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In vitro growth inhibitory efficacy of some target specific novel drug molecules against *Theileria equi*

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ABSTRACT

The *in vitro* growth inhibitory efficacies of five drug molecules against *Theileria equi* were evaluated in *in vitro* cultured parasites. A continuous microaerophilic stationary-phase culture (MASP) system was established for propagation of *T. equi* parasites. This *in vitro* culture system was used to assess the growth inhibitory effect of harmaline hydrochloride dihydrate (HHD), hexadecyltrimethylammonium bromide (HDTAB), hesparidin methyl chalcone (HMC), andrographolide and imidocarb dipropionate against *T. equi*. The 50% inhibitory concentration value of HHD, HDTAB, HMC, and imidocarb dipropionate for *T. equi* growth were 17.42 μM, 14.00 μM, 246.34 μM and 0.279 μM (equivalent to 0.139 μg/ml), respectively ($P < 0.05$). The andrographolide was not effective in inhibiting *in vitro* growth of *T. equi* in the present study. Furthermore, the *in vitro* cytotoxicity of these five drugs was evaluated on horse PBMC. At 2000 μM concentration of HHD, HDTAB, HMC, andrographolide and imidocarb dipropionate were 8.34, 46.44, 58.53, 31.06, 15.14% cytotoxic on PBMC, respectively. Out of our four tested drug molecules, HHD was having low IC_{50} value along with least cytotoxicity, as compared to reference drug imidocarb dipropionate. The difference in IC_{50} value of HDTAB and HHD was significant, but HDTAB was moderately more cytotoxic on PBMC cell lines. HHD and HDTAB are selective inhibitor for heat shock protein 90 (Hsp90) and choline kinase pathway. It can be concluded that HHD and HDTAB are potential drug molecules against *T. equi* parasite by acting on Hsp90 and choline kinase pathway.

1. Introduction

Equine piroplasmosis is a vector-borne disease, caused by two intraerythrocytic protozoan parasites, *Theileria equi* (formerly called as *Babesia equi)* (Mehlhorn and Schein, 1998) and *Babesia caballi* (Ristic, 1988). These parasites can affect all equids—horse, donkey, mule, ass and zebras and are endemic in tropical and subtropical regions of the world, where suitable vector-tick population exist (Bruning, 1996). This disease is considered of major economic significance to equids industry as international movement of affected equids is restricted and affected horse exhibit decreased working capacity (Kuttler, 1988; Kumar et al., 2007). Disease condition caused by *T. equi* infections is clinically severe, more pathogenic and endemic than that by *B. caballi* (de Waal and van Heerden, 2004). Also, sero-prevalence of *T. equi* infection is quite high in many parts of the world and latency rate of >35% has been reported from various countries *viz.*, India (Kumar et al., 2009); Pakistan (Rashid et al., 2009); Mongolia (Munkhjargal et al., 2013) and China (Xu et al., 2003). Until now there is no chemotherapeutic drug which can help in clearing *T. equi* parasite from latently infected equids (Bruning, 1996). Many babesicidal drugs are available for control *T. equi* infection, but none of these drugs can sterilize the infection from *T. equi* infected animals (Bork et al., 2004; Kumar et al., 2003) and also those drugs have harmful effect on host health (Vial and Gorenflot, 2006).

Most of the apicomplexa groups of parasites possess unique organelle and metabolic pathways (Vial and Gorenflot, 2006), which have been the drug targets. Heat shock proteins (Hsp) are conserved group of proteins and play vital role in stabilization of parasite protein during its life cycle. Hsp90 perform chaperone function and are noble target for developing antiprotozoal therapy (Pratt and Toft, 2003; Johnson, 2012). Florin-Christensen et al. (2000) and Ambawat et al. (1999) observed increase in phospholipid in *Babesia* infected erythrocytes. Choline kinase enzyme is essential for biosynthesis of phosphatidylcholine for *Plasmodium* parasite growth (Vial et al., 2003). Hence, the choline kinase is a

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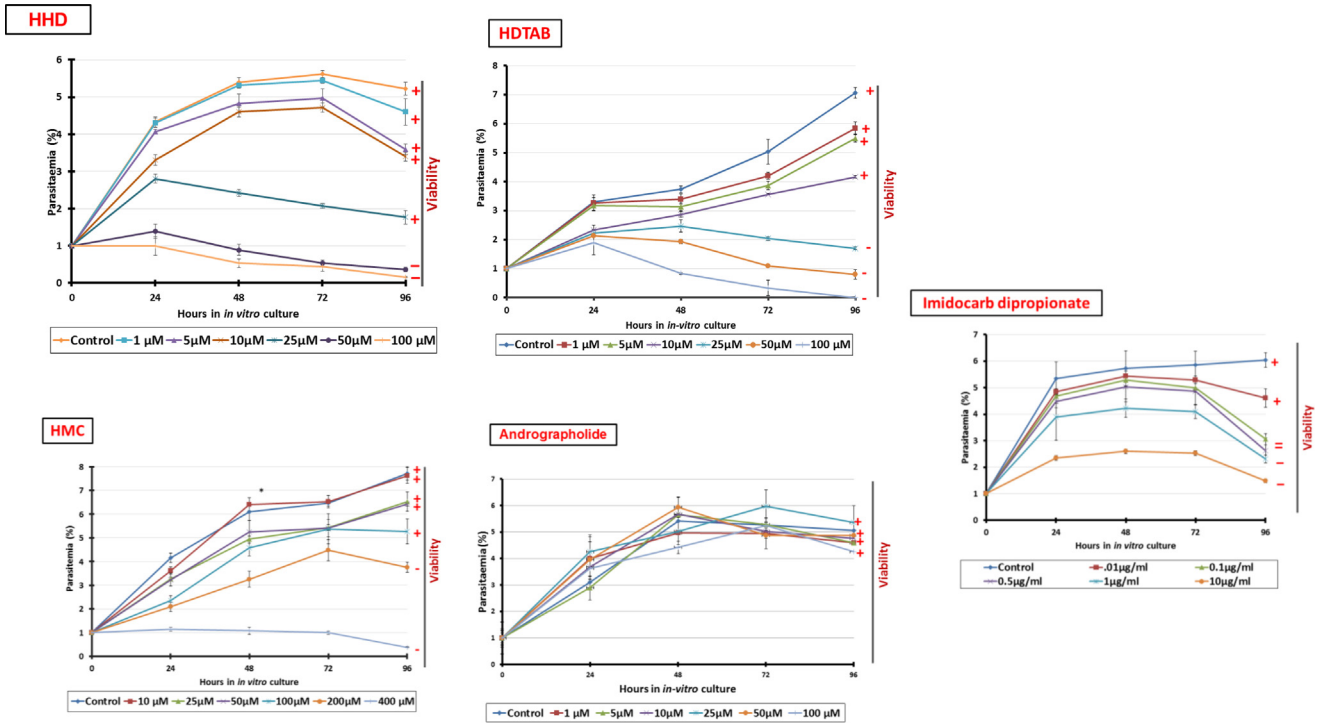

Fig. 1. *Theileria equi in vitro* growth inhibitory efficacy of harmaline hydrochloride dihydrate (HHD), hexadecyltrimethylammonium bromide (HDTAB), hesperidin methylchalcone (HMC) and andrographolide at different concentration. Imidocarb dipropionate was used as positive controls. Each value in the individual graph represent per cent parasitaemia (mean ± SD) observed at each respective concentration of the specific drug molecule experimented in triplicate. '+' ; '−' numeral on right hand side of the graph represented viability status of the *T. equi* parasite after 96 h of *in vitro* treatment trial.

specific drug target for apicomplexa group of parasites. The parasite infected erythrocytes generates reactive oxygen species (ROS) during its erythrocytic asexual life cycle (Griffiths et al., 2001; Mishra et al., 1994) and infected erythrocytes are highly susceptible to oxidative stress (Becker et al., 2003). Nuclear factor—κB (NF-κB) is important transcription factor which is playing pivotal role in many parasitic infections namely *Plasmodium falciparum* (Tripathi et al., 2009); *Toxoplasma gondii* (Shapira et al., 2005); *Theileria parva* (Palmer et al., 1997; Guergnon et al., 2003). NF-κB may be specific target site for control parasitic infection. In this study, we selected specific drug molecules targeting Hsp90, choline kinase, NF-κB and oxidative stress inhibitors and evaluated their *T. equi* growth inhibition properties and cytotoxicity effects, if any. The imidocarb dipropionate was included as a reference control drug against *T. equi*.

2. Materials and methods

2.1. Parasites

The Indian strain of *T. equi* (Gopalakrishnan et al., 2015), isolated from a latently infected pony was used in this study.

2.2. In vitro *cultivation of* equi *and evaluation of parasite growth*

T. equi parasitized erythrocytes were maintained by microaerophilous stationary phase (MASP) culture techniques, in *in vitro* cultivation medium which consisted of Medium M199 (Sigma–Aldrich, India) supplemented with 40% defibrinated horse serum, antibiotic solution (containing 60 U/ml penicillin and 60 mg/ml streptomycin) and 200 μM hypoxanthine (Igarashi et al., 1998; Bork et al., 2004). The cultures were maintained at a temperature of 37 °C with micro-aerophilic atmosphere of 5% CO_2, 3% O_2, and 95% N.

Evaluation of *T. equi* parasite growth in the MASP culture system was monitored by blood smears examination under oil immersion lens of microscope at 1000× magnification. Qualitative and quantitative counting of *T. equi* infected erythrocytes was performed and for this purpose at least 1000 erythrocytes were counted in different field.

2.3. Chemical reagents

Harmaline hydrochloride dihydrate (HHD), hexadecyltrimethylammonium bromide (HDTAB), hesperidin methylchalcone (HMC) and andrographolide were purchased commercially (Sigma–Aldrich, India). A working stock solution of 1000 μM of HHD, HDTAB and HMC were prepared by dissolving in deionized distilled water and stored at −20 °C until further use. Andrographolide was dissolved in 0.005% dimethyl sulfoxide (DMSO) and working stock solution of 1 mM was prepared and stored at 4 °C. Imidocarb dipropionate was purchased commercially (Sigma–Aldrich, India) and used as standard control drug. A working stock solution of 100 μg/ml of imidocarb dipropionate was prepared and stored at −20 °C until use. The possible toxicity of DMSO (used to dissolve andrographolide drug molecule) was tested at concentrations of 0.005% and 0.5% (as a 100 times higher concentrate than control concentrate) on its toxic effect, if any on the growth of *T. equi* parasite in an *in vitro* culture.

2.4. In vitro *growth inhibition assay*

The *in vitro* growth inhibitory assay was performed as described by Igarashi et al. (1998) and Bork et al. (2004). *T. equi* parasites were obtained from MASP cultures with parasitemias of 7.0–10%. *T. equi* parasite-infected red blood cells (RBCs) were diluted with uninfected RBCs (collected from a healthy horse) to obtain a RBCs stock with 1% initial parasitemia. Evaluation of *in vitro* growth inhibitory

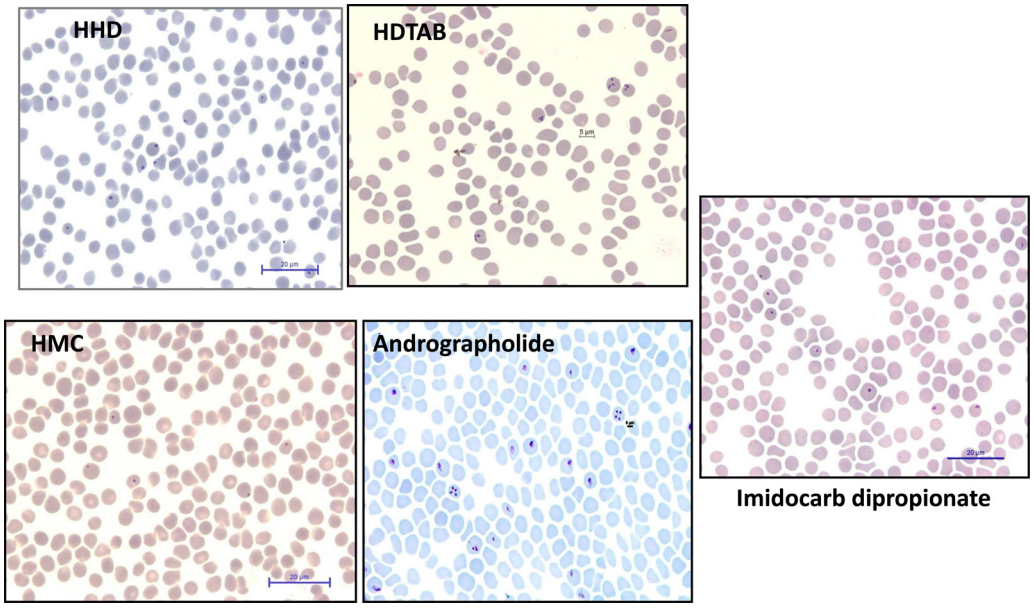

Fig. 2. Microphotographgraphs showing Harmaline hydrochloride dihydrate (HHD), hexadecyltrimethylammonium bromide (HDTAB), hesparidin methylchalcone (HMC), andrographolide and Imidocarb dipropionate treated *Theileria equi* parasites in an *in vitro* culture. Microphotographgraphs were after 48 h of *in vitro* treatment. HHD, HDTAB, HMC treated cultures showed a higher number of degenerated parasites as in imidocarb dipropionate treated cultures. Scale bars = 5 μm, and 20 μm.

assay was performed in 48-well culture plates. About 50 μl of the 1% *T. equi* parasite infected RBCs were dispensed per well (in triplicate) together with 500 μl of the culture complete medium containing the indicated drug concentration(s). HHD, HDTAB and andrographolide were tested at 1 μM, 5 μM, 10 μM, 25 μM, 50 μM, and 100 μM against the *T. equi* parasites. While, HMC was tested at concentration of 10 μM, 25 μM, 50 μM, 100 μM, 200 μM and 400 μM against the *T. equi* parasites. Imidocarb dipropionate (standard) drug was diluted at 0.01 μg/ml, 0.1 μg/ml, 0.5 μg/ml, 1.0 μg/ml and 10.0 μg/ml and tested against *T. equi* parasites. For experimental control, cultures without drug molecule and cultures containing only DMSO (0.005% and 0.5%) were prepared and tested against *T. equi*. These *in vitro* cultures with respective drug molecules concentrations were incubated at 37 °C in an atmosphere of 5% CO_2, 3% O_2, and 95% N, for a period of 96 h. The overlaid culture medium was replaced daily with fresh medium containing indicated drug molecule concentration.

Blood smears from different drug molecules treated wells or control wells were prepared after every 24 h and *T. equi* parasitemia was monitored. Morphological changes in the drug molecule treated parasites were observed and recorded. IC_{50} (50% inhibitory concentration) value for each drug molecules was calculated by interpolation using the curve-fitting technique (Bork et al., 2004). This curve-fitting technique is usually based on parasitemia values on 72 h or 96 h, when control wells showed maximum *T. equi* parasitaemia.

2.5. In vitro *viability test*

In vitro viability test of *T. equi* parasites was performed on different drug molecules treated RBCs, collected after completion of 96 h of treatment experiment. Briefly, 20 μl of RBCs treated with a particular concentration of drug molecule were transferred to a fresh culture well, having 30 μl of fresh naive RBCs suspended in 500 μl of complete medium (without any drug molecule). The culture plate wells were incubated for another 72 h and overlaid medium was replaced after every 24 h with fresh complete medium. Recrudescence of *T. equi* parasite was determined by examining Giemsa stained smears and observing the presence/absence of parasites and its multiplication in the fresh RBC's (Bork et al., 2004).

2.6. In vitro *cytotoxicity assay on PBMC*

Cytotoxicity assay was performed as per the standard resazurin method in 48 well culture plates. Normal horse blood was collected aseptically in EDTA vials and was diluted with equal volume of PBS (pH 7.4). Equal volume of horse blood was overlaid on histopaque-1077 (Sigma–Aldrich, India) and centrifuged at 1800 × *g* for 20 min. A clear white ring formed at the interface was collected and transferred aseptically to another tube. Washed three times with PBS (pH 7.4) and the peripheral blood mononuclear cell (PBMC) in the white pellet were re-suspended in 1 ml of growth medium which consisted of RPMI-1640 medium (Sigma Aldrich India) supplemented with 2 mM L-glutamine, 60 μg/ml penicillin, 100 μg/ml streptomycin, and 10% foetal bovine serum (Sigma Aldrich India)). PBMC in the suspension were counted using haemocytometer and final cell concentration was adjusted to 3×10^7/ml. 100 μl of suspended PBMC were added to each 96 well the culture plates and 50 μl phytohaemoglutinin—A (2 μg/ml) was also added. The culture plate was incubated for 48 h in CO_2 incubator at 37 °C and 5% CO_2 in air. Different concentrations (1 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM and 2000 μM) of drug molecules—HHD, HDTAB, HMC, andrographolide and imidocarb dipropionate were prepared in complete culture medium and 100 μl of each respective drug molecule concentration was added to the triplicate wells and incubated for another 24 h in CO_2 incubator. 25 μl of resazurin dye (150 μg/ml) was added to each well (at 10% of total well volume) and culture plate was again incubated for another 4 h. The blue color of the resazurin dye changed to pink after reduction and optical density (OD) was measured at 570 nm and 650 nm. The OD value each well at 570 nm was deducted from OD value at 650 nm and this calculated OD value of each well was used for determining cytotoxicity percentage as per below formula.

$$\%\ \text{Cytotoxicity} = \frac{\text{OD of negative control} - \text{OD of test sample}}{\text{OD of negative control}} \times 100$$

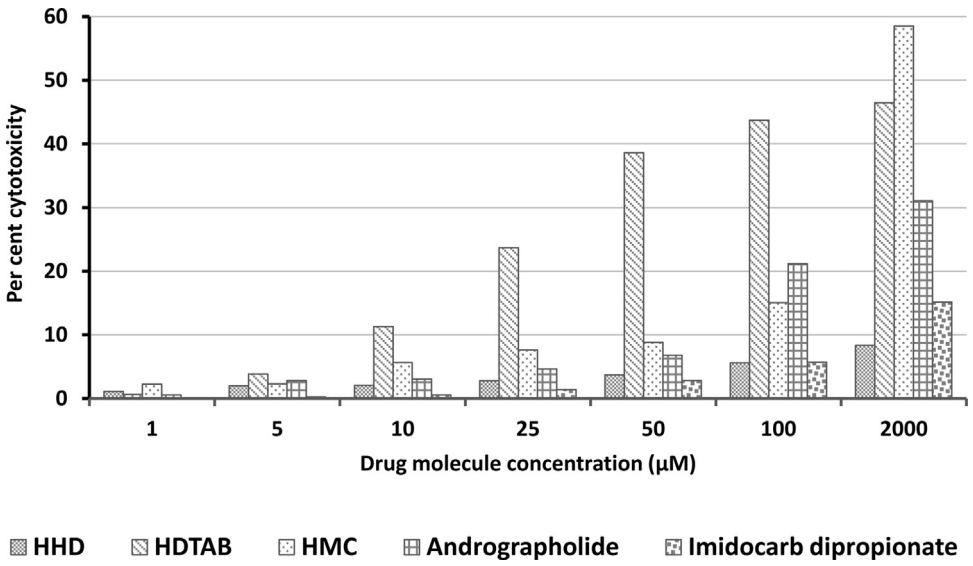

Fig. 3. Per cent cytotoxicity of harmaline hydrochloride dihydrate (HHD), hexadecyltrimethylammonium bromide (HDTAB), hesparidin methylchalcone (HMC), andrographolide and imidocarb dipropionate at different concentration. At highest concentration (2000 μM) HHD drug molecule was least toxic than the control drug imidocarb dipropionate on PBMC of horse origin.

Table 1
Different drug molecules tested against *T. equi* for *in vitro* growth inhibitory efficacy and their respective IC_{50} concentration.

	Drug molecules	IC_{50} concentration
1	Harmaline hydrochloride dihydrate (HHD)	17.42 μM
2	Hexadecyltrimrthylammonium bromide (HDTAB)	14.00 μM
3	Hesperidin methyl chalcone (HMC)	246.34 μM
4	Andrographolide	Not effective
5	Imidocarb dipropionate	0.279 μM

2.7. Statistical analysis

Statistical analysis was performed using Graphpad prism version 4.00 software (San Diego California, USA). The two-way ANOVA followed by Bonferroni Post-hoc test ($P < 0.05$) was computed to know the anti-piroplasmic activity of these novel drug molecules against *T. equi* 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 4.00 software (San Diego California, USA).

3. Results

3.1. Drug molecule's in-vitro growth inhibitory efficacy

Theileria equi parasite *in vitro* growth was significantly inhibited ($P < 0.05$) at 25 μM, 10 μM, 200 μM concentrations of HHD, HDTAB, HMC drug molecules on 48 h, 96 h and 72 h of treatment, while andrographolide drug molecule failed to inhibit *T. equi* growth at 100 μM or its higher concentrations (Fig. 1). Imidocarb dipropionate inhibited *T. equi* growth significantly ($P < 0.05$) at 0.5 μg/ml concentrations on 72 h of treatment (Fig. 1). In the presence of 50 μM, 100 μM and 400 μM concentration of HHD, HDTAB and HMC drug molecules, the *in vitro* growth of *T. equi* parasites was completely repressed, while Imidocarb dipropionate was able to absolutely inhibit *T. equi* growth at a concentration of 10 μg/ml. The IC_{50} value of HHD, HDTAB, HMC, for *T. equi* growth inhibition were 17.42 μM, 14.00 μM, 246.34 μM, respectively ($P < 0.05$). Imidocarb dipropionate inhibited *T. equi* growth at IC_{50} value of 0.279 μM (equivalent to 0.139 μg/ml) (Table 1).

3.2. Viability test after treatment with tested drug molecules

T. equi failed to multiply and infect naïve horse RBCs when infected drug treated RBCs collected at 96 h of experiment from different concentrations of HHD (50 μM and 100 μM), HDTAB (25 μM, 50 μM, 100 μM), HMC (400 μM) were tested for viability. Imidocarb dipropionate treated *T. equi* infected erythrocytes collected at 0.5 μg/ml, 1.0 μg/ml, 10.0 μg/ml concentration showed no viability and failed to further infected horse RBCs, while andrographolide treated *T. equi* parasites started multiplying and successfully infected naïve horse erythrocytes indicating that these were viable after drug treatment (Fig. 1).

3.3. Morphological changes of drug treated parasites

The morphological changes of *T. equi* were observed in Giemsa-stained thin smear prepared from HHD, HDTAB, HMC and imidocarb dipropionate treated cultures. Different drug treated cultures showed a high number of degenerated parasites with condensed nucleus (except andrographolide), which appeared as dot shaped when compared to those in control viable cultures (Fig. 2).

3.4. Effect of in vitro cytotoxicity of tested compounds

Concentration of all drug molecules were positively significantly correlated with toxicity on horse PBMC. At 2000 μM concentration of HHD, HDTAB, HMC, andrographolide and imidocarb dipropionate showed 8.34%, 46.44%, 58.53%, 31.06%, 15.14% cytotoxicity on PBMC, respectively. Different concentrations of different drug molecules and their respective percentage of cytotoxicity on PBMC have been depicted in Fig. 3.

4. Discussion

T. equi in vitro growth inhibition efficacy of four drug molecules—HHD, HDTAB, HMC and andrographolide, followed by cytotoxicity studies was the prime aim of this present study. HHD is a harmaline derivatives extracted from seeds of *Peganum harmala* plant. This compound has shown anti-bacterial (Gaviraj et al., 1998), anti-oxidative (Liu and Zhao, 2005), anti-tumoral (Chen et al., 2005) and anti-leishmanial activity (Di Giorgio et al., 2004)

in some previous studies. Harmine and harmaline derivatives have also shown anti-*P. falciparum* activity with IC_{50} values of 8.0 μg/ml and 25.1 μg/ml, respectively (Astulla et al., 2008) targeting parasite's Hsp90 (Shahinas et al., 2010, 2012). HHD compound showed IC_{50} of 17.42 μM against *T. equi* cultured *in vitro* in this study indicating that Hsp 90 pathway can also be a potential target for this parasite. Further, *in vitro* cytotoxicity assay with this drug molecule showed 8.34% cytotoxicity at 2000 μM concentration, which is the least value as compared to other drug molecules tested in this study.

The IC_{50} value of HDTAB against *T. equi* parasite was 14.0 μM. Choubey et al. (2007) reported IC_{50} concentration of 10.0 μM against *P. falciparum*. Further, Choubey et al. (2007) documented that HDTAB compound are effective inhibitor of choline kinase enzyme, which is essential for biosynthesis of phosphatidylcholine for *Plasmodium* parasite growth. Choline kinase enzyme might have been the target for HDTAB in *T. equi* for inhibiting its *in vitro* growth, but we observed moderately high cytotoxicity of 46.44% on horse PBMC.

HMC compound is a bioflavonoid compound with anti-oxidative properties, isolated from *Citrus aurantium* plant fruit (Kerry and Abbey, 1997). The IC_{50} value of HMC against *T. equi* parasite was quite high (246.34 μM) along with more cytotoxicity (58.53%) to host cells. *Plasmodium* parasites generate reactive oxygen species (ROS) during their erythrocytic life stage and are highly susceptible to oxidative stress (Becker et al., 2003; Mishra et al., 1994; Griffiths et al., 2001). Oxidative damage of horse erythrocytes infected with *T. equi* correlating with increasing parasitaemia has been observed (Gopalakrishnan et al., 2015). Lipid peroxidation of *T. equi* infected erythrocytes as indicated by increased erythrocytic membrane lipids and plasma malondialdehyde levels in experimentally infected donkeys has also been observed (Ambawat et al., 1999). This prompted us to test the efficacy of anti-oxidative drug molecules—HMC, but it failed to inhibit the growth of *T. equi* parasites at low concentration and found to be more cytotoxic. HMC has properties of osmotic resistance on erythrocytes (Vacca et al., 1955) and hence be less effective in diminishing formation of superoxide anion radicals. Chandrakant (2012) also concluded that the hesperidin have moderate growth inhibitory effect against resistant strain of *P. falciparum* at IC_{50} value of 91.06 μM.

Andrographolide did not show any *T. equi* growth inhibiting efficacy in *in vitro* culture and high toxicity of 31.06% at 2000 μM. Siti Najila et al. (2002) reported *in vitro* anti-malarial activity of andrographolide (*Andrographis paniculata*) against *P. falciparum* at IC_{50} of 45.74 μg/ml. Andrographolide target at transcription pathway of the cell (Mishra et al., 2009) by acting on nuclear transcription factor-kappaβ (NF-$\kappa\beta$) in *Plasmodium* parasites (Tripathi et al., 2009). Apicomplexan family parasites share similar bio-machinery pathway. The finding of this study requires further validations by taking more anti- NF-$\kappa\beta$ drug molecules against *T. equi* parasites.

Imidocarb dipropionate is the drug of choice for treatment of clinically *T. equi* infected equids and hence included in this study as a reference control. Imidocarb dipropionate showed *in vitro* growth inhibition of *T. equi* at 0.139 μg/ml (0.279 μM or 279.9 nM) and was moderately cytotoxic (15.14%). Hines et al. (2015) tested imidocarb dipropionate against *in vitro* cultured *T. equi* Florida strain (FL) and Texas isolate (TX) and reported IC_{50} of 24 nM and 6.4 nM, respectively. A very high variation in imidocarb dipropionate *in vitro* efficacy against these two *T. equi* strains was observed and similar variation in *in vivo* susceptibility of imidocarb dipropionate in natural host has also been reported (Hines et al., 2015). It seems that Indian strain of *T. equi* requires high concentration of imidocarb dipropionate.

Out of four molecules tested, HHD was effective at low IC_{50} value and least cytotoxic as compared to our standard reference drug imidocarb dipropionate. The HDTAB and HMC showed significant high IC_{50} value and moderate high cytotoxicity. So HHD and HDTAB are potential drug molecules for further validation and Hsp 90 and choline kinase pathway of *T. equi* can be the novel targets for anti-theilerial drug development.

Conflict of interest

The authors declare that they have no conflict of interest.

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