



Evaluation of The *In vitro* Inhibitory Effects of Etoposide On The Growth of *Babesia* and *Theileria* Parasites

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ETOPOSIDE EXHIBITS antibacterial, antimalarial, and anticancer properties. Three *Babesia* parasites namely, *B. bovis*, *B. bigemina*, and *B. caballi* and *Theileria equi* were used to assess the remedial impacts of etoposide *in vitro*. Etoposide's impact on the transcription of the type II DNA topoisomerase, the DNA gyrase subunits A and B genes, has been studied using reverse-transcription PCR. Etoposide exhibited significant growth inhibition, having IC₅₀ estimates of 3.5 for *B. bovis*, 4 for *B. bigemina*, 3.7 for *B. caballi*, and 4 for *T. equi* nM. It suppressed growth in *B. bovis* and *T. equi*, *B. bigemina*, and *B. caballi* at concentrations, including 25 μM and 10 μM, respectively. Throughout the viability assay, it inhibited the regrowth of the four piroplasms at 10 μM. RT-PCR revealed etoposide therapy repressed DNA gyrase transcription in *B. bovis*. Therefore, the DNA gyrase is the target of etoposide in *B. bovis*. Etoposide showed promise in treating babesiosis and theileriosis *in vitro* but required further studies *in vivo* evaluation for mouse babesiosis

Keywords: Etoposide; *In vitro*; RT-PCR; DNA gyrase.

Introduction

Blood parasites infect erythrocytes and present an obstacle to animal production. They are transmitted by hard ticks. The major species of bovines and equines around the world include *Babesia bovis*, *B. bigemina*, *B. caballi*, and *Theileria equi*. Clinical signs comprise weakness, hemolytic anemia, pyrexia, hemoglobinuria, and icterus [1]. Disease

The apicoplast was obtained through a flat exchange (auxiliary endosymbiosis) from eukaryotic algae [3]. In the rounded 33 kbp genome of the *B. bovis* apicoplast, there is a small- as well as a large-subunit rRNA gene, an entire suite of tRNA genes, then 32 putative protein-coding genes [4]. It is vital for the abiding parasite's life. The majority of typical biological practices, including DNA replication,

control is determined by the diagnosis and administration of chemotherapeutic drugs. The currently available medications have certain drawbacks, including host poisonousness [2]. In this way, advancement regarding innovative therapeutic medications opposing babesiosis and theileriosis with low harmfulness to the animals is critical.

transcription, translation, posttranslational adjustment, catabolism, and anabolism, ensue within the organelle [5]. It is an alluring focus for the advancement of parasitocidal treatments since the biochemical pathways found within are of cyanobacterial source and vary noticeably from the parallel paths in mammals, that are eukaryotic [6].

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Etoposide is a significant chemotherapeutic agent which is trusted in treating a vast range of human malignancies. It was used for more than 20 years in the clinical sector and is still one of the most extensively utilized anticancer medicines globally. DNA gyrase subunits A and B are the two subunits that make up DNA gyrase, which is coded within the nuclear genome. It hinders bacterial DNA replication via blocking DNA gyrase, a type II topoisomerase in the prokaryotes [7] involved in the replication of untangling DNA, resulting in the linearization of circular DNA, which leads to deaths in prokaryotic species [8]. Etoposide has anticancer [9], antiviral [10], antibacterial [11, 12], and antimalarial [13,14] effects. DNA gyrase was found in *B. bovis* and *T. equi* genome databases [4, 15]. Therefore, etoposide may have a suppressing impact on the *T. equi* and *Babesia* species' growth. Thus, the present research sought to assess the *in vitro* repressive effects of etoposide on the growth of *T. equi* and *Babesia* species.

Material and Methods

Chemicals

Etoposide of Sigma-Aldrich in the United States was bought, and a 100-millimolar stock suspension in dimethyl sulfoxide (DMSO) was established afterward, reserved at -20°C . As a positive control drug, diminazene aceturate, Known as Ganaseg, from Ciba-Geigy Japan Ltd. in Tokyo, Japan was employed. At -20°C , a stock suspension made up of 10 millimolar of DDW was assembled and deposited.

In vitro culture

Etoposide had been assessed regarding its curative impact on *B. bovis* (Texan strain) [16], *B. bigemina* (Argentine strain) [17], *B. caballi* [18], and *T. equi* (The U.S.D.A. strains) [19]. Parasites had been developed in cattle or horse red blood cells utilizing a continuous micro-aerophilous stationary phase culture technique [20]. The medium M199 was improved by the use of 40% cattle otherwise stallion serum, 60 U/ml of penicillin G, sixty g/ml of streptomycin, and 150 mg/ml of amphotericin B to support *B. bovis*, *B. bigemina*, and *T. equi*, purchased coming out of Sigma-Aldrich in Tokyo, Japan. At 0.136 g/ml dilution, hypoxanthine (ICN Biomedicals, Inc., Ohio, USA), an indispensable enhancement, had been included in *T. equi* medium. *B. caballi* was grown within medium RPMI 1640 that was enriched with 40% stallion serum, antibacterials, and antifungals [21].

In vitro growth inhibition assay

Following earlier research, the *in vitro* propagation inhibition experiment was achieved [22, 23]. For the experiments, the parasites were received from cultures that had 5% parasitemia before being mixed with non-infected erythrocytes to a beginning parasitemia of 1%. Using 96-well dishes with twenty

microliters of packed RBCs combined with 200 microliters of a suitable growth medium comprising 0.005, 0.05, 0.1, 1, 5, 10, and 25 μM of etoposide for *B. bovis* and the same concentrations except the 25 micromolar of etoposide for the *Babesia* spp., the development inhibition test was implemented. The concentrations utilized depended on an initial study. Diminazene aceturate was utilized in cultures at concentrations of 0.005, 0.01, 0.05, 0.1, 1, and 2 μM as an affirmative standard [24]. Media were generated as a negative standard with only DMSO (0.025%, for etoposide) or DDW instead of the drug (0.02%, for diminazene aceturate). The experiments were completed in triplicate and three distinct trials. At 37°C , cultures were incubated in an environment that contained 90% N_2 , 5% CO_2 , and 5% O_2 . For four days, 200 μl of the newly-fitted medium was swapped out for the cultivated medium each day. Based on 1,000 RBCs in a Giemsa-tainted smear, parasitemia was observed. Utilizing light microscopy, the structure of remedied *Babesia* species was compared to the standard. During 3rd day concerning *in vitro* cultivation, a curve-fitting procedure was used to interpolate the 50 percent inhibitory concentration (IC_{50}) values [25].

Viability test

Next to 4 days of medication, 14 μl of infected RBCs from previously drug-cured cultures were treated with 6 μl of fresh cattle or horse red blood cells in 200 μl of new media devoid of etoposide. Every day, the medium was exchanged for the subsequent ten days then piroplasms revival was once resolved every day following the exclusion of the chemical in Giemsa-stained films [21].

Effect of etoposide on animal red blood cells

As previously shown, the cytotoxicity of etoposide on animal RBCs was investigated [21]. Cow and horse RBCs were raised for 3 hours at 37°C with 10 and 25 μM etoposide, respectively. Erythrocytes were then used to cultivate *Babesia* parasites for 72 hours after being cleansed three times with medication-free media. Similar procedures were operated on the pretreated cells and the control untreated cells. Compared to non-treated cells, the development of parasites in pretreated erythrocytes was observed.

Reverse-transcription PCR (RT-PCR)

RT-PCR had been utilized for assessing possible influences related to remedy using etoposide against translating the previously named gyrase A and gyrase B components of the type II topoisomerase found in *B. bovis* DNA as earlier designated with a few adjustments [22]. Following the aforementioned instructions, *B. bovis* was cultivated in 24-well culture dishes with cattle erythrocytes and given an 8-hour course of etoposide treatment with 99% inhibitory dilution (IC_{99}). It has been determined

depending on the IC₅₀ value acquired through the results of the propagation restraint experiment, multiplied by two, then 99/100, which eradicates 99% of the parasites. For negative control, cultures

deprived of the etoposide made up of only DMSO (0.001%). Three wells of erythrocytes were assembled and cleansed thrice with phosphate-buffered saline (PBS) for five minutes at a speed of 3000 rpm. TRI® reagent was used to extract the total RNA (Sigma-Aldrich, USA). The NanoDrop 2000, from Thermo Fisher Scientific, Inc., the USA, was operated to estimate the amount of RNA. The PrimeScript™ Kit from Takara, Japan was used for conducting RT-PCR. The *B. bovis* gyrase A and B

subunits and tubulin beta-chain were amplified using 150 ng of total RNA from the control and treated cultures with primers (Table 1) belonging to a previous study [22]. The reverse transcription reaction for 30 minutes to complete within a 50 µL reaction capacity at 50 °C. Then, the PCR was accomplished in reaction conditions similar to the previous investigation [22]. Ethidium bromide from Sigma-Aldrich, Japan was used to recolor 5 µL of PCR results after they had been electrophoresed in a 2% agarose gel beside a thousand-bp DNA standard scale indicator of Takara Bio Inc. in Tokyo, Japan.

TABLE 1. Primers used in this study

Gene	Sequence	Product size (bp)
Gyrase Subunit A	F 5 [▢] -CTGGTTTATTATATATGAACCC-3 [▢] R 5 [▢] -CATTITAGCAAAT ATTAGTTA-3 [▢]	2822
Gyrase Subunit B	F 5 [▢] -CCCGCCTCACTGCTACTGGCAGATG-3 [▢] R 5 [▢] -CTACCTATCTAGGTCATGTAGC-3 [▢]	3275
Tubulin beta-chain	F 5 [▢] -ATGAGAGAA ATCGTACACATCC-3 [▢] R 5 [▢] -TCAATAATCATTACCATG TCATCGG-3	1400

Statistical analysis

The distinctions between the parasitemia *in vitro* cultures were assessed utilizing an independent student's t-test in JMP statistical program from SAS Institute, Inc., the USA. It was decided that a P value of 0.05 or lower became significant statistically.

Results

In vitro growth inhibition assay

The proliferation of the treated parasites was considerably slowed ($P < 0.05$) on day 2 of treatment with five nM etoposide for *B. bovis* (Fig. 1A), *B. bigemina* (Fig. 1B), *B. caballi* (Fig. 1C), and *T. equi* (Fig. 1D). The *in vitro* development was substantially ($P < 0.05$) repressed with five nM diminazene aceturate administration. Development was suppressed with twenty-five µM for *B. bovis* and ten micromoles for *B. caballi*, *B. bigemina*, and *T. equi* (Fig. 1). The parasites were cultured without medication for 10 days and there was no proliferation of parasites at 10 µM concentration for the four parasites. On day 3, parasites were dissipated from *B. bovis*, *B. caballi*, and *T. equi*, and by day four, from

B. bigemina cultures. Parasites treated with lesser etoposide dilutions continued development after the medication was eliminated. In the following viability test, parasite regrowth was inhibited by diminazene aceturate at 0.05 micromole for *B. caballi* along with *B. bigemina* besides 1 micromole for *B. bovis* as well as *T. equi* (data not presented). Table 2 shows the IC₅₀ estimates for diminazene along with etoposide toward various piroplasms. Parasites within the cultures treated just with DMSO developed similarly to the control. Morphologic changes were compared between *Babesia* parasites from treated and untreated cultures. In etoposide-exposed *B. bovis* cultures, piroplasms had dot-appearance (Fig. 2B) compared to typical piroplasms inside the DMSO negative standard cultivation (Fig. 2A). In etoposide-cured *B. bigemina* (Fig. 2D), *B. caballi* (Fig. 3B), and *T. equi* (Fig. 3D) cultivations, a comparable appearance was detected. Etoposide was not harmful to cattle or horse erythrocytes at the maximum dilution (25 µM and 10 µM), since the pretreated RBCs had the same parasite peaks equally to the uncured ones (data not shown).

TABLE 2. IC₅₀ values of etoposide and diminazene aceturate for Three *Babesia* species and *Theileria equi*

	IC ₅₀ (nM) ^a	
	Etoposide	Diminazene
<i>Babesia bovis</i>	3.75 ± 0.4	300 ± 30
<i>Babesia bigemina</i>	4.21 ± 0.6	190 ± 20
<i>Babesia caballi</i>	4 ± 0.8	10 ± 2
<i>Theileria equi</i>	4 ± 0.5	710 ± 15

^a The IC₅₀ values are expressed as drug concentrations in the nanomolar of the growth medium and were determined using a curve fitting technique on day 4 of *in vitro* culture. IC₅₀ values denote the mean and standard deviation of 3 independent experiments.

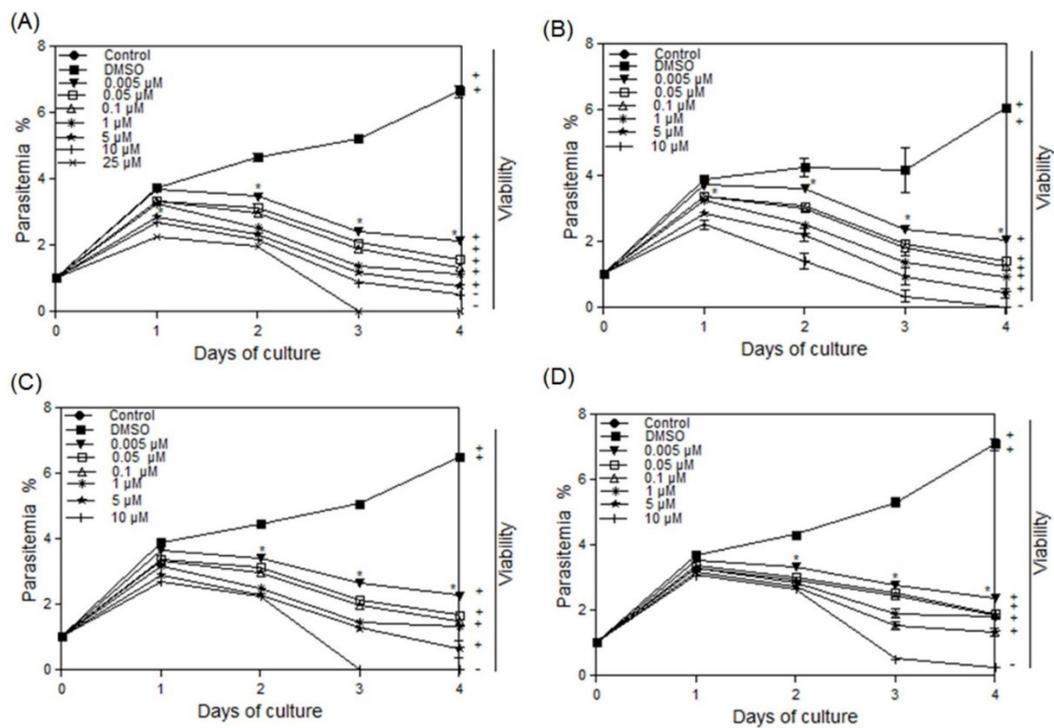


Fig. 1. Inhibitory effects of varying etoposide concentrations on *in vitro* growth. *Babesia bovis* (A), *B. bigemina* (B), *B. caballi* (C), and *Theileria equi* (D). Each value indicates the mean ± standard deviation in triplicate. These curves show the results obtained from three sets of experiments conducted in triplicate. Significantly divergent results between the drug-treated and control cultures are denoted by asterisks (* $P < 0.05$), as determined by Student's *t*-test. The regrowth status after 10 days is denoted as viable (+) and dead (-).

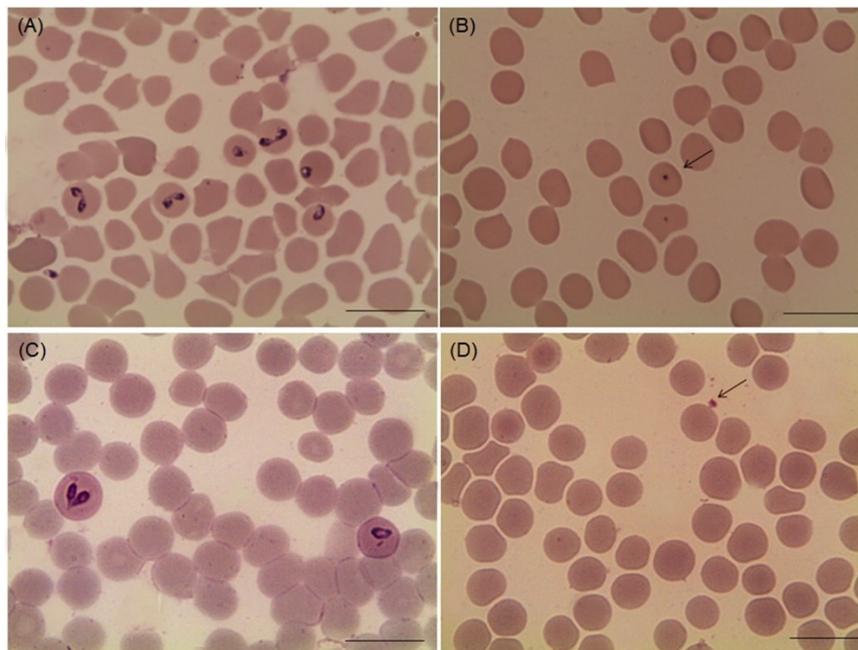


Fig. 2. Light micrographs of *Babesia bovis* and *B. bigemina* cultures subjected to *in vitro* treatment with etoposide. *B. bovis* control (A), *B. bovis* etoposide-treated (B), *B. bigemina* control (C), and *B. bigemina* etoposide-treated (D) cultures. The drug-treated cultures presented greater numbers of degenerated parasites, denoted by arrows, in comparison to the control cultures. The scale bars represent 10 μm .

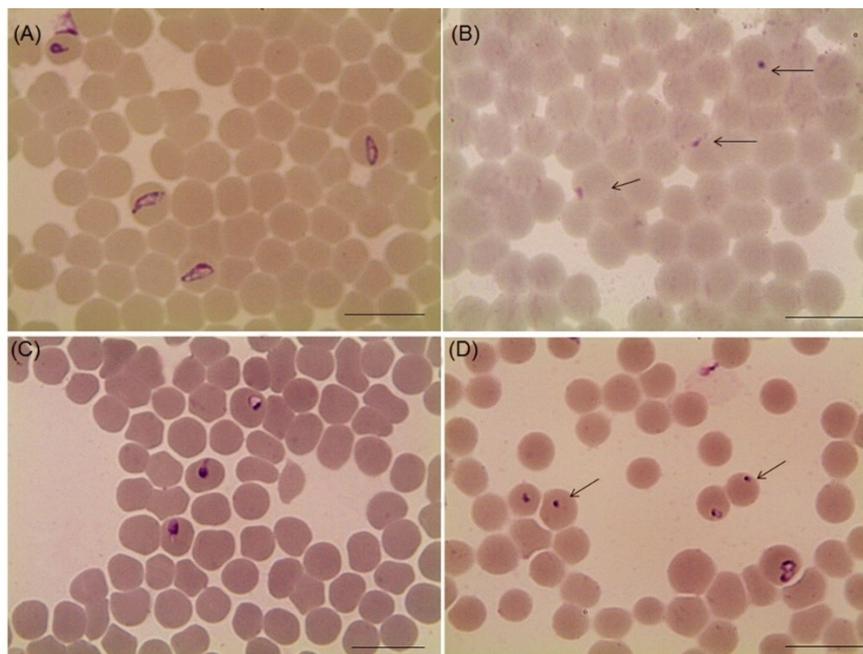


Fig. 3. Light micrographs illustrate *Babesia caballi* and *Theileria equi* *in vitro* cultures following treatment with etoposide. *B. caballi* control (A), etoposide-treated (B), *T. equi* control (C), and *T. equi* etoposide-treated (D) cultures were depicted. The etoposide-treated cultures presented greater numbers of degenerated parasites, denoted by arrows compared to the control cultures. Bars, 10 μm .

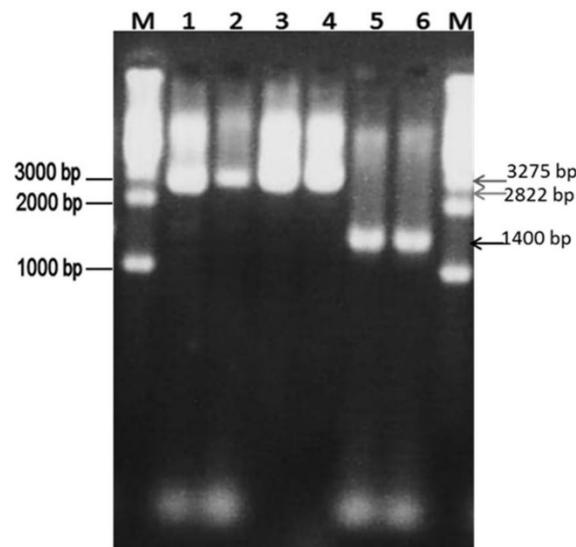


Fig. 4. Reverse-transcription PCR results for DNA gyrase A and gyrase B subunits, as well as tubulin beta chain genes, obtained from *Babesia bovis* cultures exposed to an IC₉₉ concentration (8 nM) of etoposide for 8 hours. The lanes represent DNA gyrase A from control (lane 1) and treated (lane 2) cultures, DNA gyrase B subunit from control (lane 3) and treated (lane 4) cultures, and tubulin beta chain from control (lane 5) and treated (lane 6) cultures. The 1000-bp DNA ladder-size marker is labeled as M.

T-PCR

The IC₉₉ dose of etoposide restrained the mRNA transcripts relevant to the gyrase A gene (Fig. 4, lane 2) than the gyrase B gene (Fig. 4, lane 4). The medication did not influence the tubulin beta chain gene transcripts (Fig. 4, lane 6).

Discussion

Babesia and *Theileria* parasites have a detrimental impact on animal production. The drugs commonly used for treatment, based on availability in the market, include quinuronium sulphate, imidocarb dipropionate, pentamidium, diminazene aceturate, amicarbalide, and buparvaquone. These drugs may have side effects when administered for treatment, ranging from cholinergic effects to hepatotoxicity. Therefore, in the current study, we used etoposide, a known inhibitor of the DNA gyrase enzyme.

Etoposide hindered the *in vitro* development of the four piroplasm parasites. The experiment's control verified that the outcomes were attributable to etoposide. The four piroplasms had a similar sensitivity to etoposide.

The IC₅₀ values of etoposide reported in this study were 3.75 ± 0.4 , 4.21 ± 0.6 , 4 ± 0.8 , and 4 ± 0.5 nM for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*, respectively. These IC₅₀ estimates of etoposide for the four parasites were lesser than the values described in this study for diminazene aceturate. In another study, etoposide IC₅₀ values for *P. falciparum* inhibition were 40 μ M for the 3D7 clone [14] and 350 μ M for the NF54 clone [13], which were very

high compared with those for the four parasites in the present study. This may be due to the differences between the parasites and/or host erythrocytes. The IC₅₀ values of etoposide for the four piroplasms were very low contrasted to the previous studies for *Trypanosoma brucei* at 2.6 μ M [26]. This may be due to the differences between the parasites. The IC₅₀ estimates of etoposide for the four parasites had been also lower than the chemical drugs evaluated as babesicidal drugs such as Luteolin [27], miltefosine [28], thiostrepton [22], nerolidol [25], (-)-epigallocatechin-3-gallate [21], trans-chalcone and chalcone 4 hydrate [29], ivermectin [30], Ellagic acid [31], heparin [32], thymoquinone [33], 17-DMG [34], enoxacin [35], N-acetyl-L-cysteine [36], fusidic acid [37], clofazimine [38], and allicin [39]. In this investigation, the IC₅₀ estimates of etoposide had been in the same range as those for enrofloxacin and quercetin [40, 41]. In this investigation, the IC₅₀ estimates of etoposide were comparable to the ones of other antibabesial medications, including imidocarb dipropionate [42], quinuronium sulphate [43], atovaquone [44], and epoxomicin [24]. Etoposide may be non-toxic for remedying babesiosis in mammals as the IC₅₀ estimates relevant to etoposide for *Babesia* spp. and *T. equi* are exceptionally small contrasted to the 50% inhibitory concentration values of 92.61 micromoles for renal TK10 cells [45], >100 μ M for melanoma UACC62 cells [45], >100 μ M for breast MCF7 cells [45], 17.1 μ M for HCT-116 cells [46], 16.5 μ M for Caco-2 cells [46], 19.30 μ M for HT-29 cells [46], and 43.2 μ M for CCD-18Co cells [46].

Etoposide treatment with the 99% inhibitory concentration suppressed the mRNA transcription belongs to the *B. bovis* DNA gyrase, which is in agreement with the result of a previous study that it suppresses the topoisomerase II in *P. falciparum* [14] as well as *T. brucei* [26]. Moreover, development restraint might be the result of inhibition of *Babesia* DNA topoisomerase II. Therefore, more studies are essential to elucidate the process of inhibition.

Conclusions

In conclusion, etoposide restrained the propagation of *Babesia* and *Theileria* piroplasms *in vitro*, besides affecting topoisomerase II expression in *B. bovis*. Therefore, the DNA gyrase is a target of etoposide in *B. bovis*. Etoposide showed promise to treat babesiosis and theileriosis *in vitro* but requires further studies *in vivo* evaluation for mouse babesiosis before being applied as a chemotherapeutic medication for diseases brought about by *Babesia* species and *Theileria equi*.

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Conflicts of interest

No competing interests are declared.

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تقييم التأثيرات المثبطة للإيتوبوسيد على نمو طفيليات البابيزيا والتيليريا معمليا

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أظهر الإيتوبوسيد خصائص مضادة للبكتيريا والملاريا ومكافحه للسرطان. تم استخدام ثلاثة أنواع من البابيزيا للإيتوبوسيد خصائص مضادة للبكتيريا والملاريا ومكافحه للسرطان. تم استخدام ثلاثة أنواع من البابيزيا، بابيزيا بوفيز، بابيزيا بيجيمينا، بابيزيا كابالي والتيليريا ايكوي لتقييم التأثيرات العلاجية للإيتوبوسيد في المختبر. تم دراسة تأثير الإيتوبوسيد على نسخ الـ دي إن إيه توبوايزوميراز من النوع الثاني (جينات وحدتي جايراز الـ دي إن إيه أيه و بي) التي تكون الانزيم باستخدام تقنية الـ سي آر العكسي. أظهر الإيتوبوسيد تثبيطاً كبيراً للنمو بتركيزات نانومولارية صغيرة و كان التركيز القاتل لنصف الطفيليات (IC_{50}) 3.5 للبابيزيا بوفيز، 4 للبابيزيا بيجيمينا ، 3.7 للبابيزيا كابالي، و 4 للتيليريا ايكوي نانومولار. قام الإيتوبوسيد بقمع النمو في البابيزيا بوفيز و التيليريا ايكوي عند تركيز 25 ميكرومول/لتر و البابيزيا بيجيمينا و البابيزيا كابالي عند تركيز 10 ميكرومول/لتر. خلال اختبار القابلية للبقاء على قيد الحياة، قام الإيتوبوسيد بتثبيط إعادة نمو الأربعة بروتيازومات عند 10 ميكرومول/لتر. أظهرت تقنية الـ سي آر العكسي أن علاج الإيتوبوسيد منع نسخ جين جايراز الـ دي إن إيه في البابيزيا بوفيز لذلك، جايراز الـ دي إن إيه هو هدف الإيتوبوسيد في البابيزيا بوفيز. أظهر الإيتوبوسيد نجاحاً مباشراً في علاج البابيزيا والتيليريا في المختبر ولكنه يحتاج إلى تقييم إضافي في الحيوانات لعلاج طفيليات البابيزيا في الفئران.

الكلمات الدالة: الإيتوبوسيد و بابيزيا و تيليريا ايكوي و التقييم المعمل و جايراز الـ دي إن إيه و الـ سي آر العكسي.