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Editorial Board

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Detection of *Mycoplasma* and *Hepatozoon* spp. in Philippine Dogs

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ABSTRACT

Tick-borne diseases (TBD) in dogs have been an emerging issue worldwide. In the Philippines, most reports are in the northern areas, which include *Babesia*, *Ehrlichia*, *Anaplasma,* and *Hepatozoon* spp. The *Mycoplasma* spp., a suspected TBD pathogen, has not been reported in Philippine dogs while *Hepatozoon* spp. has not been previously documented in the southern Philippines. The present study aimed to evaluate the presence of *Mycoplasma* and *Hepatozoon* spp. in dogs in Cebu, Philippines. A total of 100 dogs from four veterinary clinics and hospitals were tested for *Hepatozoon* and *Mycoplasma* spp. using PCR. The inclusion criteria used were presence or history of ticks, anemia and/or thrombocytopenia. Clinical signs of the dogs were also obtained. There were four and two dogs found positive for *Hepatozoon* and *Mycoplasma* spp., respectively using PCR, and two dogs were found positive for *Hepatozoon* spp. using PBSE. Clinical signs such as lethargy, inappetence, fever, weight loss, and paleness were observed in most of the subjects. This study is the first of *Mycoplasma* spp. detection in Philippine dogs, and the initial report of *Hepatozoon* spp. in the southern Philippines.

Keywords: *Hepatozoon* spp.; *Mycoplasma* spp.; PBSE; PCR; tick-borne diseases

INTRODUCTION

Tick-borne diseases (TBDs) pose a serious threat to the health and welfare of dogs. TBDs are considered to be the most significant subcategory of canine vector-borne infectious diseases worldwide because of the broad geographic spectrum of many tick species, the capability of tick-borne pathogens to induce infections, and the highly zoonotic potential of some of these pathogens (Elsheikha, 2016). The occurrence and detection of these diseases in dogs have a multi-factorial cause, which includes improved animal care, faster and more efficient diagnostic tools, and a wider distribution of the vectors in a favorable environment through population migrations (Chomel, 2011).

Among the TBD pathogens is the *Hepatozoon canis*, which is an apicomplexan protozoa with a worldwide distribution affecting the health of domestic dogs and wild canids (Smith, 1996; Baneth, 2011). It is transmitted differently compared to other arthropod-borne pathogens since the ticks containing mature oocysts have to be ingested and later resulting in infection in leukocytes and parenchymal tissues (Baneth et al., 2001). Its vector is the ubiquitous brown dog tick, *Rhipicephalus sanguineus*. Meanwhile, *Mycoplasma haemocanis* is a pleomorphic bacterium that can be visualized in the peripheral blood smear of the host either singly or in chains appearing like a "violin-bow" form (Lumb, 1961). *Mycoplasma* spp. was already identified as a canine hemoplasma of worldwide distribution (Compton et al., 2012; Hamel et al., 2013; Torkan et al., 2014). Its implicated vector is still the *R. sanguineus*.

In the Philippines, the only recorded tick vector in dogs is the *R. sanguineus* (Ybañez, 2013; Ybañez et al., 2015a; Ybañez et al., 2016; Ybañez et al., 2017). The presence of several TBD pathogens has already been documented in the Philippines, but *Hepatozoon* spp. has only been reported in the northern part of the country (Baticados et al., 2010; Ybañez, 2013; Corales et al., 2014; Ybañez et al., 2017; Adao et al., 2017). On the other hand, the detection of *Mycoplasma* spp. in dogs in the Philippines is yet to be confirmed. *Mycoplasma* spp. in dogs were already detected in other ASEAN countries, including Thailand and Cambodia (Inpankaew et al., 2016; Liu et al., 2016). Hence, the present study aimed to document the presence of *Hepatozoon* spp. in the southern area of the Philippines and confirm the presence of *Mycoplasma* spp. in dogs in the country.

MATERIALS AND METHODS

Ethical considerations

The procedures performed in this study were guided by the principles of animal welfare, Animal Welfare Act of the Philippines (RA 8485) and Administrative Order No. 45 of the Bureau of the Animal Industry of the Philippines. Prior to sampling, this study was approved by the Institutional Animal Care and Use Committee (IACUC) and was given an IACUC Clearance with approval no. 2018-003. Owners of the dogs from where the samples were obtained gave consent.

Blood Sample Collection

A total of 100 dogs were sampled from four veterinary clinics and hospitals in Cebu, Philippines. Three milliliters of blood sample was collected from the cephalic vein using a BD K3 EDTA Vacutainer® tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and were stored at –20 ºC until DNA extraction. Peripheral blood smear examination (PBSE) was performed to evaluate the presence of the selected tick-borne pathogens. Complete blood count (CBC) was also performed.

DNA Extraction and PCR

DNA was extracted from the blood samples using a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, U.S.A.), following the protocol recommended by the company. After measuring their concentrations, the DNA samples were stored at -20 ºC until use.

Previously described PCR assay was used to detect the selected tick-borne pathogens. Detection for *Mycoplasma* and *Hepatozoon* spp. were respectively performed using PCR based on 16S rRNA and 18S rRNA genes (Ybañez et al., 2015b; Inokuma et al., 2002). The PCR assay was carried out in a final volume of 25 μl reaction mixture containing 5 μl of each DNA template. Double distilled water (DDW) was used as a negative control. Amplification cycles included initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s, and extension at 72 °C for 90 s followed by a final extension at 72 °C for 5 min. The PCR amplicons were visualized using a 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer stained with ethidium bromide under the UV transilluminator.

Data Processing and Analysis

Gathered data were manually encoded in Microsoft Excel, and imported to statistical software. Descriptive statistics were employed.

RESULTS AND DISCUSSION

PCR analysis revealed that four and two dogs were positive for *Hepatozoon* spp. and *Mycoplasma* spp., respectively. The low detection rate of *Hepatozoon* spp. in the area is similar to other reports in the northern Philippines (Baticados et al., 2010; Adao et al., 2017), but it is lower than those reported by nearby countries like Thailand (Liu et al., 2016) and Cambodia (Inpankaew et al., 2016). PBSE revealed two of the four *Hepatozoon* spp.-positive PCR to have inclusion bodies, while none was observed for the *Mycoplasma* spp.-positive PCR samples. The negative detection using PBSE may have been affected by the low level of parasitemia during the time of sample collection or maybe due to the chronic stage of the disease (Rani et al., 2011). High parasitemia level is usually seen in acute stages (Sakuma et al., 2009), which can be evident in the blood smears. However, PBSE is considered to be less reliable as PCR-positive animals may have negative PBSE results (Ybañez, 2013).

Due to the low detection rates reported in this study, performing statistical analysis on profile, clinical signs, complete blood count and PCR positivity was not ideal. Instead, summarized information on the positive cases is hereby presented.

Upon presentation, clinical signs such as lethargy, inappetence, fever, weight loss, and paleness were observed in most of the subjects. However, not all dogs had high neutrophil counts and not all were anemic and thrombocytopenic (3/4) as shown in their complete blood count findings (data not shown). These results indicate that the dogs, upon the time of presentation, were not in the acute stage of the disease and the similar clinical signs and blood values may have been caused by other pathogens present in the animal (Rojas et al., 2014). *Hepatozoon* spp.-infected dogs exhibited lethargy, paleness of mucous membranes, anemia, and thrombocytopenia. The observed lethargy and inappetence may be due to the inflammatory reactions caused by *Hepatozoon* spp. in the organs such as the bone marrow, lymph nodes, spleen, and liver. Since *Hepatozoon* spp. infects the bone marrow, liver, and spleen, paleness of the mucous membranes can be an expected clinical finding, since these organs are involved in the production of red blood cells (Duncan and Prasse, 1977). Anemia was observed in most of the positive dogs (3/4) *w*hich was similar to previous

studies (Elias and Hommans, 1988; Sakuma et al., 2009; Rojas et al., 2014). Anemia can be due to the necrosis of the spleen and liver in *Hepatozoon* spp infection (Tsachev et al., 2008). *Hepatozoon* spp. can also cause dysmegakaryopoiesis and dysgranulopoiesis in the bone marrow of affected dogs which may lead to the development of anemia in *Hepatozoon* spp. infections (De Tommasi et al., 2014). It should be taken into account that merogony of *Hepatozoon* spp. occurs in the bone marrow (Baneth et al., 2007).

Fever, which would have been an expected finding, was only observed in one patient. One out of the four positive dogs had no clinical signs seen during presentation proving that *Hepatozoon* spp. infection can also be subclinical, and clinical manifestations can only be found when dogs are immunocompromised. Thrombocytopenia in this study can be most likely due to co-infection with other diseases causing thrombocytopenia such as ehrlichiosis and babesiosis, as the same vector, *R. sanguineus*, carries these diseases (Yabsley et al., 2008; De Tommasi et al., 2013). *Mycoplasma* spp.-infected dogs exhibited inappetence, weight loss, and pale mucous membrane. The pathogen seldom causes anemia unless the dogs are immunocompromised or splenectomized. Another factor is co-infection with other TBDs such as babesiosis and ehrlichiosis which could be the cause of the manifestation of pallor or anemia and other clinical signs (Sasanelli et al., 2009; Rojas et al., 2014). Thrombocytopenia was also observed.

An increased neutrophil count was detected in half of the dogs in this study which is attributed to the secondary necrosis of spleen, liver, lymph nodes (Tsachev et al., 2008). Increased neutrophil count was also reported in the studies of Voyvoda et al. (2004) and Kaur et al. (2012). Basophilia was also witnessed in most dogs which might be an indication of chronic infection in TBDs. Other blood values in this study were found to be within the normal range.

The present study reports the presence of *Hepatozoon* spp. in dogs in the southern Philippines and the first detection of *Mycoplasma* spp. in Philippine dogs. Infection with the pathogens mentioned above must be considered as among the differential diagnoses by local practitioners, especially when presenting clinical signs resemble the disease. The results of this study is a valuable addition to the TBD pathogen diversity in the Philippines. Moreover, further studies at the genetic analysis level will have to be endeavoured in the future for species confirmation and for analysis of their relatedness to pathogens isolated in dogs from other countries.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that the work described has not been published previously and that it is not under consideration for publication elsewhere. All the authors approve the publication of this manuscript and that, if accepted, it will not be published elsewhere in the same form, in English or any other language, including electronically without the written consent of the copyright holder.

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Anti-piroplasmic potential of the methanolic *Peganum harmala* seeds and ethanolic *Artemisia absinthium* leaf extracts

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ABSTRACT

The available drugs against piroplasmosis are insufficient and faced with several challenges, such as drug-resistant parasites and toxicity to treated animals. Therefore, the discovery of new drug compounds is necessary for the effective control of babesiosis and theileriosis. Methanolic *Peganum harmala* seed extract (MPHSE) and ethanolic *Artemisia absinthium* leaf extract (EAALE) have several medicinal properties. In the present study, the growth-inhibition effects of MPHSE and EAALE were evaluated *in vitro* and *in vivo*. The half-maximal inhibitory concentration (IC_{50}) values for MPHSE against *Babesia bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *Theileria equi* were 24.9 ± 1.2, 77.1 ± 2.3, 61.1 ± 2.9, 80.8 ± 4.1, and 11.3 ± 2.1 μg/mL, respectively. EAALE exhibited IC_{50} values of 43.3 ± 3.1, 39.2 ± 2.7, 38.5 ±3.7, 50.3 ± 2.1, and 28.2 ± 2.1 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 MPHSE affected the viability of MDBK, NIH/3T3, and HFF cell lines with half maximum effective concentration (EC_{50}) values of 611.7± 10.9, 870 ± 22, and >1500 μg/mL, respectively, while EAALE exhibited EC_{50} values of 340.7 ± 8.5, 736.7 ± 9.3, and 1371.5 ± 17.3 μg/mL against MDBK, NIH/3T3, and HFF cell lines, respectively. In the *in vivo* experiment, MPHSE and EAALE oral treatments at 150 mg/kg inhibited the growth of *Babesia microti* in mice by 60% and 55.1%, respectively. These findings suggest that MPHSE and EAALE have the potential to be alternative remedies for treating piroplasmosis.

Keywords: *Peganum harmala*; *Artemisia absinthium*; *Babesia*; *Theileria*; *In vitro*; *In vivo*

INTRODUCTION

Piroplasmosis caused by hematotropic parasites of the genus *Babesia* and *Theileria* is a tick- transmitted disease (Bock et al., 2004). *Babesia bovis*, *B. bigemina*, and *B. divergens* infect cattle, causing bovine babesiosis. *B. bovis* is a much more virulent organism than *B. bigemina* or

B. divergens, and the pathogenic effects of the parasites relate more directly to erythrocyte destruction, a hypotensive shock syndrome, combined with generalized nonspecific inflammation, coagulative disturbances, and erythrocytic stasis in capillaries (Vos and Bock, 2006). *Babesia caballi* and *Theileria equi* infect horses, causing equine piroplasmosis. *T. equi* parasitizes leucocytes and erythrocytes for the completion of their life cycle, causing anemia, weight loss, lethargy, and fever (Bock et al., 2004). *B. divergens* and *B. microti* infect humans, especially immunocompromised persons, causing human babesiosis, characterized by hemolytic anemia due to high parasitemia that may exceed 70% (Homer et al., 2000; Weiss, 2002).

Unfortunately, many reports documented that some *B. gibsoni* strains have shown resistance to atovaquone (AQ) (Korsinczky et al., 2000; Matsuu et al., 2006). Previous reports have shown the toxic effects of, and parasite resistance to, drugs currently used for animals, namely diminazene aceturate (DA) and imidocarb dipropionate (Mosqueda et al., 2012; Moti et al., 2015). Moreover, Wormser et al. (2010) documented the therapeutic failure of some severe human cases infected with human babesiosis during the treatment with azithromycin-AQ combination therapy because of the development of *B. microti* resistance parasite. The antiparasitic drugs that have been evaluated against the pathogenic protozoa often have severe side effects. Therefore, low toxicity and more effective chemotherapeutic agents are urgently needed against *Babesia* and *Theileria* parasites, possibly through research on medicinal plants.

Plants have been used to treat common infectious diseases since ancient times, and some of these traditional medicines are still part of the habitual treatment of various maladies (Batiha et al., 2019a; Ríos and Recio, 2005). Because of the side effects and the resistance that pathogenic microorganisms build against the antibiotics and most drugs on the market today, much attention has been paid to the extracts and biologically active compounds isolated from plant species that are used in herbal medicine (Batiha et al., 2019b; Beshbishy et al., 2019a). Plant extracts contain several terpenoids with numerous uses, specifically monoterpenes, diterpenes, and sesquiterpenes. Terpenes are among the active chemicals in plants used for medicinal, culinary, and aromatic purposes (Dorman and Deans, 2000). Known for their antiseptic, i.e., bactericidal, virucidal, fungicidal, and medicinal properties as well as their fragrance, they are used in embalmment, in food preservation, and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic, and local anesthetic remedies (Bakkali et al., 2008).

Peganum harmala is a widely used medicinal plant from the family Nitrariaceae. The main medicinal part of the plant is the seed (Moloudizargari et al., 2013). The pharmacologically active compounds of *P. harmala* are several alkaloids, which are found especially in the seeds and roots, including β-carbolines such as harmine, harmaline, harmalol, and harman and the quinazoline derivatives vasicine and vasicinone, which illustrate well the diversity of antiprotozoal compounds found in this plant (Mirzaie et al., 2007). Their composition was evaluated for phenolics (gallic acid equivalent 2.48 to 72.52 g/kg), tannins (catechin equivalent 0 to 25.27 g/kg), anthocyanins (cyanidin equivalent 0 to 20.56 mg/kg), and flavonoids (quercetin equivalent 0 to 3.12 g/kg) (Chabir et al., 2014).

Artemisia absinthium is an important perennial shrubby plant belonging to the genus *Artemisia*, commonly known as wormwood in the United Kingdom and absinthe in France (Nahrevanian et al., 2012). *A. absinthium* leaves have been of great botanical and pharmaceutical interest and are employed in folk medicine against various ailments (Rodríguez-Pérez et al., 2006; Valdés et al., 2008). The plant has been widely used, mainly for its neuroprotective, antifungal, antimicrobial, insecticidal, acaricidal, anthelmintic, antimalarial, hepatoprotective, and antidepressant proprieties (Beigh and Ganai, 2017). *A. absinthium* leaves have been used

successfully for many years by the French army in Algeria as a remedy against malaria (Beigh and Ganai, 2017), and its leaf extracts have been reported to exhibit antiprotozoal potential against *Trypanosoma brucei*, *T. cruzi*, and *Leishmania infantum*. The antileishmanial activity of *A. absinthium* extracts is attributed to its constituent flavonoids (Beigh and Ganai, 2017).

Despite the many pharmacologic investigations of *P. harmala* seeds and *A. absinthium* leaves, there are no reports on the anti-piroplasmic activity of *P. harmala* and *A. absinthium* crude extracts. Therefore, this study investigated the anti-piroplasmic activity of methanolic *Peganum harmala* seed extract (MPHSE) and ethanolic *Artemisia absinthium* leaf extract (EAALE) against the growth of bovine *Babesia*, *B. bovis*, *B. bigemina*, and *B. divergens*, and equine piroplasm parasites, *B. caballi,* and *T. equi* using *in vitro* cultures. Furthermore, we evaluated the chemotherapeutic effect of the two extracts on rodent *B. microti* in mice.

MATERIALS AND METHODS

Ethical statement

The experiments described in this study were conducted according to the rules of care and 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 of the animal experiment: 28-111-2/28-110).

Plant material

P. harmala seeds and *A. absinthium* leaves were purchased from a local market in Damanhour, Egypt, and dried at 30°C in an electric drying oven (Sanyo Electric Co., Ltd., Osaka, Japan). The dried seeds and leaves were milled into a fine powder using a 60–80 mm mesh. Fifty grams (50 g) of the finely ground plant powder was dissolved in 250 mL of methanol and ethanol and then incubated at 30°C for 72 h. The obtained solutions were filtrated through sterile gauze and cotton. Next, the extracts were concentrated under reduced pressure at 40°C to remove solvents using a rotary evaporator (BUICHI® RotavaporR-200/205, Flawil, Switzerland). After that, lyophilization was performed using a Freeze Dry Vacuum System (Labconco, Kansas City, MO, USA) as previously described (Sulaiman et al., 2019; Valdés et al., 2008; Gordanian et al., 2014; Mohammad et al., 2017). Crude extracts were weighed, and 1 mL of dimethyl sulfoxide (DMSO) was added to 100 mg of the extract and stored at -30°C.

Chemical reagents

To obtain MPHSE and EAALE, 99.8% methanol (Wako Pure Chemical Industrial, Ltd., Osaka, Japan, Cat. No. 67-56-1), 99.5% ethanol (Chameleon Reagent, Osaka, Japan, Cat. No. 64-17-5), and DMSO (Wako Pure Chemical Industrial, Ltd., Osaka, Japan, Cat. No. 67-68-5) were used to prepare stock solutions of 100 mg (crude extract) /1 mL (DMSO). DMSO was used to prepare stock solutions of 10 mM clofazimine (CF) (Sigma-Aldrich, Japan, Cat. No. 2030-93-9), DA (Ciba-Geigy Japan, Ltd., Tokyo, Japan, Cat. No. 908-54-3), and AQ (Sigma-Aldrich, Japan, Cat. No. 95233-18-4). The SYBR Green 1 nucleic acid stain (SG1, 10,000x, Lonza, USA, Cat. No. 50513) was stored at -20°C until 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 stored at 4°C until use.

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 with 5% CO_2. The MDBK cell line was maintained in a 75 cm^2 culture flask with Eagle's Minimum Essential Medium (EMEM, Gibco, Thermo Fisher Scientific, Carlsbad, California, USA), while Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Thermo Fisher Scientific, Carlsbad, California, USA) was used for NIH/3T3 and HFF cell line cultivation. Each medium was supplemented with 10% inactivated 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 to 4 days and incubated until approximately 80% confluent. The cells were free from mycoplasma contamination after being checked by staining with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich Corp., St. Louis, MO, USA). Detachment of the cells from the culture flask was done using TrypLETM Express (Gibco, Thermo Fisher Scientific, Carlsbad, California, USA) after washing two times with Dulbecco's phosphate-buffered saline (DPBS). Subsequently, the counting of viable cells was carried out using a Neubauer improved C-Chip (NanoEntek Inc., Korea) after staining with 0.4% trypan blue solution.

Cytotoxicity assay of MPHSE and EAALE on MDBK, NIH/3T3, and HFF cells

A drug-exposure viability assay was performed in accordance with the recommendation for Cell Counting Kit-8 (CCK-8, Dojindo, Japan). Briefly, the assay was carried out using a 96-well plate at 37°C in a humidified incubator with 5% CO_2 and 95% air. One hundred microliters of cells at a density of 5 x 10^4 cells/mL were seeded per well and allowed to attach to the plate for 24 h. For the two extracts, 10 μL of twofold dilutions were added to each well to a final concentration of 15.8 to 1500 μg/mL in triplicate, while for DA, AQ, and CF, 10 μL of twofold dilutions were added to each well to a final concentration of 100 μg/mL in triplicate. The wells with only a culture medium were used as blanks, while the wells containing cells and a medium with 0.4% DMSO were used as a positive control. The drug exposure 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.

Parasites

A German bovine strain of *B. divergens*, the Texas strain of *B. bovis*, the Argentine strain of *B. bigemina*, and the United States Department of Agriculture (USDA) strains of *B. caballi* and *T. equi* (Guswanto et al., 2018) were used for *in vitro* studies. The Munich strain of *B. microti* (Tayebwa et al., 2018) was used to infect 8-week-old female BALB/c mice (Clea, Japan) to conduct the *in vivo* experiments.

Culture conditions

Bovine and equine parasites were cultivated in purified bovine and equine red blood cells (RBCs) using a micro-aerophilic stationary phase culture system (Guswanto et al., 2018). For *B. bovis* and *B. bigemina*, medium 199 (M199) (Sigma-Aldrich, Tokyo, Japan) supplemented with 40% cattle serum was used, while for *B. caballi* cultivation, GIT medium supplemented with 40% horse serum was used (Sigma-Aldrich, Tokyo, Japan). RPMI 1640 medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 40% cattle serum was used for the cultivation of *B. divergens*. For *T. equi* cultivation, medium 199 (M199) supplemented with 40% horse serum was used. 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, USA) were added to the culture media. Then 13.6 μg of hypoxanthine (MP Biomedicals, USA) per mL was added as a vital supplement for the *T. equi* culture.

Evaluation of the effect of MPHSE and EAALE on bovine and equine RBCs

The hemolytic effect of MPHSE and EAALE on RBCs was conducted according to the previously described protocol (Guswanto et al., 2018). In two separate experiments, 200 μg/mL of MPHSE and EAALE were used to treat fresh cattle and horse RBCs for 3h at 37ºC. Then the treated RBCs were washed with phosphate-buffered saline three times and used to culture bovine and equine parasites. Giemsa-stained blood smears were prepared daily to determine the parasitemia in treated and untreated RBCs.

***In vitro* growth-inhibitory effects**

The growth-inhibitory effects were examined via fluorescence assay as previously described (Beshbishy et al., 2019b). The parasite-infected RBCs were diluted with uninfected RBCs to obtain a stock supply of RBCs with 1% parasitemia. 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. We dispensed 2.5 μL of RBCs at 1% parasitemia for *B. bovis* and *B. bigemina* with 97.5 μL of the medium into a 96-well microtiter plate. Meanwhile, 5 μl of RBCs at 1% parasitemia for *B. divergens*, *B. caballi*, and *T. equi* with 95 μL of the medium was added into a 96-well microtiter plate. The media used contained various concentrations of the test extracts, and each concentration was dispensed in triplicate. The herbal extract concentrations were 0.24, 1.2, 6, 30, and 150 μg/mL. 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, while the CF concentrations were 0.117, 0.234, 0.468, 0.94, 1.89, 3.75, 7.5, and 15 μg/mL. The wells containing only media were used as a negative control, and wells 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 for 4 successive days with an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. On the fourth day of culture, 100 μL of a lysis buffer containing 2 × SG1 was added to each well on the culture plate. 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 LabSystems, Oceanside, California, USA) at 485 nm and 518 nm excitation and emission wavelengths, 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.

Viability test and morphological changes

A microscopy assay was used to evaluate the viability of MPHSE- and EAALE-treated parasites. Two hundred microliters of media containing 0.25 ×, 0.5 ×, 1 ×, 2 ×, and 4 × the IC_{50} of MPHSE or EAALE and 20 μL of infected RBCs at 1% parasitemia were cultivated by changing the media daily for 4 successive days. On the fourth day, 6 μL of infected, treated RBCs 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 37ºC with an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for the next 8 days. The parasite growth was detected by microscopy in order to evaluate the parasite viability as previously described by Batiha et al. (2019c). Each experiment was made in triplicate in three separate trials. The morphological changes were observed under a

microscope, and micrographs were captured using Nikon Digital Sight ® (Nikon Corporation, Tokyo, Japan).

Combination treatment *in vitro*

At a constant ratio (1:1), the drugs were combined as previously described (Chou, 2006), and the combination effect was detected by using the fluorescence assay as previously described (Guswanto et al., 2018). Three sets of duplicate wells with five selected concentrations, 0.25 ×, 0.5 ×, 1 ×, 2 ×, and 4 × the IC_{50} of MPHSE or EAALE and DA, were set up in a 96-well plate. In a separate experiment, the combination effects with AQ and CF were studied. The first set of wells contained concentrations of extract mono treatments; the second set contained concentrations of DA, AQ, or CF mono treatments; and the third set contained a combination of the extract with DA, AQ, or CF. The experiment was repeated in three separate trials. For cultivation, 100 μL of growth media containing the drug concentrations was cultured for 4 days with 2.5% infected RBCs for *B. bovis* and *B. bigemina*; 5% infected RBCs were used for the culture of *B. divergens*, *B. caballi*, and *T. equi*. On the fourth day, 100 μL of the lysis buffer containing SG1 was added. The plate was covered with aluminum foil for protection against light and incubated in a dark place at room temperature for 6 h. The fluorescence value was detected using a fluorescence spectrophotometer reader at 485 and 518 nm excitation and emission wavelengths, 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 harvested growth-inhibition values were entered into CompuSyn software, and the combination index (CI) values were obtained as previously described (Chou, 2006). The CI values of the drug combination were obtained using the formula $((1 \times IC_{50}) + (2 \times IC_{75}) + (3 \times IC_{90}) + (4 \times IC_{95}))/10$, and the results were explained using the reference combination index scale; < 0.90 (synergism), 0.90–1.10 (additive), and > 1.10 (antagonism) developed previously (Chou, 2006).

Chemotherapeutic effects of MPHSE and EAALE on *B. microti* in mice

Eight-week-old female BALB/c mice were used to evaluate the growth inhibition of MPHSE and EAALE against *B. microti* as previously described (Tayebwa et al., 2018). Twenty-five mice were separated into five equal groups. Four of the groups were injected intraperitoneally with 1×10^7 *B. microti*–infected RBCs, while one of the groups was left uninfected to act as the negative control. When the parasitemia in the infected mice reached 1%, the mice were injected daily with each specific drug for 5 days. MPHSE and EAALE were administrated orally at a dose of 150 mg kg^{-1} to the first and second groups, respectively. DA was used as a reference antibabesial drug and administrated intraperitoneally to the third group at a dose of 25 mg kg^{-1}. The fourth group was administered with double-distilled water (DDW) as an untreated control group. The levels of parasitemia in all mice were examined daily using Giemsa-stained thin blood smears prepared from venous tail blood every 2 days until day 32 post-infection. The parasitemia was calculated by counting infected RBCs among 2,000 RBCs and the inhibition rate was calculated by dividing the peak parasitemia of the herbal extract treated group /the untreated-infected group -1%. The hematocrit (HCT) percentage was monitored, and 10 µL of blood was collected from each mouse every 96 h and measured with a Celltac α MEK-6450 automatic hematology analyzer (Celltac α MEK-6450, Nihon Kohden Corporation, Tokyo, Japan). The experiment was repeated twice.

Data analysis

The nonlinear regression (curve fit), available in GraphPad Prism (GraphPad Software Inc., USA), was used to calculate the IC_{50} of extracts, DA, AQ, and CF from the percentage of inhibition.

RESULTS

Growth-inhibitory effect of MPHSE and EAALE *in vitro*

A growth-inhibitory assay was conducted on *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*. MPHSE (Fig. 1) and EAALE inhibited the growth of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* (Fig. 2). The IC_{50} values of MPHSE on *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* were 24.9 ± 1.2, 77.1 ± 2.3, 61.1 ± 2.9, 80.8 ± 4.1, and 11.3 ± 2.1 μg/mL, respectively. The IC_{50} values of EAALE on *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* were 43.3 ± 3.1, 39.2 ± 2.7, 38.5 ± 3.7, 50.3 ± 2.1, and 28.2 ± 2.1 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 ± 0.02, 0.11 ± 0.01, 0.35 ± 0.03, 0.003 ± 0.001, and 0.37 ± 0.01 μg/mL, respectively. AQ inhibited the growth of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* with IC_{50} values of 0.015 ± 0.001, 0.26 ± 0.03, 0.014 ± 0.002, 0.038 ± 0.01, and 0.035 ± 0.01 μg/mL, respectively. CF inhibited the growth of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* with IC_{50} values of 0.015 ± 0.001, 0.26 ± 0.03, 0.014 ± 0.002, 0.038 ± 0.01, and 0.035 ± 0.01 μg/mL, respectively (Table S1). There was no significant difference in inhibition between the wells containing DMSO and the untreated wells.

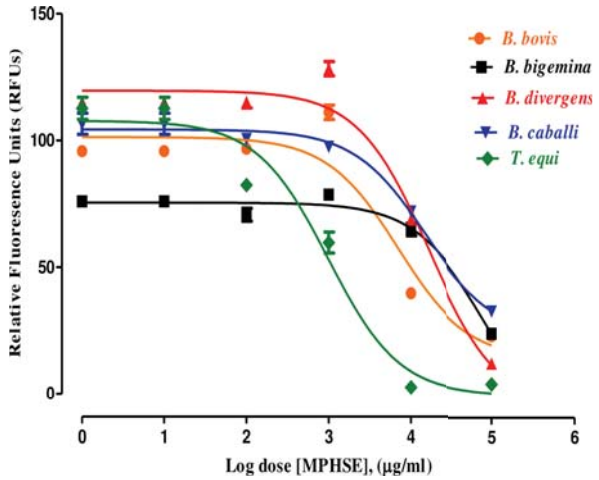

Fig. 1. Dose-response curves of methanolic *Peganum harmala* seed extract (MPHSE) against *Babesia* and *Theileria* parasites *in vitro*. Curves showing the growth inhibition of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* treated with Log concentrations of MPHSE. The result was determined by the fluorescence assay after 96 h of incubation. The percentage of inhibition is expressed as the percentage of inhibited parasites compared with the positive control (untreated wells) after subtracting the negative control (uninfected RBC). The data were the mean and S.D. from triplicate experiments.

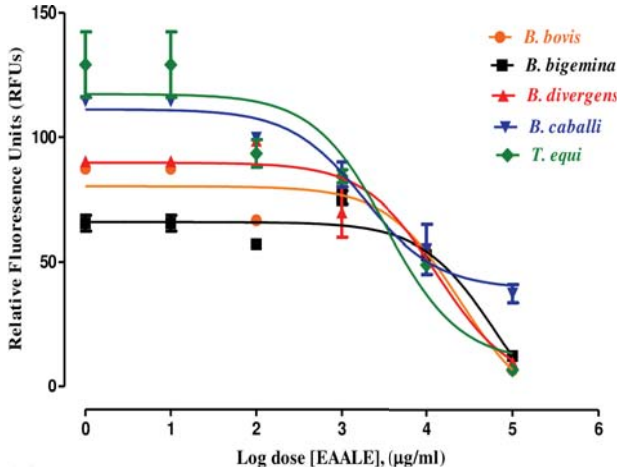

Fig. 2. Dose-response curves of ethanolic *Artemisia absinthium* leaf extract (EAALE) against *Babesia* and *Theileria* parasites *in vitro*. Curves showing the growth inhibition of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* treated with Log concentrations of EAALE. The result was determined by the fluorescence assay after 96 h of incubation. The percentage of inhibition is expressed as the percentage of inhibited parasites compared with the positive control (untreated wells) after subtracting the negative control (uninfected RBC). The data were the mean and S.D. from triplicate experiments.

Table 1. IC_{50} and selectivity index of methanolic *Peganum harmala* seed extract (MPHSE) and ethanolic *Artemisia absinthium* leaf extract (EAALE)

Crude extracts	Parasites	IC_{50} (µg/mL)[a]	EC_{50} (µg/mL)[b] cell lines			Selective indices[c]		
			MDBK	NIH/3T3	HFF	MDBK	NIH/3T3	HFF
Methanolic *Peganum harmala* seed extract	***B. bovis***	24.9 ± 1.2	611.7± 10.9	870 ± 22	>1500	24.6	34.9	> 60.2
	B. bigemina	77.1 ± 2.3	611.7± 10.9	870 ± 22	>1500	7.9	11.2	> 19.4
	B. divergens	61.1 ± 2.9	611.7± 10.9	870 ± 22	>1500	10	14.2	> 24.5
	B. caballi	80.8 ± 4.1	611.7± 10.9	870 ± 22	>1500	7.5	10.7	> 18.5
	T. equi	11.3 ± 2.1	611.7± 10.9	870 ± 22	>1500	54.1	76.9	> 132.7
Ethanolic *Artemisia absinthium* leaf extract	***B. bovis***	43.3 ± 3.1	340.7± 8.5	736.7 ±9.3	1371.5	7.9	17	31.6
	B. bigemina	39.2 ± 2.7	340.7± 8.5	736.7 ±9.3	1371.5	8.7	18.8	34.9
	B. divergens	38.5 ±3.7	340.7± 8.5	736.7 ±9.3	1371.5	8.8	19.1	35.6
	B. caballi	50.3 ± 2.1	340.7± 8.5	736.7 ±9.3	1371.5	6.8	14.6	27.2
	T. equi	28.2 ± 2.1	340.7± 8.5	736.7 ±9.3	1371.5	12.1	26.1	48.6

[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 fit 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 fit 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.

Effect of MPHSE and EAALE on cattle and horse RBCs

To detect the effect of MPHSE and EAALE on cattle and horse RBCs, the RBCs of cattle and horses were incubated with MPHSE and EAALE at 200 µg/mL for 3 h before the subculture of *B. bovis* and *T. equi*. Growth and parasitemia of *B. bovis* and *T. equi* did not significantly differ between the MPHSE-treated RBCs and the untreated RBCs. No significant difference was observed between the EAALE-treated RBCs and the untreated RBCs (data not shown).

Viability of parasites treated with MPHSE and EAALE

A viability assay was performed to determine whether the extracts could completely clear the parasites after 4 days of successive treatment followed by withdrawal of the drug pressure. *Babesia bovis*, *B. divergens*, *B. caballi*, and *T. equi* treated with MPHSE could not regrow at an extract concentration of 4 × IC_{50} (99.6, 244.4, 323.2, and 45.2 μg/mL, respectively), while *B. bigemina* could regrow at 4 × IC_{50} (308.4 μg/mL). EAALE inhibited the regrowth of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* at 4 × IC_{50} (173.2, 156.8, 154, 201.2, and 112.8 μg/mL, respectively) (Table 2).

Table 2. The viability of *Babesia* and *Theileria* parasites treated with methanolic *Peganum harmala* seed extract (MPHSE) and ethanolic *Artemisia absinthium* leaf extract (EAALE)

Drugs	Conc. of compounds	Parasites				
		B. bovis	*B. bigemina*	*B. divergens*	*B. caballi*	*T. equi*
Methanolic *P. harmala* seed extract	**0.25 × IC_{50}**	+	+	+	+	+
	0.5 × IC_{50}	+	+	+	+	+
	1 × IC_{50}	+	+	+	+	+
	2 × IC_{50}	+	+	+	+	+
	4 × IC_{50}	-	+	-	-	-
Ethanolic *A. absinthium* leaf extract	**0.25 × IC_{50}**	+	+	+	+	+
	0.5 × IC_{50}	+	+	+	+	+
	1 × IC_{50}	+	+	+	+	+
	2 × IC_{50}	+	+	+	+	+
	4 × IC_{50}	-	-	-	-	-
	Untreated control	+	+	+	+	+

The positive (+) shows the regrowth of parasites, and the negative (-) shows the total clearance of parasites on day 8 after withdrawing the drug pressure, according to the microscopy assay.

Morphological changes in parasites treated with MPHSE and EAALE

Micrographs of *B. bigemina* treated with MPHSE and EAALE at 308.4 and 156.8 μg/mL, respectively (Fig. 3), and micrographs of *B. caballi* treated with MPHSE and EAALE at 323.2 and 201.2 μg/mL, respectively (Fig. 4), were taken. The micrographs showed spindle-shaped, dividing parasites at 24 h as compared to the piriform shape of normal *B. bigemina* and *B. caballi*, while at 72 h, drug-treated cultures showed higher numbers of degenerated parasites than did the control cultures.

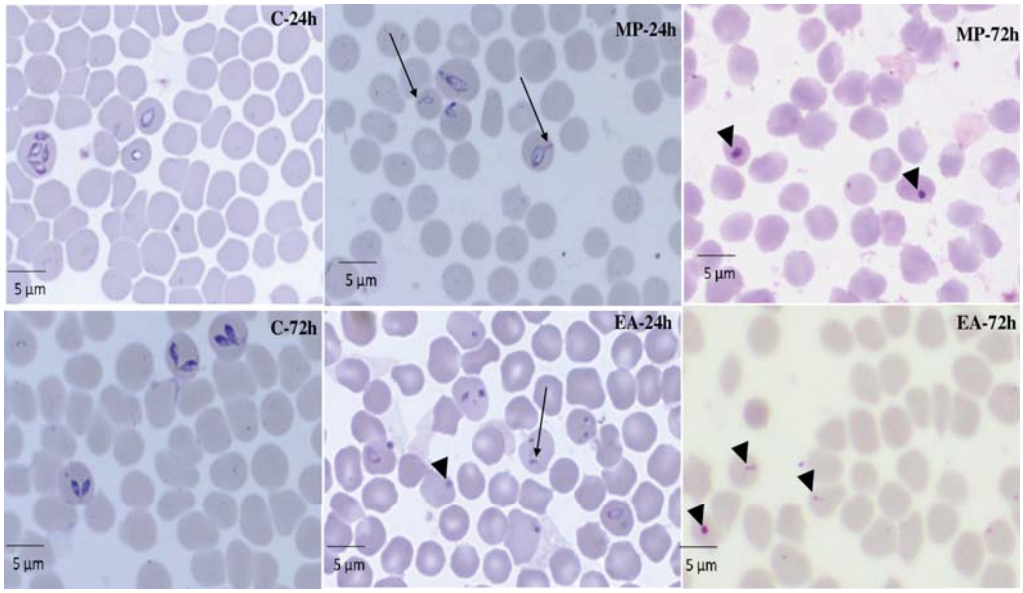

Fig. 3. Morphological changes observed in *B. bigemina* treated with extracts from methanolic *Peganum harmala* seeds (MP) and ethanolic *Artemisia absinthium* leaves (EA). Light micrographs of *B. bigemina* treated with 308.4 and 156.8 µg/mL of extracts taken from MP and EA, respectively, in an *in vitro* culture taken after 24 and 72 h. The arrows show the spindle shapes of dividing parasites of MP- and EA-treated cultures at 24 h as compared to the normal piriform shape shown in the untreated control *B. bigemina* (C), while the arrowheads showed the degenerated parasites in EA-treated cultures at 24 h and 72 h and in MP-treated cultures at 72 h.

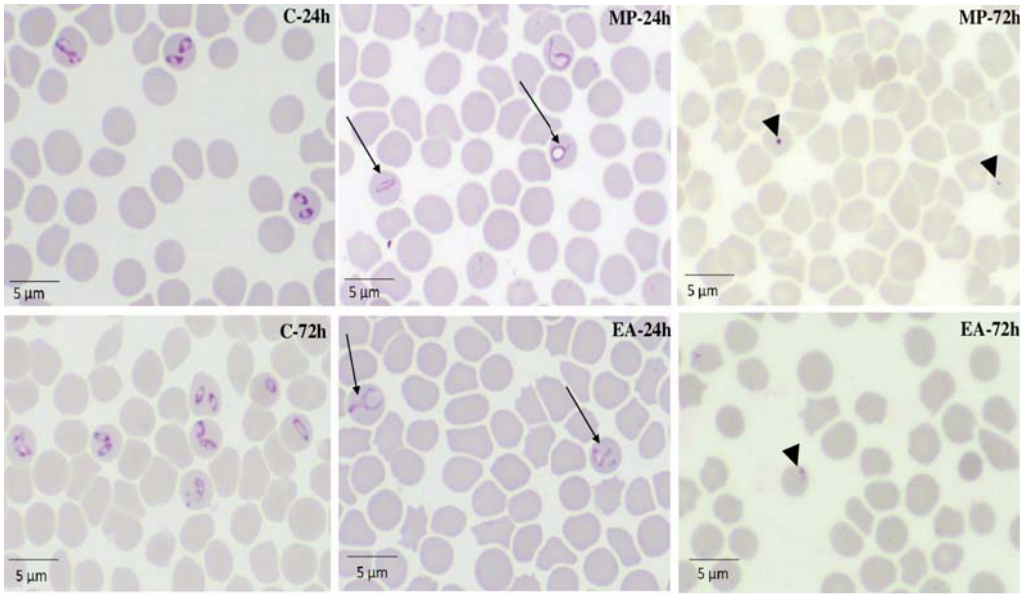

Fig. 4. Morphological changes observed in *B. caballi* treated with extracts from methanolic *Peganum harmala* seeds (MP) and ethanolic *Artemisia absinthium* leaves (EA). Light micrographs of *B. caballi* treated with 323.2 and 201.2 µg/mL of extracts from MP and EA, respectively, in an *in vitro* culture taken after 24 h and 72 h. The arrows show the spindle shapes and vacuolation of dividing parasites at 24 h as compared to the normal piriform shape shown in the untreated control *B. caballi* (C), while at 72 h, the arrowheads showed higher numbers of degenerated parasites in the drug-treated cultures than did the control cultures.

Toxicity of MPHSE and EAALE to MDBK, NIH/3T3, and HFF cell lines

MPHSE and EAALE showed an inhibitory effect on the *in vitro* culture of *Babesia* and *Theileria* parasites. The effect of MPHSE and EAALE on the host cells was evaluated using MDBK, NIH/3T3, and HFF cell lines. The EC_{50} values of MPHSE on MDBK, NIH/3T3, and HFF cell lines were 611.7 ± 10.9, 870 ± 22, and >1500 μg/mL, respectively. The EC_{50} values of EAALE on MDBK, NIH/3T3, and HFF cell lines were 340.7 ± 8.5, 736.7 ± 9.3, and 1371.5 ± 17.3 μg/mL, respectively (Table 1). In a separate assay, DA and AQ at a concentration of 100 μg/mL did not show any inhibition of MDBK, NIH/3T3, or HFF cell viability, while CF showed inhibition only of MDBK with an EC_{50} value of 34 ± 3.4 μM (Table S1). The selectivity index, defined as the ratio of the cell line EC_{50} to the parasite IC_{50}, is shown in Table 1. For the MDBK cell line, the highest selectivity index of MPHSE was 54.1 times higher than the IC_{50} for *T. equi*. The highest selectivity index of EAALE was 12.1 times higher than the IC_{50} for *T. equi*. For the HFF cell line, the highest selectivity index of EAALE was 48.6 times higher than the IC_{50} for *T. equi*. The selectivity index of MPHSE and EAALE for the NIH/3T3 cell line was also determined for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* (Table 1).

Combination treatment *in vitro*

Combinations of MPHSE and EAALE with DA, AQ, and CF were performed on the *in vitro* cultures of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*. The MPHSE/DA combined treatment showed an antagonistic effect against *B. bovis*, *B. bigemina*, *B. divergens*, and *T. equi*, while an additive effect was shown against *B. caballi*. The EAALE/DA combination showed a synergistic effect against *B. bovis*, *B. bigemina*, and *B. divergens*, while an additive effect was shown against *B. caballi* and *T. equi* (Table 3). The combination of MPHSE with AQ showed a synergistic effect against all tested parasites. The combination of EAALE with AQ showed a synergistic effect against *B. bovis* and an additive effect against *B. bigemina*, *B. divergens*, and *T. equi*. The combination of MPHSE with CF showed a synergistic effect against all tested parasites. The combination of EAALE with CF showed a synergistic effect against *B. bovis* and an additive effect against *B. bigemina*, *B. divergens*, and *T. equi*.

Table 3. Effect of methanolic *Peganum harmala* seed extract (MPHSE) and ethanolic *Artemisia absinthium* leaf extract (EAALE) with diminazene aceturate, atovaquone, and clofazimine against *Babesia* and *Theileria* parasites *in vitro*

		Parasites				
Combined treatments		***B. bovis***	***B. bigemina***	***B. divergens***	***B. caballi***	***T. equi***
MPHSE + DA	**CI values**	3.328	7.102	9.002	1.071	5.023
	Degree of association	**Antagonism**	**Antagonism**	**Antagonism**	**Additive**	**Antagonism**
EAALE + DA	**CI values**	0.730	0.873	0.636	1.089	1.0814
	Degree of association	**Synergism**	**Synergism**	**Synergism**	**Additive**	**Additive**
MPHSE + AQ	**CI values**	0.847	0.552	0.781	0.783	0.903
	Degree of association	**Synergism**	**Synergism**	**Synergism**	**Synergism**	**Synergism**
EAALE + AQ	**CI values**	0.799	1.097	1.003	0.9903	1.075
	Degree of association	**Synergism**	**Additive**	**Additive**	**Additive**	**Additive**
MPHSE + CF	**CI values**	0.638	0.9932	0.9845	1.1189	0.202
	Degree of association	**Synergism**	**Additive**	**Additive**	**Additive**	**Synergism**
EAALE + CF	**CI values**	0.0098	0.9756	0.0956	0.2370	0.7200
	Degree of association	**Synergism**	**Additive**	**Synergism**	**Synergism**	**Synergism**

DA: diminazene aceturate; AQ: atovaquone; CF: clofazimine; CI: combination index value

Chemotherapeutic effect of MPHSE and EAALE on *B. microti* in mice

The promising efficacy of MPHSE and EAALE *in vitro* prompted further research to evaluate the antibabesial effects of the two extracts against *B. microti* in mice. In treated groups, the level of parasitemia increased by a significantly lower percentage than in the control group ($p < 0.05$) from days 6 to 12 post infection (p.i.). The peak parasitemia level in the treated groups reached 24%, 28.1%, and 5% with 150 mg kg^{-1} MPHSE, 150 mg kg^{-1} EAALE, and 25 mg kg^{-1} DA, respectively, at day 8 p.i. as compared with 62.6% in the untreated-infected control group (Fig. 5).

Comparison of the hematocrit percentage (Fig. 6) showed a significant difference in the MPHSE- and EAALE-treated groups as compared to the untreated-infected group. On the other hand, there was no significant reduction ($p < 0.05$) of hematocrit percentage in the MPHSE- and EAALE-treated groups as compared to the DA-treated group.

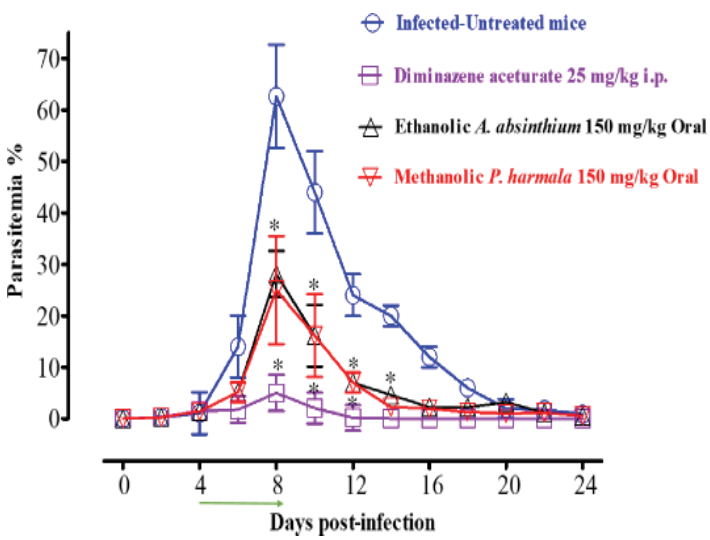

Fig. 5. Growth inhibition of methanolic *Peganum harmala* seed extract (MPHSE) and ethanolic *Artemisia absinthium* leaf extract (EAALE) on *B. microti in vivo*. Inhibitory effect of extracts from MPHSE and EAALE on the growth of *B. microti* in mice, based on observations of five mice per experimental group. The arrow indicates 5 consecutive days of treatment. Asterisks indicate statistically significant ($p < 0.05$) differences of parasitemia between the treated groups and the untreated control group based on unpaired *t*-test analysis. The data were the mean and standard deviation from two separate experiments.

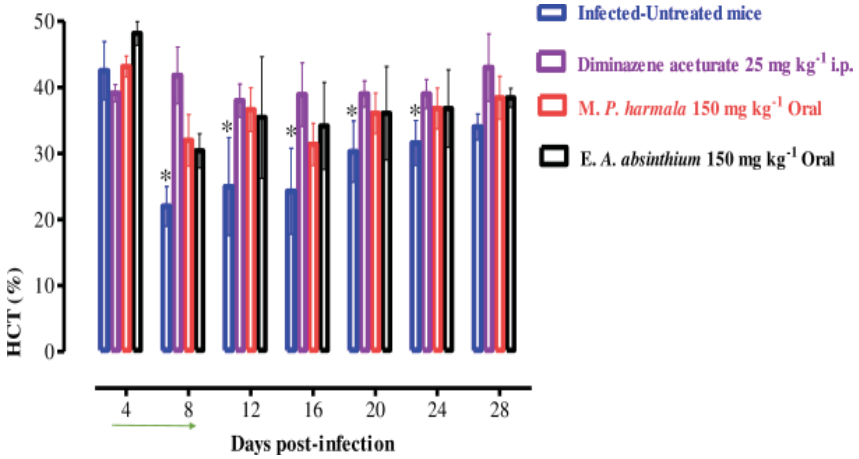

Fig. 6. Changes in the hematocrit percentage in mice treated with methanolic *Peganum harmala* seed extract (MPHSE) and ethanolic *Artemisia absinthium* leaf extract (EAALE) *in vivo*. Graph showing the changes in hematocrit (HCT) percentage in mice treated with DA and extracts from MPHSE and EAALE. The arrow indicates 5 consecutive days of treatment. Asterisks indicate statistical significance ($p < 0.05$) based on unpaired *t*-test analysis. The data were the mean and standard deviation from two separate experiments (five mice per group).

DISCUSSION

Available chemotherapeutic agents against piroplasmosis utilize several undesirable consequences on host organs and are unable to control the recrudescence of parasites. Therefore, drugs of herbal origin are considered safe for use in human and animal medicine as they are less toxic and easily metabolized compared to synthetic chemicals (Ghosh and Nagar, 2014). The current study documented the effectiveness of MPHSE and EAALE against *Babesia* and *Theileria* parasites.

MPHSE and EAALE have a growth-inhibitory effect against *Babesia* and *Theileria* parasites (Figs. 1 and 2). Interestingly, the growth-inhibitory effect of MPHSE was consistent with observations by Ravindran and Ghosh. (2017), who reported that the total alkaloids of *P. harmala* reduced the peak parasitemia level in cattle naturally infected with *B. bigemina*. Additionally, the growth-inhibitory effect of EAALE against *Babesia* and *Theileria* was consistent with findings by (Valdés et al., 2008), who reported that EAALE has shown *in vitro* activity against the erythrocyte stages of *Plasmodium falciparum*. The familiarity observed could be attributed to the fact that *Babesia* and *Plasmodium* parasites belong to the same Apicomplexa phylum. Moreover, Mirzaie et al. (2007) and Valdés et al. (2008) revealed that MPHSE and EAALE showed a growth-inhibitory effect against *Trypanosoma* and *Leishmania* (protozoan parasites). In the present study, the viability assay showed that MPHSE and EAALE prevent the regrowth of *Babesia* and *Theileria in vitro*. One possible reason for the observed growth-inhibitory effects of MPHSE and EAALE is the many bioactive molecules present in the crude extract. In comparison with previous studies, the IC_{50} values of MPHSE against *Babesia* were lower than those of gedunin against *B. bovis* and *B. bigemina* (Azirwan et al., 2013) and of clindamycin phosphate against *B. bovis*, *B. bigemina*, and *T. equi* (AbouLaila et al., 2012). On the other hand, the IC_{50} values of EAALE against *Babesia* were also lower than those of fusidic acid against *B. bovis* and *B. caballi* (Salama et al., 2013), clodinafop-propargyl against *B. bovis* and *B. bigemina* (Bork et al., 2003), and clindamycin phosphate against *B. bovis* and *B. bigemina* (AbouLaila et al., 2012). Thus, MPHSE and EAALE could be alternative antiparasitic drug candidates for piroplasmosis treatment.

Micrographs showed that MPHSE- and EAALE-treated parasites were unable to egress and, subsequently, died within the infected RBCs at 72 h (Figs. 3 and 4). Previous studies of MPHSE and EAALE against other protozoan parasites showed that *P. harmala* and *A. absinthium* crude extracts restricted the growth of the *Leishmania* parasite with multiple promastigote changes (Beigh and Ganai, 2017; Niroumand et al., 2015).

Toxicity studies of various cell lines showed that MPHSE affected the viability of MDBK and NIH/3T3 cell lines, while EAALE affected the viability of MDBK, NIH/3T3, and HFF cell lines. The EC_{50} values shown by MPHSE and EAALE against cell lines were higher than the IC_{50} values shown against the four tested *Babesia* species and *T. equi*. Hence, a highly selective index was achieved. This finding is consistent with observations documented by Lamchouri et al. (2013), who showed that the *P. harmala* extract significantly decreased the growth rate and cell survival of cancer cell lines without cytotoxic activity against normal cell lines. *P. harmala* extracts induced higher growth inhibition of MDA-MB-231 and Mcf-7 cancer cell lines. Additionally, *P. harmala* extracts significantly decreased

the growth rate of the Hep-2 (human laryngeal carcinoma cell) cancer cell line without cytotoxic activity against normal cell lines (Seyed et al., 2014). The cytotoxic activity of *A. absinthium* extracts against the MCF7 cancer cell line was 40% greater than that against the HEK293 cancer cell line and showed no cytotoxic activity against normal cell lines, revealing that *A. absinthium* extracts may have great potential in the exploration of new anticancer drugs (Gordanian et al., 2014). Interestingly, previous reports revealed that the cancer cells act as metabolic parasites in which they obtain nutrients from host cells (Pavlides et al., 2009). Therefore, MPHSE and EAALE will more likely to affect *Babesia* and *Theileria* parasites than the host cell. Inferring from the above, MPHSE and EAALE might be safe for use in animals and humans following further *in vivo* clinical studies.

The combination chemotherapy has been recommended against drug-resistant protozoal and bacterial pathogens, and it is not only aimed to enhance the potency of the drugs but also reduce their dose that subsequently led to reducing their toxic side effects. The combined application of MPHSE and EAALE with DA and AQ showed antagonistic, additive and synergetic effects against tested parasites. One possible explanation for this is that herbal extracts contain many bioactive ingredients that may interact differently with multiple pathways in combination treatment (Khlifi et al., 2013). Therefore, further studies are needed to confirm the exact mechanism employed by the different active molecules found in MPHSE and EAALE and how they interact with other drugs, such as DA and AQ.

Oral administration of MPHSE at a dose of 150 mg kg^{-1} significantly ($p < 0.05$) inhibited the growth of *B. microti* in mice and resulted in 61.7% inhibition of the parasitemia level at day 8 p.i., which is higher than that with the untreated-infected group and lower than the 92% inhibition with the presence of 25 mg kg^{-1} DA at day 8 p.i. (Fig. 5). The chemotherapeutic effect shown by MPHSE against *B. microti* is higher than the 60.8% inhibition shown by 500 mg kg^{-1} fusidic acid (Salama et al., 2013). On the other hand, oral administration of EAALE at a dose of 150 mg kg^{-1} significantly ($p < 0.05$) inhibited the growth of *B. microti* in mice and resulted in 55.1% inhibition of the parasitemia level at day 8 p.i. The inhibitory effect of EAALE on the growth of *B. microti* was compatible with Nahrevanian et al. (2012), who showed that the aqueous and alcoholic leaf extracts of *A. absinthium* showed antimalarial activity against *Plasmodium berghei* in mice. Therefore, the chemotherapeutic effects produced by MPHSE and EAALE on *B. microti* indicate the presence of a potential compound with high antibabesial activity.

In conclusion, the present study documents that MPHSE and EAALE showed inhibitory effects against *Babesia* and *Theileria* parasites and no apparent adverse effects were observed in mice. These findings are compatible with previous reports (Lamchouri et al., 2002; Khanjani Jafroodi et al., 2015), which documented the safety of *P. harmala* and *A. absinthium* extracts in mice and rats *in vivo*. Therefore, they could be useful for the treatment of bovine babesiosis and equine piroplasmosis. Thus, identifying the active compound is necessary for contriving a higher chemo-suppression effect from these extracts for the future discovery of a novel potential drug against piroplasmosis.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

This manuscript has been approved by all of the authors, and if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically, without the written consent of the copyright holder.

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Improvement of SYBR Green I–based fluorescence assay reading procedure for anti–babesial drugs screening *in vitro*

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ABSTRACT

Drug susceptibility of the *Babesia* parasite *in vitro* has been used to assess the anti-babesial activities of new candidates. Recently, the *in vitro* drug susceptibility assay has been performed by SYBR Green I (SG I) -based fluorescence assay. Herein, we investigated the validity of a fluorescence assay for the shipping process under various culture conditions. The highest fluorescence signal was observed in freeze-thaw of *Babesia* and *Theileria* parasites cultures incubated with lysis buffer containing SG I for a half hour. This result indicates the ability of this additional step to lysis of infected RBCs completely with releasing of parasite nucleic acid. Such that, the nucleic acid can interact with SG I stain. All tested drugs showed potential anti-babesial activity and slightly high selective index against the *in vitro* growth of the *Babesia* and *Theileria* parasites. The IC_{50} values generated using the fluorescence assay with additional freeze-thaw step is comparable with those of IC_{50} values obtained from the unmodified fluorescence assay and microscopy method.

Keywords: Fluorescence; *Babesia*; SYBR Green; Freeze–thaw

INTRODUCTION

Babesiosis is a tick-borne protozoan disease caused by genus *Babesia*. The disability and the poverty associated with this disease constitute large burdens on the health and economic development of low- and middle-income countries in the world. Strategies to control this disease are based on surveillance, early diagnosis, vector control, and treatment. So far, there are a few drugs on the market to treat parasitic disease, and they are not universally available in the affected areas. Currently available drugs are inefficiency, toxicity, prolonged treatment schedule, and high cost. Therefore, there is an urgent need for new treatments

which are safe, effective, cheap, and easy-to-administer, and for a lead compound with novel mechanisms of action.

The SYBR green I (SG) dye-based fluorescence-based assay is a fast, inexpensive, readily growth assessment of the parasite and broadly applicable for use in drug screening. The SG dye binds to the double-stranded DNA of parasites in infected erythrocytes and the calculated fluorescence signals used as a measure of parasite growth and propagation or growth inhibition by antiparasitic drugs into the culture (Smilkstein et al., 2004; Rizk et al., 2015).

Although, fluorescence assay was employed for mass drug screening against the *in vitro* growth of *Plasmodium* (Smilkstein et al., 2004) and *Babesia* parasites (Rizk et al., 2015) is reported to have inherent limitations such as low fluorescence reading. It seems to be the presence of hemoglobin in the parasite culture correlated with the level of fluorescence as previously reported for a similar fluorochrome (Quashie et al., 2006). Therefore, there is a need to improve SG assay to overcome these shortages.

Recently, Rizk et al., (2015) optimized fluorescence assay with corresponding HCTs without daily replacement of the culture medium for *Babesia* and/or *Theileria* parasites, which offers a new approach for simplicity, robust performance and rapid detection of the parasites as well as large-scale screening of anti-babesial drugs in an *in vitro* culture. However, the method is not valid for the shipping process. Therefore, in the current study, we have modified the method to be consistence for the shipping process. To achieve this purpose, the effect on fluorescence when the incubation period of culture with lysis buffer containing SG was extended up to 24 h at fixed time intervals and the effect of freeze-thaw on fluorescence intensity was ascertained. Further, this study investigated the anti-babesial and cytotoxic activities of four candidate compounds compared to the standard anti-babesial drug (diminazene aceturate) *in vitro* using fluorescence assay with or without freeze-thaw effect and traditional microscopy method.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine, in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine.

Chemical reagents

SYBR Green I (SG) nucleic acid stain (Lonza, 10.000x) was stored at -20°C until use. A lysis buffer consisting of Tris (130 mM; pH 7.5), Ethylenediaminetetraacetic acid (EDTA) (10 mM), saponin (0.016%; W/V), and TritonX-100 (1.6%; V/V) was prepared in advance and stored at 4°C. Three histone deacetylase inhibitors namely, apicidin, trichostatin A, and valproic acid, and medical plant, cepharanthine (Sigma-Aldrich, Japan) were used as tested

drugs prepared as 10 mM stock solutions. Standard drugs, diminazene aceturate (DA) and fluorouracil (Sigma-Aldrich, Japan) were prepared as 100 mM stock solutions.

Parasites

The Texas T_2Bo strain of *B. bovis* (Hines et al., 1992), Argentine strain of *B. bigemina* (Hotzel et al., 1997), and U.S. Department of Agriculture (USDA) strains of *B. caballi* (Avarzed et al., 1997) and *T. equi* (El-Sayed et al., 2015) were maintained in bovine and equine red blood cells (RBCs) with medium using a microaerophilic stationary–phase culture system (Munkhjargal et al., 2012).

Determination of the relationship between parasite DNA and SYBR Green I fluorescence

DNA was extracted from the *B. bovis*-, *B. bigemina*-, *B. caballi*-, and *T. equi*-infected erythrocytes using a commercial DNeasy Blood and Tissue Kit (Qiagen). The concentration of the purified DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). After determining the concentration of the parasite DNA it was serially diluted with RPMI1640 medium to generate a concentration range. One hundred microliters of each concentration was dispensed into a 96-well plate followed by addition of 100 µl lysis buffer containing 2×SG. Control wells consisting of 100 µl RPMI1640 and an equal volume of lysis buffer containing SG were also set up. Plates were incubated in the dark at room temperature for 1 h and the fluorescence intensity was measured at 485 nm excitation and 528 nm emission using a fluorescence plate reader (Fluoroskan Ascent, Thermo Scientific). Fluorescence units were then plotted against parasite DNA concentration. Each data point was performed in triplicate and three independent experiments were performed.

Determination of the effect of hemoglobin on SYBR Green I fluorescence

Bovine and equine uninfected blood was centrifuged and washed in RPMI 1640 to remove the plasma and buffy coat. The pellet was diluted with culture medium to generate a hematocrit (HCT) range of 0.03-10 %. Each hematocrit was spiked with parasite DNA (prepared above) at a fixed concentration (0.2 ng/µl). A hundred microliters of each mixture was dispensed into the wells of a microtiter plate and an equal volume of lysis buffer containing 2×SG was added. The plate was incubated in the dark for at least 1 h and fluorescence was measured as previously described (Dery et al., 2015). Fluorescence readings were plotted against HCT and the correlation between them was determined.

Maximum time of incubation with lysis buffer containing SG for optimum fluorescence signals and the effect of freeze–thaw

The incubation period of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* cultures with lysis buffer containing SG that yields the best fluorescence signal and the effect of freeze–thaw fluorescence intensity were ascertained (Dery et al., 2015). Briefly, 100 µl of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* cultures at an initial parasitemia of 4-8 % was serially diluted in a 96-well microtiter plate with blood at 2.5 % and 5 % HCTs for bovine and equine *Babesia* parasites, respectively (Rizk et al., 2015) to give a concentration range of 0.04-8 %

parasitemia. Direct lysis of the RBCs to release the parasite DNA was done by adding 100 µl of lysis buffer containing 2×SG (10.000x) to each well. After incubating the plates in the dark for 20 min, fluorescence signals were measured as previously described. Thereafter, the plate was kept in the dark and the fluorescence intensity was determined after 0.5, 1, 3, 6, and 24 h incubation in the dark. A second plate containing the same parasite concentration range was prepared in parallel to that described above. However, this plate was frozen immediately at -30 °C for 1 h after which the culture was thawed and 100 µl lysis buffer containing SG was added. The plate was then incubated in the dark and the emitted fluorescence signals were determined after 0.5, 1, 2, 3, 6, and 24 h. Fluorescence readings were plotted against parasitemia.

Determination of assay quality

To determine the assay quality, statistical parameters, the Z' factor (Z'), signal to noise (S/N) ratio, coefficient of variation at the maximum signal (% CV_{max}, positive control), and coefficient of variation at the minimum signal (% CV_{min}, negative control) were calculated for the fluorescence assay after freeze-thaw step according to Zhang et al., (1999).

Determination of inhibitory concentration 50 % (IC_{50}) values of anti-babesial drugs

An *in vitro* growth-inhibitory effect of apicidin, trichostatin A, cepharanthine, valproic acid, and DA on *Babesia* and *Theileria* parasites was evaluated by current and modified fluorescence-based assays as described previously (Rizk et al., 2015). DA was used as standard anti-babesial drug (0.01, 0.05, 0.1, 0.5, 1, and 5 µM). *B. bovis*-, *B. bigemina*-, *B. caballi*-, and *T. equi*-infected RBCs (iRBCs) were cultivated at 1% parasitemia in double 96-well plates using the previously published optimal HCT for each parasites (Rizk et al., 2015) and M199 or RPMI 1640 media alone or with 0.01, 0.05, 0.1, 0.5, 1, and 5 µM concentrations of each drug except of DA. For the control, cultures without drugs and cultures containing only DMSO (0.01%) were prepared. The iRBCs were cultivated for 4 days without replacement of the medium in triplicate wells for each concentration of the drug. On the fourth day of culture, a lysis buffer containing 2×SG was added into each well of the first 96-well plate and incubated in dark place for 6 h. After incubation, fluorescence values were determined using a fluorescence plate reader at 485 nm and 518 nm of excitation and emission wavelengths, respectively. A second plate containing the same drug concentration range was prepared in parallel to that described above. However, on the fourth day of culture, this plate was frozen at -30 °C for 1 h after which the culture was thawed and 100 µl lysis buffer containing 2×SG added. The plate was then incubated in the dark and the fluorescence reading was taken after 0.5 h. All experiments were repeated three times, and the mean fluorescence values were then plotted against the logarithm of drug concentrations. In addition, for all tested drug, thin blood smears were prepared on the fourth day and stained with Giemsa to calculate the parasitemia using the microscopy-based method. The IC_{50} values were calculated for each drug using the fluorescence-based method with and without freeze-thaw effect and microscopy method.

Cytotoxicity assay

Cytotoxicity of each drug was evaluated against Madin Darby Bovine Kidney (MDBK) cells using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution. Briefly, $3x10^4$ MDBK cells were seeded in double 96 well plates with or without 10, 50, 100, 500, 1000, 5000 μM concentrations of each drug and incubated at 37°C, 5 % CO_2 for 72 h. Fluorouracil was used as a positive control. Subsequently, 10 μl of MTT solution (a final concentration of 5 mg/ml) was added to each well of the first plate and incubated at 37°C for 4 h. To dissolve to crystals, the medium was subsequently replaced with 100 μl of DMSO was added, and the absorbance was measured at 570 nm with an automatic ELISA plate reader. Numbers of cells were evaluated as % of control without drugs. The selective index (SI) was calculated as the 50 % cytotoxic concentration on MDBK cells divided by the IC_{50} of the compound for *Babesia* parasites.

Statistical analysis

Data analysis was performed using GraphPad Prism ver. 5 for windows (GraphPad Software, Inc., USA) using the one-way ANOVA and an unpaired *t*-test (Rizk et al., 2015). The IC_{50} values were calculated using GraphPad Prism ver. 5. $P < 0.05$ was considered to be statistically significant.

RESULTS

Relationship between parasite DNA and SYBR Green I fluorescence

The relationship between the concentrations of parasite DNA and the fluorescence units determined after incubation with lysis buffer containing SG in the dark showed a well–correlated linear relationship with r^2 values of 0.9531, 0.9624, 0.9492 and 0.9283 for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*, respectively (Fig. 1).

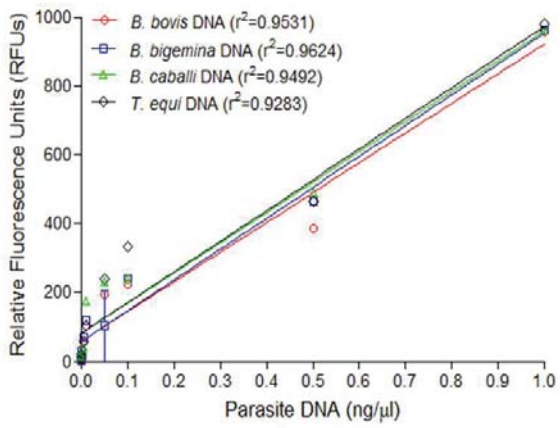

Fig. 1. DNA titration using SYBR Green I. Background fluorescence, defined as fluorescence detected in the absence of DNA, was subtracted from each data point. Fluorescence is measured as relative fluorescence units (RFUs). Data show average from three replicate experiments. Error bars indicate standard deviations.

Effect of hemoglobin on fluorescence

The effect of hemoglobin on fluorescent readings was tested by mixing different amounts of lysed erythrocytes (0.03-10% HCT) with a fixed amount of DNA. The fluorescence emission of SG-DNA adducts, shown in Figure 2, was highly quenched in the presence of red cells. Control samples, containing DNA without hemoglobin, rendered fluorescence readings around 20-30 fold as compared with samples containing blood, suggesting a heavy interference of hemoglobin. Additionally, the Z' factor, S/N ratio, % CVmax, and % CVmin were calculated in comparison with the fluorescence assay method without freeze-thaw effect (Rizk et al., 2015). Our results for bovine Babesia parasites revealed that S/N ratios showed the highest values at 2.5% HCT with freeze-thaw step (Table 1a). Regarding the equine *Babesia* and *Theileria* parasites, S/N ratios showed the highest values at 5%HCT for both parasites with freeze-thaw step (Table 1b). These results revealed the accuracy of the fluorescence-based assay with freeze-thaw effect at 2.5% and 5% HCTs for bovine and equine parasites, respectively.

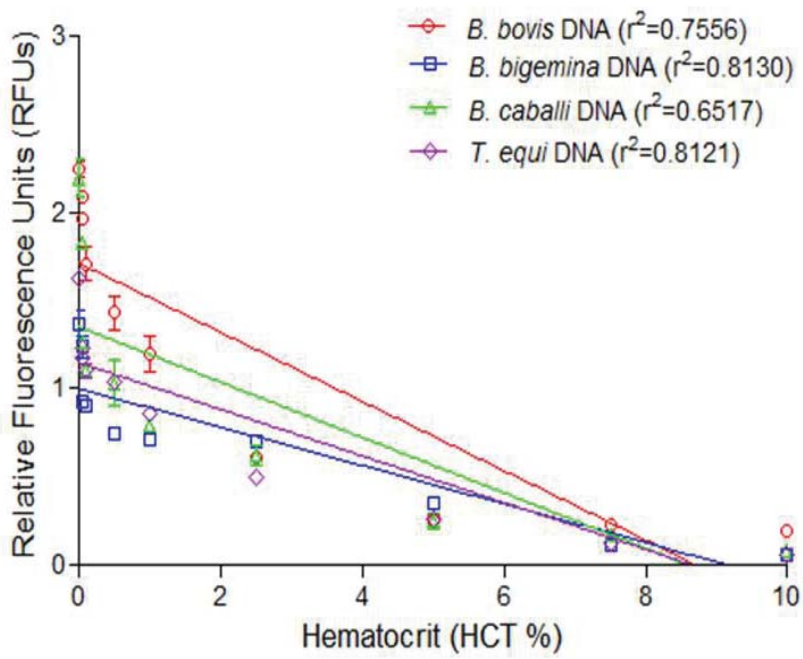

Fig. 2. Effect of hemoglobin on fluorescence emission. Fluorescence emission of SG of 0.2 ng/ml parasite DNA, in the presence of different amounts of bovine or equine RBCs. Fluorescence at 0.1 % hematocrit was obtained from a 0.2 ng/ml solution of parasite DNA in the culture medium.

Table 1a. Statistical parameters for determining the quality of the high–throughput screening (HTS) assay in bovine *Babesia* parasites with different percentages of HCTs without and with freeze–thaw step.

Parameters	*B. bovis*						*B. bigemina*					
	without freeze–thaw step			with freeze–thaw step			without freeze–thaw step			with freeze–thaw step		
	HCT %			HCT %			HCT %			HCT %		
	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10
Z' factor	-1.1	-0.79	-0.4	0.9	0.43	-0.99	-19.4	-7.9	1.04	0.9	0.35	-12.4
S/N ratio	2.9	5.2	5.7	34.1	13.8	4.9	0.76	0.6	-152.9	76.5	6.8	0.25
% CV_{max}	2.5	5.6	5.2	0.7	3.3	8.4	1.5	2.9	2.3	1.04	1.9	2.3
% CV_{min}	2.7	3.1	3.8	2.1	2.8	4.5	6.24	1.9	0.5	1.02	5.6	25.1

Table 1b. Statistical parameters for determining the quality of the high–throughput screening (HTS) assay in equine *Babesia* and *Theileria* parasites with different percentages of HCTs without and with freeze–thaw step.

Parameters	*B. caballi*						*T. equi*					
	without freeze–thaw step			with freeze–thaw step			without freeze–thaw step			with freeze–thaw step		
	HCT %			HCT %			HCT %			HCT %		
	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10
Z' factor	0.62	0.47	0.28	0.81	0.84	0.45	0.46	0.75	0.76	0.75	0.87	0.63
S/N ratio	16.2	12.8	10.6	38.5	37.1	88.4	6.6	18.9	72.9	16.03	24.4	10.3
% CV_{max}	0.52	2.12	3.5	0.58	0.43	6.03	0.2	0.9	0.99	1.4	2.6	0.7
% CV_{min}	0.54	2.1	3.03	0.48	0.51	0.61	1.1	2.2	0.9	2.8	0.4	3.5

Maximum time of incubation with lysis buffer for optimum SYBR Green1 fluorescence and effect of freeze-thaw cycle

The high fluorescence intensity was observed in freeze-thaw cycle followed by incubation with SG for 0.5 h gave the highest fluorescence intensity (Fig. 3). The results showed a well-correlated linear relationship (r^2 values of 0.9183, and 0.9874 for *B. caballi*, and *T. equi*, respectively) for freezing the samples for 0.5 h followed by thawing before the addition of lysis buffer containing SG (Fig. 3C, and D, respectively). However, a correlation was found between the emitted fluorescence signals and freezing the sample for 0.5 h followed by thawing before the addition of lysis buffer containing SG with 0.8861 and 0.8749 goodness of fit (r^2) for *B. bovis* and *B. bigemina*, respectively (Fig. 3A and B, respectively). Though there was a significant increase in fluorescence signal when the incubation time was at 0.5 h, there appears to be not much gain in fluorescence when the incubation time was extended 1 h to overnight. A similar trend was observed in the fluorescence assay without freeze-thaw step (Fig. 4). The difference between fluorescence signal for freezing samples for at least 1 h followed by a 0.5 h incubation with lysis buffer containing SG and incubating the samples 6 h after the addition of lysis buffer containing SG without freezing was significant ($P < 0.0001$ and F values for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were 47.8, 108.2, 55.3, and 23.6, respectively) (data not shown). This trend was consistently observed in 3 independent experiments.

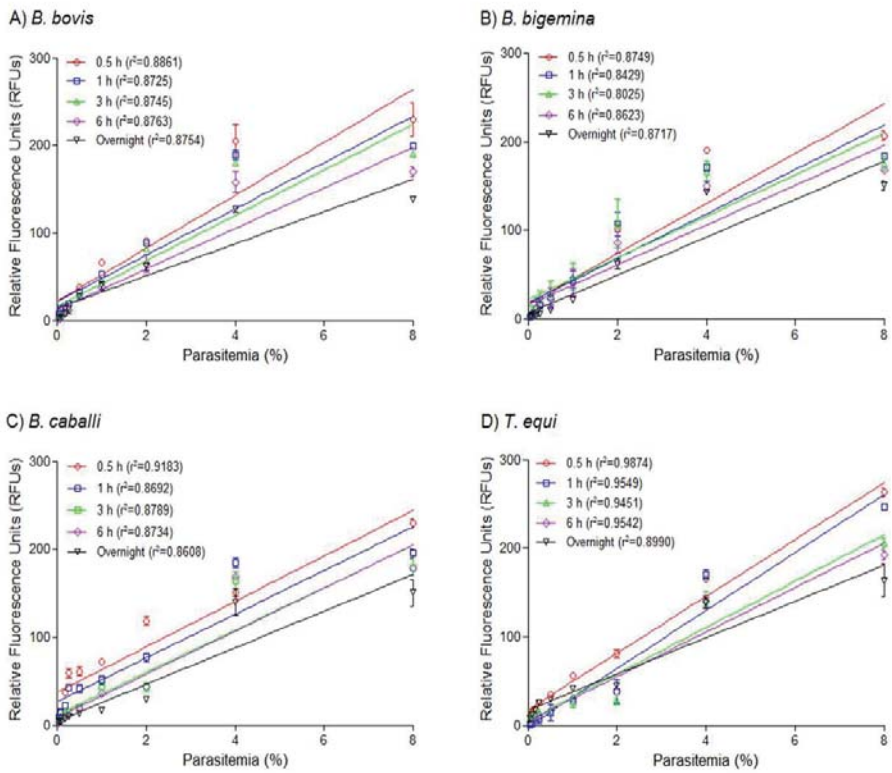

Fig. 3. Effect of length of incubation with lysis buffer containing SG after freeze-thaw effect on the intensity of fluorescence signal. Fluorescence at 2.5 % and 5 % hematocrit for bovine and equine *Babesia* parasites, respectively, to give a concentration range of 0.04-8 % parasitemia in the culture medium. Fluorescence was measured at 485 nm excitation and 528 nm emission.

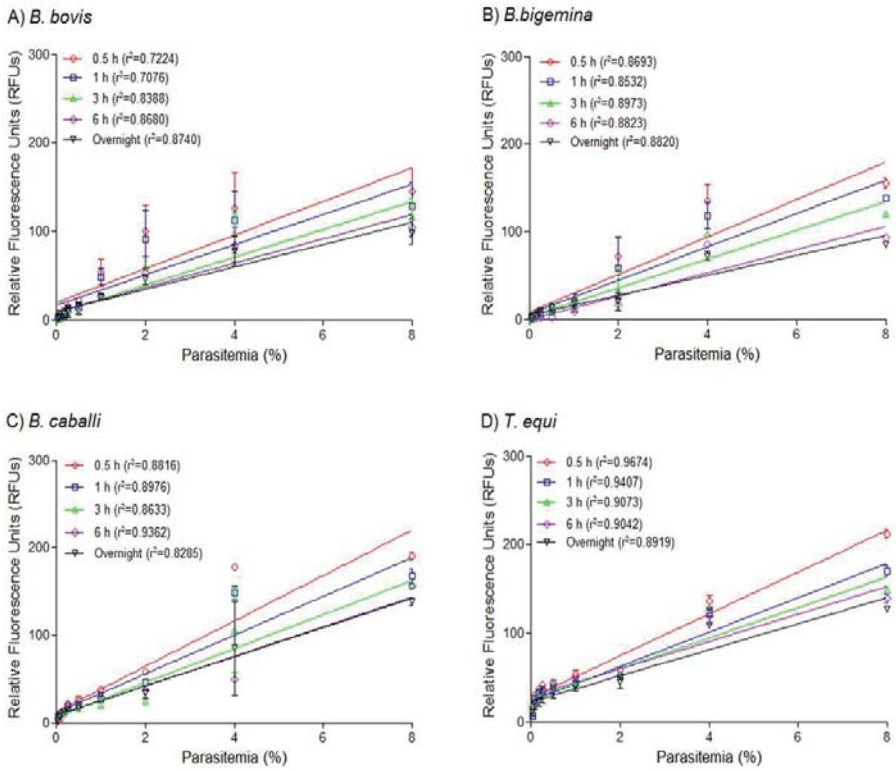

Fig. 4. Effect of length of incubation with lysis buffer containing SG without freeze-thaw step on the intensity of fluorescence signal. Fluorescence at 2.5 % and 5 % hematocrit for bovine and equine *Babesia* parasites, respectively, to give a concentration range of 0.04-8 % parasitemia in the culture medium. Fluorescence was measured at 485 nm excitation and 528 nm emission.

Table 2a. Comparison of inhibitory concentration 50 % (IC_{50}) values obtained from fluorescence assay without and with freeze−thaw effect and microscopy method. IC_{50}s of all tested drugs for bovine *Babesia* parasites

Drugs	IC_{50} values (nM)[a]									
	B. bovis					*B. bigemina*				
	Fluorescence			Microscopy	*P* value[c]	Fluorescence			Microscopy	*P* value[c]
	without freeze−thaw	with freeze−thaw	*P* value[b]			without freeze−thaw	with freeze−thaw	*P* value[b]		
Apicidin	42±6.7	43±5.4	0.9959	32.1±5.6	0.9333	19.8±8.9	16.8±10.2	0.9061	12.5±8.2	0.5077
Trichostatin A	37.4±5.2	36.5±8.1	0.9671	35.5±4.9	0.9290	68.4±35	68.5±33.6	0.9810	54.9±30.2	0.7293
Cepharanthine	45±20.5	49.2±20.1	0.9295	40.2±18.2	0.7933	19.5±11.5	20.1±12.1	0.8902	22.1±9	0.9494
Valproic acid	87.3±46.6	92.5±52.2	0.8678	110.2±78.2	0.4852	240.5±180	191.2±156	0.4049	265±160.2	0.5536
Diminazene aceturate	46.4±14.1	47.2±19	0.9064	35.1±12.1	0.9403	25.9±18	22.6±20.2	0.6626	25±10.2	0.9275

Table 2b. Comparison of inhibitory concentration 50 % (IC_{50}) values obtained from fluorescence assay without and with freeze−thaw effect and microscopy method. IC_{50}s of all tested drugs for equine *Babesia* and *Theileria* parasites.

Drugs	IC_{50} values (nM)[a]									
	B. caballi					*T. equi*				
	Fluorescence			Microscopy	*P* value[c]	Fluorescence			Microscopy	*P* value[c]
	without freeze−thaw	with freeze−thaw	*P* value[b]			without freeze−thaw	with freeze−thaw	*P* value[b]		
Apicidin	43±25.4	30±23.5	0.5104	29.1±21.4	0.7940	28.4±11.4	21.1±13	0.7907	25.5±9.8	0.9520
Trichostatin A	96.7±25.1	93.1±28.4	0.9978	84.8±26.9	0.9204	21.7±9.2	15.7±10.4	0.7575	14.7±7.1	0.6882
Cepharanthine	75.5±15.2	59.3±14	0.8390	55.5±10.1	0.9154	15.4±5.8	11.2±5.5	0.7259	18.2±6.1	0.6272
Valproic acid	468±155	453.1±162	0.9869	430.2±149.4	0.9381	70.6±55.4	72±59	0.8259	75.5±46.6	0.8052
Diminazene aceturate	35±8.4	25.4±9.7	0.8133	34.5±6.8	0.8002	33±29	37±34	0.2137	42±24	0.8098

[a]IC_{50}s for each drug were calculated on the fourth day based on the growth inhibitions determined using fluorescence− and microscopy− based methods in three separate experiments. [b]The differences between the IC_{50}s calculated using current and modified fluorescence assay were not statistically significant for all drugs analyzed. [c]The differences between the IC_{50}s calculated using present fluorescence− and microscopy-based methods were not statistically significant for all drugs analyzed.

The quality of fluorescence assay after a freeze−thaw step

To evaluate the quality of the assay, the Z' factor, S/N ratio, % CV_{max}, and % CV_{min} were calculated for previously optimized HCTs (2.5 % and 5 %) (Rizk et al. 2015). The Z' factor of additional freeze-thaw plus 0.5 h incubation was higher than 0.5 at a fixed HCT for each parasite (Table 1). Additionally, after freeze-thaw step, the assay exhibited high S/N ratios in bovine *Babesia* and *T. equi* parasites. On the contrary, S/N ratio showed low value in *B. caballi* for the assay after freeze-thaw step (Table 1).

Comparison of IC50 values obtained by fluorescence assay with or without freeze−thaw step

The correlation of the drug-induced growth inhibition and emission of fluorescence signal after freeze-thaw step was confirmed by the commonly used anti-babesial drug, DA which was inversely correlated with the emission of fluorescence signals in a dose-dependent manner (Fig. 5). The *in vitro* growth of the *Babesia* and *Theileria* parasites including *B. bigemina, B. caballi*, and *T. equi* was significantly inhibited ($P < 0.05$) by a 0.01 µM DA treatment (Fig. 5). While 0.05 µM DA significantly inhibited the *in vitro* growth of *B. bovis* (Fig. 5). Notably, a similar growth inhibition for bovine *Babesia* and equine *Babesia* and *Theileria* parasites was obtained by the fluorescence assay with and without freeze-thaw step and microscope (Fig. 6).

Further, the outcome of the *in vitro* test of susceptibilities of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* to apicidin, trichostatin A, cepharanthine, and valproic acid using the fluorescence method with or without freeze-thaw step is showed in Figs. 7. Data showed that fluorescence values were inversely correlated with drug concentrations in *in vitro* parasite cultures with and without freeze-thaw step (Figs. 7). After the freeze-thaw step, the numbers of fluorescence units slightly increased without substantive changes in the IC_{50} values. The *in vitro* growth of the three parasites, *B. bigemina, B. caballi*, and *T. equi* was significantly

inhibited ($P < 0.05$) by a 0.01 μM apicidin treatment (Fig. 7). Whilst, the *in vitro* growth of *B. bovis was* significantly inhibited by 0.05 μM apicidin (Fig. 7). The growth of the cultured parasites was significantly inhibited ($P < 0.05$) by cepharanthine at 0.01 μM for *B. bigemina* and 0.05 μM for *B. bovis* and *B. caballi*, and *T. equi*. Whereas, 0.01 μM treatment by trichostatin A was significantly inhibited ($P < 0.05$) the *in vitro* growth of *B. bovis* and *T. equi*. 0.05 μM of trichostatin A was sufficient for the significant inhibition ($P < 0.05$) of *B. bigemina and B. caballi* (Fig. 7). In contrast, the *in vitro* growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* was significantly inhibited by 0.05, 0.1, 0.5, and 1 μM valproic acid, respectively. Similar findings observed in *in vitro* parasite culture with freeze-thaw step (Fig. 7).

Microscopy- and SG-based fluorescence method with or without additional freeze-thaw step was used to determine the IC_{50} values of apicidin, trichostatin A, cepharanthine, valproic acid, and DA (Table 2). Importantly, the IC_{50} values obtained from the fluorescence assay with and without freeze-thaw effect were significantly concordant with those obtained by traditional microscopy method ($P < 0.05$) in all tested drugs for the bovine *Babesia* and equine *Babesia* and/or *Theileria* parasites (Table 2).

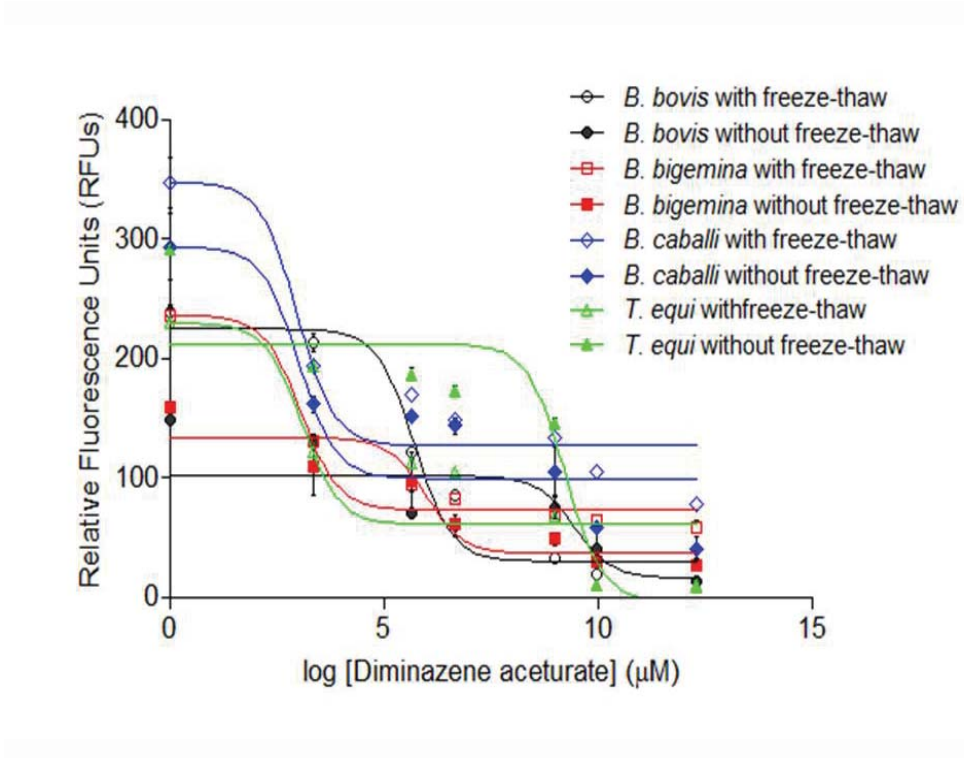

Fig. 5. Correlation between diminazene aceturate concentrations and RFUs on *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* at 0.5 and 6 h incubation with and without freeze-thaw step, respectively. Each value represents a mean of triplicate wells after subtraction of the background fluorescence for non-parasitized RBCs. Statistically, significant differences are indicated by asterisks (*P<0.05) between the drug-treated cultures and the control cultures.

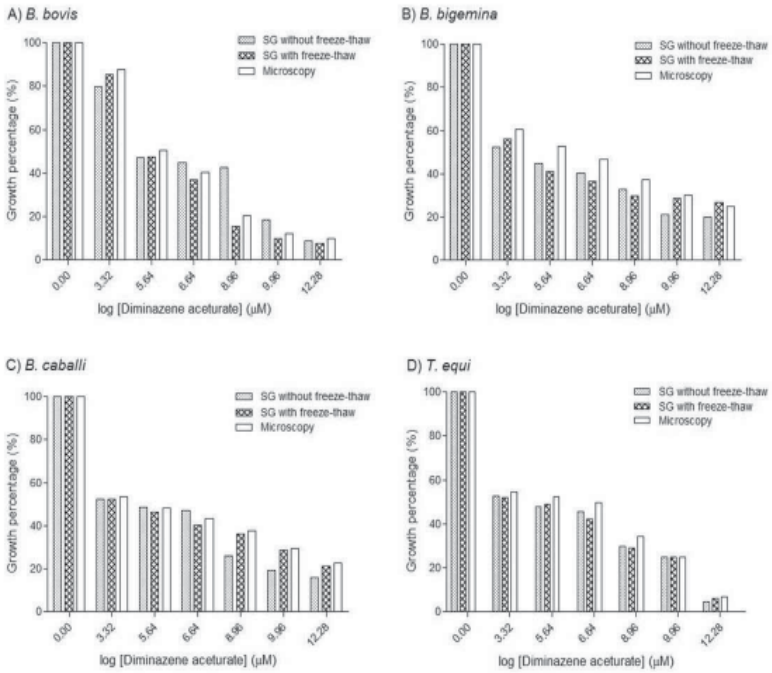

Fig. 6. Comparison of the inhibitory effects of diminazene aceturate on bovine and equine *Babesia* and *Theileria* parasites by fluorescence- and microscopy-based methods. Growth percentage of the parasite (y-axis) and log concentration of drugs (x-axis). Each value is represented as the mean of three triplicate wells ± SD.

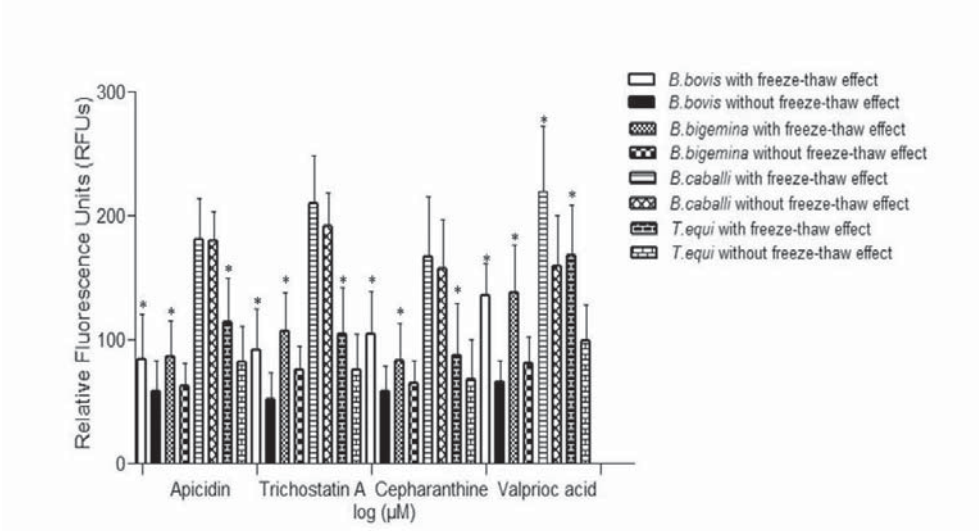

Fig. 7. Fluorescence-based monitoring of apicidin-, trichostatin A-, cepharanthine-, and valproic acid-induced growth inhibition of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* on the fourth day of treatment. Correlation between apicidin, trichostatin A, cepharanthine, and valproic acid concentrations and RFUs on *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* with and without freeze-thaw step. Each value represents a mean of triplicate wells after subtraction of the background fluorescence for non-parasitized RBCs. Statistically, significant differences are indicated by asterisks (*P<0.05) between the drug-treated cultures and the control cultures.

Cytotoxicity of anti–babesial drugs

The results of the cytotoxic activity of apicidin, trichostatin A, cepharanthine, valproic acid, and DA on MDBK cells are shown in Fig. 8. For all drugs' concentrations as high as 2400 μM, no cytotoxic effect was found which was assessed using MTT assay (Table 3). The SI was employed to compare the toxicity for mammalian cells and the activity against the parasites. The best anti-babesial activity was obtained with apicidin against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* with IC_{50} ranged between 1.3 and 35.7 nM and the SI values greater than 90 that generated by MTT assay results (Tables 2 and 4). In contrast, cepharanthine was showed activity (SI > 90 and IC_{50}s< 50) against *B. bigemina* and *T. equi*, whereas trichostatin A exhibited activity with SI=204.1 and IC_{50}s < 50 against only *T. equi* (Table 2b). Although, IC_{50}s of valproic acid great than 50 nM, SI values less than 90. Conversely, DA was less active (SI < 90 and IC_{50}s < 50) against the test all parasites compared with those of other drugs (Table 4).

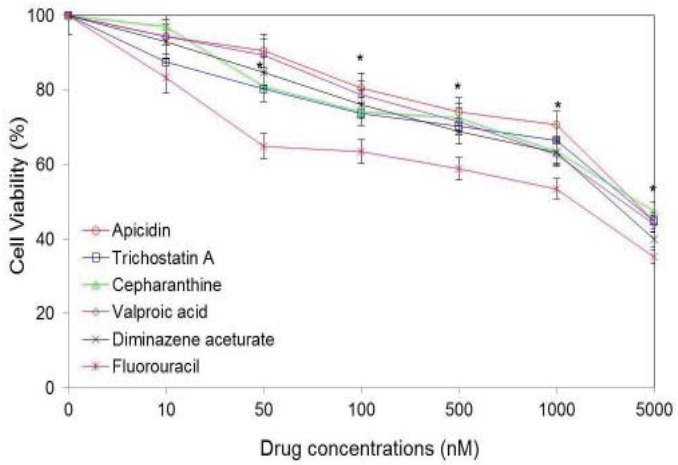

Fig. 8. Effect of apicidin, trichostatin A, cepharanthine, valproic acid, diminazene aceturate, and fluorouracil on MDBK cell viability. Cells were cultured for 48 h in the presence of different concentrations of each drug. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described in Materials and Methods.

Table 3. Cytotoxic concentration 50 % (CC_{50}) values of each drug against MDBK cells

Organism	CC_{50} values drugs (μM)[a]						
	Apicidin	Trichostatin A	Cepharanthine	Valproic acid	Diminazene aceturate	Fluorouracil	References
MDBK cells	3300±3200	3000±2800	3300±3100	2700±2400	3500±2900	1500±1300	Present study
Jurkat cells	10	0.1	ND	10.000	ND	ND	29
Esophageal squamous cell carcinoma	ND	ND	ND	2150±1020	ND	ND	30

[a] The CC_{50} was defined as the concentrations (μM) of apicidin, trichostatin A, cepharanthine, valproic acid, diminazene aceturate, and fluorouracil required for the reduction of cell viability by 50%, which were calculated by regression analysis.

Table 4. Selectivity index (SI) values for each drug against *Babesia* and *Theileria* parasites

Organism	Apicidin	Trichostatin A	Cepharanthine	Valproic acid	Diminazene aceturate
B. bovis	>90	>90	>90	>90	>90
B. bigemina	>90	>90	>90	>90	>90
B. caballi	>90	>90	>90	>90	>90
T. equi	>90	>90	>90	>90	83.3

The results were obtained from the average of three independent experiments performed in duplicate. Selectivity Index (CC_{50}/IC_{50}).

DISCUSSION

Previously, fluorescence assay using SYBR Green I for assessing the susceptibility of parasites to anti-babesial compounds was reported (Guswanto et al., 2014; Rizk et al., 2015). The assay detects the presence of parasite DNA in infected erythrocytes as a measure of parasite propagation and the inhibition in parasite growth by antiparasitic drugs. However, SG assay for testing parasite susceptibility to antiparasitic drugs needs further improvement which avoids of low fluorescence signal due to the presence of hemoglobin in the parasite culture, which could be responsible for quenching of fluorescence (Bennett et al., 2004).

Recently, cold treatment of *B. bovis in vitro* culture was found to induce an effective release of merozoites from the infected RBCs (Ishizaki et al., 2016) which may support to ensures complete lysis of the RBC and release the parasite leading to an increased availability of parasite DNA for staining with fluorescence dye. The present study reports the effect of a SG-based fluorescence assay under different culture conditions and compared the assay results to those of previously published SG method in order to determine the best fluorescence intensity. Our result showed that freezing and thawing for at least 1 h, followed by incubation with lysis buffer containing SG in the dark for 0.5 h gave the highest fluorescence signal on bovine (Table 1a) and equine *Babesia* and *Theileria* parasites (Table 1b). A similar pattern was observed in the *Plasmodium falciparum* (*P. falciparum*) culture that was frozen at -30°C for over than 1 h, followed by thawing. Moreover, additional incubation with SG for 3 h provided a significant improvement in fluorescence signal (Smilkstein et al., 2004; Dery et al., 2015). In contrary, freezing the plates before or after the addition of lysis buffer containing SG couldn't lead to a significant increase in fluorescence signal (Johnson et al., 2007). With inference to the above, the modified fluorescence-based assay with the freeze-thaw effect is considered as the best assay with the best fluorescence intensity.

Herein, linearity between the concentrations of parasite DNA and the fluorescence units was observed with high r^2 values. In contrast, a decreasing trend in fluorescence with increasing HCT at fixed parasite DNA concentration was observed in this study, suggesting that may be attributed to the quenching effect of the red blood cells as previously reported (Moneriz et al., 2009; Dery et al., 2015). The quality of the fluorescence signal was assessed by calculating the signal to noise ratio (S/N) (Makler et al., 1991; Zhang et al., 1999), based on the measurement of the fluorescence activity of parasite exposed to previously optimized

HCT (Rizk et al., 2015). Both the signal and the background were shown to remain relatively stable. Interestingly, after the freeze-thaw effect, the results of the SG assay for bovine *Babesia* and *T. equi* parasites with previously optimized HCT (2.5 % and 5 %) generated high S/N ratios. However, other studies are required to clarify the low S/N ratio generated for *B. caballi* after freeze-thaw step. Further, the suitability of the assay for high throughput screening was determined using the Z' factor, a dimensionless statistical parameter combining the difference between the positive and negative controls, and their respective variations (Bennett et al., 2004). The Z' factor value is ≥ 0.5 generally indicates a good assay (Zhang et al., 1999). Our results showed that the Z' factors of SG assay with freeze-thaw step were higher than 0.5 at 2.5 % and 5 % HCT for bovine and equine *Babesia* parasites, respectively.

Furthermore, the SG based fluorescence assay with and without additional freeze-thaw step and microscopy methods were used to determine the IC_{50} values of apicidin, trichostatin A, cepharanthine, valproic acid, and DA. The IC_{50} values obtained using the current optimized conditions with freeze-thaw effect appear to be within the expected range and comparable to those of IC_{50} values obtained using fluorescence assay without freeze-thaw effect and microscopy method. There was no significant difference ($P > 0.05$) in the IC_{50} values of all tested drugs for bovine and equine *Babesia* and *Theileria* parasites. In addition, apicidin, trichostatin A and cepharanthine showed a marked *in vitro* anti-*Babesia* and/or -*Theileria* activity, with low IC_{50} values. In contrary, valproic acid showed activity levels 2-6 times higher than those of apicidin, trichostatin A, and cepharanthine in *B. bigemina* and *B. caballi* parasites.

As view previous reports, apicomplexan histone deacetylase (HDAC) enzymes have suggested as an attractive drug target. HDAC inhibitors, apicidin, trichostatin A, and valproic acid have broad-spectrum antiviral, anticancer, antiproliferative, and antiparasitic activities against chronic myeloid leukemia cells (Park et al., 2009), hela cells (Darkin-Rattray et al., 1996), and apicomplexan parasites (Desgrouas et al., 2014). Cepharanthine, a natural compound isolated from *Stephania cepharantha* Hayata possess many pharmacological effects such as antiparasitic, anti-inflammation, anti-allergic, and anticancer (Desgrouas et al., 2014). The IC_{50} values of cepharanthine against the growth of FCM29, W2, 3D7, and K1 *P. falciparum* have been reported to be 3059 nM, 927 nM, 2276 nM, and 1803 nM, respectively (Desgrouas et al., 2014). These values were higher than those for three *Babesia* and *T. equi* parasites determined by the SG with or without freeze-thaw effect and microscopic methods. Moreover, the IC_{50} values of apicidin and trichostatin A for *Babesia* or *Theileria* parasites were higher than those calculated for *Toxoplasma gondii* (*T. gondii*) but lower than *P. falciparum* (Darkin-Rattray et al., 1996). In contrast, the IC_{50} values of valproic acid for *T. equi* and three *Babesia* parasites were lower than that calculated for *T. gondii* (Strobi et al., 2007). Interestingly, for inhibitors of HDAC, apicidin, and trichostatin A showed IC_{50} values lower than those of currently available anti-babesial drugs including; 17-DMAG, diminazene aceturate, and Atovaquone (Guswanto et al., 2018) suggested that this candidate drug might be a potent anti-babesial drug.

Finally, we determined the CC_{50} of all tested drugs by MTT assay. The results revealed that the CC_{50} values of all drugs are higher than those of IC_{50} values obtained from the present study, suggesting that drugs are no toxic. Hit activity criteria for protozoa are

considered when the IC_{50} is < 0.2 μg/ml and the SI is >100 (Nwaka and Hudson, 2006). In this study, apicidin with IC_{50} is less than 31.2 μg/ml and the SI is higher than 100 that generated by MTT assay results, met this requirement. Cepharanthine (for *B. bigemina* and *T. equi*) and trichostatin A (for *T. equi*) also classify as a hit according to the WHO Special Programme for Research & Training in Tropical Diseases (TDR).

Herein we reported that evidence obtained in this study gives further credence to the fact that the SG method is consistent and rapid for the routine assessment of parasites sensitivity to drugs with the enhanced signal intensity. Current results showed that freezing and thawing of culture, followed by incubation with SG in the dark for 0.5 h gives the highest fluorescence signal. It may be useful for attaining the full benefit of using the recently introduced SG method in the anti-babesial drug monitoring system. Importantly, there is no need to carrying delicate equipment to the field in order to perform the assay and a freezing step permits long-term storage and shipping of assay plates. Because of the test plates can be frozen and later transported to the main laboratory for fluorescence reading. This has proved to be quite beneficial and has allowed the assay to be conducted in remote regions, where access to a fluorometer is limited.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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Malaria infections among pregnant women attending antenatal clinic at Bududa hospital, eastern Uganda

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ABSTRACT

Pregnancy-associated malaria is a major health concern in Bududa district. To assess the prevalence of malaria infection in pregnancy in Bududa District; a hospital-based, cross-sectional study was carried out. Two hundred fifty two participants were selected using consecutive simple non probability sampling on consenting. Capillary blood samples were collected. Thick and thin smears were made, stained using 3% Giemsa staining technique and examined under the light microscope for malaria parasites and species typing. Data was cleaned and entered using Epi information version 3.5.3, later converted by Microsoft access, excel and word and descriptive data analysed using SPSS. Prevalence of malaria was 8.73 % (22/252) (95% CI 3.8-13.7). Only *P. falciparum* species was identified and malaria infections were high in primigravid with 16.28% (14/86) than in multigravid (P-value < 0.001). Malaria infection still remains a health challenge among pregnant women with a prevalence of 8.73% (22/252); *P. falciparum* was the only species identified, primigravid pregnant women are most affected. Routine malaria screening in symptomatic and asymptomatic malaria infections in pregnancy for early diagnoses and prevent transmission is vital. It is crucial to intensify and sensitize mothers and communities about malaria control programmes.

Keywords: Malaria, prevalence, gravidae, pregnancy, Bududa

INTRODUCTION

Malaria remains an important treat to global public health. According to the latest World malaria report, 2018, there were 219 million cases of malaria in 2017, up from 217 million cases in 2016. African Region continues to carry a disproportionately high share of the global malaria burden. Malaria is endemic in more than 90% parts of Uganda with a national prevalence of 27% (Colon-Gonzalez et al., 2016); prevalence of 9% in central Uganda (Namusoke et al., 2010) and at 32.70% (95% CI 9.3–56.2%) Northern Uganda (Maziarz et al., 2018) and 27% in Mbarara, South Western Uganda by De Beaudrap et al., (2013).

Globally, 125 million women are at risk of malaria every year (Conroy et al., 2012). In sub-Saharan Africa, the area most burdened by malaria, the disease is thought to cause as many as 10,000 cases of malaria-related deaths in pregnancy, mainly due to severe maternal anemia (Takem & D'Alessandro, 2013). Studies by Takem & D'Alessandro, (2013) in sub-Saharan Africa among pregnancy showed a malaria prevalence of 29.50% in East and Southern Africa and 35.10% in West and Central Africa. Malaria makes a large but unquantifiable contribution to low birth weight in infants in the developing world, a major cause of morbidity and mortality in infants (Desai et al., 2007).

Observations made in December 2015, antenatal records at Bududa Hospital, indicated that in the last six months, out of 1122 pregnant women who attended antenatal clinic 115 were found positive for malaria (Un published) and verbal expressions from the midwives at Bududa Hospital suggested that the problem could worsen (Personal communication). Therefore the knowledge of prevalence of malaria infections creates awareness to health authorities about the infection rate and dangers of the disease.

MATERIALS AND METHODS

Study area and design

A cross sectional descriptive laboratory based study to determine the prevalence of malaria infection among pregnant women attending antenatal clinic at Bududa hospital located in Eastern Uganda was used.

Sample size and study population.

Two hundred fifty two (252) pregnant women who attended antenatal clinic during the study period were recruited into the study.

Sampling technique

Consecutive non-probability sampling technique was used to recruit 252 pregnant women attending antenatal clinic upon consent.

Sample collection and processing

Supplies or reagents and materials were purchased from Joint Medical Stores, Uganda. Capillary blood samples were obtained aseptically from 252 pregnant women by a finger prick using a spring loaded lancet, thick and thin smears were prepared on separate slides. Air dried and thin smears were fixed for three minutes using absolute methanol. Both dried thin and thick smears were stained using 3% Giemsa staining protocol and examined under a light microscope using an oil emersion objective lens. Thick smears were used to detect the parasites and thin smears for species typing and identification.

Data analysis and management.

Data obtained was entered into MS Excel spreadsheets; Cross-checking and data cleaning was done. Statistical Package of Social Sciences was used for descriptive data analysis and outputs in form of frequency tables, figures and percentages presented. The

prevalence was calculated as the number of individuals with malaria divided by the total number of participants (n=252) multiplied by 100 using 95% confidence interval and distribution of cases by gravidae was considered significant with P. value less than 0.05.

Ethical considerations

The study was approved by Research Ethics Committee of Mbarara University Science and Technology and participants' consent was obtained before recruitment. Participant's results were treated with strict confidentiality by using codes known only to the investigator.

RESULTS

Socio-demographic characteristics for study participants

Two hundred fifty two (252) study participants were recruited into the study and data collection guide was used to capture socio-demographic characteristics.

Study participant enrollment was grouped into four age group categories in years as follows; 18 – 27 years 67.46% (170/252), 28 – 37 years 29.37% (74/252), 38 – 47 years 3.18% (8/252), there were no participant from ≥ 48 age group. Majority of study participant were between 18 – 27 age group

Study participants enrollment was also categorized based on gravidity into two groups that is primigravid with 34.13% (86/252) and multigravid with 65.87% (166/252) of study participants. Majority of participants belonged to multigravid (Fig. 1).

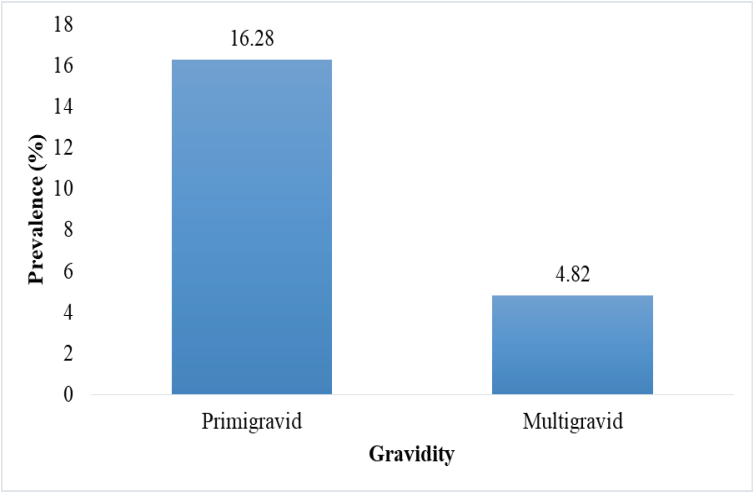

Fig 1. A bar graph showing the distribution malaria cases by gravidity

General prevalence of malaria and isolated plasmodium species among pregnant women

Malaria prevalence of 8.73% (22/252) (95% CI: 5.6 – 11.4) was obtained and *P. falciparum* was the only *Plasmodium* species found contributing to 100% (22/22) of the cases (Fig.2).

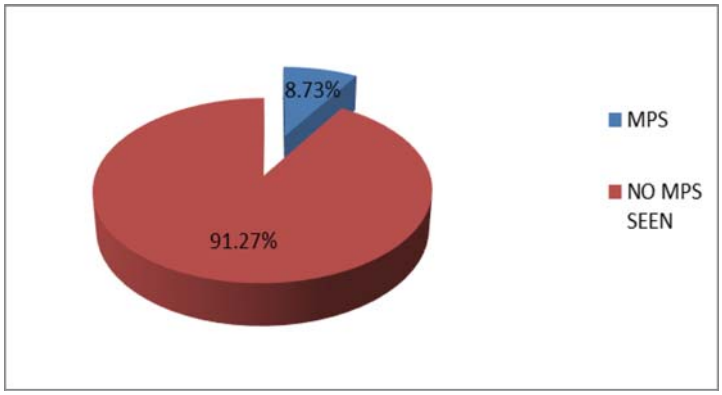

Fig 2. Laboratory findings for malaria parasites in peripheral blood smears

Distribution of malaria cases among participants by age groups and gravidity

In relation to gravidity, Percentage prevalence of malaria infections decline in the study population as gravidity increased; being highest in the primigravid with 16.28% (14/86) of infections and low in multigravid with 4.82% (8/166) of the infections (P-value < 0.001) (Table 1).

Table 1. A table showing the percentage distribution of malaria cases by gravidity and age group

Variable	No of pregnant women tested (n=252)	No of women infected (cases)	No of women tested negative	Prevalence (%)
a) **Age group (Years)**				
18 – 27	170	16	154	9.41
28 – 37	74	6	68	8.12
38 – 47	8	0.0	8	0.0
Total	**252**	**22**	**230**	**5.84**

b) **Gravidity**				
Primigravid	86	14	72	16.28
Multigravid	166	8	158	4.82
Total	**252**	**22**	**230**	**10.55**

DISCUSSION

Malaria infection during pregnancy is a major public health problem in Bududa with a general prevalence of 8.73% (22/252) with primigravid having the highest malaria infections. This calls for improved efforts in controlling and management if the quality of life is improved in pregnancy.

The prevalence of malaria was 8.73% (22/252). The study results are in agreement with the study at Mulago National Referral Hospital, Uganda by Namusoke et al., (2010) that gave a prevalence of 9%. This could be due to the similarities in geographical location sharing similar climatic conditions, study design, diagnostic methods and control interventions. These study findings are contrary to those by Maziarz et al., (2018) from the study carried out in Northern Uganda with prevalence of 32.70% and 27% in Mbarara, South Western Uganda by De Beaudrap et al., (2013), Raimi O & Kanu C, (2010) and Falgunee et al., (2007) in Nigeria that gave a significantly higher prevalence.

The lower prevalence from this study could be attributed to the differences in climatic conditions in study areas where in Bududa the received intermittent rainfall patterns during the study period and surrounding districts which could have contributed to the reduction of breeding grounds for mosquitoes. High prevalence in Northern and south Western Uganda is attributed to hot climate and intermittent wet seasons could have been receiving too much rain and hence increased breeding grounds for mosquitoes and the differences in the sensitivities of the diagnostic methods used. The differences in the findings is attributed to the differences in study designs used in these studies where our study used a cross sectional study design whereas the study in South Western was a prospective study design. This is also attributed to differences in levels of implementation of preventive measures like use of insecticide treated mosquito nets used due to national wide malaria control campaigns to alleviate malaria burden and varied study site settings and diagnostic approaches used.

Malaria infections still remains a challenge in pregnancy in Bududa, therefore preventing and treating malaria in pregnancy can be a key intervention to improving maternal, foetal and child health and to minimize effects of malaria infection like maternal anaemia, abortions, still birth, intrauterine growth retardation, intrauterine foetal death need special measures to ensure their survival and improve birth outcome (Agomo et al., 2009).

This area is in rural setting with a higher incidence rate of malaria yet have little access to Health information and services than those living in urban areas (Yeka, 2012).

In this study, *P. falciparum* was the only species contributing 100% of all positive cases. This is in agreement with the study findings by Maziarz et al., (2018) in Northern Uganda and De Beaudrap et al., (2013) in Mbarara South Western Uganda. This is because *P. falciparum* is the most common species in tropical and subtropical Africa (Cheesbrough

M, 1994) and the absence of other plasmodium species could be attributed to their great rarity in the above parts of the world.

This study findings defers to the study by Falgunee et al., (2007) in a suburb of Lagos, Nigeria, where two Plasmodium species were *P. falciparum* and *P. malariae* were identified in different proportions. *P. falciparum* was evident to be more prevalent with a prevalence of 84.60% compared to 15.40% for *P. malariae*.

The study also revealed that malaria is more prevalent in primigravid with 16.28% (14/86) of the infections than in multigravid with 4.82% (8/166) (P value < 0.001). These findings are in agreement with those by Maziarz et al., (2018) in Northern Uganda. This observation is attributed to the partial pregnancy immunity or sensitization that develops due to frequent exposures to malaria infections in multigravidae unlike those in primigravidae, this immunity accumulates with successive pregnancies provided there is exposure to malaria infection and this is common in places where malaria is endemic

These study findings are not in line with the study by Brennan et al., (2005) that showed that there is no difference in the level of specific immunity to malaria, this could be support by the fact that even multigravida pregnant women without prior exposure to malaria in previous pregnancies, malaria treats them equally like the primigravidae since there is no immunity development unless exposed in previous pregnancies hence all are vulnerable to the infection.

CONCLUSION

Malaria infection still remains a burden in pregnancy with *P. falciparum* species as the main cause of malaria infections in Bududa. This observation highlights the need to broaden and enhance control interventions to prevent malaria during pregnancy regardless of the gravidae. The study recommends routine malaria screening for symptomatic and asymptomatic malaria infections for early diagnoses and prevent transmission. The district health authority should intensify enlightenment campaigns to health educate women of child-bearing age and the community about the preventive and control measures of malaria.

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CONFLICT OF INTEREST

All authors declare no conflict of interest in the study.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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Lepidosomes acquire fluorescence after encystation: Including additional notes of morphological events during encystation and reconsideration of the morphological features in the ciliate *Colpoda cucullus*

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ABSTRACT
The resting cyst formation (encystment) is a survival strategy against environmental stressors, and is found in many species of single-celled eukaryotic organisms. The process incorporates cell differentiation accompanied by drastic morphological changes. This study presents observation via fluorescence microscopy on the encystation process of *Colpoda cucullus*. We confirmed that *C. cucullus*, which was identified by 18S rRNA analysis, contains lepidosomes, which are cyst-specific cell structures. The existence of these structures in *Colpoda* species is controversial at present, so we present background information concerning the controversy. Moreover, we reveal that lepidosomes acquire autofluorescence after formation, that is, encysting cells contain lepidosomes lacking autofluorescence while mature cysts contain lepidosomes exhibiting autofluorescence. In addition, we describe the process of formation of nuclear surrounding particles (NSPs), which are cyst-specific cell structures.

Keywords: *Colpoda,* cyst, *cryptobiosis,* lepidosome, autofluorescence

INTRODUCTION
The survival of microorganisms depends on their ability to sense changes in the environment and respond to new situations (Gutiérrez et al., 2003). Encystment is a common occurrence among free-living ciliates, which often undergo this process under adverse environmental conditions (Verni and Rosati, 2011). Encystment represents a strategy against various environmental stressors such as starvation (Gutiérrez et al., 2001), desiccation (Taylor and Stickland, 1936), freezing (Uspenskaya and Lozina-Lozinski, 1979), high and low temperatures (Taylor and Stickland, 1936), ultraviolet irradiation (Uspenskaya and Lozina-Lozinski, 1979; Matsuoka et al., 2017), and acidity (Sogame et al., 2011). Therefore, an important part of the life cycle of most ciliates is the formation of resting cysts (Benčaťová and Tirjaková, 2018).

The process of encystment in the ciliate genus *Colpoda* was reported to be controlled by an intracellular signaling pathway (Matsuoka et al., 2010) triggered by an increase in intracellular Ca^{2+} concentration caused by Ca^{2+} inflow (Sogame and Matsuoka, 2013), which leads to protein phosphorylation (Sogame et al., 2012a) and alteration of protein expression (Izquierdo et al., 2000; Chessa et al., 2002; Sogame et al., 2012b, 2014). It also involves progressive and drastic morphological changes (Gutiérrez et al.,2003). The morphological events during the encystment process of *Colpoda cucullus* have been described and include mitochondrial fragmentation, expulsion of net-like globules, synthesis of ectocysts and endocysts, and chromatin extrusion (Funatani et al., 2010). The net-like globules (Foissner 1993) were defined as lepidosomes (Foissner et al., 2003) and are reported to have autofluorescence (Matsuokaet al., 2017). The main scope of this study was to reveal whether lepidosomes acquire fluorescence after their formation. In addition, nuclear surrounding particles (NSPs) were previously reported to be cyst-specific fluorescent materials surrounding the nucleus (Matsuoka et al., 2017); however, details on their formation have not yet been elucidated. We observed the NSP formation process by double visualization (nuclei were visualized with Propidium Iodide (PI) staining and NSPs were visualized by their autofluorescence) by fluorescence microscopy.

As stated above, the *C. cucullus* cysts in our culture cell line (*C. cucullus* R2TTYS) expelled lepidosomes. However, *C. cucullus* was previously reported to contain no lepidosomes (Foissner, 1993), although *C. cucullus* Nag-1, whose partial 18S rRNA sequence is closest to that of *C. lucida*, was reported to have lepidosomes (Funadani et al., 2016). Another aim of this study was to reconsider the presence of lepidosomes as a taxonomic characteristic of *C. cucullus*.

MATERIALS AND METHODS

Cell culture

Colpoda cucullus R2TTYS was cultured in an infusion of dried wheat leaves supplemented with bacteria (*Klebsiella pneumoniae* NBRC13277 strain) as food. *K. pneumoniae* was cultured on agar plates containing 1.5% agar, 0.5% polypeptone, 0.5% yeast extract, and 0.5% NaCl.

Human liver adenocarcinoma cell line SK-HEP-1cells were kindly provided from Prof. T. Nakatsura and Dr. M. Shimomura in National Cancer Center, Japan. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich Japan LLC., Tokyo, Japan) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin, and 1% streptomycin for 3–4 days.

Encystment induction

Encystment was induced according to Sogame et al., 2019. The cultured vegetative cells were corrected by centrifugation (1500 g, 1min) and suspended at high cell density (> 10,000 cells/mL) in encystment-inducing medium [1 mM Tris-HCl (pH 7.2), 0.1 mM $CaCl_2$].

Microscopy

Vegetative cells, cysts, and dry cysts were observed using the Avart A1 system (Carl Zeiss Microscopy Co., Ltd., Tokyo, Japan; Fig. 1 A-1, A-3) or Axio Scope A1 system (Carl Zeiss Microscopy Co., Ltd.; Fig. 1 A-2).

To visualize lepidosomes, cysts at 6 h ('immature cysts', Fig. 2 A-1, 2B a1–a3, 2C 'Immature cysts') and more than 1 week ('mature cysts', Fig. 2 A-2, 2B b1–b3, 2C 'Mature cysts') after the onset of encystment induction were stained with Congo Red solution [0.25% Congo Red in 1mM Tris-HCl (pH 7.2) (Fig. 2)] for 30 min, washed with 1 mM Tris-HCl (pH 7.2) (Fig. 2A), and observed under an optical microscope (Axio Scope A1; Fig. 2A) or a confocal laser microscope (FluoView 10i, Olympus Corporation, Tokyo, Japan; Fig. 2B) using a PI filter (emission maximum, 620 nm) to visualize Congo Red fluorescence (Fig. 2B a-2, b-2, 2C '620 nm') or DAPI filter (emission maximum, 460 nm) to visualize autofluorescence (Fig. 2B a-1, a-2, 2C '460 nm') with 405 nm and 473 nm laser diode (LD) excitation peaks. The rates of fluorescing cells were directly calculated under the confocal laser microscope. The significance of differences among samples was evaluated prepared by the Mann-Whitney U test.

For 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) double staining (Fig. 3), SK-HEP-1 cells (for control), *Colpoda* vegetative cells, and *Colpoda* cysts were prepared following two protocols (short-fix protocol and long-fix protocol) as follows. In the short-fix protocol, cells were fixed by 2% (final concentration) paraformaldehyde in Phosphate buffered saline (PBS) for 5 min, washed with PBS, and suspended in 1% (final concentration) NP40 in PBS for 1 h. Second, in the long-fix protocol, cells were fixed by 2% (final concentration) paraformaldehyde in PBS for 1 week, washed with PBS, and suspended in 1% (final concentration) NP40 in PBS for 1 h. Subsequently during both protocols, the cells were washed with PBS and stained with DAPI (final concentration 0.001%) and PI (final concentration 0.002%) in PBS for 15 min, and observed using a confocal laser microscope with a DAPI filter (emission maximum, 460 nm) with 405 nm LD excitation peak and PI filter (emission maximum, 620 nm) with 473-nm LD excitation peak.

For double-visualization of nuclei by PI staining and NSPs (Fig. 4), cells were stained with PI following a long staining protocol and subsequently observed by a confocal laser microscope under a DAPI filter (emission maximum, 460 nm) to visualize autofluorescence, and a PI filter (emission maximum, 620 nm) to visualize PI fluorescence with 473-nm LD excitation peak.

Amplification and sequencing of 18S rRNA and phylogenetic analysis

Total genomic DNA was extracted from *C. cucullus* R2TTYS using the DNeasy Blood and Tissue Kit (Qiagen K. K., Tokyo, Japan) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification for the partial 18S rRNA gene (1623 base-pairs, bp) was performed using the genomic DNA as a template. The PCR product was purified with a PCR Clean-Up Kit (Nippon Gene CO. LTD, Japan) and sequenced via BigDye Terminator v3.1 Cycle Sequencing Kit analyzed on a 3730 xl DNA analyzer (Thermo Fisher Scientific K. K., Tokyo, Japan). The primer sets has been previously described (Funadani et al., 2016). The nucleotide sequence was assembled using CLC Main

Workbench software (Filgen Inc., Aichi, Japan), and was used to search International Nucleotide Sequence Database Collaboration (INSDC). The sequence determined in this study have been deposited in INSFC under accession numbers LC441007. The estimation of the best evolutionary model and construction of a neighbor-joining (NJ) tree using the T92 model (Tamura, 1992) were performed in MEGA 6.0 (Tamura et al., 2013). A sequence of *Cyrtolophosis mucicola* (EU039898.1) was used as outgroup. The tree probability was assessed using bootstrap resampling of 1000 replicates.

RESULTS AND DISCUSSION

The vegetative cells, cysts, and dry cysts of our culture cell line of *C. cucullus* R2TTYS are shown in Fig. 1A. The partial nucleotide sequence of the 18S rRNA gene in our strain (LC441007/INSDC) was 99% the same as that in *C. cucullus* EU039893.1 (1620/1623 bp), *C. cucullus* Nag-1 strain AB918716.1 (1591/1601 bp), *C. inflata* PABBc-30 strain KJ60798.1 (1615/1623 bases), and *C. lucida* EU039895.1 (1609/1622 bp). The NJ tree showed that the partial sequence of 18S rRNA gene of the strain was most closely related to that of *C. cucullus* EU0398983.1 (Fig. 1B); we therefore identified our culture cell line as *C. cucullus*. However, it seemed that the cysts of the *C. cucullus* R2TTYS strain expelled small particles outside the cyst wall (Fig. 1A-2), which appeared to be lepidosomes (Foissner et al., 2005); the presence of lepidosomes disagrees with a previous report on *C. cucullus* (Foissner, 1993). To identify whether the expelled particles were lepidosomes, immature cysts (i.e., 6 h after the onset of encystment) and mature cysts (i.e., more than 1 week after the onset of encystment) were stained with Congo Red and observed by optical microscopy, because lepidosomes are reported to be visibly stained by this dye (Funadani et al., 2016). The observations (Fig. 2A) indicated that the *C. cucullus* R2TTYS strain expelled lepidosomes around their cyst wall. This result agreed with reports of *C. cucullus* Nag-1 strain (Funadani et al., 2016), *C. inflata* (Foissner, 1993), and *C. lucida* (Foissner, 1993), but disagreed with that of the *C. cucullus* strain reported by Foissner (1993). Molecular phylogenetic analysis showed that the partial sequence of the 18S rRNA gene of *C. cucullus* R2TTYS was most closely related to that of *C. cucullus* EU0398983.1 (Fig. 1B), although the *C. cucullus* R2TTYS strain contained lepidosomes. A previous study reported that *C. cucullus* Nag-1, which does have lepidosomes, was most closely related to *C. lucida*, although its morphological features agreed with those of *C. cucullus* in a 1993 report by Foissner (Funadani et al., 2019). These results suggest the possibility that lepidosomes are an intraspecific variation or that *C. lucida* might actually be a synonymic relationship of *C. cucullus*.

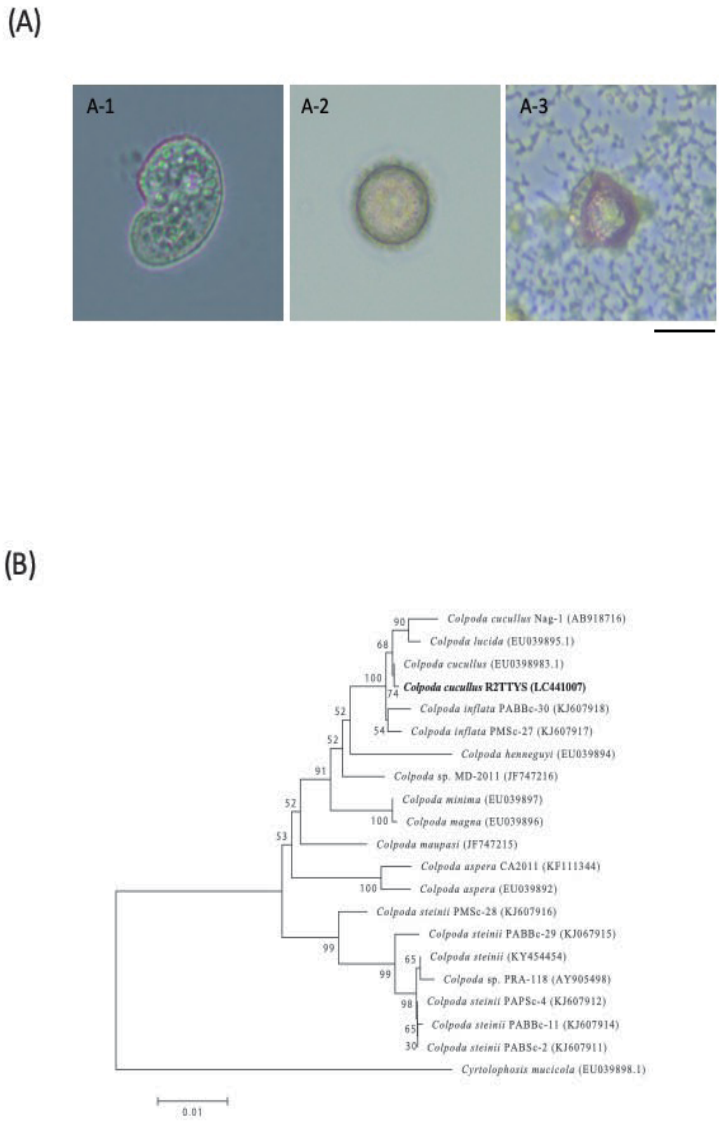

Fig. 1. Optical microscopy analysis and phylogenetic analysis of *C. cucullus* R2TTYS used in this study. (A) Optical microscopy images: vegetative cell (A-1), 1-week-old cyst (A-2), and dry cyst (A-3). The scale bar represents 20 μm. (B) Neighbor-joining (NJ) tree of the species of *Colpoda*, which was constructed by partial 18S rRNA gene sequences (1623 bp). The scale indicates genetic distance of T92 model. Numbers near the internal branches are percentage of bootstrap value. The bold letter shows haplotype of strain of *C. cucullus* R2TTYS determined in this study. The accession number of each sequence is indicated in parentheses.

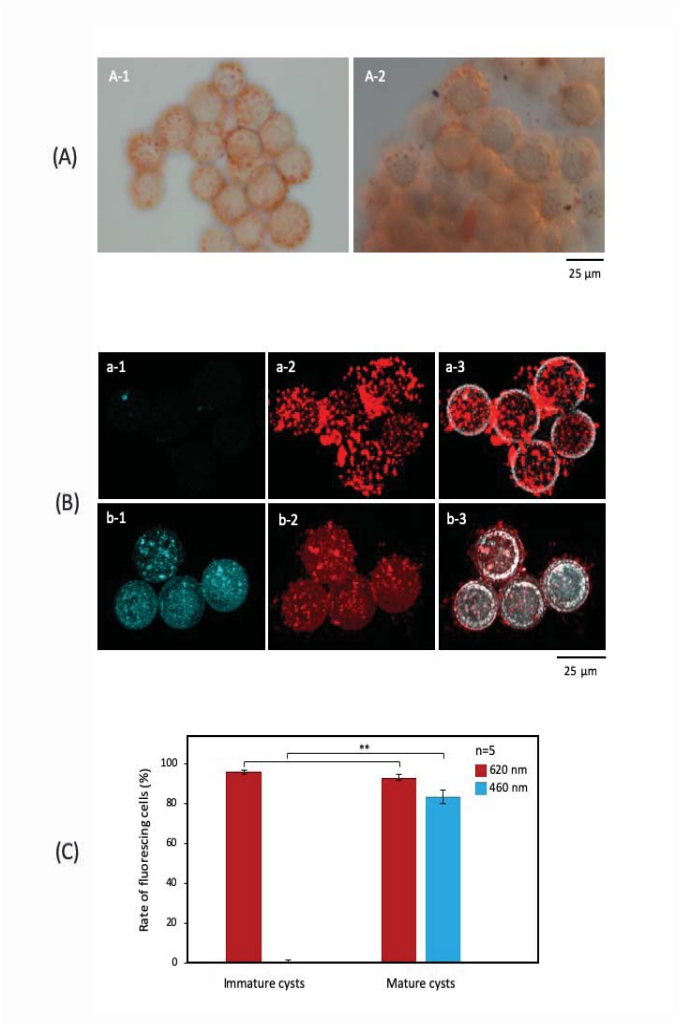

Fig. 2. Congo Red staining of encystation (at 6 h; A-1) and more than 1-week-old cysts (A-2) observed by optical microscopy. The scale bar represents 25 μm. Observation of lepidosomes by confocal laser microscopy (B). Observation of lepidosomes of encysting cell (a) and 1-week-old cysts (b). They were stained with Congo Red. Detection of autofluorescence of lepidosomes (a-1, b-1), fluorescence of Congo Red (b-2, b-3), and merged bright field images (a-3, b-3). The scale bar represents 25 μm. The rates of fluorescing cysts were calculated (C). Cysts 6 h after the onset of encystment induction 'Immature cysts' and more than 1 week after the onset of encystment induction 'Mature cysts' were stained with Congo Red and detected autofluorescence at an emission maximum of 460 nm '460 nm' and fluorescence of Congo Red at an emission maximum of 620 nm '620 nm', respectively. Columns and bars correspond to the means and their standard errors, respectively, of five measurements. Double asterisks indicate significant differences among columns at $p < 0.01$ (Mann-Whitney U test).

According to a previous report (Matsuoka et al., 2017), lepidosomes of mature cysts exhibit autofluorescence. In this study we found new information about lepidosomes. Indeed, the lepidosomes of mature cysts exhibited autofluorescence (Fig. 2B b-1); however, lepidosomes of immature cysts did not show autofluorescence (Fig. 2B a-1). To further clarify this, we prepared a double visualization of lepidosomes, that is, Congo Red-stained lepidosomes were visualized by both autofluorescence (at a wavelength of 460 nm) and fluorescence from Congo Red (at a wavelength of 620 nm). Congo Red staining can be observed with excitation (Clement and Truong, 2014). Our observations showed that lepidosomes in cells during encystation showed fluorescence at a wavelength of 620 nm (Fig. 2B a-2) but not at an emission maximum of 460 nm (Fig. 2B a-1). We confirmed the existence of lepidosomes in cells during encystation, however they did not show autofluorescence. In contrast, the fluorescence of lepidosomes in mature cysts was detected at both 620 nm (Fig. 2B b-2) and 460 nm (Fig. 2B b-1). Overlaying showed that the two types of fluorescence almost merged (Fig. 2B b-3). Subsequently, the rate of cysts which emitted fluorescence were confirmed (Fig 2C). In immature cysts, 96.1 % of cysts emitted fluorescence at a wavelength of 620 nm but only 1.0 % of cysts emitted fluorescence at a wavelength of 460 nm (Fig 2C 'Immature cysts'). On the other hand, 93.2 % and 83.4 % of cysts emitted fluorescence at a wavelength of 620 nm and 460 nm, respectively in mature cysts (Fig 2C 'Mature cysts'). The rate of fluorescing cells by Congo Red staining (fluorescence at a wavelength of 620 nm) of immature cysts was almost same as much as it of mature cysts ($p > 0.05$), however, the rate of autofluorescing (fluorescence at a wavelength of 460 nm) cells of immature cysts was significantly lower than that of mature cysts ($p < 0.01$). These results indicated that lepidosomes are produced in the early phase of the encystment process and acquire fluorescence later in the process.

In addition to lepidosomes, NSPs have also been reported as fluorescent structures of cysts (Matsuoka et al., 2017); however, detailed information about the formation process of NSPs has not been elucidated. However, we revealed the process of NSP formation in relation to the positions of nuclei (Fig. 4). NSPs were visualized as autofluorescence (emission maxima, 460 nm) and nuclei were stained with PI and observed at an emission maximum of 620 nm. The double visualization was performed using a general PI staining protocol (short-fix protocol; see Materials and Methods), however, nuclei were not stained with PI− (Fig. 3C 'S-PI'). To find the cause of the result, double staining of nuclei with DAPI and PI was performed. In consequence, nuclei in both *Colpoda* vegetative cells (Fig. 3B 'S-DAPI') and cysts (Fig. 3C 'S-DAPI') were stained with DAPI although both of them were not stained with PI (Fig. 3B, 3C 'S-PI'). Hence, PI was ineffective in both *Colpoda* vegetative cells (Fig. 3B 'S-PI') and cysts (Fig. 3C 'S-PI'). On the other hand, the fluorescent signals of DAPI (Fig. 3A 'S-DAPI') and PI (Fig. 3A 'S-PI') were almost co-localized in SK-HEP-1 cells via the same protocol (Fig. 3A 'S-Merge'). By extending the fixation time, the DAPI fluorescence in *Colpoda* vegetative cells (Fig. 3B 'L-DAPI') and cysts (Fig. 3C 'L-DAPI') and that of PI in *Colpoda* vegetative cells (Fig. 3B 'L-PI') and cysts (Fig. 3C 'L-PI') were co-localized in both *Colpoda* vegetative cells (Fig. 3B 'L-Merge') and cysts (Fig. 3C 'L-Merge'), respectively. Nevertheless, the long-fix protocol was problematic in SK-HEP-1 cells (Fig. 3A 'L-Merge'). In this case, PI did not work (Fig. 3A 'L-PI') although DAPI was

effective (Fig. 3A 'L-DAPI'). Since PI and DAPI bind strongly to DNA, nuclei must be stained with them if they got inside of cells and nuclei by surfactant preparation. PI was possibly trapped by cell structure or substances outside of nuclei because nuclei were apparently darkened (Fig. 3C 'S-PI').− Unfortunately, we cannot explain why PI did not work in *Colpoda* cells, but we were at least able to establish a staining protocol. Then, vegetative cells and cysts at 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 1 week, 2 weeks, and 3 weeks after the onset of encystment induction were prepared by long-fix protocol and double visualization was performed. The nuclei of all cells were stained with PI and the NSP formation process revealed the positional relationship between nuclei and NSPs (Fig. 4). At 12 h after the onset of encystment induction, a phenomenon which appeared to be chromatin extrusion (Akematsu and Matsuoka, 2008) occurred and the NSPs began to form around the nuclei (Fig. 4, 12 h, arrowhead). Thereafter, NSPs were gradually formed around the macro- and micronuclei. The photos shown as Fig. 4 were representative from repeated experiments, and the time course of NSP formation was similar among the repeated experiments.

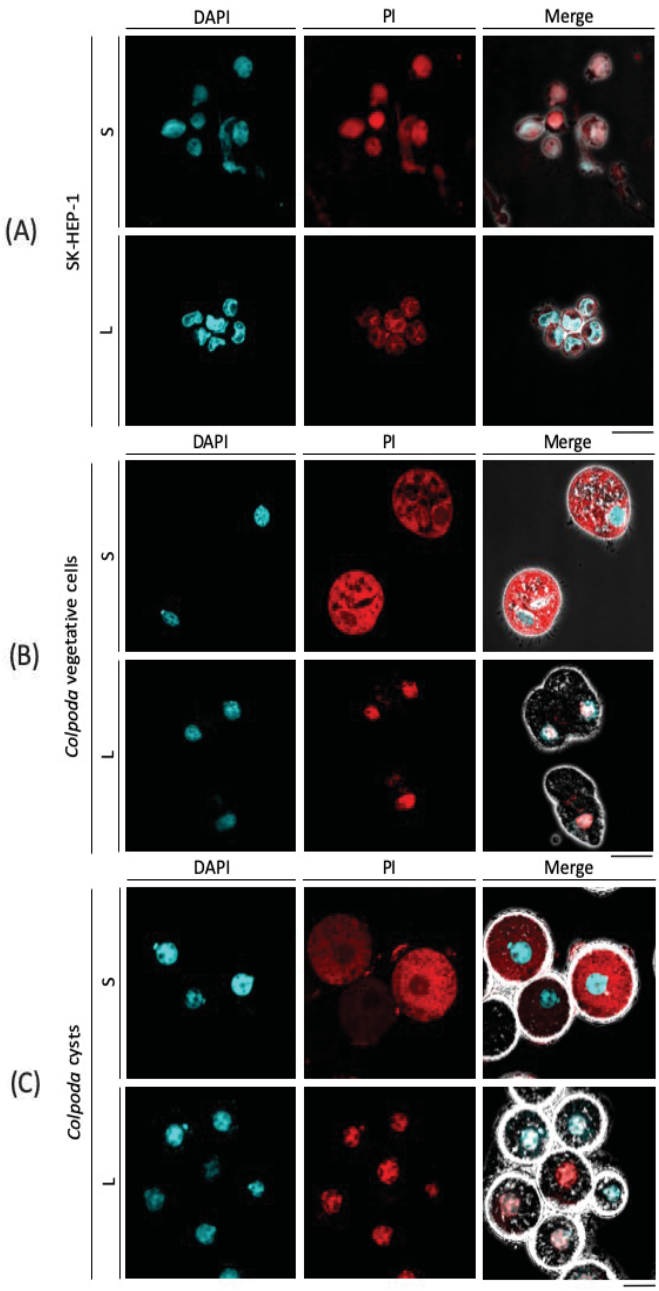

Fig. 3. Images of double staining. SK-HEP-1 cells (A), *Colpoda* vegetative cells (B), *Colpoda* cysts (C) were stained with DAPI and PI and their nuclei were visualized by fluorescence via DAPI ('DAPI') and ('PI'), by a short-fix protocol ('S') or long-fix protocol ('L'). The scale bars of (A), (B), and (C) represent 30 μm, 30 μm, and 15 μm, respectively.

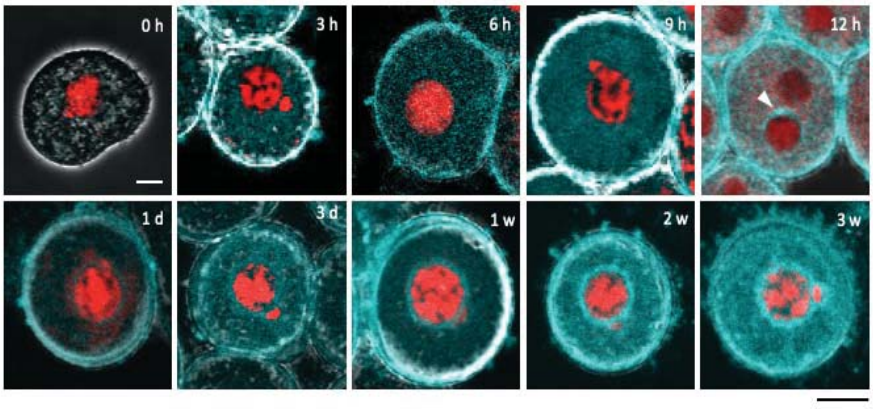

Fig. 4. Observations of NSPs with PI stained nuclei in vegetative cells '0 h' and cysts at 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 1 week, 2 weeks, and 3 weeks after the onset of encystment induction. The arrowhead of 12 h indicates NSP. The scale bar for vegetative cells is shown within the '0 h' image and for other samples it is shown on the bottom right of the entire figure. Each of them represents 10 μm.

Cyst-specific fluorescent structures such as lepidosomes and NSPs may contain a kind of pigment that perhaps absorbs the partial energy of radiation and might be responsible for antioxidant activity, as reported for *Deinococcus radiodurans*. The pigment deinoxanthin that was isolated from *D. radiodurans* (Lemee et al., 1997) was reported to be responsible for antioxidant activity (Carbonneau et al., 1989). If cells are irradiated by radiation rays such as ultraviolet or gamma rays, they can be damaged by reactive oxygen species (ROS) (Azzam et al., 2012). In this situation, their fluorescent materials protect the cells by absorbing some of the energy via scavenging to protect against radiation. The ability to tolerate radiation rays, including the activities of fluorescent materials, probably evolved to allow tolerance against desiccation for survival in terrestrial environments over long periods of time because ROS stress is also caused by desiccation (França et al., 2007).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

This manuscript is approved by all authors and, if accepted, will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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Nakayama A. 1978. Rhythm of the body temperature. pp. 140-154. In: Biorhythm and its Mechanisms. Suda M, Hayashi O, Nakagawa H. (Eds), Kodansha, Tokyo (in Japanese).

f-3) Books: Author(s), year, title, edition (edn) if appropriate, publisher as above.

Example (NOTE THE PUNCTUATIONS):
Bloom W, Fawcett DW. 1969. A Text Book of Histology, 9th ed., B. W. Sauders, Philadelphia.

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Examples:
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This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Permit Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

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