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Original article

Lumefantrine and o-choline – Parasite metabolism specific drug molecules inhibited *in vitro* growth of *Theileria equi* and *Babesia caballi* in MASP culture system

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ABSTRACT

Theileria equi and *Babesia caballi* are tick-borne apicomplexan haemoprotozoan parasites of equines and are responsible for considerable economic losses to stakeholders. Chemotherapeutic drugs that are available not only require multiple dosages but also prompt multiple organ toxicity in treated host though incapable of clearing parasitaemia completely. In this study, we have screened the *in vitro* inhibitory efficacy of four different drug molecules (o-choline, DABCO®, lumefantrine and eugenol) against *T. equi* and *B. caballi*, targeting different parasite metabolism pathways. Imidocarb dipropionate and diminazene aceturate were used as reference control drugs. The 50% *in vitro* growth inhibitory concentration (IC_{50}) of lumefantrine, o-choline, DABCO® and eugenol for *T. equi* were: 30.90 µM; 84.38 µM; 443 µM; 120 µM and for *B. caballi* growth inhibition were: 5.58 µM; 135.29 µM; 150 µM; 197.05 µM, respectively. Imidocarb dipropionate inhibited the *in vitro* growth of *T. equi* at IC_{50} of 257.5 nM, while diminazene aceturate inhibited the *in vitro* growth of *B. caballi* at IC_{50} of 22 nM. DABCO® and eugenol were not so effective in inhibiting the *in vitro* growth of *T. equi* and *B. caballi*, while lumefantrine and o-choline significantly ($p \leq 0.05$) inhibited the *in vitro* growth of these piroplasms targeting haem digestion and parasite membrane phospholipid synthesis.

1. Introduction

Equine piroplasmosis is an OIE-notified (World Organisation for Animal Health) disease of equids (horse, pony, donkey, zebra and mule) caused by two distinct intra-erythrocytic protozoan parasites - *Theileria equi* (Mehlhorn and Schein, 1998) and/or *Babesia caballi* (Nuttall and Strickland, 1912). It is transmitted by ticks of the genera Dermacentor, Hyalomma, and Rhipicephalus (Scoles and Ueti, 2015). This disease is responsible for economic losses in horses (Preston, 2001; Uilenberg, 2001). The disease condition caused by these parasites (individually or in combination) is endemic in most of the tropical and subtropical countries (Wise et al., 2013). The equids recovered from piroplasmosis usually becomes a lifelong latent carrier of *T. equi* parasite (De Waal, 1992) that enables the transmission of the parasite to naïve animals. The western and the northwestern part of India are the highly endemic zones for *T. equi* infection as reported in recent seroprevalence studies (Kumar et al., 2009; Sumbria et al., 2016).

Currently, imidocarb dipropionate is the only available drug recommended for the treatment of *T. equi* infected equids, nevertheless incapable of clearing parasitaemia completely from the affected host. Even after four sequential intramuscular injections of imidocarb dipropionate (@ 4 mg/kg body weight) *T. equi* parasites persist in the experimentally infected horse (Grause et al., 2013). The repeated dosage of imidocarb dipropionate may further deteriorate the clinical condition of equids (Adams, 1981). Numerous drug compounds have been successfully tested against *in vitro* cultured *T. equi* and *B. caballi* parasites in MASP system succeeding to identify drug targets (Bork-Mimm, 2011; Mosqueda et al., 2012 and Silva et al., 2018). Phosphatidylcholine, necessary for parasite membrane synthesis (Florin-Christensen et al., 2000); cyclin-dependent kinase inhibitors (Nakamura et al., 2007); transfection system (Suarez and McElwain, 2010); L-lactate dehydrogenase (Bork et al., 2004) are the few drug targets which have been identified against *Babesia* parasites of animals. Therefore, there is a need to identify more novel drug targets for *T. equi*

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and *B. caballi* parasites.

During the intraerythrocytic developmental cycle, apicomplexan parasite utilizes host molecules for its metabolism purpose and these molecules have been a drug target for indirect intervention of parasitic growth. *Babesia* and *Theileria* species employ host choline for *de novo* synthesis of membrane phospholipids (AbouLaila et al., 2014). O-choline (octa-decylphosphocholine), a phosphocholine (PC) analogue, is a potential PC biosynthesis inhibitor (Posse de Chaves et al., 1995). Plasmodial protozoa also catabolise host erythrocyte's haem and convert it into its non-toxic haemozoin form (Chugh et al., 2013). Lumefantrine, mefloquine and quinine, all are clinically important antimalarials drugs, which inhibit haemozoin formation in the parasite cell and arrest the further growth of plasmodial parasites (Combrinck et al., 2013). Lumefantrine (also known as benflumetol) that belongs to amino alcohol class (WHO, 1990), is generally used in combination with artemether (an artemisinin derivative) for the treatment of *Plasmodium falciparum* (Basco et al., 1998). L-lactate dehydrogenase (LDH) is essential for anaerobic growth phase of the apicomplexan parasites and helps in catalysis of L-lactate to pyruvate, a source of ATP generation pathway (Gomez et al., 1997). Eugenol is one of the important active components of *Ocimum sanctum* plant extract (Prakash and Gupta, 2005) that is reported to possess bactericidal (Nascimento et al., 2000), anti-fungal (Kumar et al., 2010) and anti-malarial (van Zyl et al., 2006) efficacy. DABCO (1,4-diazabicyclo[2.2.2]octane derivative) is a quaternary ammonium compound (QAC) known for its anti-microbial, fungicidal, anti-viral (Burakova et al., 2016), and anti-protozoal efficacy (Thomas, 2013). QAC also inhibits PC by way of impairment of transport of choline into *P. falciparum*-infected erythrocytes (Ancelin and Vial, 1986). DABCO interacts not only with intracellular targets but also with DNA (Zinchenko et al., 2004). In this study we have investigated *in vitro* growth inhibitory efficacy of drug molecules – lumefantrine, o-choline, eugenol and DABCO, targeting parasite metabolism against *T. equi* and *B. caballi* parasites.

2. Materials and methods

2.1. Parasites

Theileria equi (Indian isolate, Gopalakrishnan et al., 2015; GenBank accession numbers: MG874693, MG874694, MG874695, MG874696) and *Babesia caballi* [Indian isolate from a horse seropositive in cELISA (VMRD, USA) and IFAT (Fuller Laboratories, California)] were continuously grown in microaerophilus stationary phase (MASP) as described in 2.2 section of this manuscript. A stock of *T. equi* or *B. caballi* parasitized erythrocytes were cryopreserved in sucrose-dextrose media (Schmid et al., 2011). These cryopreserved *T. equi* and *B. caballi* parasites were further used for culture initiation and *in vitro* drug trials.

2.2. MASP in vitro culture system

Cryopreserved *T. equi* and *B. caballi*, parasites were taken out from -80 °C and immediately transferred at 37 °C water bath followed by washing with Vega Y Martinez phosphate buffer saline solution (Vega et al., 1985) until haemolysed supernatant was removed. After that, the pelleted infected erythrocytes were mixed with fresh normal horse erythrocytes and initiated in MASP culture system under controlled atmosphere (5% CO_2, 3% O_2, 92% N_2 and 37 °C temperature) as described by Igarashi et al. (1994) and Bork et al. (2004). *T. equi* parasitized red blood cells (RBC) were cultured in M-199 medium supplemented with 40% defibrinated uninfected horse serum, penicillin-streptomycin mixed antibiotic solution (containing 100 IU/ml penicillin G and 100 µg/ml streptomycin) and 0.2 mM Hypoxanthine. A modified HL-1 medium enriched with 40% horse serum (as above), penicillin-streptomycin antibiotic solution, 0.2 mM hypoxanthine and 2 mM L-glutamine were used for culture of *B. caballi* in MASP system (Avarzed et al., 1997).

2.3. Drug molecules

The drug molecules, O-(Octadecylphosphoryl) choline (o-choline, cat. no. 04449-100MG), eugenol (cat. no. E51791-5 G), DABCO® 33-lv (cat. no. 290734-100ML), lumefantrine (cat. no. L5420-5MG), imidocarb dipropionate (cat. no. 33441-50MG) and diminazene aceturate (cat no. D7770-1 G) were purchased commercially (Sigma-Aldrich, India). O-choline, lumefantrine, eugenol, imidocarb dipropionate and diminazene aceturate were dissolved in deionized distilled water, whereas DABCO® 33-lv was dissolved in DMSO for preparing 1 M working stock solution of each of these drug molecules and stored at -20 °C until further *in vitro* trial.

2.4. In vitro growth inhibition assay

The growth inhibition of *T. equi* and *B. caballi* treated with different concentration of four above drug molecules were assayed in 48 well culture plate as per the method described by Igarashi et al. (1998) and Bork et al. (2004). The parasites, *T. equi* and *B. caballi*, were initiated in MASP culture system at 1% parasitaemia in 500 µl (containing 50 µl infected erythrocytes) of complete medium supplemented with respective drug molecule tested concentration. Each drug molecule concentration was tested in triplicate well for 96 h. Overlaid respective culture media supplemented with different drug molecule concentration was replaced every 24 h. O-choline and lumefantrine drug molecules were tested at 200 µM, 100 µM, 50 µM, 20 µM and 10 µM and 100 µM, 50 µM, 20 µM, 10 µM and 5 µM concentrations, respectively, for *in vitro T. equi* growth inhibition studies. While, DABCO® and eugenol drug molecules were tested at the concentrations of 10 mM, 1 mM, 0.5 mM and 0.05 mM. Imidocarb dipropionate drug was taken as reference drug control and *in vitro* tested in MASP culture at 10.0 µg/ml, 1.0 µg/ml, 0.5 µg/ml, 0.1 µg/ml, and 0.01 µg/ml concentrations. *In vitro B. caballi* growth inhibition studies with different concentrations of lumefantrine and o-choline (200 µM, 100 µM, 50 µM, 20 µM, and 10 µM), eugenol (500 µM, 200 µM, 100 µM, 50 µM, and 10 µM) and DABCO® compound (1000 µM, 500 µM, 100 µM, and 50 µM) were also undertaken. Diminazene aceturate drug was taken as reference drug control and *in vitro* testing in *B. caballi* MASP culture at 0.1 µM, 0.075 µM, 0.05 µM, 0.02 µM, 0.01 µM concentrations. All working concentrations of these different drug molecules were prepared in respective complete media defined for *T. equi* or *B. caballi* from 1 M stock concentration (as described above).

Percentage of parasitized erythrocytes (PPE) and parasite's morphological alterations (*T. equi* or *B. caballi*) in these drugs treated wells was monitored at a regular interval of 24 h, by examining Giemsa stained smears under oil immersion lens. Parasites (*T. equi* or *B. caballi*) with a condensed nucleus and adhered cytoplasm membrane were recorded as dead parasites. Fifty per cent Inhibitory concentration (IC_{50}) of each drug molecules were calculated separately for *T. equi* and *B. caballi* using interpolation curve-fitting technique described by Bork et al., 2004.

2.5. In vitro parasite recrudescence studies

In vitro viability assay for each drug-treated *T. equi* or *B. caballi* parasite cultures were performed as described previously (Gopalakrishnan et al., 2016). Briefly, after 96 h of drug molecules *in vitro* treatment, 20 µl of erythrocytes collected from all the drug molecule concentration treated wells were transferred into a new well having 500 µl fresh complete medium (for *T. equi* or *B. caballi*) and 30 µl normal horse erythrocytes. The complete culture medium (as defined for *T. equi* and *B. caballi*) without any drug molecule was replaced after every 24 h for 96 h. Smears were examined daily under a light microscope for the observation of recrudescence and multiplication of *T. equi* or *B. caballi* parasites.

2.6. In vitro cell viability assay on horse PBMCs

Effect of different drug molecules at various concentration was assayed on peripheral blood mononuclear cells (PBMCs) and was determined by resazurin-based cell viability assay (Gopalakrishnan et al., 2016). The PBMCs were separated from whole blood of a healthy horse. Briefly, freshly collected PBMCs were suspended in 1 ml complete growth medium consisting of RPMI-1640 medium supplemented with 2 mM L-glutamine, 60 µg/ml penicillin, 100 µg/ml streptomycin and 10% foetal bovine serum (Sigma Aldrich, India). Enriched PBMCs suspension with complete growth media was adjusted to a final concentration of 1×10^6 cells/100 µl and distributed to each well (100 µl) in 96 well culture plate. Simultaneously, phytohaemagglutinin–A (at the concentration of 2 µg/ml) in 50 µl volume was also added to each of these well. The culture plate was incubated at 37 °C with 5% CO_2 in air for 48 h. After 48 h, PBMCs in cultured wells were treated with 100 µl volume of the different respective concentration of drug molecules *i.e.* lumefantrine, o-choline, eugenol, DABCO® (2000 µM, 1000 µM, 500 µM, 200 µM, 100 µM, 50 µM, 20 µM, and 10 µM). The 96 well culture plate was again incubated (as above) for another 24 h, followed by addition of 25 µl of resazurin dye (150 µg/ml) and culture plate was kept in an incubator further for 4 h. The change of dye colour was monitored by measuring optical density (OD) at 570 nm and 650 nm. The effective OD value for each well was calculated by deducting OD_{570} value from its respective OD_{650} value. The IC_{50} of each drug molecule on PBMCs was calculated from a regression equation based on the effective OD value, as mentioned above. Effect of drug molecules on PBMCs in terms of per cent viable cell population was determined as below:

$$PBMCs\ viability\ (\%) = \frac{\text{OD of test sample} - \text{OD of positive control}}{\text{OD of negative control} - \text{OD of positive control}} x \frac{}{100}$$

The drug molecules specific selectivity index (SI) was also calculated, which represents the ratio of IC_{50} of each drug molecule on the mammalian cell line (PBMCs) to the protozoan parasite (*T. equi* or *B. caballi*) as per the method of Lenta et al., 2007.

$$Selectivity\ Index\ (SI) = \frac{\text{IC50 of drug molecule on horse PBMCs}}{\text{IC50 of drug molecule on protozoan parasite}}$$

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 software (San Diego California, USA). The two-way ANOVA followed by Bonferroni post-hoc test ($p < 0.05$) was computed to know the anti-piroplasm activity of these novel drug molecules against *T. equi* and *B. caballi* in *in vitro* culture. The 'p' values < 0.05 were considered statistically significant differences between the treated groups and control cultures. Correlation between drug molecule concentration and cytotoxicity was evaluated by using GraphPad Prism version 5.00 software (San Diego California, USA).

3. Results

3.1. T. equi and B. caballi in vitro growth inhibitory efficacy of different drug molecules

Significant ($p < 0.05$) inhibition in the *in vitro* growth of *T. equi* was initially observed at 5 µM, 50 µM and 50 µM respective concentration of lumefantrine, o-choline and eugenol on 72 h of treatment and this inhibitory response also continued in higher concentrations of these above drug molecules (Fig. 1). Similarly, *in vitro* growth of *B. caballi* was significantly ($p < 0.05$) inhibited on 72 h of treatment with lumefantrine, o-choline and eugenol at a respective initial concentration of 10 µM, 100 µM and 10 µM (Fig. 1). DABCO® was able to inhibit *in vitro* growth of *T. equi* and *B. caballi* at an initial concentration of 500 µM and 50 µM on 48 h and 96 h of treatment (Fig. 1). Although, eugenol able to clear *T. equi* parasitized erythrocytes in < 24 h (Fig. 1), but at very high concentration (10,000 µM or 10 mM). Simultaneously, lumefantrine cleared the *B. caballi* parasites from the cultures in < 48 h at 200 µM (Fig. 1). The IC_{50} value of lumefantrine, o-choline, DABCO® and eugenol for *T. equi* growth inhibition was – 30.90 µM; 84.38 µM; 443 µM; 120 µM and for *B. caballi* growth inhibition was – 5.58 µM; 135.29 µM; 150 µM; 197.05 µM (Fig. 2). Imidocarb dipropionate inhibited the *in vitro* growth of *T. equi* at IC_{50} of 257.5 nM, while diminazene aceturate inhibited the *in vitro* growth of *B. caballi* at IC_{50} of 22 nM.

3.2. T. equi and B. caballi parasite recrudescence after in vitro drug molecules treatment

Theileria equi and *B. caballi* failed to infect and multiply naïve horse erythrocytes, when drug-treated infected erythrocytes collected at 96 h of *in vitro* experiment from different concentrations of lumefantrine (50 µM, 100 µM); DABCO® and eugenol (10 mM, 100 mM) and o-choline (100 µM, 200 µM) and were initiated in a fresh drug free medium in a new culture plate. *Theileria equi* and *B. caballi* MASP cultured parasites treated with rest of drug molecules concentrations were able to infect and multiply the naïve horse's erythrocytes indicating recrudescence of parasitaemia.

3.3. Parasite's morphological alterations after in vitro drug treatment

Parasites (*T. equi* and *B. caballi*) morphology at IC_{90} of different drug molecules (as above) was observed by microscopy of stained blood smears over a period of 96 h (Fig. 3). The protozoan parasites with the clear silhouette of cytoplasm were indicative of live healthy parasites, while dead parasites were pyknotic with condensed nuclear material. *T. equi* treated at IC_{90} of lumefantrine, eugenol, o-choline and DABCO® was alive up to 48 h, 72 h, 96 h and 96 h after treatment, respectively (Fig. 3). *Theileria equi* was marked dead on 72 h interval onward of treatment with IC_{90} of lumefantrine. *B. caballi* parasites remained live at IC_{90} of o-choline and eugenol up to 96 h of treatment duration. Dead *B. caballi* were observed with IC_{90} of lumefantrine and DABCO® at 96 h and 48 h (Fig. 3). In *T. equi* and *B. caballi* control culture well, parasites were live, healthy and dividing throughout the 96 h of *in vitro* treatment period.

3.4. Horse PBMCs viability assay after treatment with different drug molecules

Effect of different drug molecules at various concentrations (0 µM to 2000 µM) on horse PBMCs cell line was analysed by resazurin-based assay (Fig. 4A). Selectivity index (SI) was also calculated for individual drug molecule (Fig. 4B) with the help of its IC_{50} on horse's PBMCs (mammalian cell line) in relation with corresponding *in vitro* growth inhibitory concentration on *T. equi* or *B. caballi* (protozoan parasite). A drug molecule's concentration-dependent positive correlation was observed on PBMCs viability. It has been observed that DABCO (cell viability ranged from 99.97% to 96.11%; SI: *T. equi* 66.62, *B. caballi* 196.74) was least cytotoxic to PBMCs cell line followed by o-choline (cell viability ranged from 93.03% to 85.90%, SI: *T. equi* 147.02, *B. caballi* 91.69). The horse PBMCs viability in lumefantrine treated cultures ranged from 92.32% to 31.55%, while its SI values for *T. equi* and *B. caballi* were 45.49 and 51.95, respectively. Eugenol exhibited the least SI values for *T. equi* (12.57) and *B. caballi* (7.65); whereas PBMCs viability ranged from 99.46% to 34.24%. These observations (Fig. 4) indicated that lumefantrine was safe to PBMCs (cell viability > 70%) at 743.44 µM concentration (extrapolated from regression analysis) and exhibited lesser growth inhibitory IC_{50} against *T. equi* (30.90 µM) and *B.*

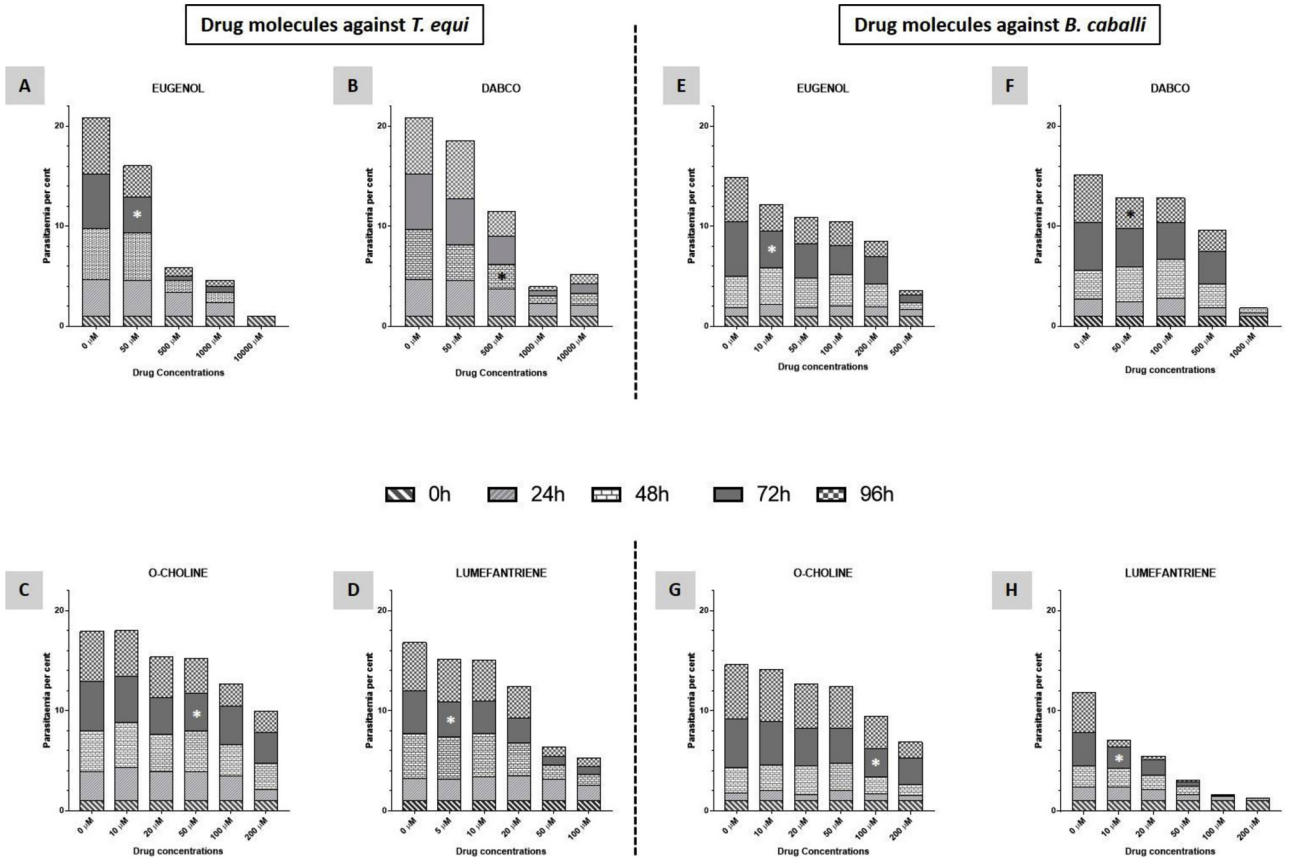

Fig. 1. *Theileria equi* (A, B, C, D) and *Babesia caballi* (E, F, G, H) *in vitro* growth inhibitory efficacy of eugenol, DABCO®, o-choline and lumefantrine at different concentration. Each stacked column represents mean parasitaemia at a respective concentration of the specific drug molecules at 0 h–96 h treatment duration. The asterisk mark (*) embedded in the stacked column of respective drug molecule concentration indicates initiation of its significant ($p < 0.05$) difference in parasitaemia with reference to control.

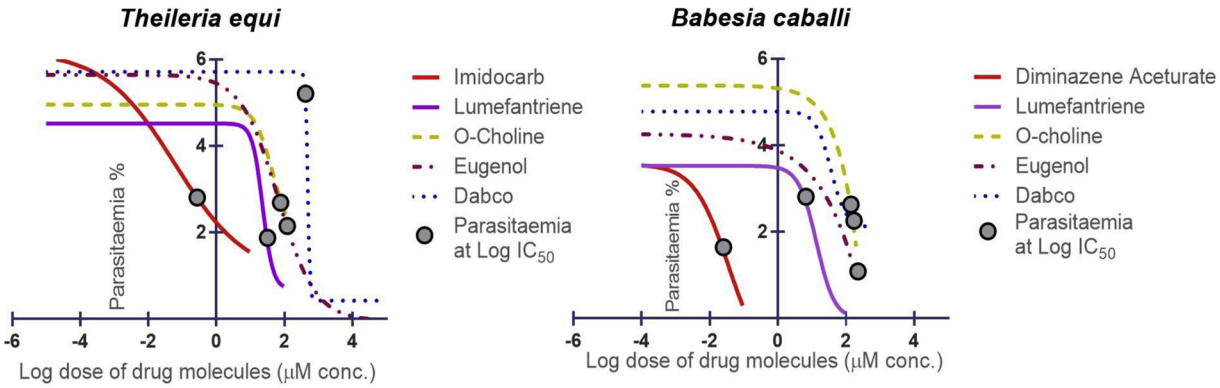

Fig. 2. Variable log dose-response curve of eugenol, DABCO®, o-choline, lumefantrine, imidocarb dipropionate and diminazene aceturate at different concentration against *Theileria equi* and *Babesia caballi* at 96 h of treatment generated by non-linear regression. Respective IC_{50} is calculated using GraphPad Prism by interpolation curve fitting technique.

caballi (5.58 μM).

4. Discussion

Available chemotherapeutics against equine piroplasmosis employ different untoward consequence on host organs and are unable to control recrudescence of parasites. Target based *in vitro* screening of drug molecules for their growth inhibitory efficacy is one of the validated ways in drug discovery (Vasaikar et al., 2016). Apicomplexan haemoprotozoan necessitates *de novo* biosynthesis of membrane phospholipid for its viability and further multiplication within infected erythrocytes (Ramakrishnan et al., 2013). Intracellular protozoa utilize host choline for synthesizing the phosphatidylcholine (PC) that is a crucial component of its membrane phospholipids (Ancelin et al., 1991). In our study, o-choline revealed significant ($p < 0.05$) *in vitro* growth inhibition of *T. equi* and *B. caballi* at IC_{50} of 84.38 μM and 135.29 μM, respectively. It has a comparatively good PBMCs viability effect (86.26% at 1000 μM concentration) among four drug molecules screened in this study. Richier et al. (2006) synthesized choline analogue drug molecule - bisthiazolium (T16) and used it in *in vitro* treatment experiment against *Babesia divergens* and *Babesia canis* in MASP culture system. They observed IC_{50} of this drug molecule as

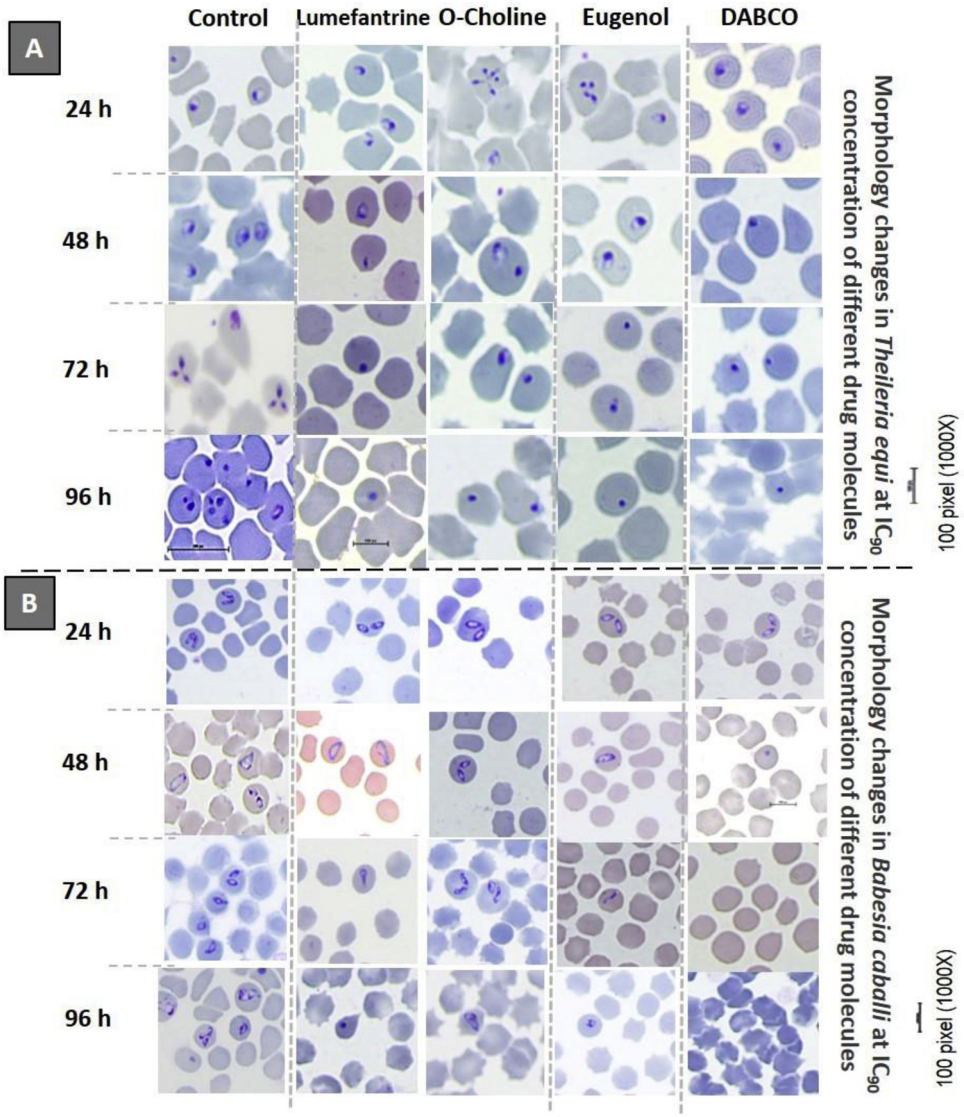

Fig. 3. Microphotograph showing morphological alteration in *Theileria equi* (A) and *Babesia caballi* (B) parasites at IC_{90} of different drug molecules at 0 h–96 h treatment duration. Change of morphology (pear-shaped form to dot, pyknotic form) and division pattern was well marked at different time intervals between drug-treated parasite and control parasite.

28 nM and 7 nM, respectively. Gopalakrishnan et al. (2016) used hexadecyltrimethylammonium bromide (HDTAB) as choline kinase inhibitor and reported inhibition of *T. equi in vitro* growth. These observations indicated that the PC can be a potential drug target and its inhibitors can be further investigated for anti-equine piroplasm's activity.

DABCO® has been found microbicidal at concentrations of 10–50 ppm (Gerba, 2015). Keeping these efficacies in view, we tested it *in vitro* growth inhibition efficacy on *T. equi* and *B. caballi*. DABCO® was able to inhibit *in vitro* growth of these protozoa in MASP culture system at relatively high concentration (IC_{50} - 443 µM). Ancelin and Vial (1986) tested different QAC −HC3, DM and DTMA for *in vitro* growth inhibition efficacy of *P. falciparum* and reported IC_{50} in the range of 1 µM to 700 nM. In our experiment DABCO® was incompetent in *in vitro* growth of *T. equi* or *B. caballi* at such lower IC_{50}, indicating that this compound cannot be a promising growth inhibitor for these protozoa.

Eugenol has been reported to inhibit the *in vitro* growth of a chloroquine-resistant strain of *P. falciparum* at IC_{50} of 753.7 µM (van Zyl et al., 2006). While in our study, its IC_{50} against *T. equi* and *B. caballi* was 120 µM and 197.05 µM, respectively. Eugenol has shown 63.52% PBMCs viability at 1000 µM concentration. Eugenol's specific mechanism of action is unknown, although Panchal et al. (2013) predicted (by the drug-docking study) probable activity of this compound against plasmodial lactate dehydrogenase (LDH).

Previously, *in vivo* trial in *T. equi* infected experimental donkeys indicated that individually arteether (another artemisinin derivative) was ineffective in clearing the parasite infection, while its synergistic efficacy was observed in combination with buparvaquone (Kumar et al., 2003). In the present study, lumefantrine was most effective in inhibiting *in vitro T. equi* and *B. caballi* growth with respective IC_{50} of 30.90 µM and 5.58 µM. This drug has been effective for inhibiting *in vitro* growth of *P. falciparum* and *Babesia gibsoni* at IC_{50} of 147.7 nM (Mungthin et al., 2010) and 480 nM (Iguchi et al., 2015), respectively. Further, the recrudescence of *T. equi* and *B. caballi* parasites was not observed in this study at more than 50 µM and 20 µM concentration of lumefantrine, respectively. The per cent viability of horse PBMCs was 70% at 743.44 mM concentration, indicated its safety to mammalian cell lines (ISO, 2009). The mechanism of action of lumefantrine against *Babesia* or *Theileria* parasites has not been established. While digesting haemoglobin, Plasmodial parasites convert ferriprotoporphyrin IX (haemin, ferric state, toxic to the parasite) to non-toxic haemozoin form. It has been hypothesized that lumefantrine binds to

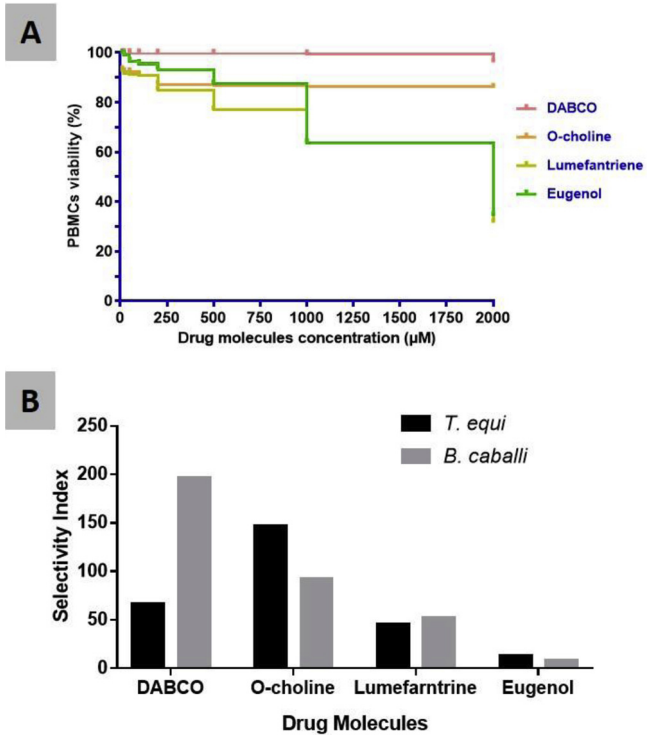

Fig. 4. Effect of different concentrations of eugenol, DABCO®, o-choline and lumefantrine on the viability of horse's peripheral blood mononuclear cells (PBMCs) after 96 h of culture. (A): Viability was assessed through colorimetric assay by standard resazurin reduction method. (B): Respective selective index (SI) was calculated to assess the selectivity of test drug molecules on host cells.

ferriprotoporphyrin IX and disrupts its further breakdown to haemozoin (Warhurst et al., 2001; Xue et al., 2013). *Theileria equi* and *B. caballi* also possess food vacuole and cytostome as observed in plasmodial parasite (Kawai et al., 1999; Guimarães et al., 2003). These organelles are responsible for the digestion of host haemoglobin. In our study, lumefantrine may be able to inhibit the *in vitro* growth of *T. equi* and *B. caballi* by this mechanism, which requires further elucidation and confirmation.

5. Conclusions

We tested four drug molecules as *in vitro* growth inhibitors targeting different parasite metabolism pathways. Lumefantrine and o-choline (a phosphocholine analogue), drug molecules are responsible for disturbing parasite haemoglobin digestion and phospholipid membrane synthesis, respectively. The above two drug molecules were found most effective in *in vitro* growth inhibition of *T. equi* and *B. caballi* in MASP culture system. These drug molecules were safe in mammalian (horse PBMCs) cell line.

Conflict of interest

The author declares that they have no conflict of interest.

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