti* for observations of five mice per experimental group. Each value represents the mean ± S.D for two experiments. Asterisks indicate a significant difference (* $P < 0.01$) from days 4 to 8 post-inoculation between quercetin-treated and dimethyl sulfoxide (DMSO) control group.

2.5. Viability test

After four days of remedy, 6 µL of fresh host erythrocytes was supplementary to 14 µL of full RBCs from the formerly drug-treated cultures in 200 µl of a medium free of the drug. The new growth medium was substituted daily for the succeeding 10 days, and parasite recurrence was observed daily after the exclusion of the medication by using a light microscope (Aboulaila et al. 2010c).

2.6. Effect of quercetin on host erythrocytes

The toxicity of quercetin to host erythrocytes was assessed as formerly designated(Aboulaila et al. 2010c). Bovine and equine erythrocytes were raised in the existence of 100 µM quercetin (the highest concentration used in this study) for 3 hours at 37 ˚C; then erythrocytes were cleaned thrice with media and were utilized for the parasites' cultures for 72 hours. The control untreated cells handled in the identical method as the pretreated cells. The outline of parasite growth in pretreated erythrocytes was witnessed and compared with control untreated cells.

2.7. In vivo growth inhibition assay

The quercetin *in vivo* inhibition assay for *B. microti* was performed twice as formerly designated (Aboulaila et al. 2012; Tasdemir et al. 2006). with some alterations. Three groups of five females BALB/c mice (8-week-old) were intraperitoneally injected with 1×10^7 *B. microti*-infected RBCs. When the inoculated mice had approximately 1 % parasitemia, mice were delivered daily injections for five days.

Drugs were melted in DMSO (3 % for quercetin) and DDW (12.5 % for diminazene aceturate), then thinned in PBS or DDW prior to inoculation. In the negative control, DMSO was given in PBS (0.03 %). In the first group, quercetin was injected intraperitoneally at a dose rate of 14.5 mg/kg in 0.3 ml of PBS (Aboulaila et al. 2012). A 0.3 ml PBS (0.03 % DMSO) was intraperitoneally injected to the control cluster. Diminazene aceturate (Ganaseg) at a dosage of 25 mg/kg was subcutaneously injected to the third experimental group in 0.1 ml DDW (Aboulaila et al. 2012). The parasitemia heights in all mice were checked every day until day 22 by inspection of 1,000 erythrocytes in smears made from the venous tail blood.

2.8. Statistical analysis

The differences in the parasitemia percentage for the *in vitro* and *in vivo* experiments were explored using JMP statistical software (SAS Institute, Inc., USA) by the Student's *t*-test. A *P* value of < 0.05 revealed statistical significance.

3. Results

In vitro growth inhibition assay

Quercetin significantly ($P < 0.05$) subdued the *in vitro* progress of the cultured parasites at 10 nM for *B. bovis* (Fig. 1A), *B. caballi* (Fig. 1C), and *T. equi* (Fig. 1D) at day 1 of remedy and *B. bigemina* at day 2 of remedy (Fig. 1B). After 10 days from drug removal in the viability test, no re-raise in parasitemia at concentrations of 50 µM for *B. caballi* and *T. equi* and 100 µM for *B. bovis* and *B. bigemina* (Fig. 1). Diminazene aceturate significantly repressed the *in vitro* development of the 4 parasites at the 5 nM concentration. Regrowth of *B. bigemina* and *B. caballi* was inhibited at a level of 100 nM diminazene aceturate while *T. equi* and *B. bovis* at 1 µM. While lower drug concentrations permitted regrowth in the ensuing viability test (data not shown). The values of 50 % inhibitory concentrations of quercetin and diminazene for different *Babesia* species are shown (Table 1). DMSO did not disturb the parasites' growth associated with the control cultures.

Quercetin Affected on the shape of treated parasites. The parasites appeared dot-shaped in quercetin-treated *B. bovis* culture (Fig. 2B) relative to normal parasites in the DMSO negative control culture (Fig. 2A). A similar effect was observed in quercetin-treated *B. bigemina* (Fig. 2D), *B. caballi* (Fig. 3B), and *T. equi* (Fig. 3D) cultures. Pretreatment of the cattle and horse erythrocytes at 100 µM quercetin, highest concentration, resulted in no deviations in the erythrocyte morphology and parasite growth for 3 days indifference to the normal erythrocytes (not shown).

In vivo effect of quercetin on B. microti infection

In vivo suppressive influence of quercetin was estimated for *B. microti* in mice. Quercetin significantly subdued the growth in treated mice in dissimilarity to the DMSO control group from day 4 to 8 p.i. (Fig. 4). Highest parasitemia points in the drugs injected groups reached an average of 6.2 % in the occurrence of 25 mg/kg diminazene aceturate at 5 days p.i. and 12.4 % in the presence of 14.5 mg/kg quercetin at 7 days p.i., compared with 42.12 % in the DMSO control group (DMSO) at 7 days p.i. (Fig. 4).

4. Discussion

Quercetin repressed the *in vitro* advance of *T. equi, B. caballi, B. bigemina*, and *B. bovis*. The control for the experiment confirmed that the outcomes were because of quercetin. Bovine *Babesia* was more sensitive to quercetin than *B. caballi* and *T. equi*.

The IC_{50} values of quercetin for *T. equi* and *Babesia* species were inferior to those of diminazene aceturate described in this study. The IC_{50} values of quercetin for *B. caballi*, *B. bovis*, *T. equi*, and *B. bigemina* were very small matched with those for *P. falciparum* was 15 µM (Lehane and Saliba 2008), *Encephalitozoon intestinalis* 15 µM (Mead and McNair 2006), *Entamoeba histolytica* 348.2 µM (105.2 µg/ml) (Cimanga et al. 2006), *Trypanosoma brucei* 27.5 µM (8.3 µg/ml) (Tasdemir et al. 2006) , *Trypanosoma cruzi* > 99.3 µM (>30 µg/ml) (Tasdemir et al. 2006), *Leishmania donovani* 3.31 µM (1 µg/ml) (Tasdemir et al. 2006). The quercetin IC_{50} values for *T. equi* and *Babesia* species were very small matched with those assessed as antibabesial drugs (AbouLaila et al. 2014; Aboulaila et al. 2012; Aboulaila et al. 2010a; AbouLaila et al. 2010b; Aboulaila et al. 2010c; Bork et al. 2004; Salama et al. 2013; Salama et al. 2014). The quercetin IC_{50} values for *T. equi* and *Babesia* species were smaller than the IC_{50} values of other commercial drugs: imidocarb dipropionate (Rodriguez and Trees 1996) and quinuronium sulfate (Brockelman and Tan-ariya 1991). Quercetin will be safe for curing piroplasmosis because the IC_{50} values of quercetin for *T. equi* and *Babesia* species are very low linked with the IC_{50} value of 122.8 µM (37.1 µg/ml) for L6 cells (Tasdemir et al. 2006) and 47 µM (14.2 µg/ml) for MT-4 cells (Cimanga et al. 2006).

Quercetin inhibits topoisomerases I (Das et al. 2006) and topoisomerase II (Cantero et al. 2006). Topoisomerase I gene was found in *B. bovis* (accession no.: XM_001609514) and *T. equi* (accession no.: XM_004828676). Moreover, DNA gyrase subunits A and B, a type II Topoisomerase, genes were found in the gene bank; *B. bovis* subunit A (accession No.: AAXT01000004) and subunit B (accession No.: XM_001611055) and *T. equi* subunit A (accession No.: CP001669) and subunit B (accession No.: XM_004833696). Therefore, growth inhibition might be a result of inhibition of parasite DNA topoisomerases I and/or II. Further study is necessary to explain the method of inhibition, especially after the characterization of the DNA topoisomerases from *Babesia* species and *T. equi.*

Quercetin showed well *in vitro* inhibitory effects for *Babesia* and *T. equi* and *in vivo* hindering properties on *L. donovani*, thus, we were encouraged to value the *in vivo* inhibitory outcomes of quercetin on *B. microti* in a mouse model. The inhibitory outcome of quercetin on the development of *B. microti* was evident. *B. microti*-infected mice, which were treated with 14.5 mg/kg had 70.6 % inhibition compared with 85.3% inhibition for diminazene aceturate. Mice in the quercetin-treated group did not show signs of toxicity and were active during and after the experiment, which agreed with a preceding study where quercetin at 75 mg/kg had no adverse effects (Molina et al. 2003). Quercetin enhanced lymphocyte propagation and phagocytosis by macrophages. Furthermore, it had effective immune-modulatory properties (Liang et al. 1997; Lyu and Park 2005) and it induces significant gene expression and production

of Th-1-derived interferon IFN-γ moreover declined Th-2-directed IL-4 production (Nair et al. 2002). Moreover, immunity to *Babesia* is of Th-1 type immune response which includes augmented manufacture of IFN-γ and IL-12 (Homer et al. 2000); therefore, quercetin might have an immunomodulatory influence on *Babesia* infection and may be utilized as a cure for such infection.

In conclusion, quercetin reserved the development of *Babesia* species and *T. equi in vitro* and the *in vivo* development of *B. microti* in BALB/c mice. Quercetin may be utilized as a drug for Babesiosis and Theileriosis.

Competing Interests

The authors have no conflict of interest.

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