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Original article

Inhibitory effects of *Syzygium aromaticum* and *Camellia sinensis* methanolic extracts on the growth of *Babesia* and *Theileria* parasites

Gaber El-Saber Batiha[a,b,1], Amany Magdy Beshbishy[a,1], Dickson Stuart Tayebwa[a,c], Hazem Mohammed Shaheen[b], Naoaki Yokoyama[a], Ikuo Igarashi[a,*]

[a] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2 Sen-11, Inada-cho, Obihiro, Hokkaido, 080-8555, Japan

[b] Department of Pharmacology and Therapeutics, Faculty of Veterinary Medicine, Damanhour University, Al Beheira, Damanhour, 22511, Egypt

[c] Research Center for Tick and Tick-borne diseases, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, PO BOX 7062, Kampala, Uganda

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ABSTRACT

Currently, chemotherapeutics against piroplasmosis are also associated with toxicity and the emergence of drug-resistant parasites. Therefore, the discovery of new drug compounds is necessary for the effective control of bovine and equine piroplasms. *Syzygium aromaticum* (clove) and *Camellia sinensis* (green tea) have several documented medicinal properties. In the present study, the growth-inhibiting effects of *S. aromaticum* and *C. sinensis* methanolic extracts were evaluated *in vitro* and *in vivo*. The half-maximal inhibitory concentration (IC_{50}) values for methanolic *S. aromaticum* against *Babesia bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *Theileria equi* were 109.8 ± 3.8, 8.7 ± 0.09, 76.4 ± 4.5, 19.6 ± 2.2, and 60 ± 7.3 µg/ml, respectively. Methanolic *C. sinensis* exhibited IC_{50} values of 114 ± 6.1, 71.3 ± 3.7, 35.9 ± 6.8, 32.7 ± 20.3, and 60.8 ± 7.9 µg/ml against *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. The toxicity assay on Madin–Darby bovine kidney (MDBK), mouse embryonic fibroblast (NIH/3T3), and human foreskin fibroblast (HFF) cell lines showed that methanolic *S. aromaticum* and methanolic *C. sinensis* affected only the viability of the MDBK cell line with half-maximal effective concentrations (EC_{50}) of 894.7 ± 4.9 and 473.7 ± 7.4 µg/ml, respectively, while the viability of NIH/3T3 and HFF cell lines was not affected even at 1000 µg/ml. In the *in vivo* experiment, methanolic *S. aromaticum* and methanolic *C. sinensis* oral treatments at 150 mg/kg inhibited the growth of *Babesia microti* in mice by 69.2% and 42.4%, respectively. These findings suggest that methanolic *S. aromaticum* and methanolic *C. sinensis* extracts have the potential as alternative remedies for treating piroplasmosis.

1. Introduction

Babesiosis is a tick-borne zoonotic disease caused by intraerythrocytic protozoa parasites of the genus *Babesia* (Bock et al., 2004). Human babesiosis is caused mainly by *Babesia divergens* and *Babesia microti* (Vannier et al., 2015). Bovine babesiosis is caused by *Babesia bovis, Babesia bigemina,* and *B. divergens* (Bork et al., 2004), while equine piroplasmosis is caused by *Theileria equi* and *Babesia caballi* (Ueti et al., 2008).

Previous reports showed the toxic effects and drug-resistance of parasites to the drugs currently used for animals, namely diminazene aceturate (DA) and imidocarb dipropionate (Mosqueda et al., 2012; Moti et al., 2015). In humans, babesiosis is managed with combination therapies consisting of atovaquone (AQ)–azithromycin or clindamycin–quinine (Krause et al., 2000; Weiss, 2002; Vannier et al., 2015). Unfortunately, a previous report documented the persistence or relapse of *B. divergens* and *B. microti* infection in splenectomized patients after treatment with clindamycin and quinine (Gonzalez et al., 2014). Another study by Hatcher et al. (2001) showed that quinine treatment failed to cure patients severely infected with babesiosis in addition to provoking an adverse reaction. Even though combination therapy of AQ and azithromycin was effective, the parasitemia persisted for over 20 days after treatment. Furthermore, the resistance of AQ to *Babesia gibsoni* has already been documented (Matsuu et al., 2006). Therefore, the development of alternative treatment remedies against babesiosis is urgently required. Our study focused on screening extracts

* Corresponding author at: National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, 080-8555, Japan.
E-mail address: igarcpmi@obihiro.ac.jp (I. Igarashi).
[1] Equal contributor.

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from medicinal plants because they have been a vital source of novel products of medicinal importance over the past years (Shakya, 2016).

Syzygium aromaticum (clove) is a medium-sized tree (8–12 m) of the Myrtaceae family native to the Maluku Islands in east Indonesia (Rusmana et al., 2015). The main active constituent of *S. aromaticum* buds is eugenol. However, there are several other bioactive molecules not limited to acetyl eugenol, beta-caryophyllene vanillin, crategolic acid, bicornin, gallotannic acid, methyl salicylate, eugenin, kaempferol, rhamnetin, eugenitin, oleanolic acid, stigmasterol, campesterol, and sesquiterpenes (Nassar et al., 2007). *S. aromaticum* extracts have antioxidant, antibacterial, antifungal, antimicrobial, anthelmintic, anticancer, anti-mutagenic, anti-diabetic, and anti-*Plasmodium* properties (Bagavan et al., 2011; Gustavo et al., 2015).

Camellia sinensis (green tea) is one of the most popular beverages in the world and is deeply rooted in the cultures of China and Japan. Most of the beneficial effects of *C. sinensis* are attributed to the presence of polyphenols. These polyphenols are mainly comprised of catechins and catechin derivatives, including (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-gallocatechin gallate (Hsu et al., 2011). *C. sinensis* reportedly possesses anticancer, antitrypanosomal, and anti-*Plasmodium* properties (Paveto et al., 2004; Thipubon et al., 2015). Despite many pharmacologic investigations on *S. aromaticum* and *C. sinensis*, there have been no reports on their antibabesial activity. Therefore, this study investigated the anti-piroplasmic activity of *S. aromaticum* and *C. sinensis* methanolic extracts against the growth of bovine *Babesia* parasites (*B. bovis*, *B. bigemina*, and *B. divergens*) and equine piroplasms (*B. caballi* and *T. equi*) using *in vitro* culture. Furthermore, we evaluated the effect of the two extracts on zoonotic *B. microti* using a mouse model.

2. Materials and methods

2.1. Ethical statement

The experiments described in this study were conducted according to the rules of care and description of animal use in research published by Obihiro University of Agriculture and Veterinary Medicine, Japan. The protocol was approved by the Animal Experimentation Ethics Committee at Obihiro University of Agriculture and Veterinary Medicine (accession number: 28-111-2/28–110).

2.2. The chemical reagents

To obtain *S. aromaticum* and *C. sinensis* methanolic extracts, 99.8% methanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used to prepare stock solutions by dissolving 100 mg (crude extract) in 1 ml of DMSO. DMSO was also used to prepare stock solutions of 10 mM of DA (Ciba-Geigy Japan Ltd., Tokyo, Japan) and AQ (Sigma-Aldrich Japan, Tokyo, Japan). SYBR Green 1 (SG1) nucleic acid stain (10,000x, Lonza America, Alpharetta, Georgia, USA) was stored at −20 °C and thawed before use. Tris-HCl (130 mM; pH 7.5), 10 mM ethylenediaminetetraacetic acid (EDTA), saponin (0.016%; W/V), and Triton X-100 (1.6%; V/V) were used to prepare a lysis buffer, which was then stored at 4 °C for future use.

2.3. The plant material

S. aromaticum buds and *C. sinensis* leaves were purchased from a local market in Egypt and dried at 30 °C in an electric drying oven (Sanyo Electric Co., Ltd., Osaka, Japan). The dried leaves were milled into fine powder using a 60–80 mm mesh. Ten grams (10 g) of the finely ground plant powder was dissolved in 50 ml of methanol and then incubated at 30°C for 72 h. The obtained slurry was filtrated with Whatman filter paper No. 1. The extracts were concentrated using rotary evaporator (BUICHI®RotavaporR-200/205, Flawil, Switzerland) under reduced pressure at 40°C. After that, lyophilization was performed using a freeze-dry vacuum system (Labconco, Kansas City, MO, USA) as previously described (Kamkar et al., 2013; Kalyani, 2014; Paveto et al., 2004). Crude methanolic extracts were weighed, and 1 ml of DMSO was added to 100 mg of the extract and stored at -30°C.

2.4. The parasites

The Texas strain of *B. bovis*, the Argentine strain of *B. bigemina*, the United States Department of Agriculture (USDA) strains of *B. caballi* and *T. equi*, and a German bovine strain of *B. divergens* (Lengauer et al., 2006) were used for *in vitro* studies, while for the *in vivo* experiments, the rodent *B. microti* (Munich strain) (Lu et al., 2012) was used to infect 8-week-old female BALB/c mice (CLEA Japan, Inc., Tokyo, Japan) (Guswanto et al., 2018; Tayebwa et al., 2018).

2.5. Culture conditions

In this study, bovine and equine parasites were cultivated in bovine and equine red blood cells (RBCs) using a previously established microaerophilic stationary phase culture system (Igarashi et al., 1998). For *B. bovis*, *B. bigemina*, and *T. equi* cultivation, medium 199 (M199, Sigma-Aldrich, Tokyo, Japan) was used, while for *B. caballi* cultivation, GIT medium was used (Sigma-Aldrich Japan, Tokyo, Japan), whereas RPMI 1640 medium (Sigma-Aldrich Japan, Tokyo, Japan) was used for the cultivation of *B. divergens*. The media were supplemented with 40% cattle or horse serum. To prevent bacterial contamination, 60 µg/ml of streptomycin, 0.15 µg/ml of amphotericin B, and 60 U/ml of penicillin G (Sigma-Aldrich Corp., St. Louis, MO, USA) were added to the culture media. As a vital supplement for the *T. equi* culture, 13.6 µg of hypoxanthine (MP Biomedicals, Santa Ana, CA, USA) was added per ml.

2.6. Evaluation of the effect of S. aromaticum and C. sinensis methanolic extracts on cattle and horse RBCs

To determine the effect of *S. aromaticum* and *C. sinensis* methanolic extracts on the host RBCs, pre-treated RBCs were used for cultivation of piroplasms as described previously (Guswanto et al., 2018; Tayebwa et al., 2018). In two separate experiments, 400 µg/ml of *S. aromaticum* or *C. sinensis* methanolic extract was used to treat fresh cattle or horse RBCs for 3 h at 37 °C. The treated RBCs were then washed three times with phosphate-buffered saline (PBS) and used for culturing bovine and equine parasites. Giemsa-stained blood smears were prepared daily to determine the parasitemia in treated and untreated RBCs.

2.7. In vitro growth-inhibiting effects

The growth-inhibiting effects were examined using fluorescence assay as previously described (Guswanto et al., 2014; Rizk et al., 2015). Briefly, we dispensed 2.5 µl of RBCs at 1% parasitemia for *B. bovis* and *B. bigemina* with 97.5 µl of the medium (2.5% hematocrit) into a 96-well microtiter plate, while 5 µl for *B. divergens*, *B. caballi*, and *T. equi* with 95 µl of the medium (5% hematocrit) was added to a 96-well microtiter plate. The 60 inner wells of a 96-well plate were used in the assay, while the peripheral wells were filled with sterile distilled water to reduce evaporation during incubation. The media used contained various concentrations of the extracts, and each concentration was dispensed in triplicate. The herbal extract concentrations were 1.9, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 µg/ml, and the DA, and AQ concentrations were 0.0012, 0.0025, 0.012, 0.025, 0.051, 0.25, 0.5, and 1.1 µg/ml. Wells containing only media were used as a negative control, while those containing DMSO (0.3%) and media were used as a positive control. Thereafter, the *in vitro* culture for all parasites was incubated at 37 °C in a humidified multi-gas water-jacketed incubator at 5% CO_2, 5% O_2, and 90% N_2 for 4 successive days. On the fourth day, 100 µl of lysis buffer containing 2 × SG1 was added into each well. The

plates were incubated for 6 h in the dark at room temperature, and fluorescence values were determined using a fluorescence plate reader (Fluoroskan Ascent; Thermo Scientific, San Diego, CA, USA) at excitation and emission wavelengths of 485 and 518 nm, respectively. Gain values were set to 100. Non-parasitized bovine or equine RBCs were loaded into each well in triplicate and used as blank controls. Each experiment was repeated three times.

2.8. Viability test and the morphological changes

Microscopy assay was used to evaluate the viability of parasites treated with *S. aromaticum* and *C. sinensis* methanolic extracts. The media were changed daily for 4 successive days to cultivate 200 µl of media containing 0.25 ×, 0.5 ×, 1 ×, 2 ×, and 4 × the IC_{50} of *S. aromaticum* and *C. sinensis* methanolic extracts and 20 µl of infected RBCs at 1% parasitemia. On the fourth day, 6 µl of RBCs from all cultures including the treated, the non-treated (positive control) and DMSO treated was mixed with 14 µl of fresh RBCs and supplemented with 200 µl of growth medium without extracts and cultured in a new well plate. The plates were incubated at 37 °C with an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for the next 6 days. The medium was changed daily. Every 2 days, Giemsa stained slides were prepared and the parasite viability was detected by microscopy as previously described (Tayebwa et al., 2018). Each experiment was conducted in three separate trials. During the 4 day course of treatment, Giemsa stained smears were prepared at 24 h, 48 h, 72 h, and 96 h to monitor drug-induced morphological changes. The morphological changes were observed under a microscope, and micrographs were captured using Nikon Digital Sight® (Nikon Corporation, Tokyo, Japan).

2.9. Cell cultures

Madin–Darby bovine kidney (MDBK), mouse embryonic fibroblast (NIH/3T3), and human foreskin fibroblast (HFF) cell lines were cultured continuously at 37 °C in a humidified incubator at 5% CO_2. In 75 cm^2 culture flasks, MDBK cell line was maintained with Minimum Essential Medium Eagle (MEM, Gibco, Thermo Fisher Scientific, Carlsbad, California, USA), while NIH/3T3 and HFF cell lines were maintained with Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Thermo Fisher Scientific, Carlsbad, California, USA). Each medium was supplemented with 10% fetal bovine serum, 0.5% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, Carlsbad, California, USA), and an additional 1% glutamine. The medium was changed every 3–4 days and incubated until approximately 80% confluent. The cells were checked for mycoplasma contamination by staining with 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich Corp., St. Louis, MO, USA). TrypLE™ Express (Gibco, Life Technologies, Grand Island, New York, USA) was added to allow the cell detachment from the culture flask after washing twice with Dulbecco's phosphate-buffered saline. Subsequently, the counting of viable cells was carried out using a Neubauer improved C-Chip (NanoEnTek Inc., Seoul, Korea) after staining with 0.4% trypan blue solution.

2.10. Cytotoxicity assay of S. aromaticum and C. sinensis methanolic extracts, diminazene aceturate, and atovaquone on MDBK, NIH/3T3, and HFF cell lines

The drug-exposure viability assay was performed in accordance with recommendations for the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, the assay was carried out using a 96-well plate at 37 °C in a humidified incubator with 5% CO_2. One hundred microliters of cells at a density of 5×10^4 cells/ml was seeded per well and allowed to attach to the plate for 24 h. For the two extracts, 10 microliters of twofold dilutions was added to each well to a final concentration of 15.8 to 1000 µg/ml in triplicate, while for DA and AQ, 10 microliters of twofold dilutions was added to each well to a final concentration of 100 µg/ml in triplicate. Wells with only culture medium were used as blanks, while wells containing cells and medium with 0.4% DMSO were used as positive controls. The exposure to drugs was carried out for 24 h, followed by the addition of 10 µl of CCK-8. The plate was further incubated for 3 h, and the absorbance was measured at 450 nm using a microplate reader as previously described by Guswanto et al. (2018).

2.11. The in vitro effect of combination treatment

At a constant ratio (1:1), the drugs were combined as previously described (Chou, 2006), and the effect of the combination was detected by using the fluorescence assay as previously described (Tayebwa et al., 2018). Three sets of duplicate wells with five selected concentrations at 0.25 ×, 0.5 ×, 1 ×, 2 ×, and 4 × the IC_{50} of *S. aromaticum* and *C. sinensis* methanolic extracts and DA were set up in a 96-well plate. In a separate experiment, the effect of a combination with AQ was studied. The first set of wells contained concentrations of extracts for mono treatment, while the second set contained concentrations of DA or AQ mono treatments, and the third set contained a combination of the extract with DA or AQ. The experiment was repeated in three separate trials. For the cultivation, 100 µl of growth media containing a combined concentration of the drugs was cultured with 2.5% hematocrit infected RBCs for *B. bovis* and *B. bigemina*, and 5% hematocrit infected RBCs were used for cultures of *B. divergens*, *B. caballi*, and *T. equi* for 4 days. On the fourth day, 100 µl of lysis buffer containing 2 × SYBR Green I® (SG1) nucleic acid stain was added. The plate was covered with aluminum foil and incubated in a dark place at room temperature for 6 h. The fluorescence value for each well was detected using a fluorescence spectrophotometer reader at excitation and emission wavelengths of 485 and 518 nm, respectively. The harvested fluorescence values were calculated as percentages after subtraction of the mean values of the negative control. To determine the degree of association, the growth inhibition values harvested were entered into CompuSyn software, and the combination index (CI) values were obtained as previously described (Chou, 2006). The CI values of the drug combinations were obtained using the formula, $[(1 \times IC_{50}) + (2 \times IC_{75}) + (3 \times IC_{90}) + (4 \times IC_{95})]/10$, and the result was interpreted using a previously developed reference combination index scale: < 0.90 (synergism), 0.90–1.10 (additive), and > 1.10 (antagonism) (Chou, 2006).

2.12. The chemotherapeutic effects of S. aromaticum and C. sinensis methanolic extracts on B. microti in mice

The growth inhibition of *S. aromaticum* and *C. sinensis* methanolic extracts against *B. microti* was evaluated using 8-week-old female BALB/c mice as previously described by Tayebwa et al. (2018). Initially, two BALB/c were injected intraperitoneally with 200 µl containing 1×10^7 *B. microti*-infected RBCs. The parasitemia was monitored every 2 days until it was over 40% (Day 6-Day 7 post infection (p.i.)). The two mice were sacrificed and all the blood was harvested by venipuncture and diluted in PBS. Twenty-five mice were divided equally into five groups. Four of the groups were injected intraperitoneally with 200 µl containing 1×10^7 *B. microti*-infected RBCs, while one group was left uninfected to act as the negative control. Every 2 days, Giemsa-stained thin blood smears were prepared from the venous tail blood and parasitemia was determined by counting infected cells among 2000 RBCs. When the parasitemia in the infected mice reached 1% (after 4 days p.i.), the mice were treated daily with each specific drug for 5 days. Methanolic *S. aromaticum* and *C. sinensis* extracts were administrated orally at a dose of 150 mg kg^{-1} to the first and second groups, respectively (Agbaje et al., 2009; Chan et al., 2010; Kamkar et al., 2013). DA was used as a reference antibabesial drug and administrated intraperitoneally to mice in the third group at a dose of 25 mg kg^{-1}. The fourth group was injected with 90% double-distilled water and 10% DMSO as the vehicle used in this study. Every 2 days

until day 32 p.i., blood collected from the tip of the tail was used to prepare Giemsa stained thin smears. The smears were then viewed under a light microscope to monitor the parasitemia. The parasitemia was then calculated by counting infected RBCs among 2000 RBCs. The experiment was repeated twice.

The hematocrit (HCT), hemoglobin (HGB), and RBC counts were determined. Ten µl of blood from each mouse was collected every 96 h and used to monitor the parameters using the Celltac α MEK-6450 automatic hematology analyzer (Celltac α MEK-6450, Nihon Kohden Corporation, Tokyo, Japan).

2.13. Data analysis

The nonlinear regression (curve fitting), available in GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA), was used to calculate the IC_{50} values of extracts, DA and AQ, while the differences in the parasitemia and hematology profiles among the treated and control groups were analyzed using the Student's *t*-test, available in the GraphPad Prism software. The difference was considered statistically significant if $P < 0.05$ was obtained.

3. Results

3.1. In vitro growth inhibition

The growth inhibition assay was conducted on *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*. Methanolic *S. aromaticum* extract (Fig. 1) and methanolic *C. sinensis* extract (Fig. 2) inhibited the growth of all parasites tested. The IC_{50} values of methanolic *S. aromaticum* extract on *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi,* and *T. equi* were 109.8 ± 3.8, 8.7 ± 0.09, 76.4 ± 4.5, 19.6 ± 2.2, and 60 ± 7.3 µg/ml, respectively. The IC_{50} values of methanolic *C. sinensis* extract on *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi,* and *T. equi* were 114 ± 6.1, 71.3 ± 3.7, 35.9 ± 6.8, 32.7 ± 20.3, and 60.8 ± 7.9 µg/ml, respectively (Table 1). DA inhibited the growth of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* with IC_{50} values of 0.25, 0.11, 0.35, 0.003, and 0.37 µg/ml, respectively. AQ inhibited the growth of *B. bovis*, *B. bigemina*, *B. divergens, B. caballi,*and *T. equi* with IC_{50} values at 0.015, 0.26, 0.014, 0.038, 0.035 µg/ml, respectively **(Table S1)**. The effectiveness of methanolic *S. aromaticum* and methanolic *C. sinensis* crude extracts was not influenced by the diluent since there was no significant difference in the inhibition between the wells containing DMSO and untreated wells.

3.2. Effect of S. aromaticum and C. sinensis methanolic extracts on cattle and horse RBCs

To detect the effects of methanolic *S. aromaticum* extract and methanolic *C. sinensis* extract on cattle and horse RBCs, the RBCs of cattle and horses were incubated, respectively, with *S. aromaticum* and *C. sinensis* methanolic extracts at 400 µg/ml for 3 h before subcultures of *B. bovis* and *T. equi* were taken. The growth and parasitemia of *B. bovis* and *T. equi* did not differ significantly between methanolic *S. aromaticum* extract-treated RBCs and untreated RBCs. Also, no significant difference was observed between methanolic *C. sinensis* extract-treated RBCs and untreated RBCs (data not shown).

3.3. Viability assay

A viability assay was performed to determine the concentration of the extracts that could completely clear parasites after treating for four successive days and then withdrawing the drug pressure. The results (Figs. S1 and S2) on the 6th day of monitoring following the withdraw of drug pressure showed that *B. bigemina* and *B. caballi* cultures treated with methanolic *S. aromaticum* could not regrow at the concentration of 2 × IC_{50} (17.4 and 39.2 µg/ml, respectively), while *B. bovis*, *B. divergens*, and *T. equi* cultures did not regrow at 4 × IC_{50} (439.2, 305.6, and 240 µg/ml, respectively). Methanolic *C. sinensis* extract at 2 × IC_{50} (65.4 µg/ml) completely cleared *B. caballi* parasites. *B. bovis*, *B. divergens*, and *T. equi* cultures could not regrow at 4 × IC_{50} (456, 143.6, and 243.2 µg/ml, respectively). On the contrary, *B. bigemina* regrowth was observed in wells treated with 4 × IC_{50} (Table 2). In the untreated wells (positive control), the growth of the parasites was not affected at all.

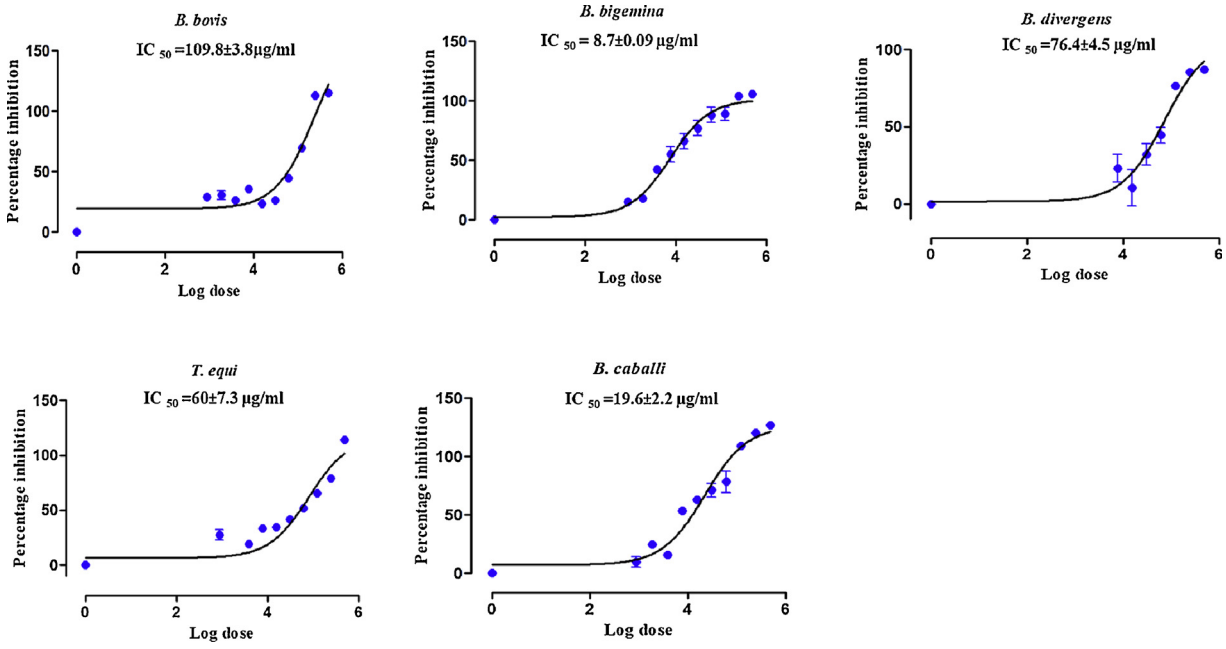

Fig. 1. The dose-response curves of methanolic *Syzygium aromaticum* against *Babesia* and *Theileria* parasites *in vitro*. The curves show the growth inhibition of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* treated with various concentrations of methanolic *S. aromaticum*. The result was determined by fluorescence assay after 96 h of incubation. The values obtained from three separate trials were used to determine the IC_{50} values using nonlinear regression (curve fitting analysis) in GraphPad Prism software.

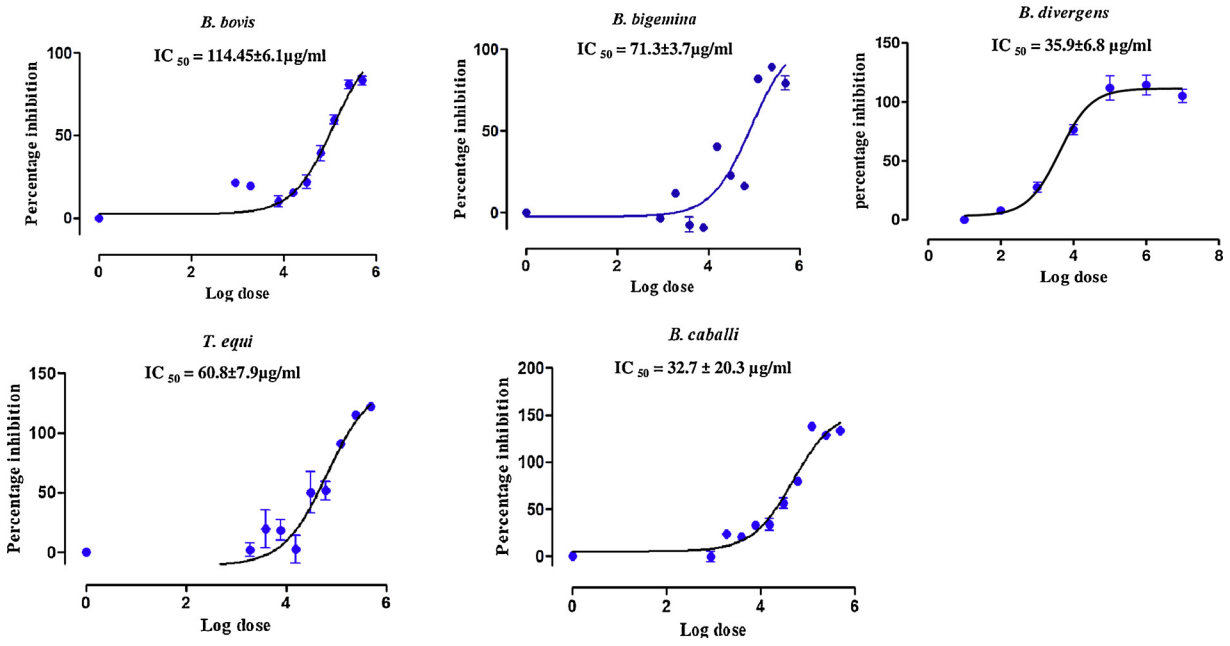

Fig. 2. The dose-response curves of methanolic *Camellia sinensis* against *Babesia* and *Theileria* parasites *in vitro*. The curves show the growth inhibition of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* treated with various concentrations of methanolic *C. sinensis*. The result was determined by fluorescence assay after 96 h of incubation. The values obtained from three separate trials were used to determine the IC_{50} values using nonlinear regression (curve fitting analysis) in GraphPad Prism software.

3.4. Morphological changes

The parasitemia was examined for all parasites after 24 h, 48 h, 72 h and 96 h of incubation with the two crude methanolic extracts in Giemsa-stained blood smears (Figs. S1 and S2). Additionally, the micrographs were taken at 24 h, 48 h, 72 h and 96 h of incubation following treatment with the two crude methanolic extracts. The micrographs of *B. bigemina* and *B. caballi* treated with methanolic *S. aromaticum* extract at 17.4 and 39.2 µg/ml, respectively are shown in Fig. 3, and micrographs of *B. bigemina* and *B. caballi* treated with methanolic *C. sinensis* extract at 142.6 and 65.4 µg/ml, respectively are shown in Fig. 4. The observations showed degeneration of the parasites as compared to the piriform shape of untreated *B. bovis*, *B. bigemina, B. divergens*, and *B. caballi*. Additionally, drug-treated parasites at 24 h and 48 h were spindle-shaped and the degenerated parasites increased in numbers at 72 h and 96 h of treatment. *T. equi* parasites treated with methanolic *S. aromaticum* and methanolic *C. sinensis* were smaller and pyknotic at 24 h and 48 h as compared to the oval shape of the untreated *T. equi* parasites in the control group. Subsequently, the drug-treated *T. equi* parasites showed higher numbers of dot-shaped degenerated parasites at 72 h and 96 h of treatment.

3.5. Toxicity of S. aromaticum and C. sinensis methanolic extracts on MDBK, NIH/3T3, and HFF cell lines

The effects of *S. aromaticum* and *C. sinensis* methanolic extracts on host cells were evaluated using MDBK, NIH/3T3, and HFF cell lines. The EC_{50} values of *S. aromaticum* and *C. sinensis* methanolic extracts on MDBK cells were 894.7 ± 4.9 and 473.7 ± 7.4 µg/ml, respectively. The two extracts did not affect the viability of NIH/3T3 and HFF cell lines until the highest concentration of 1000 µg/ml. In a separate assay, DA and AQ at concentrations of 100 µg/ml did not show any inhibition to MDBK, NIH/3T3, or HFF cell viability **(Table S1)**. The selectivity index, defined as the ratio of cell line EC_{50} to the parasite IC_{50}, is shown

Table 1
The IC_{50} and selectivity indices of methanolic *Syzygium aromaticum* and methanolic *Camellia sinensis*.

Crude extracts	Parasites	IC_{50} (µg/ml)[a]	EC_{50} (µg/ml)[b]			Selective indices[c]		
			MDBK	NIH/3T3	HFF	MDBK	NIH/3T3	HFF
Methanolic *Syzygium aromaticum*	*B. bovis*	109.8 ± 3.8	894.7 ± 4.9	> 1000	> 1000	8.1	> 9.1	> 9.1
	B. bigemina	8.7 ± 0.09				102.8	> 114.9	> 114.9
	B. divergens	76.4 ± 4.5				11.7	> 13.1	> 13.1
	B. caballi	19.6 ± 2.2				45.6	> 51.1	> 51.1
	T. equi	60.0 ± 7.3				14.9	> 16.7	> 16.7
Methanolic *Camellia sinensis*	*B. bovis*	114.0 ± 6.1	473.7 ± 7.4	> 1000	> 1000	4.2	> 8.8	> 8.8
	B. bigemina	71.3 ± 3.7				6.6	> 14.1	> 14.1
	B. divergens	35.9 ± 6.8				13.2	> 27.9	> 27.9
	B. caballi	32.7 ± 20.3				14.5	> 30.6	> 30.6
	T. equi	60.8 ± 7.9				7.8	> 16.4	> 16.4

[a] Half-maximal inhibition concentration of extracts on the *in vitro* culture of parasites. The value was determined from the dose-response curve using nonlinear regression (curve fitting analysis). The values are the means of triplicate experiments.

[b] Half-maximal effective concentration of extracts on cell lines. The values were determined from the dose-response curve using nonlinear regression (curve fitting analysis). The values are the means of triplicate experiments.

[c] Ratio of the EC_{50} of cell lines to the IC_{50} of each species. High numbers are favorable.

Table 2
The viability of *Babesia* and *Theileria* parasites treated with methanolic *S. aromaticum*, methanolic *C. sinensis,* DA and AQ.

Parasites	Concentrations of extracts (µg/ml)	M. *S. aromaticum*	M. *C. sinensis*
B. bovis	0.25 × IC_{50}	+	+
	0.5 × IC_{50}	+	+
	1 × IC_{50}	+	+
	2 × IC_{50}	+	+
	4 × IC_{50}	–	–
B. bigemina	0.25 × IC_{50}	+	+
	0.5 × IC_{50}	+	+
	1 × IC_{50}	+	+
	2 × IC_{50}	–	+
	4 × IC_{50}	–	+
B. divergens	0.25 × IC_{50}	+	+
	0.5 × IC_{50}	+	+
	1 × IC_{50}	+	+
	2 × IC_{50}	+	+
	4 × IC_{50}	–	–
B. caballi	0.25 × IC_{50}	+	+
	0.5 × IC_{50}	+	+
	1 × IC_{50}	+	+
	2 × IC_{50}	–	–
	4 × IC_{50}	–	–
T. equi	0.25 × IC_{50}	+	+
	0.5 × IC_{50}	+	+
	1 × IC_{50}	+	+
	2 × IC_{50}	+	+
	4 × IC_{50}	–	–

The positive (+) shows the regrowth of the cultures of the parasites, and the negative (-) shows the total clearance of parasites on day 6 after withdrawing the drug pressure.

in Table 1. For MDBK cell line, the highest selectivity index of *S. aromaticum* extract was found to be 102.8 times higher than the IC_{50} of *B. bigemina*, while the highest selectivity index of C. *sinensis* extract was found to be 14.5 times higher than the IC_{50} of *B. caballi*. For NIH/3T3 and HFF cell lines, the highest selectivity index of *S. aromaticum* extract was found to be > 114.9 times higher than the IC_{50} of *B. bigemina*, while the highest selectivity index of C. *sinensis* extract was found to be > 30.6 times higher than the IC_{50} of *B. caballi* (Table 1).

3.6. Combination treatment in vitro

Combination treatments of *S. aromaticum*, *C. sinensis* methanolic extracts with DA or AQ were performed on the *in vitro* cultures of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* **(Table S2)**. The methanolic *S. aromaticum* extract-DA combination treatment showed synergism against *B. bovis, B. divergens*, and *T. equi*, while it was antagonistic against *B. bigemina*. On the other hand, the methanolic *S. aromaticum* extract-DA combination showed an additive relationship against *B. caballi*. Methanolic *C. sinensis* extract-DA combination treatment showed synergism against *B. bovis*, *B. bigemina*, *B. divergens*, and *T. equi*, whereas it was antagonistic against *B. caballi* **(**Table 3**)**. Similarly, the effects of the combination treatments of *S. aromaticum* and *C. sinensis* methanolic extracts with AQ were determined on *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*. The methanolic *S. aromaticum* extract-AQ combined treatment showed a synergetic effect against *B. bovis, B. bigemina, B. divergens*, *B. caballi,* and *T. equi.* The methanolic *C. sinensis* extract-AQ combination treatment was synergetic against *B. bovis* but additive against *B. bigemina*, *B. divergens*, *B. caballi,* and *T. equi* (Table 3).

3.7. Chemotherapeutic effect of S. aromaticum and C. sinensis methanolic extracts on B. microti in mice

The promising efficacy of *S. aromaticum* and *C. sinensis* methanolic extracts *in vitro* prompted further research to evaluate the antibabesial effects of the two extracts against *B. microti* in mice. In treated groups, the parasitemia increased at a significantly lower rate than the control group ($P < 0.05$), from days 6–12 p.i. The peak parasitemia level in treated groups reached 16%, 30%, and 7% in 150 mg kg^{-1} of

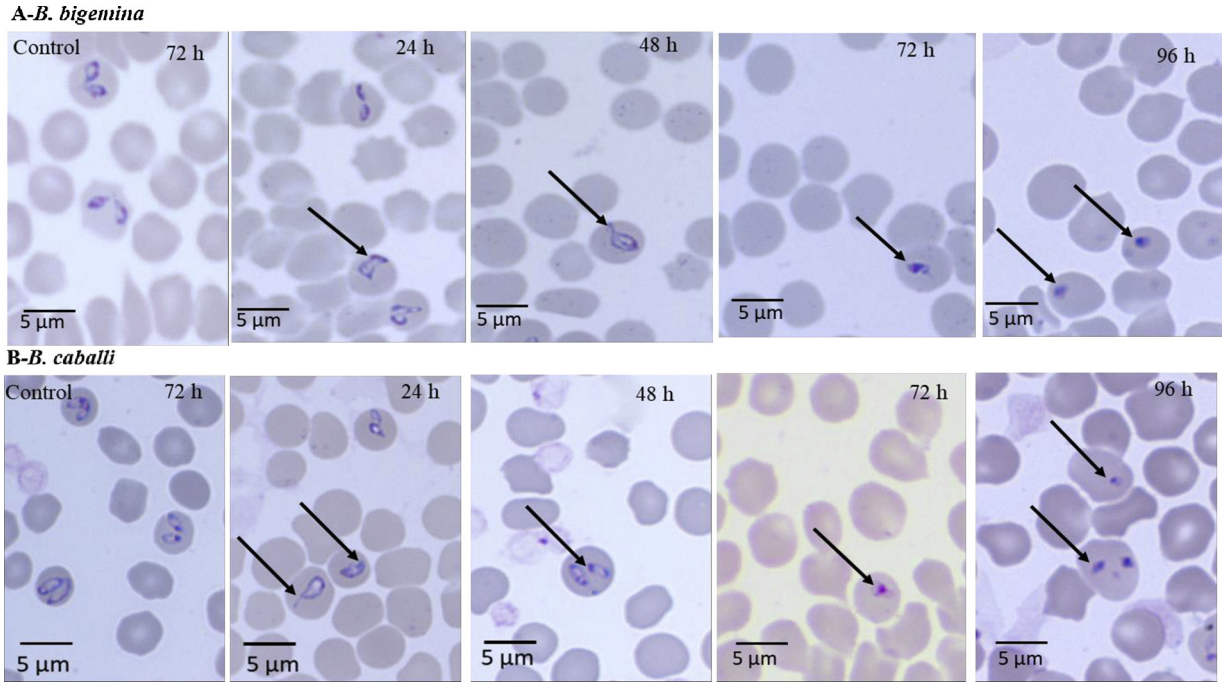

Fig. 3. The morphological changes observed in methanolic *Syzygium aromaticum*–treated *B. bigemina* and *B. caballi.* Light micrographs of methanolic *S. aromaticum*–treated *B. bigemina* and *B. caballi* at 17.4 and 38.8 µg/ml, respectively, in an *in vitro* culture taken after 24, 48, 72, and 96 h. The arrows show the spindle shapes of dividing parasites observed at 24 and 48 h as compared to the piriform shape of normal *B. bigemina* and *B. caballi* (control), while at 72 and 96 h, drug-treated cultures showed higher numbers of degenerated parasites than did the control cultures.

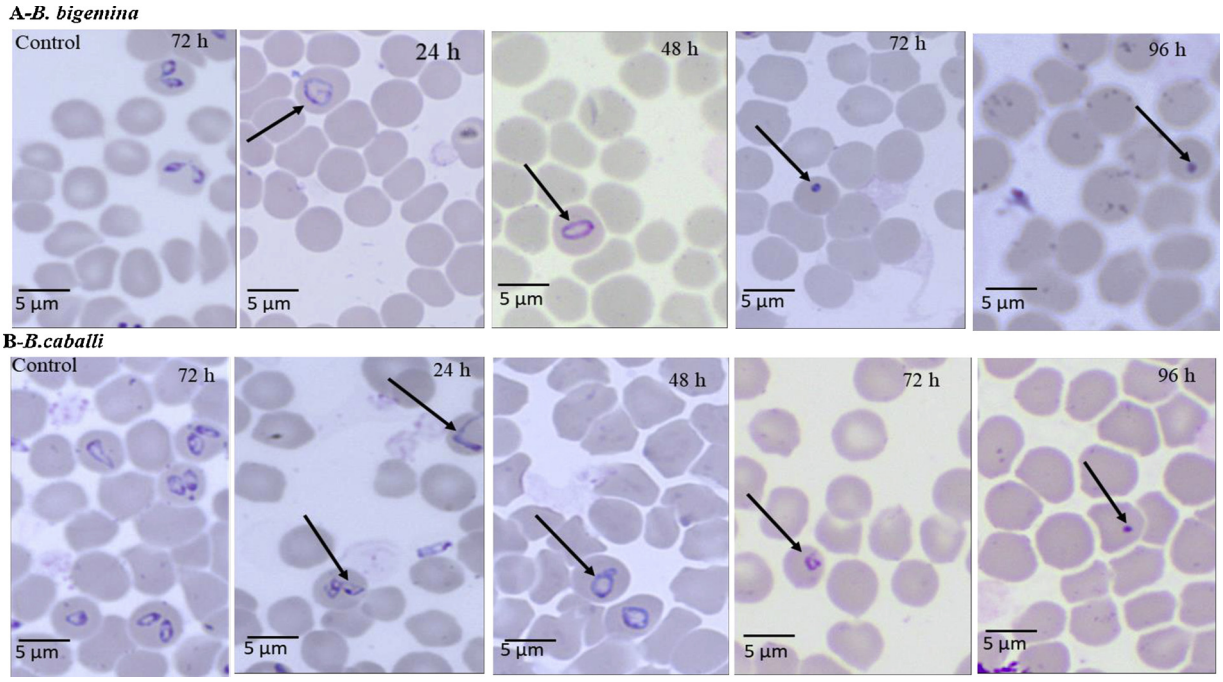

Fig. 4. The morphological changes observed in methanolic *Camellia sinensis*–treated *B. bigemina* and *B. caballi.* Light micrographs of methanolic *C. sinensis*–treated *B. bigemina* and *B. caballi* at 142.6 and 65.4 µg/ml, respectively, in an *in vitro* culture taken after 24, 48, 72, and 96 h. The arrows show the spindle shapes of dividing parasites observed at 24 and 48 h as compared to the piriform shape of normal *B. bigemina* and *B. caballi* (control), while at 72 and 96 h, drug-treated cultures showed higher numbers of degenerated parasites than did the control cultures.

Table 3
The effect of methanolic *S. aromaticum,* methanolic *C. sinensis* with DA and AQ against *Babesia* and *Theileria* parasites *in vitro.*

Parasites	Drug combinations[a]	CI values (µg/ ml)				Weighted average CI values[b]	Degree of association[c]
		IC_{50}	IC_{75}	IC_{90}	IC_{95}		
B. bovis	M. *S. aromaticum* + DA	1.298	0.873	0.802	0.878	0.892	Synergism
	M. *C. sinensis* + DA	0.200	0.425	0.582	0.626	0.530	Synergism
	M. *S. aromaticum* + AQ	0.722	0.691	0.768	0.503	0.642	Synergism
	M. *C. sinensis* + AQ	0.685	0.397	0.513	0.473	0.491	Synergism
B. bigemina	M. *S. aromaticum* + DA	3.406	3.794	5.954	8.968	6.502	Antagonism
	M. *C. sinensis* + DA	0.003	0.007	0.003	0.001	0.003	Synergism
	M. *S. aromaticum* + AQ	1.398	0.372	0.526	0.425	0.542	Synergism
	M. *C. sinensis* + AQ	0.949	0.877	1.289	0.950	0.987	Additive
B. divergens	M. *S. aromaticum* + DA	0.922	0.857	0.878	0.808	0.8502	Synergism
	M. *C. sinensis* + DA	0.374	0.472	0.438	0.432	0.436	Synergism
	M. *S. aromaticum* + AQ	0.754	0.707	0.882	0.772	0.7902	Synergism
	M. *C. sinensis* + AQ	1.853	0.981	1.001	1.053	1.103	Additive
B. caballi	M. *S. aromaticum* + DA	1.627	0.898	0.765	0.998	0.971	Additive
	M. *C. sinensis* + DA	4.482	13.052	9.372	12.572	10.899	Antagonism
	M. *S. aromaticum* + AQ	2.084	0.782	0.662	0.799	0.883	Synergism
	M. *C. sinensis* + AQ	0.239	1.053	1.291	0.953	1.003	Additive
T. equi	M. *S. aromaticum* + DA	0.561	0.682	0.299	0.552	0.503	Synergism
	M. *C. sinensis* + DA	0.036	0.023	0.002	0.013	0.014	Synergism
	M. *S. aromaticum* + AQ	1.215	0.637	0.491	0.442	0.573	Synergism
	M. *C. sinensis* + AQ	0.447	0.791	0.838	1.358	0.9975	Additive

CI value, combination index value; IC_{50}, 50% inhibition concentration; DA, diminazene aceturate; AQ, atovaquone.

[a] Two-drug combination between M. *S. aromaticum*, M. *C. sinensis* with DA, and AQ at a concentration of approximately 0.25 x IC_{50}, 0.5 x IC_{50}, IC_{50}, 2 x IC_{50}, and 4 x IC_{50} (constant ratio).

[b] The higher inhibition is preferable, thus the weighted average CI value was calculated with the formula [(1 x IC_{50}) + (2 x IC_{75}) + (3 x IC_{90}) + (4 x IC_{95})]/10.

[c] The degree of synergism was determined based on the following CI value: < 0.90 (synergism), 0.90–1.10 (additive), and > 1.10 (antagonism).

methanolic *S. aromaticum* extract, 150 mg kg^{-1} of methanolic *C. sinensis*, and 25 mg kg^{-1} of DA, respectively, at 8 days p.i., as compared to 52.1% peak parasitemia in the control group (Fig. 5).

The hematology parameters that included the number of RBCs (Fig. 6A), HGB concentration (Fig. 6B) and HCT percentage (Fig. 6C) were significantly different in the methanolic *S. aromaticum* and *C. sinensis* methanolic extract-treated groups as compared to the infected-untreated group. On the other hand, there was no significant reduction ($P < 0.05$) in the number of RBCs, HGB concentration, and HCT percentage in the methanolic *S. aromaticum* and *C. sinensis* methanolic extract treated groups in comparison to the DA-treated group.

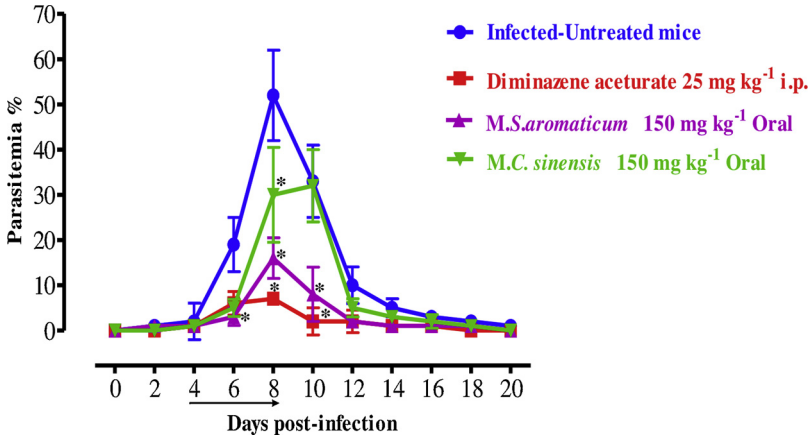

Fig. 5. The growth inhibition of methanolic *Syzygium aromaticum* and methanolic *Camellia sinensis* on *B. microti in vivo*. Inhibitory effects of methanolic *S. aromaticum* and methanolic *C. sinensis* extracts on the growth of *B. microti* in mice, based on observations taken from five mice per experimental group. The arrow indicates 5 consecutive days of treatment. Asterisks indicate statistically significant ($P < 0.05$) differences of parasitemia between treated groups and the untreated control group based on unpaired *t*-test analysis. Parasitemia was calculated by counting infected RBCs among 2000 RBCs using Giemsa-stained thin blood smears. The data is presented as the mean and standard deviation from two separate experiments.

4. Discussion

The pathology of babesiosis is related to hemolytic anemia as a consequence of the intravascular destruction of erythrocytes either by the immune system or by the direct damage caused by the parasite (Ajith et al., 2017). Current drugs used against bovine and equine piroplasmosis have shown toxic effects on treated animals, and the parasites have developed resistance (Mosqueda et al., 2012). Therefore, there is an urgent need to develop novel antibabesial drug candidates. Previous reports have shown that extracts obtained from plants possess significant therapeutic effects and yet have low toxic side effects (Paveto et al., 2004; Bagavan et al., 2011). As such, *S. aromaticum* and *C. sinensis* have been used in traditional medicine to treat several ailments (Kamkar et al., 2013; Mota et al., 2015). Many reports have shown that *S. aromaticum* and *C. sinensis* extracts are rich in numerous bioactive compounds (Santoro et al., 2007; Kalyani, 2014; Nassar et al., 2007; Hsu et al., 2011). Despite the diverse pharmacological effects of *S. aromaticum* and *C. sinensis*, they have not been evaluated against babesiosis. Therefore, this study examines the effectiveness of *S. aromaticum* and *C. sinensis* methanolic extracts against *Babesia* and *Theileria in vitro* and *in vivo*.

Methanolic extracts of *S. aromaticum* and *C. sinensis* exhibited growth-inhibitory effects against *Babesia* and *Theileria* parasites. Several reports documented the inhibitory effect of *S. aromaticum* and *C. sinensis* methanolic extracts against the growth of other apicomplexan parasites including *Plasmodium* which is closely related to *Babesia* and *Theileria* (Bagavan et al., 2011; Sannella et al., 2007). Santoro et al. (2007) reported that *S. aromaticum* eugenol has strong trypanocidal activity. Additionally, Paveto et al. (2004) reported that the polyphenolic groups present in *C. sinensis* catechins have a trypanocidal effect and showed IC_{50} values lower than IC_{50} values observed in the present study, indicating a higher sensitivity of *Trypanosoma cruzi* to *C. sinensis* catechins than *Babesia* parasites. Interestingly, the growth-inhibiting effect of methanolic *C. sinensis* extract is consistent with that reported by AbouLaila et al. (2010b, and 2011), who described that EGCG mainly extracted from tea leaves inhibited the growth of *B. bovis,*

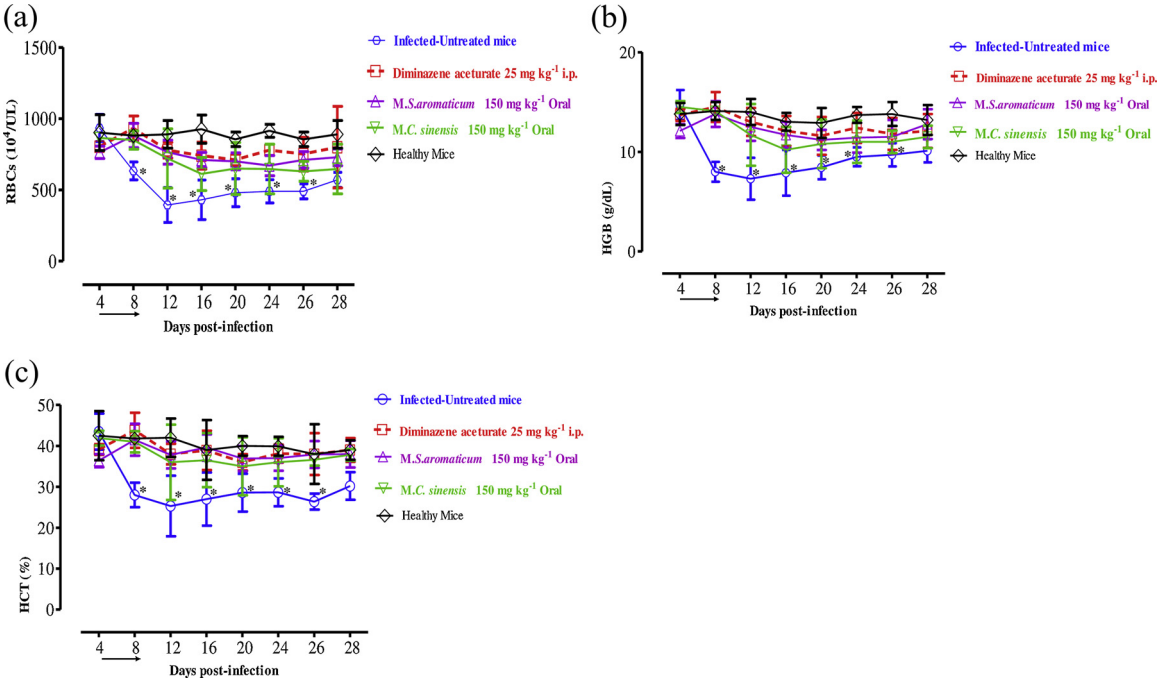

Fig. 6. The effects of *Syzygium aromaticum* and *Camellia sinensis* methanolic extracts treatment on blood parameters of mice. The changes in the number of red blood cells (RBCs) (A), hemoglobin concentration (HGB) (B), and hematocrit percentage (HCT) (C) in mice treated with methanolic *S. aromaticum*, methanolic *C. sinensis* extracts and DA (Control). The arrowhead indicates the last day of successive treatment (Day 5), while the asterisks indicate statistical significance ($P < 0.05$) of the based on unpaired *t*-test analysis. The data shown is the mean and standard deviation from two separate experiments (five mice per group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

B. bigemina, B. caballi, and *T. equi*. Additionally, the viability assay revealed that *B. bovis, B. bigemina, B. caballi,* and *T. equi*cultures could not regrow after withdrawing of the EGCG compound (AbouLaila et al. 2010b, and 2011). The growth-inhibiting effects of the two methanolic extracts observed in the current study suggest that more than one bioactive ingredients might be active against the growth of *Babesia* and *Theileria* parasites. Therefore, there is a need for further studies to isolate and test for the efficacy of the different bioactive molecules from *S. aromaticum* and *C. sinensis* methanolic extracts against *Babesia* and *Theileria* parasites.

Micrographs showed that *S. aromaticum* and *C. sinensis* methanolic extract-treated parasites were unable to egress and, subsequently, died within the infected RBCs **(**Figs. 3 and 4**)**. Previous studies of *S. aromaticum* and *C. sinensis* extracts against other protozoan parasites by Ueda-Nakamura et al. (2006) showed that eugenol extracted from *S. aromaticum* extract restricted the growth of *Leishmania amazonensis* with multiple morphological changes. Paveto et al. (2004) reported that *C. sinensis* crude extract strongly inhibited *T. cruzi* arginine kinase enzymatic activity, resulting in numerous morphological changes in the parasite.

Our study of MDBK, NIH/3T3, and HFF cell lines showed that methanolic *S. aromaticum* and methanolic *C. sinensis* affected only the viability of the MDBK cell line with a high selectivity index. This finding implied that *S. aromaticum* and *C. sinensis* methanolic extracts' bioactive ingredients were more likely to affect *Babesia* and *Theileria* than the host cells. This finding was consistent with that of Bagavan et al. (2011), who showed that methanolic *S. aromaticum* extract had no cytotoxic activity against the HeLa cell line. Furthermore, Paveto et al. (2004) showed that catechins isolated from *C. sinensis* crude extract directly damage intracellular *T. cruzi* parasite forms without any visible alteration of the host cells. Accordingly, *S. aromaticum* and *C. sinensis* methanolic extracts might be safe for use in animals and humans after further clinical studies *in vivo*.

Combination chemotherapy has been recommended against drug-resistant protozoan pathogens. Combination chemotherapy does not only increase the efficacy but also reduces the dosages of the drugs combined, thereby reducing their toxic side effects (Tuvshintulga et al., 2017). Hence, the current study explored the combination of *S. aromaticum* and *C. sinensis* methanolic extracts with previously reported antibabesial drugs such as DA and AQ against *Babesia* and *Theileria* parasites *in vitro*. The findings of this study showed that the effects of *S. aromaticum* and *C. sinensis* methanolic extracts combined with DA or AQ were mainly synergistic or additive against *Babesia* and *Theileria* parasites. The fact that *S. aromaticum* and *C. sinensis* methanolic extracts were able to combine with DA and AQ is a property that can be explored in the development of chemotherapy against *Babesia* and *Theileria.*

In the *in vivo* experiment, the parasitemia in the treated groups increased at a significantly lower rate relative to the control group ($P < 0.05$), from days 6–12 p.i. Oral administration of methanolic *S. aromaticum* extract at a dose of 150 mg kg^{-1} resulted in 69.2% inhibition in the parasitemia at day 8 p.i. as compared with 86.5% inhibition in the presence of 25 mg kg^{-1} DA on day 8 p.i. **(**Fig. 5**)**. The chemotherapeutic effect shown by methanolic *S. aromaticum* extract against *B. microti* was higher than the 68.5% inhibition shown by 500 mg kg^{-1} clindamycin (AbouLaila et al., 2012). On the other hand, oral administration of methanolic *C. sinensis* extract at a dose of 150 mg kg^{-1} resulted in 42.4% inhibition of the parasitemia on day 8 p.i. The inhibitory effect of methanolic *C. sinensis* extract on the growth of *B. microti* was higher than the 36.3% inhibition shown by epoxomicin at a dose of 0.05 mg kg^{-1} (AbouLaila et al., 2010a). The chemotherapeutic effects produced by *S. aromaticum* and *C. sinensis* methanolic extracts on *B. microti* point out its chemotherapeutic potential as an antibabesial drug.

Methanolic *S. aromaticum* extract treatments yielded exceptional hematological parameters in mice (Fig. 6A-C). Interestingly, Nassar et al. (2007) reported the remarkable antioxidant activity after treatment with *S. aromaticum* extract. Moreover, Nikoui et al. (2017) reported the anti-inflammatory and antipyretic properties after clove oil treatment. In another study, Lambert and Elias (2011) reported remarkable antioxidant and pro-oxidative effects of *C. sinensis* polyphenols, resulting in cancer prevention. Such curative properties are useful because *Babesia* and *Theileria* infections are associated with the overproduction of reactive oxygen and nitrogen species, resulting in oxidative stress (Kucukkurt et al., 2014).

5. Conclusion

The results of this study suggest for the first time that methanolic *S. aromaticum* and *C. sinensis* crude extracts antagonize the growth of *Babesia* and *Theileria* parasites. However, further studies are required to identify and characterize the bioactive compounds which are responsible for the inhibitory effects of *S. aromaticum* and *C. sinensis* extracts.

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Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.04.016.

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