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Original Article

Evaluation of the *in vitro* and *in vivo* inhibitory effects of apigenin and gallic acid on the growth of *Babesia* and *Theileria* parasites

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Abstract:

Apigenin and gallic acid are naturally occurring plant flavonoids. They have antioxidant, anti-inflammatory, antitrypanosomal, and antimalarial activities. In this study, the restrictive properties of Apigenin and gallic acid were evaluated against two *Babesia* species and *Theileria equi in vitro.* Apigenin was evaluated against *B. microti* in mice. Apigenin showed significant growth inhibition for *Babesia bovis*, *Babesia caballi*, and *Theileria equi* with IC_{50} values of 125 µM, 60 µM, and 0.08 µM, respectively. IC_{50} values of gallic acid were 30, 30, and 4.5 µM for *Babesia bovis*, *Babesia caballi*, and *Theileria equi*, respectively. Apigenin at a dose rate of 5 mg/kg resulted in a 65 % restriction of *Babesia microti* progression in BALB/c mice. Apigenin may be a promising drug therapy in bovine and equine piroplasmosis.

Keywords: Apigenin; Gallic acid; *Babesia bovis*; *Babesia caballi*; *Theileria equi*; *In vitro*; *Babesia microti*; *In vivo*

INTRODUCTION

Babesia, a serious infectious agent infects erythrocytes in animals, is transmitted by ticks to vertebrates and ends up in serious economic losses within the stock trade worldwide. *B. bigemina*, *B. bovis*, and *B. divergens* are the causative agent of the disease in cattle. While *B. caballi* and *Theileria equi* are the main cause of equine piroplasmosis everywhere in the globe. The clinical signs represent fever, discomfort, jaundice, hemoglobinuria, and anemia (***Kuttler, 1988***). Humans in North America are infected with *Babesia microti*, a rodent *Babesia* (***Rozej-Bielicka et al., 2015***). Numerous babesicidal medicines, for instance, diminazene aceturate and imidocarb dipropionate have adverse properties linked with their toxicity (***Vial and Gorenflot, 2006***). Therefore, the event of novel medicines that have a chemotherapeutical result against babesiosis with little poison-ousness to the hosts is desperately required.

Apigenin and gallic acid, plant flavonoids, are extensively present in several plants, vegetables, fruits, and tea.

Apigenin has antioxidant ***(Abate et al., 2005)***, anti-inflammatory ***(Li et al., 2016)***, anti-allergic ***(Iwaoka et al., 2010)***, anticancer **(*Zhu et al., 2015*)**, antibacterial **(*Lucarini et al., 2015*)**, antiviral **(*Liu et al., 2008*)**, antitrypanosomal ***(Tasdemir et al., 2006)***, anti-amoebic **(*Cimanga et al., 2006*)**, antileishmanial **(*Tasdemir et al., 2006*)**, and antimalarial ***(Lehane and Saliba, 2008)*** activities.

Gallic acid has antioxidant (***Akanitapichat et al., 2010***), anti-proliferative (***Zhong et al., 2009***), anti-inflammatory ***(Choi et al., 2009)*** antitrypanosomal (***Tasdemir et al., 2006***), antileishmanial (***Tasdemir et al., 2006)***, and antimalarial (***Ndjonka et al., 2012***) activities. As a result of the similarities between *Plasmodium*, *Babesia*, and *Theileria* parasites apigenin and gallic acid might have inhibitory effects on *Babesia* species and *T. equi*. The aim of this study was to assess the repressing effects of apigenin and gallic acid on the *in vitro* progress of 3 *Babesia* species and *T. equi*. Moreover, analysis of the repr-essing impact of apigenin on the *in vivo* growth of *B. microti*.

MATERIALS AND METHODS

1. Chemical reagents:

Apigenin and gallic acid were gotten from Sigma-Aldrich (USA). Stock solutions of 100 mM in dimethyl sulfoxide (DMSO for apigenin) and normal saline (NS for gallic acid) were prepared and stored at −30 °C until use. Diminazene aceturate (Ganaseg), was obtained from Ciba-Geigy Japan Ltd., was used as a positive control medication. A working stock solution of 10 mM dissolved in double-distilled water (DDW) was prepared and stored at –30 ˚C until required for use.

2. Rodent *Babesia* and mice:

B. microti (Munich strain) was continued by a sequential passage in BALB/c mice **(*AbouLaila et al., 2014*).** Thirty BALB/c mice, eight weeks old females, were picked up from CLEA Japan (Tokyo, Japan) and managed for the *in vivo* experiments.

3. *In vitro* cultivation of *Babesia* parasites:

Apigenin and gallic acid were valued for their chemotherapeutical properties against *B. bovis* (Texas strain) **(*AbouLaila et al., 2014*),** *B. caballi* (AbouLaila et al., 2010b), and *T. equi* (U.S. Department of Agriculture) (***Mehlhorn and Schein, 1998***). Parasites were cultured in bovine or equine RBCs employing a continuous micro-aerophilous stationary phase culture system ***(AbouLaila et al., 2010b).*** *B. bovis* and *T. equi* were cultured in medium M199 (obtained from Sigma-Aldrich, Tokyo, Japan), was augmented with 40 % bovine or equine serum and 60 U/ml of penicillin G, 60 µg/ml of streptomycin, and 0.15 µg/ml of amphotericin B (Sigma-Aldrich). Hypoxanthine (ICN Biomedicals Inc., USA) was superimposed to the *T. equi* culture as an essential complement at 13.6 mg/ml. The medium RPMI 1640 was used for *B. caballi*, which enhanced with antibiotics, amphotericin B and 40 % horse serum **(*Aboulaila et al., 2010c*).**

4. *In vitro* growth inhibition assay:

The *in vitro* growth inhibition assay was done as stated before ***(Aboulaila et al., 2012*)**. *B. caballi*, *B. bovis*, and *T. equi* were gotten from cultures with a parasitemia of 6 % that was thinned with proper fresh erythrocytes to the first parasitemia of 1% for the assays. The growth inhibition assay was implemented in 96-well plates containing 20 µl of crowded RBCs inoculum and 200 µL of a suitable medium containing either apigenin at 0.1,1, 5, 10, 25, 50, 100, and 200 µM for *B. caballi* and *B. bovis* and at 0.1,1, 5, 10, 25, 50, and 100 µM for *T. equi* or gallic acid at 5, 10, 25, 50, and 100 µM for *B. caballi*, *B. bovis*, and *T. equi*. The concentrations used were based on a pilot experiment. Positive control cultures contain 5, 10, 50, 100, 1000 or 2000 nM of diminazene aceturate **(*Aboulaila et al., 2010a*).** For negative experimental control, cultures without the drug and cultures containing only DMSO (0.02 %, for apigenin), normal saline (0.01 %, for gallic acid), and DDW (0.02 %, for diminazene aceturate) were ready. The experiments were completed in triplicate and in three separate trials. Cultures were reared at 37 °C in an environment of 5 % O_2, 5 % CO_2, and 90 % N_2. For a period of four days, the medium was substituted on a daily basis with 200 µl of renewed medium containing the acceptable drug concentration. Parasitemia was checked based on 1,000 erythrocytes in a Giemsa-stained thin smear. Deviations in the morphology of treated *Babesia* parasites were related to the control by light microscope. The 50 % inhibitory concentrations (IC_{50}s) were estimated on the third day of *in vitro* culture by interpolation via the curve-fitting technique ***(AbouLaila et al., 2010b*).**

5. Viability test:

Once 4 days of medication, six µL of new bovine or equine red blood cells were added to 14 µL of packed RBCs from the prior drug-treated cultures in two hundred µl of a contemporary growth medium without the drug. The contemporary growth medium was replaced on a daily basis for the consequent ten days, and parasite revival was monitored daily ***(Aboulaila et al., 2010c*).**

6. Effect of apigenin and gallic acid on host erythrocytes

The toxicity of apigenin and gallic acid to host erythrocytes was evaluated as antecedently delineate ***(AbouLaila et al., 2014*).** Bovine and equine erythrocytes were incubated in the existence of a hundred µM gallic acid and two hundred µM apigenin (the highest concentrations employed in this study) for three hours at 37 $^{\circ}$C; at that point RBCs were washed thrice with media and used for the cultivation of *Babesia* parasites for 3 days. The control untreated RBCs held in the same way as the treated RBCs. The outline of parasite growth in pretreated RBCs was ascertained and paralleled with the control untreated cells.

7. *In vivo* growth inhibition assay:

The apigenin *in vivo* inhibition assay for *B. microti* in mice was performed twice following a method previously described **(*AbouLaila et al., 2010a; Abate et al., 2005*)** with some modifications. Fifteen 8-week-old BALB/c females were allocated into 3 groups, each containing five mice, and intraperitoneally inoculated with 1 × 10^7 *B. microti*-infected RBCs. Once the

infected mice displayed roughly 1 % parasitemia, mice in the investigational groups were run day-to-day shots for five days.

Drugs were liquefied in dimethyl sulfoxide (DMSO) (2 % for apigenin) and autoclaved water (DDW) (12.5 % for diminazene aceturate), at that time thinned in PBS or DDW preceding injection. In the negative control, DMSO was administered in PBS (0.03 %). In the first group, apigenin was administered intraperitoneally at a dose rate of 5 mg/kg in 0.2 ml of PBS **(*Aboulaila et al., 2012*).** A 0.2 ml PBS (0.03 % DMSO) was administered intraperitoneally to the control assemblage. Diminazene aceturate (at a dose of twenty-five mg/kg was hypodermically injected to the 3rd experimental group in 0.1 ml DDW **(*Aboulaila et al., 2012*).**

The levels of parasitemia in all mice were observed every day up to 22 days after infection by inspection of 1,000 RBCs in Giemsa-stained smears prepared from the venous tail blood.

8. Statistical analysis:

The variations in the percentage of parasitemia for the *in vitro* and *in vivo* experiments were analyzed with JMP statistical software (SAS Institute, Inc., USA) employing the independent Student's *t*-test and a *P* value of < 0.05 was deliberated statistically significant.

RESULTS

In vitro growth inhibition assay

An apigenin concentration of 0.1 µM significantly (*P* < 0.05) repressed the expansion of the cultured parasites for *B. bovis* at day 2 (Fig. 1A) and for *B. caballi* (Fig. 1B) and *T. equi* at day 1 of treatment (Fig. 1C). Gallic acid significantly subdued (*P* < 0.05) the growth of the 3 parasites at five µM on day one for *B. bovis* (Fig. 4A) and *T. equi* (Fig. 4C) and on day two for *B. caballi* (Fig. 4B). A five nanomolar concentration of diminazene aceturate significantly pent-up (*P* < 0.05) the *in vitro* expansion of the 3 parasites. Apigenin concentrations of two hundred µM for *B. bovis* and *B. caballi* and hundred µM for *T. equi* (Fig. 1) and gallic acid concentrations of fifty µM for *B. caballi* and *T. equi* and hundred µM for *B. bovis* (Fig. 4) inhibited the regrowth without the medicines in ensuing viability test. Parasites resumed growing at lesser treatment concentrations. There was no regrowth of the diminazene aceturate-treated parasites in the consequent viability test at concentrations of fifty nM (*B. caballi*) and 1000 nM (*B. bovis* and *T. equi*) (data not shown). The IC_{50} estimates of the 3 remedies are shown (Table 1). Parasites from cultures exposed to the solvents had a similar growth pattern to that from control cultures.

The morphology of parasites from drug-exposed and non-exposed cultures was compared. Apigenin treatment resulted in degenerated parasites *B. bovis* (Fig. 2B), *B. caballi* (Fig. 3B), and *T. equi* (Fig. 3D) compared with that in the DMSO negative control cultures (Fig. 2A and Fig.3A, C). Gallic acid treatment caused the parasites to appeared as dots in *B. bovis* (Fig. 5B), *B. caballi* (Fig. 6B), and *T. equi* (Fig. 6D) in divergence to normal organisms in the normal saline negative control culture (Fig. 5A and Fig.6 A, C). Apigenin and gallic acid did not produce observable toxic effects on the equine and bovine erythrocytes at the uppermost dilutions (200 and 100 µM, respectively) by light microscope. There were no alterations in erythrocyte morphology and parasitemia levels for three days of parasite

cultivation.

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

Apigenin was assessed for *in vivo* inhibitory effects on *B. microti* in BALB/c mice. The parasitemia levels in the apigenin-treated group were considerably lesser than the DMSO control group ($P < 0.05$) from days 4 to 8 p.i. (Fig. 7). Diminazene aceturate and apigenin treated groups with 25 mg/kg and 5 mg/kg for 5 days p.i., respectively had peak parasitemia levels reached an average of 6.2 % and 14.8 %, respectively, at 9 days p.i., in dissimilarity to 42.2 % in the DMSO control group (DMSO) at 7 days p.i. (Fig. 7).

DISCUSSION

Apigenin and gallic acid inhibited the *in vitro* rising of *B. caballi*, *T. equi*, and *B. bovis.* The control for the experiment confirmed that the results were due to the apigenin and gallic acid. *T. equi* was more sensitive to apigenin and gallic acid than *B. caballi* and *B. bovis*.

The IC_{50} values of apigenin for *Babesia* species and *T. equi* were greater compared to that of diminazene aceturate reported in this study. The IC_{50} values of apigenin for *B. bovis* and *B. caballi* were greater compared to that for *P. falciparum* 20 **µM *(Lehane and Saliba, 2008*)**, *Entamoeba histolytica* 47 µM (12.7 µg/ml) ***(Cimanga et al., 2006*)**, *Trypanosoma brucei* 18.9 µM (5.1 µg/ml) **(*Tasdemir et al., 2006*)**, *T. cruzi* µM 80.7 (21.8 µg/ml) **(*Tasdemir et al., 2006***), *Leishmania donovani* 7 µM (1.9 µg/ml) **(*Tasdemir et al., 2006*)**, and *Encephalitozoon intestinalis* 50 µM **(*Mead and McNair, 2006*)**. The IC_{50} values of gallic acid for *B. bovis* and *B. caballi* were lesser compared to that for *P. falciparum* 71.53 µM ***(Ndjonka et al., 2012*)**, *T. cruzi* µM 393.84 (67 µg/ml) (*Tasdemir et al., 2006*), and *L. donovani* 176.4 µM (>30 µg/ml) ***(Tasdemir et al., 2006*)** but higher than that for *T. brucei* 11.2 µM (1.6 µg/ml) ***(Tasdemir et al., 2006*)**. Conversely, the IC_{50} value of apigenin and gallic acid for *T. equi* was very low related to that for *P. falciparum*, *T. brucei*, *T. cruzi,* and *L. donovani.*

Apigenin and gallic acid IC_{50} values for *T. equi* and *Babesia* species were also lesser than that of allicin ***(Salama et al., 2014*)**. Gallic acid and apigenin IC_{50} values of for *T. equi* and *Babesia* species were a similar range with the IC_{50} values of other babesicidal drugs ***(Aboulaila et al., 2012; AbouLaila et al., 2010b; Bork et al., 2004a; Bork et al., 2004b*)**. Gallic acid and apigenin IC_{50} values of for *T. equi* and *Babesia* species were greater compared with that tested as babesicidal drugs: epoxomicin **(*Aboulaila et al., 2010a*)**, quinuronium sulfate ***(Brockelman and Tan-ariya, 1991*)**, atovaquone **(*Matsuu et al., 2008*)**, and imidocarb dipropionate ***(Rodriguez and Trees, 1996*)**. Gallic acid and apigenin will be safe for treating piroplasmosis since their IC_{50} values for *Babesia* and *T. equi* are very low paralleled with the IC_{50} value of > 925.1 µM (>250 µg/ml) and > 1469.6 µM (>250 µg/ml) for MT-4 cells ***(Cimanga et al., 2006*)**, respectively.

Apigenin inhibits the topoisomerase II enzyme ***(Azuma et al., 1995; Silva et al., 2013*)**. A DNA topoisomerase II enzyme gene was found in the gene bank for *B. bovis* (accession No. AAXT01000001) and *T. equi* (accession No. CP001669). Furthermore, apigenin inhibits *P. falciparum* serine/threonine-protein kinase (PfRIO-2 kinase) ***(Nag et al., 2013*)**. A *B.*

bovis serine/threonine-protein kinase gene homolog to PfRIO-2 kinase was found in the gene bank (accession No.: XM_001610207). Gallic acid specifically inhibits histone acetyltransferase (HAT) ***(Choi et al., 2009)*** a homologous enzyme gene was found in *B. bovis* (accession No.: AAXT01000005) and *T. equi* (accession NO.:XP_004833238). Elucidation of the mechanism of inhibition of gallic acid and apigenin for *T. equi* and *Babesia* requisites extra studies.

Gallic acid was not soluble in several solvents at the required doses for *in vivo* therapy; furthermore, apigenin exhibited worthy *in vitro* inhibitory effects for *T. equi*, thus, we were encouraged to assess the *in vivo* repressive properties of apigenin on *B. microti* in a mouse model. The repressive outcome of apigenin on the progression of *B. microti* was evident. *B. microti*-infected mice that were injected with 5 mg/kg had 65 % inhibition compared with 85.3% inhibition for diminazene aceturate. Apigenin-treated mice were normal and did not show toxic symptoms during and after the experiment that agreed with the findings of a previous study where apigenin was safely used at 50 mg/kg **(*Abate et al., 2005*)**. Therefore, apigenin is safe for treating piroplasmosis and additional research is required to conclude the best effective dose for *in vivo* therapy.

In conclusion, gallic acid and apigenin repressed the expansion of *Babesia* and *Theileria* species *in vitro* cultures and apigenin inhibited the *B. microti* growth in mice. Apigenin may be utilized as a remedy for treating *Babesiosis and Theileriosis* in different animal species.

Table (1): IC_{50} values of apigenin, gallic acid, and diminazene aceturate for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*

	IC_{50} (µM) [a]		
	Apigenin	**Gallic acid**	**Diminazene**
B. bovis	125 ± 4	30 ± 2	0.3 ± 0.03
B. caballi	60 ± 3	30 ± 4	0.1 ± 0.02
T. equi	0.08 ± 0.01	4.5 ± 0.3	0.71 ± 0.015
P. falciparum	20 ± 2 [b]	71.53 ± 8.96 [c]	ND
MT- 4 Cells[d]	> 925.1 µM	> 1469.6 µM	ND

[a] IC_{50} values expressed as drug concentration are in micromolar 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] Ndjonka et al 2012 [d] Cimanga et al. 2006 ND not determined

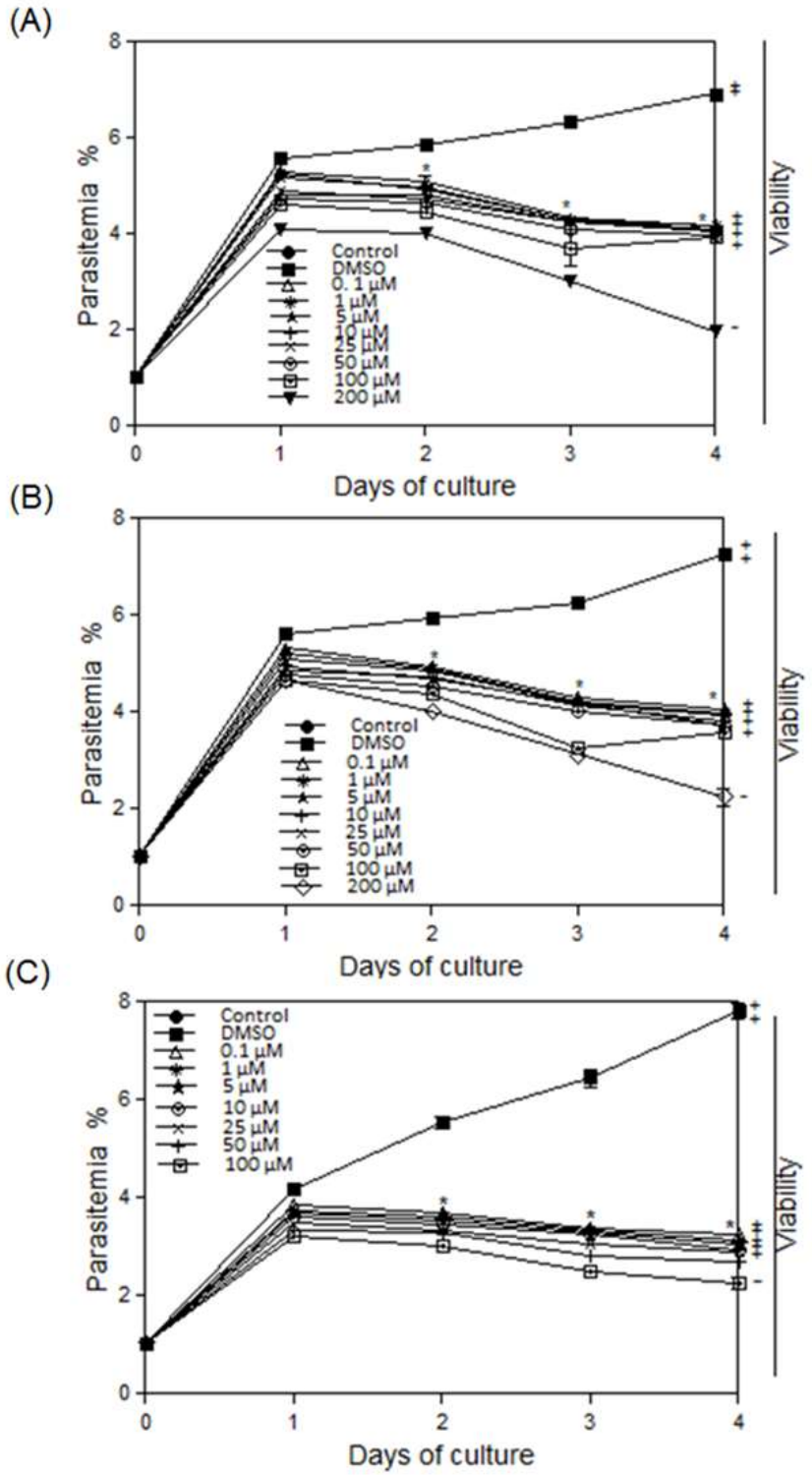

Fig. (1): Inhibitory effects of different concentrations of apigenin on the *in vitro* growth. (A) *B. bovis*, (B) *B. caballi*, and (C) *T. equi*. Each value represents the mean ± Standard deviation in triplicate. These curves represent the results of three experiments carried out in triplicate. Asterisks 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 (-).

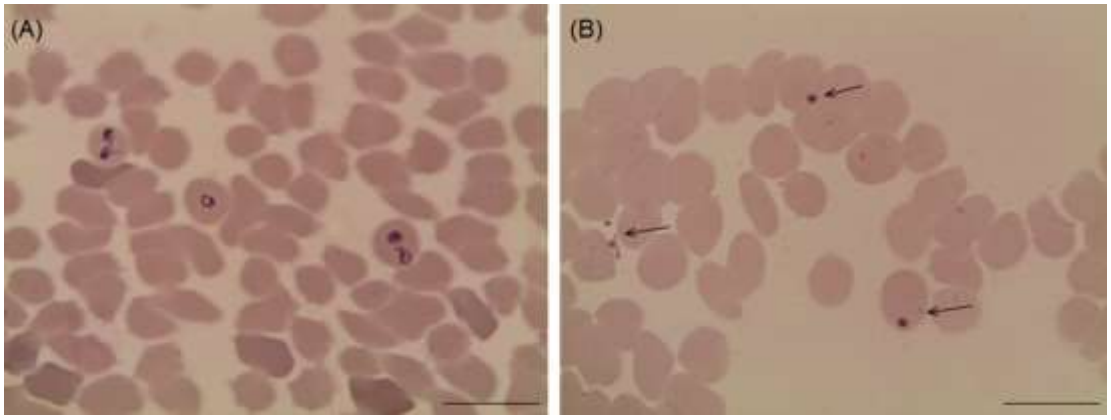

Fig. (2): Light micrographs of *Babesia bovis* treated with 25 µM apigenin *in vitro* cultures. (A) Control and (B) apigenin-treated cultures. The drug-treated cultures showed higher numbers of degenerated parasites indicated by arrows than the control cultures. Micrographs were taken on day 4 of *in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

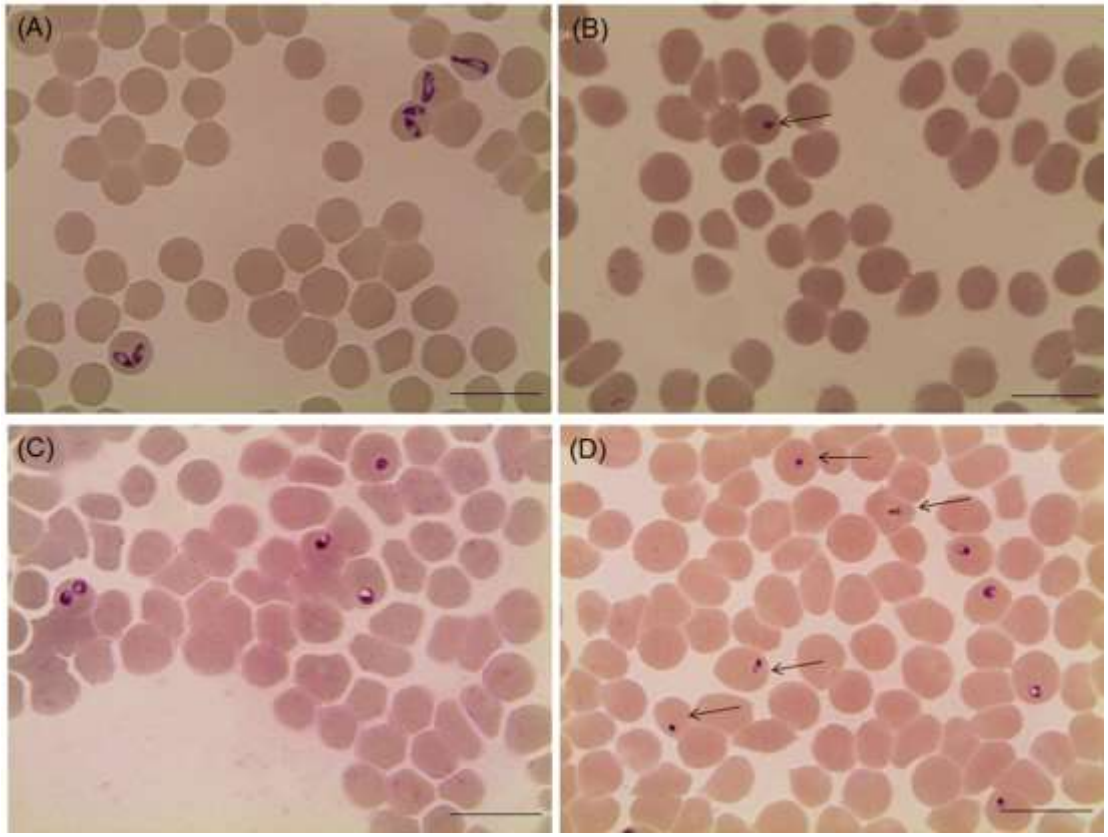

Fig. (3): Light micrographs of *Babesia caballi* and *Theileria equi* treated with 25 µM apigenin *in vitro* cultures. (A) *Babesia caballi* control, (B) apigenin-treated cultures, (C) *Theileria equi* control, and (D) apigenin-treated cultures. The drug-treated cultures showed higher numbers of degenerated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

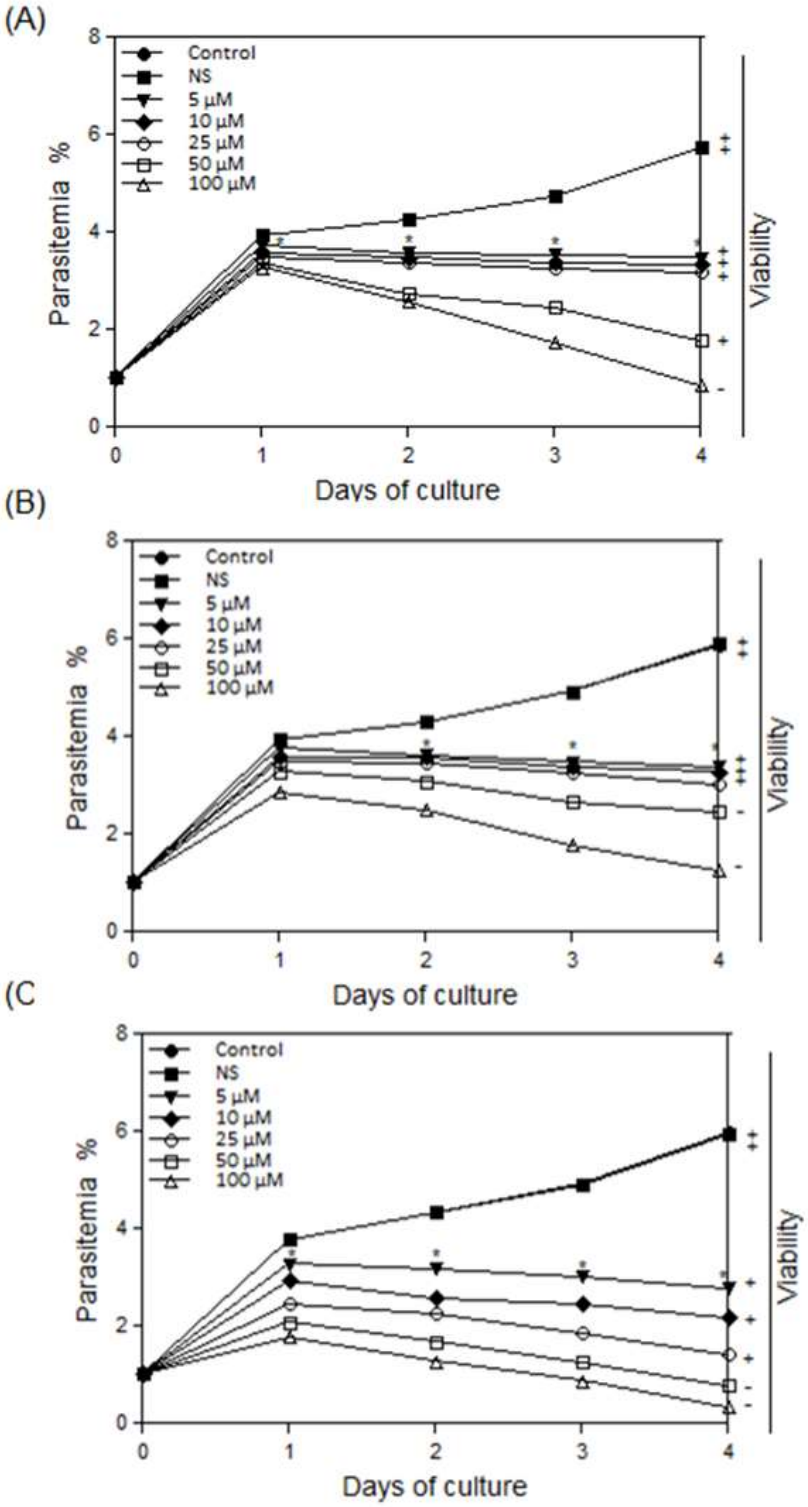

Fig. (4): Inhibitory effects of different concentrations of gallic acid on the *in vitro* growth. (A) *B. bovis*, (B) *B. caballi*, and (C) *T. equi*. Each value represents the mean ± standard deviation in triplicate. These curves represent the results of three experiments carried out in triplicate. 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 (-).

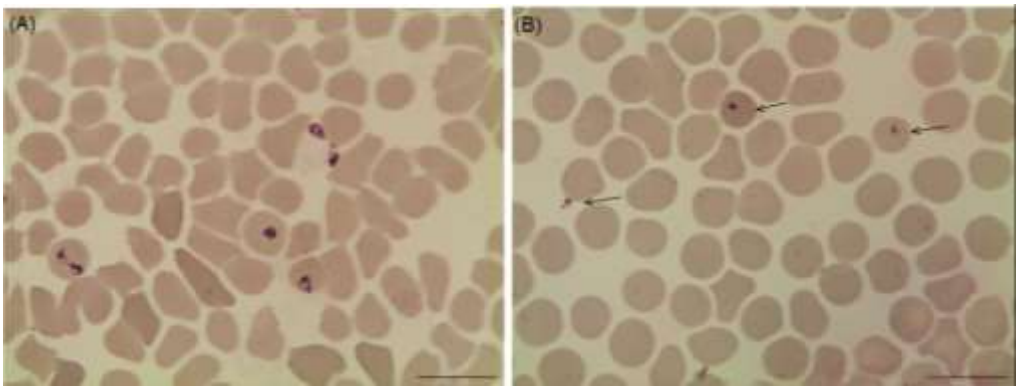

Fig. (5): Light micrographs of *Babesia bovis* treated with 50 µM gallic acid *in vitro* cultures. (A) Control and (B) gallic acid-treated cultures. The drug-treated cultures showed higher numbers of degenerated parasites indicated by arrows than the control cultures. Micrographs were taken on day 4 of *in vitro* cultures. Bars, 10 µm.

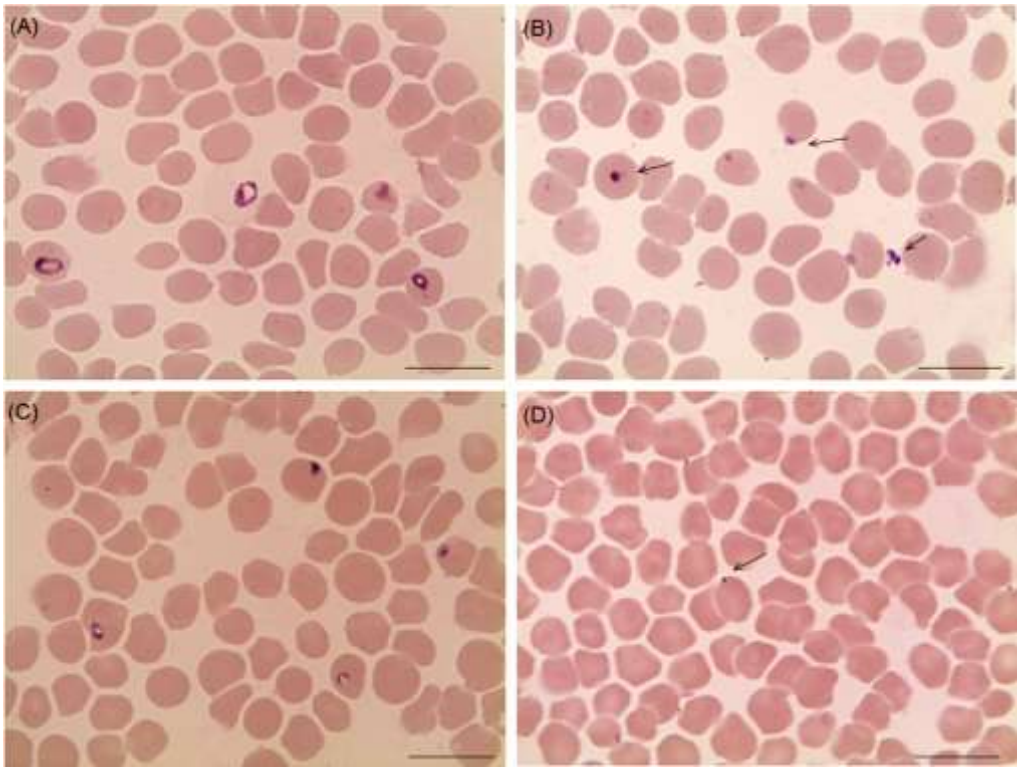

Fig. (6): Light micrographs of *Babesia caballi* and *Theileria equi* treated with 50 µM gallic acid *in vitro* cultures. (A) *Babesia caballi* control, (B) gallic acid-treated cultures, (C) *Theileria equi* control, and (D) gallic acid-treated cultures. The drug-treated cultures showed higher numbers of degenerated parasites indicated by arrows than the control cultures. Micrographs were taken on day 4 of *in vitro* cultures. Bars, 10 µm.

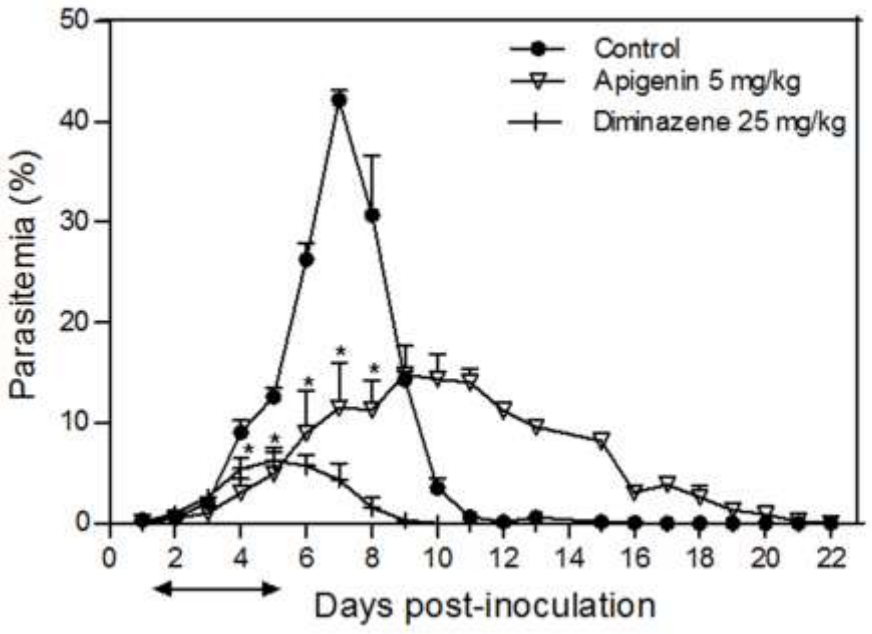

Fig. (7): Inhibitory effects of i.p. apigenin 5 mg/kg and s.c. diminazene aceturate 25 mg/kg on the *in vivo* growth of *Babesia microti* for observations of five mice per experimental group. Each value represents the mean ± S.D for two experiments. Asterisks indicate a significant difference (Student's *t*-test; * $P < 0.01$) from days 4 to 8 post-inoculation between apigenin-treated and dimethyl sulfoxide (DMSO) control group.

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الملخص العربي

مدى تأثير الأبيجينين وحمض الجاليك على نمو طفيليات البابيزيا و الثيليريا

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الأبيجينين و حمض الجاليك من الفلافونيدات النباتيه الطبيعيه. لهما تأثيرات مضاده للأكسده و مضاده للإلتهاب ومضاده للتريبانوسوما و مضاده للملاريا. تم في هذه الدراسه تقييم تأثير الأبيجينين و حمض الجاليك على البابيزيا بوفيز و البابيزيا كابالي و الثيليريا إكوي معمليا. كما تم دراسة تأثير الأبيجينين على نمو البابيزيا ميكروتي في الفئران. أظهر الأبيجينين تأثير معنوي مانع لنمو البابيزيا بوفيز و البابيزيا كابالي و الثيليريا إكوي و كان التركيز القاتل ل٥٠% من الطفيليات هو ١٢٥ و ٦٠ و ٠,٠٨ ميكرومولار على الترتيب. بينما كان التركيز القاتل لحمض الجاليك ل٥٠% من الطفيليات هو ٣٠ و ٣٠ و ٤,٥ ميكرومولار لكل من البابيزيا بوفيز و البابيزيا كابالي و الثيليريا إكوي على الترتيب. أدى علاج الفئران المعديه بالبابيزيا ميكروتي ب ٥ مليجرام / كجم وزن من الأبيجينين لوقف نمو البابيزيا ميكروتي بنسبة ٦٥%. لذلك فإن هذه الدراسة تشجع على الاستخدام المستقبلى للأبيجينين كعلاج واعد في حالات الإصابه بالبابيزيا و الثيليريا في الخيول و الأبقار.