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Luteolin: target validation in *Babesia bovis* by reverse transcription polymerase chain reaction and *in vivo* inhibition of *Babesia microti*

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Abstract

Luteolin, a naturally occurring plant flavonoid, has antioxidant, anti-amoebic, antitrypanosomal, and antimalarial activities. In this study, the inhibitory effects of luteolin were microscopically evaluated against three Babesia species and Theileria equi in vitro and against B. microti in mice. Reverse-transcription PCR was used to evaluate the effect of luteolin on transcription of DNA gyrase subunits A and B genes. Luteolin significantly inhibited the growth from an initial parasitemia of 1 % for Babesia bovis, Babesia bigemina, Babesia caballi, and Theileria equi with IC50 values of 81, 79, 90, and 99 nM, respectively. Parasite regrowth was inhibited at 100 µM in the subsequent viability test. Luteolin treatment of B.bovis cultures inhibited the transcription of the DNA gyrase subunit B gene. Luteolin at a dosage of 5 mg/kg resulted in a 77.5 % inhibition of Babesia microti growth in BALB/c mice. Luteolin might be used for drug therapy in babesiosis.

Key words: Luteolin, Babesia, Theileria equi, RT-PCR, In vitro, In vivo

Introduction

Babesia and Theileria are major protozoa infecting erythrocytes in animals. They are transferred by ticks to vertebrates and leads to serious economic losses to the farm animal business worldwide. The clinical signs embrace fever, hemolytic anemia, jaundice, and hemoglobinuria [1]. *Babesia microti* is a rodent Babesia with zoonotic importance for humans in North America [2]. The babesicidal remedies like diminazene aceturate and imidocarb dipropionate have adverse side effects related to their toxicity [3]. Therefore, the innovation of different medications that have a chemotherapeutic outcome against babesiosis with low toxicity to the hosts is immediately needed.

Flavonoids are a great assemblage of polyphenolic compounds. They are distributed into flavone and isoflavones bestowing to the linking among carbon atoms in the flavone rings. They possess a large diversity of plant minor metabolites. Flavonoids arise in brews and plants. They have many biologic activities such as antioxidants and anticancer [4].

Luteolin (3′,4′,5,7-tetrahydroxyflavone), a plant flavonoid, has antioxidant [5], anti-inflammatory [6], anti-allergic [6], anticancer [7], antibacterial [8], antiviral [9], anti-amoebic [10], antileishmanial [11], antitrypanosomal [11], and antimalarial [12,13] activities. Due to the similarities between *Babesia and Plasmodium and Theileria* parasites luteolin might have a suppressive effect on these blood parasites. The aim of the current research was to appraise microscopically the suppressive effects of luteolin on the in vitro growth of three Babesia species and *T. equi* and on the *in vivo* growth of *B. microti*. Furthermore, to study the impact of luteolin on the transcription of *B. bovis* DNA gyrase subunits A and B by Reverse transcription-PCR (RT-PCR).

Materials and Methods

Chemical reagents

Luteolin was purchased from Sigma-Aldrich (USA). A Stock solution of one hundred millimolars in dimethyl sulfoxide (DMSO) was ready and held on at −30 °C. Diminazene aceturate (Ganaseg, Ciba-Geigy Japan Ltd., Tokyo, Japan) was used as a positive control drug. An operating stock solution of a ten millimolar dissolved in DDW was ready and held on at −30 °C till needed to be used.

Rodent *Babesia* and mice

The Munich strain of *B. microti* was kept by serialized passage within the blood of BALB/c mice [14]. BALB/c mice, thirty females of 8 weeks old (CLEA Japan, Tokyo, Japan), were used for the *in vivo* studies.

In vitro cultivation of *Babesia* parasites

Luteolin was tested for its chemotherapeutical impact against *B. bovis* (Texas strain) [15], *B. bigemina* (Argentina strain [16], *B. caballi* [17], and *T. equi* (U.S. Department of Agriculture) [18]. Parasites were refined in bovine or equine red blood cells employing a continuous micro-aerophilous stationary phase culture system [17]. The medium M199 (Sigma-Aldrich, Tokyo, Japan) was applied to *T. equi, B. bigemina*, and *B. bovis* and was complemented with 40 % horse or cattle serum and 60 U/ml of penicillin G, 60 µg/ml of streptomycin, and 0.15 µg/ml of amphotericin B (Sigma-Aldrich). The *T. equi* culture was supplemented with Hypoxanthine (ICN Biomedicals, Inc., Aurora, OH) at 13.6 mg/ml. For B. caballi, the culture medium RPMI 1640 was supplemented with 40 % horse serum, antibiotics, and amphotericin B [17].

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In vitro growth inhibition assay

The in vitro growth inhibition assay was done as antecedently rumored [17]. *B. bigemina, B. caballi, B. bovis*, and *T. equi* were acquired from cultures with a parasitemia of five % that was diluted with applicable clean RBCs to a beginning parasitemia of 1 % for the assays. The growth inhibition assay was done in 96-well plates containing twenty µl of packed red blood corpuscle and 200 µL of a fitting culture medium containing 0.1,1, 5, 10, 25, 50, and 100 µM of luteolin. A preliminary study was used to set the used concentrations. Positive control cultures received 5, 10, 50, 100, 1000 or 2000 nM of diminazene aceturate [19]. Cultures without the drug and cultures containing only DMSO at 0.01 % for luteolin and DDW at 0.02 % for diminazene aceturate were set as negative controls. The experiments were administrated in triplicate wells per drug concentration and in three separate trials for every parasite species. Cultures were incubated at thirty-seven °C in an environment of 90 % N_2, 5 % O_2, and 5 % CO_2. The culture medium was exchanged every day with 200 µl of renewed medium containing the contemporary drug concentration for 4 days. Parasitemia was monitored on the premise of 1,000 erythrocytes in a Giemsa-stained smear. Changes within the morphology of treated Babesia parasites were likened with the control using light microscopy. The 50 % inhibitory concentrations (IC50) were estimated on the third day of in vitro culture by interpolation by means of the curve-fitting technique [20]

Viability test

After four days of treatment, 6 µL of parasite-free bovine or equine packed RBCs was accessorial to fourteen µL of packed red blood cells from the antecedently drug-treated cultures in two hundred µl of a new growth medium without the drug. The fresh growth medium was replaced every day for the subsequent ten days, and parasite eruption was firmed daily next removal of the drugs by microscopic examination [14].

Effect of luteolin on host erythrocytes

The toxicity of luteolin to host erythrocytes was evaluated antecedently delineated [20]. Bovine and equine erythrocytes were incubated in the existence of one hundred µM luteolin (the highest concentration utilized in this study) for three hours at thirty-seven degree Celsius; then erythrocytes were washed three times with drug-free media and used for the cultivation of Babesia parasites for seventy-two hours. The pattern of parasite growth in pretreated erythrocytes was determined and compared with control untreated cells.

RNA extraction and Reverse-Transcription PCR (RT-PCR)

Reverse-transcription PCR was accustomed to value the impact of the treatment with luteolin on the transcription of B. bovis DNA gyrase (a type II topoisomerase) subunits, gyrase A and gyrase B as antecedently delineated [17], with some modifications. B. bovis was cultured in 24-well culture plates in bovine RBCs as delineated above and treated with luteolin for 8 hours at a 99 % inhibitory concentration (IC99). The IC99 was calculated based on the IC50 concentration obtained from the growth inhibition assay results multiplied by two and 99/100. For negative control, cultures were containing only dimethyl sulfoxide (0.001 %). RBCs were collected from three wells and washed with phosphate-buffered saline (PBS) for 3 times by centrifugation at 3000 rpm for 5 minutes in TOMY MX-105 (TMP-21) centrifuge (TOMY Digital Biology Co., Ltd., Tokyo, Japan). The RNA was extracted using the TRI® reagent (Sigma-Aldrich, USA) along with the supplied protocol. The RNA concentration was measured using the Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., USA). The reverse-transcription PCR was conducted using PrimeScriptTM One-Step RT-PCR Kit Version two (Takara, Japan) along with the manufacturer's protocol. Total RNA, 150 ng, from the treated cultures and from the control was used in the amplification of B. bovis gyrase A and gyrase B subunits, with DNA gyrase subunit A foreword F 5′-CTGGTTTATTATATATGAACCC-3′ and reverse R 5′-CATTTTAGCAAAT ATTAGTTA-3′ and DNA gyrase subunit B foreword F 5′-CCCGCCTCACTGCTACTGGCAGATG-3′ and reverse R 5′-CTACCTATCTAGGTCATGTAGC-3′ primers genes. The forward and reverse primers of the B. bovis tubulin beta chain gene foreword F 5′-ATGAGAGAA ATCGTACACATCC-3′ and reverse R 5′-TCAATAATCATTTACCATG TCATCGG-3′ [17] were used as a control for transcription of the target genes from the luteolin-treated and control cultures. In a 50 µL reaction volume, the reverse-transcription reaction was carried out at 50 °C for 30 minutes. The PCR consisted of two min of denaturation at 94°C, followed by thirty cycles of denaturation at 94°C for 30 sec, primer annealing for 30 sec at 50°C for gyrase A, 62 °C for gyrase B, and 54 °C for tubulin beta chain genes; and elongation at 72°C for 3 minutes for gyrase A and gyrase B and 2 minutes for tubulin beta chain genes. Five µL of PCR products were electrophoresed in 2 % agarose gel with a 1000-bp DNA ladder size marker (Takara Bio Inc., Tokyo Japan) and stained with ethidium bromide (Sigma-Aldrich, Japan).

In vivo growth inhibition assay

The luteolin *in vivo* inhibition assay for *B. microti* in BALB/c mice was performed twice following a method antecedently delineated [19,21], with some modifications. Fifteen 8-week-old female BALB/c mice were divided into 3 groups, each containing 5 mice, and intraperitoneally inoculated with 1 × 107 B. microti-infected RBCs. When the infected mice had around 1 % parasitemia, mice in the experimental groups were administered daily injections for five days.

Drugs were dissolved in dimethyl sulfoxide (DMSO) (3 % for luteolin) and Double Distilled Water (DDW) (12.5 % for diminazene aceturate), then diluted in PBS or DDW prior to inoculation. In the negative control, DMSO was administered in PBS (0.03 %). In the first group, luteolin was intraperitoneally administered at a dose rate of 5 mg/kg in 0.3 ml of PBS [17]. A 0.3 ml PBS (0.03 % DMSO) was intraperitoneally administered to the control group. Diminazene aceturate (Ganaseg, Japan Cieba-Geigy, Ltd.) at a dose of 25 mg/kg was subcutaneously administrated to the third group in 0.1 ml DDW [17].

The levels of parasitemia in all mice were monitored daily till twenty-two days post-infection by examination of 1,000 erythrocytes in Giemsa-stained thin blood smears prepared from the venous tail blood. All animal experiments were conducted in accordance with the Standard regarding the Care and Management of Experimental Animals set by the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

Statistical analysis

The variations in the percentage of parasitemia for the in vitro cultures and among groups of the in vivo studies were analyzed by JMP statistical software using the independent Student's t-test. A P value of < 0.05 was thought of statistical significance.

Results

In vitro growth inhibition assay

Luteolin significantly ($P < 0.05$) restricted the expansion of the parasites at 0.1 µM for B. bovis at day one (Figure 1A), *B. bigemina*

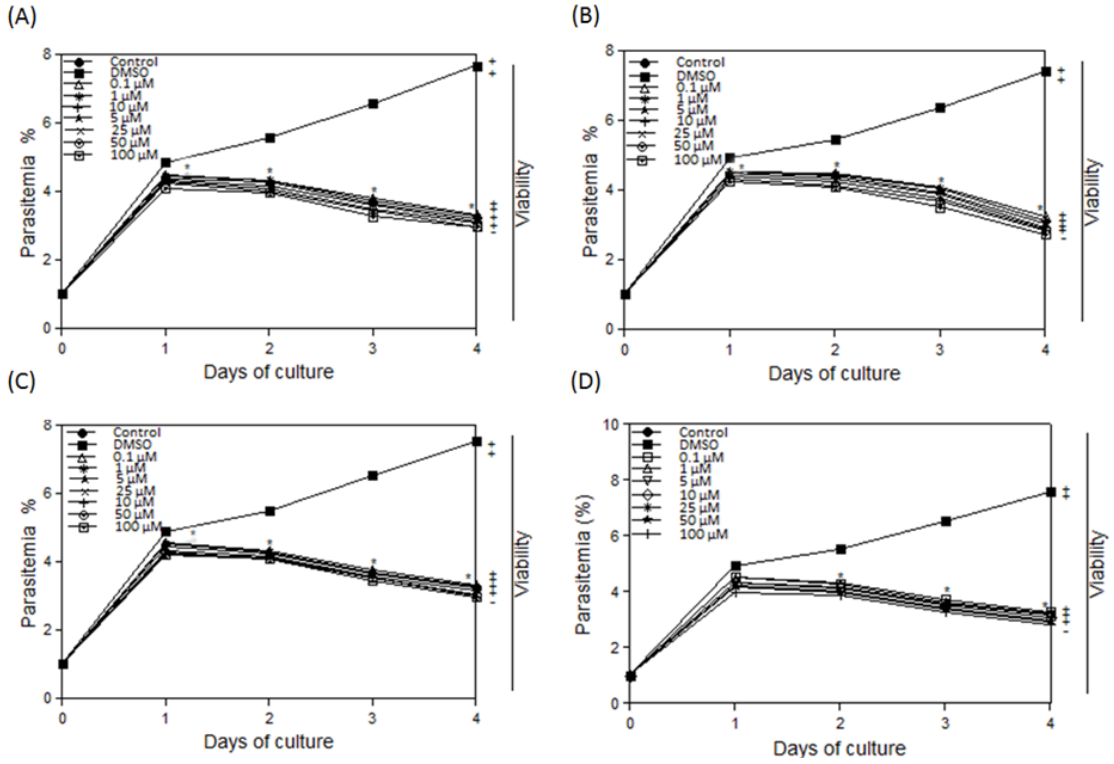

Figure 1: Inhibitory effects of different concentrations of luteolin on the in vitro growth. *(A) B. bovis, (B) B. bigemina, (C) B. caballi, and (D) T. equi*. Each value represents the mean ± standard deviation in triplicate. These curves represent the results of three experiments carried out in triplicate. Asterisks indicate a significant difference (Student's t-test; * $P < 0.05$) between drug-treated and control cultures. Regrowth after 10 days was indicated as viable (+) and dead (-).

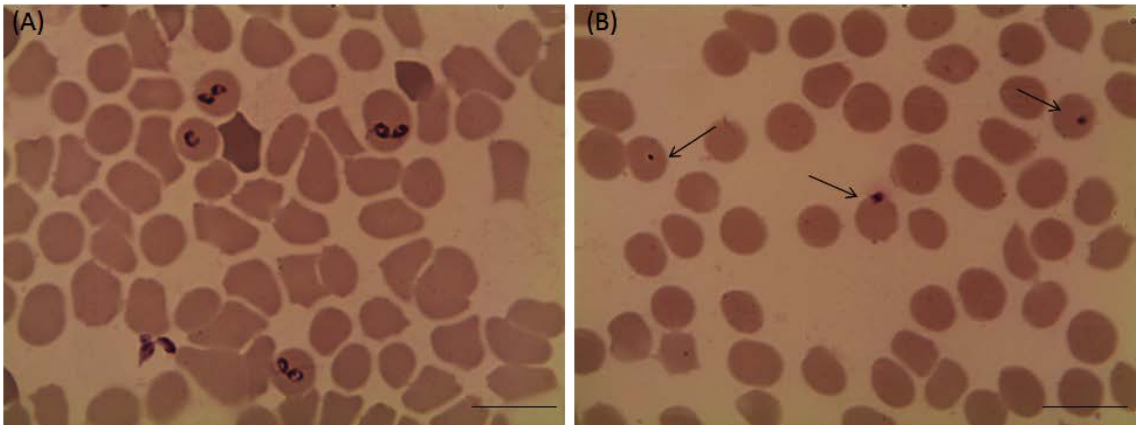

Figure 2: Light micrographs of Babesia bovis treated with 25 µM luteolin in in vitro cultures. (A) Control and (B) luteolin-treated cultures. The drug-treated cultures showed higher numbers of degenerated parasites indicated by arrows than the control cultures. Micrographs were taken on day 4 of treatment.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

(Figure 1B), *B. caballi* (Figure 1C) at day one of treatment and *T. equi* at day two of treatment (Figure 1D). Diminazene aceturate causes significant inhibition ($P < 0.05$) of the in vitro growth of the 3 *Babesia* species and *T. equi* at five nM. The consequent culture of the parasites with no drug for 10 days indicated no regrowth of parasites at a 100 µM concentration for *B. bigemina, B. bovis, T. equi, and B. caballi* (Figure 1). Parasites exposed to lower drug concentrations restarted growth when the drug was removed, as shown by increased parasitemia by microscopic examination of Giemsa-stained smears. The IC50 values of luteolin and diminazene for various Babesia species are shown in Table 1. Parasites exposed to solely solvent had similar growth pattern to the control.

The morphological changes of treated and control Babesia parasites were compared. In luteolin-treated *B. bovis* cultures, the parasites appeared dot-shaped (Figure 2B) relative to normal parasites in the DMSO negative control culture (Figure 2B). A similar effect was observed in luteolin-treated *B. bigemina* (Figure 3B) and *T. equi* (Figure

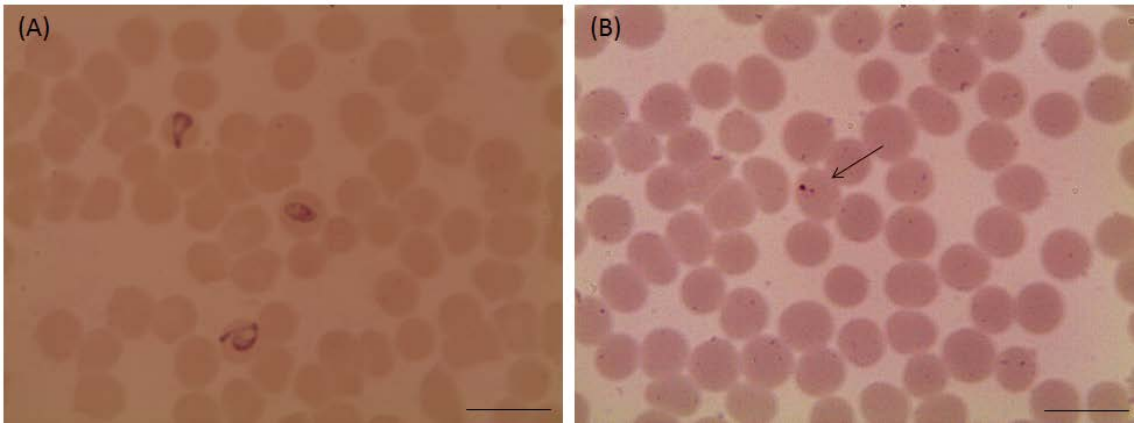

Figure 3: Light micrographs of Babesia bigemina treated with 25 µM luteolin in in vitro cultures. (A) Control and (B) luteolin-treated cultures. The drug-treated cultures showed higher numbers of degenerated parasites indicated by arrows than the control cultures. Micrographs were taken on day 4 of treatment. Bars, 10 µm.

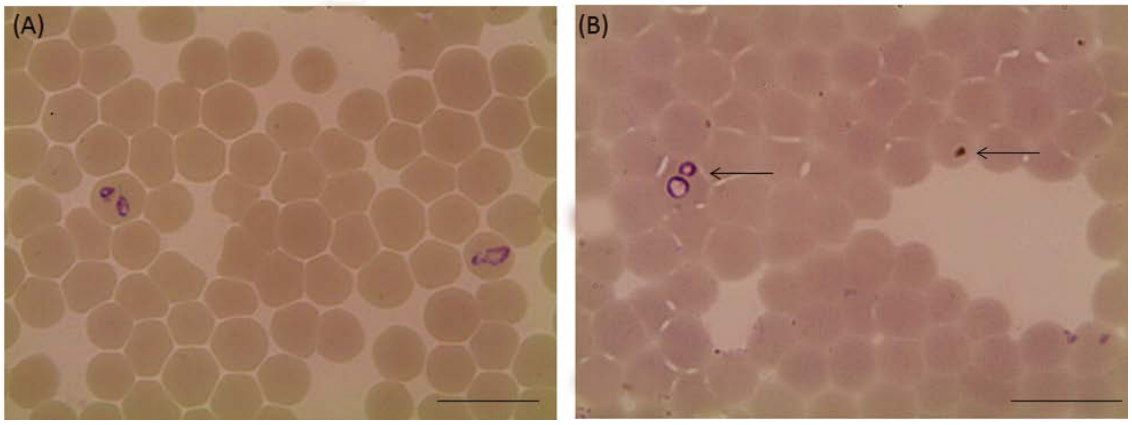

Figure 4: Light micrographs of *Babesia caballi* treated with 25 µM luteolin in *in vitro* cultures. (A) Control and (B) luteolin-treated cultures. The drug-treated cultures showed higher numbers of degenerated parasites indicated by arrows than the control cultures. Micrographs were taken on day 3 of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

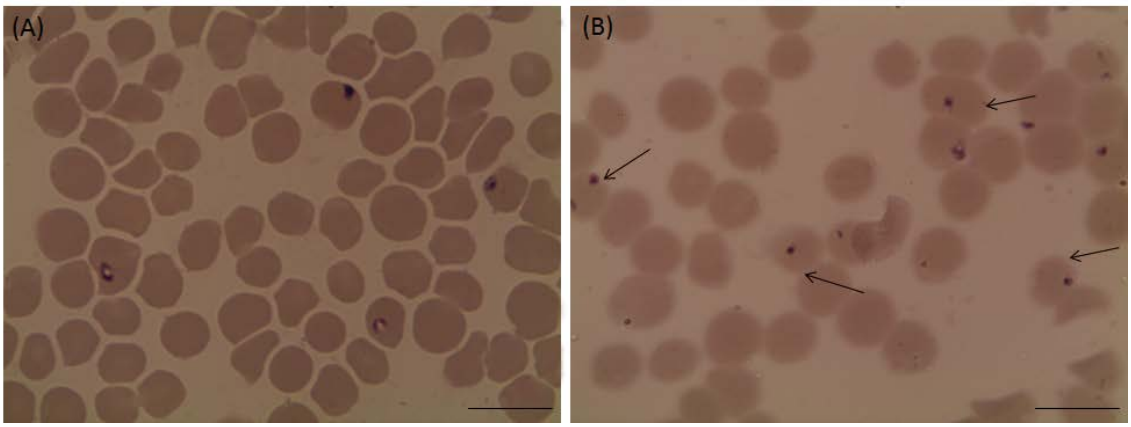

Figure 5: Light micrographs of Theileria equi treated with 25 µM luteolin in in vitro cultures. (A) Control and (B) luteolin-treated cultures. The drug-treated cultures showed higher numbers of degenerated parasites indicated by arrows than the control cultures. Micrographs were taken on day 4 of treatment. Bars,10µm.

4B) cultures. In luteolin-treated, *B. caballi* the parasites appeared either swollen or dot-shaped (Figure 5B). Luteolin was non-toxic to the bovine and equine erythrocytes at the maximum concentration (100 µM) because the pretreated erythrocytes supported a similar level of parasitemia resembling the untreated erythrocytes (data not presented).

RT-PCR

Luteolin treatment with the calculated IC99 concentration resulted in the reticence of mRNA transcripts of *B. bovis gyrase B* gene (Figure 6, lane 2) whereas the gyrase A (Figure 6, lane 1) from treated cultures not presented such inhibition (Figure 6, lane 4). Luteolin failed to have an outcome on the gene transcripts of the tubulin beta chain gene (Figure 6, lane 6).

In vivo effect of luteolin on *B. microti* infection

Luteolin was evaluated for in vivo efficacy against *B. microti* in mice. The luteolin-treated group showed significantly lower levels of parasitemia than the negative control group ($P < 0.05$) from day's 4 to 9 p.i. (Figure 7). Peak parasitemia levels in the treated groups reached an average of 6.2 % in the existence of 25 mg/kg diminazene aceturate at 5 days p.i. and 9.4% in the existence of 5 mg/kg luteolin at 7 days p.i., in contrast to 42.1 % in the negative control group (DDW) at 7 days p.i (Figure 7).

Discussion

In this study, luteolin inhibited the in vitro development of *T. equi, B. caballi, B. bovis, and B. bigemina*. The control for the experiment confirmed that the results were due to the luteolin. *Bovine Babesia* species were more sensitive to luteolin than *B. caballi and T. equi.*

The IC50 values of luteolin for Babesia species and *T. equi* were lesser than those of diminazene aceturate reported in this study except for *B. caballi.* The IC50s of luteolin for *T. equi, B. caballi, B. bovis, and B. bigemina* were very low related with those for *P. falciparum* 11-12 µM [12], Entamoeba histolytica 62.1 µM (17.8 µg/ml) [10], Trypanosoma brucei 13 µM (3.7 µg/ml) [11], T. cruzi µM 74.9 (21.4 µg/ml) [11], L. donovani 2.8 µM (0.8 µg/ml) [11]. The IC50s of luteolin for *Babesia* species and *T. equi* were lesser than those reported by a fluorescent based method [22]. The IC50 values of luteolin for *T. equi* and *Babesia* species were also lesser than those assessed as antibabesial drugs [14-25]. The IC50s of luteolin for *T. equi and Babesia* species were an identical array with the IC50s of other antibabesial drugs: epoxomicin [19], atovaquone [26], imidocarb dipropionate [27], quinuronium sulfate [28]. Luteolin will be safe for treating piroplasmosis in animals because the IC50 values of luteolin for *Babesia and T. equi* are very low matched with the IC50 value of 32.9 µM (9.4 µg/ml) for L6 cells [11] and >875 µM (>250 µg/ml) for MT-4 cells [10]. Furthermore, it prevents multiplication of normal human cells at 40 µM [29].

Luteolin repressed 3 enzymes in fatty acid biosynthesis pathway in *P. falciparum* [13] while this pathway is not found in the genome sequence database of *B. bovis* [30]. Luteolin treatment with the calculated 99 % inhibitory concentration inhibited most of the mRNA transcripts of the *B. bovis* DNA gyrase subunit B gene while not disturb the transcription of DNA gyrase subunit A gene matched with the control culture; this is in agreement with preceding studies where it suppresses the topoisomerase II in Leishmania donovani [31] and interrelates with both free enzyme topoisomerase I and substrate DNA [32]. Therefore, growth inhibition may be owing to the reserve of parasite DNA topoisomerases I and /or II. Thus, further study is required to elucidate the method of inhibition, especially after expression of the DNA topoisomerases from *Babesia species* and *T. equi.*

Luteolin showed good in vitro suppressive effects for *Babesia, T. equi, and Leishmania donovani*, thus, we were encouraged to evaluate the in vivo suppressive activities of luteolin on *B. microti* in mice. The inhibitory outcome of luteolin on the growth of *B. microti* was evident. Luteolin was not toxic for mice at 5 mg/kg, which is in agreement with the findings of a prior research where luteolin at 50 mg/kg for 2 days protected the mice from carbon tetrachloride-induced hepatotoxicity [33]. Luteolin enhanced lymphocyte proliferation and phagocytosis by macrophages. Furthermore, the potent immune-modulatory effects of luteolin [34-36] might have a role in modulating the *B. microti* contagion in mice that requires further investigations.

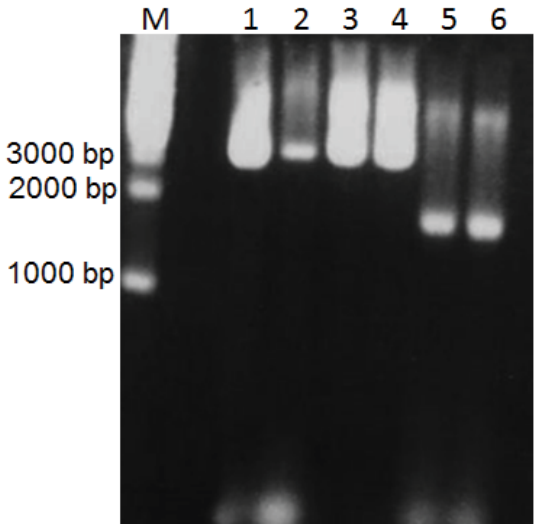

Figure 6: Reverse-transcription PCR for DNA gyrase A and gyrase B subunits and tubulin beta chain genes from Babesia bovis cultures treated with an IC99 concentration (160 nM) of luteolin for 8 hours. DNA gyrase B from control (lane 1) and treated (lane 2) cultures. DNA gyrase A subunit from control (lane 3) and treated (lane 4) cultures. Tubulin beta chain from control (lane 5) and treated (lane 6) cultures. M is 1000-bp DNA ladder size marker.

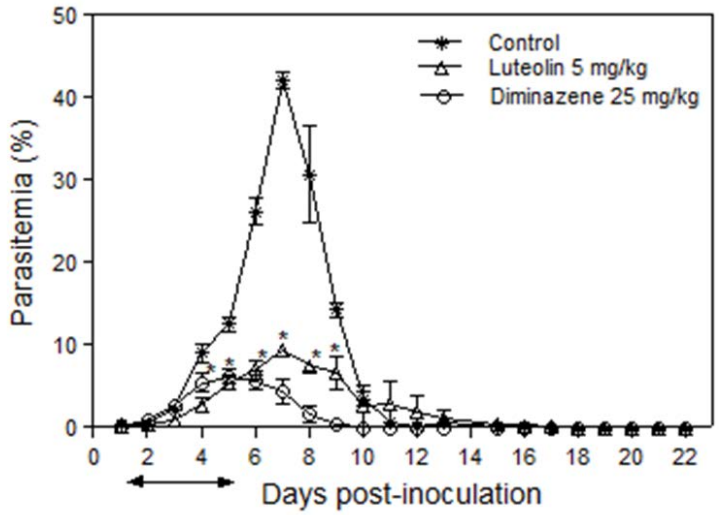

Figure 7: Inhibitory effects of I.P. luteolin 5 mg/kg and S.C. diminazene aceturate 25 mg/kg on the in vivo growth of Babesia microti for observations of five mice per experimental group. Each value represents the mean ± S.D for two experiments. Asterisks indicate a significant difference (Student's t-test; * $P < 0.01$) from days 4 to 9 post-inoculation between luteolin-treated and dimethyl sulfoxide (DMSO) control group.

In conclusion, luteolin inhibited the growth of *T. equi and Babesia* species in vitro cultures and the *in vivo* growth of *B. microti* in BALB/c mice. Luteolin may be used as a chemotherapeutic drug for infections produced by *T. equi and Babesia* species.

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