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 (IC50) 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 IC50 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 (EC50) values of 611.7 ± 10.9, 870 ± 22, and >1500 μg/mL, respectively, while EAALE exhibited EC50 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
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*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; Matsu 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, antihelminthic, antimalarial, hepatoprotective, and antidepressant properties (Beigh and Ganai, 2017). A. absinthium leaves have been used
Herbal extracts can inhibit the growth of piroplasma 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 (BUCHI® 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 I 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% CO₂. The MDBK cell line was maintained in a 75 cm² 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 TrypLE® 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% CO₂ and 95% air. One hundred microliters of cells at a density of 5 x 10⁴ 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 37°C. 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% CO₂, 5% O₂, and 90% N₂. 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 IC₅₀ 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 37°C with an atmosphere of 5% CO₂, 5% O₂, and 90% N₂ 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
Herbal extracts can inhibit the growth of piroplasma 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 IC₅₀ 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 \times 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 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.
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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\textsubscript{50} and selectivity index of methanolic Peganum harmala seed extract (MPHSE) and ethanolic Artemisia absinthium leaf extract (EAALE)

<table>
<thead>
<tr>
<th>Crude extracts</th>
<th>Parasites</th>
<th>IC\textsubscript{50} (µg/mL)\textsuperscript{a}</th>
<th>EC\textsubscript{50} (µg/mL)\textsuperscript{b}</th>
<th>Selective indices\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic Peganum harmala seed extract</td>
<td>B. bovis</td>
<td>24.9 ± 1.2</td>
<td>611.7 ± 10.9</td>
<td>&gt;1500</td>
</tr>
<tr>
<td></td>
<td>B. bigemina</td>
<td>77.1 ± 2.3</td>
<td>611.7 ± 10.9</td>
<td>&gt;1500</td>
</tr>
<tr>
<td></td>
<td>B. divergens</td>
<td>61.1 ± 2.9</td>
<td>677.7 ± 10.9</td>
<td>&gt;1500</td>
</tr>
<tr>
<td></td>
<td>B. caballi</td>
<td>80.8 ± 4.1</td>
<td>611.7 ± 10.9</td>
<td>&gt;1500</td>
</tr>
<tr>
<td></td>
<td>T. equi</td>
<td>11.3 ± 2.1</td>
<td>611.7 ± 10.9</td>
<td>&gt;1500</td>
</tr>
</tbody>
</table>

| Ethanolic Artemisia absinthium leaf extract | B. bovis    | 43.3 ± 3.1                       | 340.7 ± 8.5                      | 874.7 ± 9.3                     |
|                                           | B. bigemina | 39.2 ± 2.7                       | 340.7 ± 8.5                      | 874.7 ± 9.3                     |
|                                           | B. divergens| 38.5 ± 3.7                       | 340.7 ± 8.5                      | 874.7 ± 9.3                     |
|                                           | B. caballi  | 50.3 ± 2.1                       | 340.7 ± 8.5                      | 874.7 ± 9.3                     |
|                                           | T. equi     | 28.2 ± 2.1                       | 340.7 ± 8.5                      | 874.7 ± 9.3                     |

\textsuperscript{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.

\textsuperscript{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.

\textsuperscript{c}Ratio of the EC\textsubscript{50} of cell lines to the IC\textsubscript{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 $4 \times \text{IC}_{50}$ (99.6, 244.4, 323.2, and 45.2 μg/mL, respectively), while B. bigemina could regrow at $4 \times \text{IC}_{50}$ (308.4 μg/mL). EAALE inhibited the regrowth of B. bovis, B. bigemina, B. divergens, B. caballi, and T. equi at $4 \times \text{IC}_{50}$ (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)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Conc. of compounds</th>
<th>B. bovis</th>
<th>B. bigemina</th>
<th>B. divergens</th>
<th>B. caballi</th>
<th>T. equi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic P. harmala seed extract</td>
<td>$0.25 \times \text{IC}_{50}$</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>$0.5 \times \text{IC}_{50}$</td>
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<td>$1 \times \text{IC}_{50}$</td>
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<td>$2 \times \text{IC}_{50}$</td>
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<tr>
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<td>$4 \times \text{IC}_{50}$</td>
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<tr>
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<td>$1 \times \text{IC}_{50}$</td>
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<td></td>
<td>$2 \times \text{IC}_{50}$</td>
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<td></td>
<td>$4 \times \text{IC}_{50}$</td>
<td>-</td>
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<td>Untreated control</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
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</table>

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. 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 taken from MP and EA, respectively, in an *in vitro* culture taken after 24 h 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. caballi* (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.
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 EC₅₀ values of MPHSE on MDBK, NIH/3T3, and HFF cell lines were 611.7 ± 10.9, 870 ± 22, and >1500 μg/mL, respectively. The EC₅₀ 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 EC₅₀ value of 34 ± 3.4 μM (Table S1). The selectivity index, defined as the ratio of the cell line EC₅₀ to the parasite IC₅₀, is shown in Table 1. For the MDBK cell line, the highest selectivity index of MPHSE was 54.1 times higher than the IC₅₀ for T. equi. The highest selectivity index of EAALE was 12.1 times higher than the IC₅₀ for T. equi. For the HFF cell line, the highest selectivity index of EAALE was 48.6 times higher than the IC₅₀ 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

<table>
<thead>
<tr>
<th>Combined treatments</th>
<th>Parasites</th>
<th>B. bovis</th>
<th>B. bigemina</th>
<th>B. divergens</th>
<th>B. caballi</th>
<th>T. equi</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPHSE + DA</td>
<td>CI values</td>
<td>3.328</td>
<td>7.102</td>
<td>9.002</td>
<td>1.071</td>
<td>5.023</td>
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<td></td>
<td>Additive</td>
<td>Antagonism</td>
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<tr>
<td>EAALE + DA</td>
<td>CI values</td>
<td>0.730</td>
<td>0.873</td>
<td>0.636</td>
<td>1.089</td>
<td>1.0814</td>
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<td></td>
<td>Synergism</td>
<td></td>
<td>Additive</td>
<td>Additive</td>
</tr>
<tr>
<td>MPHSE + AQ</td>
<td>CI values</td>
<td>0.847</td>
<td>0.552</td>
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<tr>
<td>EAALE + AQ</td>
<td>CI values</td>
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<td>Additive</td>
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<td>Additive</td>
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<tr>
<td>MPHSE + CF</td>
<td>CI values</td>
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<td>0.9932</td>
<td>0.9845</td>
<td>1.1189</td>
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<td>Degree of association</td>
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<td>Synergism</td>
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<tr>
<td>EAALE + CF</td>
<td>CI values</td>
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<tr>
<td>Degree of association</td>
<td>Synergism</td>
<td></td>
<td>Additive</td>
<td></td>
<td>Synergism</td>
<td>Synergism</td>
</tr>
</tbody>
</table>

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.](image)

![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).](image)
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 IC50 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 IC50 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 EC50 values shown by MPHSE and EAALE against cell lines were higher than the IC50 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
Herbal extracts can inhibit the growth of piroplasma 

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.
ACKNOWLEDGEMENTS
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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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Herbal extracts can inhibit the growth of piroplasma polymorphisms from parasites present after atovaquone treatment. Am J Trop Med. 74: 593–597.


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