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Study of mRNA expression of thirteen genes of *Trypanosoma evansi* in response to diminazene aceturate and isometamidium chloride

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ABSTRACT

The monomorphic, non-cyclic, extracellular haemoprotozoan parasite, *Trypanosoma evansi* leads to *Surra* disease in domesticated animals. Currently, diminazene aceturate (DA) and isometamidium chloride (ISM) are the most used chemotherapeutic agents for the treatment of *Surra* in animals. There is still little knowledge on the anti-trypanosomal mechanism of action of DA and ISM. The work addresses a significant gap in the understanding of the anti-typanosomal mechanism of DA and ISM by investigating their effects on mRNA expression profiles of 13 genes of *T. evansi*. The half maximal inhibitory concentration (IC_{50}) of DA and ISM for a pony isolate of *T. evansi* was estimated as 335.3 nM and 308.6 nM, respectively. Transcript analysis of DA and ISM exposed *T. evansi* population showed its effects on the metabolic machinery of *T. evansi* by down-regulating the mRNA expression of all the 13 targeted genes. However, ISM exposure did not affect mRNA expression of Expression site-associated genes 8 (ESAG8), oligopeptidase B and ornithine decarboxylase genes. The finding provides valuable insights into the molecular action of these drugs, which is crucial for developing more effective treatment of *Surra* disease. Further, comprehensive transcriptome and proteomic analysis could provide a deeper insight into precise molecular pathway of these medications against *T. evansi*.

Keywords: Diminazene aceturate, HMI-9 medium, Isometamidium chloride, qPCR, *Trypanosoma evansi*

Trypanosoma evansi, a kinetoplastid protozoan parasite, causes lethal illness in animals worldwide. Currently, the most often utilised chemotherapeutic agents for *T. evansi* infection in animals are diminazene aceturate (DA) and isometamidium chloride (ISM). Diminazene aceturate (Berenil™, DA) is a potent babesicidal and trypanocidal drug discovered in 1954 (Giordani *et al.* 2016). It intercalates with the AT region of DNA and inhibits the replication of kinetoplastic DNA (kDNA) of trypanosomes (Newton 1975, Peregrine *et al.* 1993, Shiferaw *et al.* 2015). Likewise, ISM was developed around 1958 by the combination of homidium and DA (Giordani *et al.* 2016). It inhibits the DNA polymerase (Lantz and Van Dyke 1972), RNA polymerase (Richardson 1973), glycoprotein biosynthesis, lipid metabolism (Dixon *et al*. 1971), membrane transport (Girgis-Takla and James 1974) and intercalate between nucleotide bases of protozoa (Wagner 1971, Shapiro and Englund 1990, Kinabo 1993, Eghianruwa and Oridupa 2018). ISM causes selective cleavage of the minicircles of kDNA (Shiferaw *et al*. 2015). The kinetoplast of trypanosomes is considered as the primary site of accumulation of ISM and it is known to cleave the kDNA-topoisomerase complexes (Wilkes *et al.* 1997). However, ISM is also effective against dyskinetoplastic forms of *T. evansi* and *T. equiperdum* suggesting the multi-modal action of the drug, which is still unknown (Kaminsky *et al.* 1997, Delespaux and de Koning 2007). ISM has both therapeutic and prophylactic activities. Although scattered biochemical evidences of the mode of action are known, hitherto, molecular evidence of alteration of cellular homeostasis in trypanosomes is still lacking. With the availability and establishment of HMI-9 medium for *in vitro* cultivation of *T. evansi*, new insights on the anti-trypanosomal action of DA and ISM at mRNA expression level can be explored. To ascertain the anti-trypanosomal activity of DA and ISM against *T. evansi*, the current study examined the gene expression level of 13 potential pharmacologically significant genes, which are vital for growth, proliferation, infectivity and survivability of the *T. evansi* and are targeted in various drug discovery studies on *T. evansi* (Gupta *et al*. 2022a).

MATERIALS AND METHODS

Trypanosoma evansi cultivation in HMI-9 medium: Trypanosoma evansi stock, T. ev-India-NRCE-Horse1/Hisar/Haryana which was previously isolated from a clinically infected pony and maintained as cryostock in

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the Division of Parasitology, ICAR-NRCE, Hisar (India) was used in the current study (Gupta *et al.* 2022a). The cryostabilate was thawed, the number of live parasites was enumerated, and the contents were mixed with 1 mL HMI-9 medium and initially distributed in two wells of 48-well microtiter plate (Cellstar, Greiner) and placed in a CO_2 incubator (New Brunswick™, Germany) maintained at 37°C and 5% CO_2 for the growth and proliferation of *T. evansi*.

Trypanosoma evansi growth and proliferation inhibition assay: The diminazene aceturate (Berenil® Intervet India Pvt. Ltd.) and isometamidium chloride (Nyzom, Intas pharmaceuticals) were dissolved in DMSO and a stock solution of 5.15 mg/ml (10 mM) and 4.96 mg/ml (10 mM) was prepared and stored at 4°C, respectively. Further working solutions of different concentrations of DA (1000 nM, 2000 nM, 4000 nM, 6000 nM, 8000 nM and 10000 nM) and ISM (500 nM, 1000 nM, 2500 nM, 5000 nM, 7500 nM and 10000 nM) were prepared in HMI-9 medium for *in vitro* growth inhibitory assay. These working concentrations were prepared freshly and tested on parasite culture for their growth inhibitory activity against *T. evansi*. The 20 µM concentration of Quinapyramine methyl sulphate (QPS, TriquinS™, Vetoquinol, India) was used in the positive control. From well-adapted and steady state *in vitro* cultured *T. evansi*, an aliquot was taken, enumerated and the parasite culture was diluted with prepared HMI-9 media to bring the parasite density to 1×10^5 cells/mL and 500 µL parasite culture was seeded on a 48-well microtiter plate. For each concentration of the drug, the experiment was carried out in triplicate wells and the drug was added at every 24 h interval and incubated in CO_2 incubator at 37°C and 5% CO_2. Wells containing *T. evansi* culture material, but devoid of any drug compounds were utilised as a negative control. As a solvent control, 1% DMSO (Sigma Aldrich, USA) was added to the well. Every 24 h, *T. evansi* culture was observed under a phase contrast microscope and the density of the parasite was determined by luminous cell viability assay using Cell Titer-Glo® reagent (Promega, USA). The 50% minimal inhibitory concentration (IC_{50}) was computed by non-linear regression analysis in GraphPad Prism software (version 8.0.2).

DA and ISM effect on expression level of pharmaceutically important functional genes: For elucidation of trypanocidal mechanism of action, one step real time PCR was used to examine the expression patterns of 13 genes in HMI-9 adapted *T. evansi* parasites before and after treatment with IC_{50} of DA and ISM, respectively. The procedure and primers used for transcript analysis are as mentioned in (Gupta *et al.* 2022b). PCR efficiency of the targeted genes undertaken in the present study using custom-designed primers ranges from 96.45% to 112.99%, which is acceptable according to MIQE guidelines (Bustin *et al*. 2009, Gupta *et al.* 2023).

Statistical analysis: The non-linear regression analysis of the drug's log concentration was used to compute the IC_{50} of DA and ISM against *T. evansi* at 24 h intervals, and the inhibitory activity was determined using the curve fitting approach in GraphPad Prism software (version 8.0.2). Additionally, a two-way ANOVA post hoc Bonferroni test was used to compare the treated wells to the negative control wells. The Ct values for each target gene were normalised using ß-tubulin as housekeeping genes. The $2^{-\Delta\Delta Ct}$ method was used to assess the relative mRNA expression of genes (Livak and Schmittgen 2001). Data was expressed as fold change in mRNA expression level of target genes in drug treated *T. evansi* well in comparison to the untreated control well. To further assess the significance of the fold change data, one-way ANOVA post hoc Dunnett's multiple comparisons test was performed on the gene expression analysis data. Data generated with p-values < 0.05 was considered statistically significant differences between the drug-treated and control wells.

RESULTS AND DISCUSSION

In vitro growth inhibitory efficacy of DA and ISM against T. evansi: The half maximal inhibitory concentration of DA and ISM was estimated to be 335.3 nM and 308.6 nM at 24 h intervals with R^2 values of 0.7814 and 0. 8154, respectively (Fig. 1). Significant inhibition of growth and proliferation of *T. evansi* was detected in all treated wells at 24, 48 and 72 h interval. At 24 h interval of treatment with DA, all treated wells (1000-10000 nM) showed a significant reduction ($p<0.0001$) in *T. evansi* density in comparison to untreated control wells. After 48 h of treatment duration, highly significant ($p<0.0001$) decline in parasite density was noticed at drug concentrations of 1000 nM, with complete absence of parasites in wells treated with 2000 nM and higher concentration. At 72 h interval of treatment with DA, *T. evansi* growth inhibition was observed at drug concentrations of 1000 nM ($p< 0.0001$) and above, with absence of *T. evansi* in wells treated with 2000 nM ($p<0.0001$) and higher concentrations (Fig. 2). Likewise, at 24 h interval of treatment with ISM, all treated well (500-7500 nM) displayed significant decline ($p<0.0001$) in *T. evansi* population density from the untreated control wells, with complete absence of *T. evansi* was observed at 7500 nM concentration and higher concentration. At 48 h interval of treatment, *T. evansi* wells treated with 500 nM and higher concentration exhibited highly significant reduction ($p<0.0001$) in

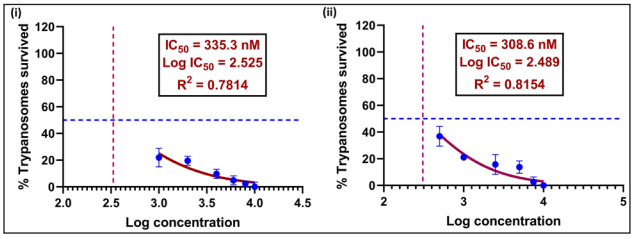

Fig. 1. Dose dependent effect of diminazene aceturate (i) and isometamidium chloride (ii) on the multiplication of *Trypanosoma evansi* cultured in the HMI-9 medium and exposed to drug for 72 h was studied. The 24 h data analysed by curve fitting technique to determine IC_{50} value is presented.

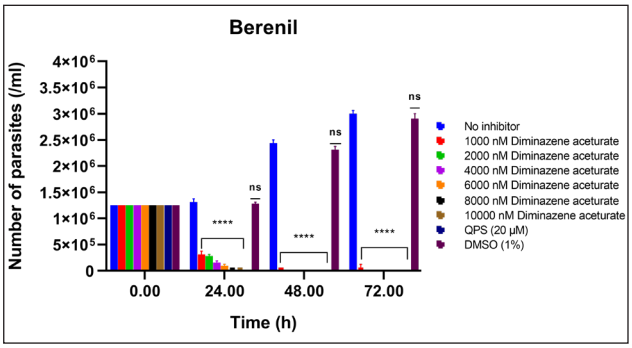

Fig. 2. Bar diagram representative of adapted viable *T. evansi* in *in vitro* culture treated with standard anti-trypanosomal drug berenil (diminazene aceturate). (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns>0.05).

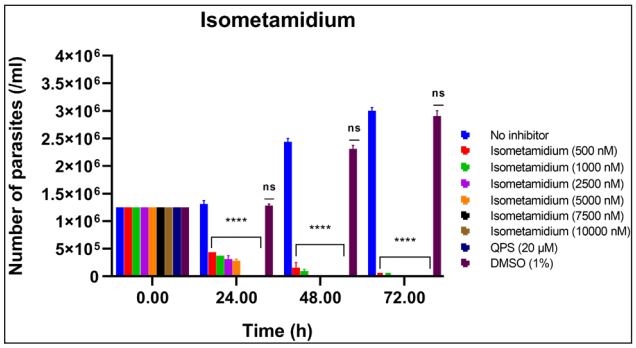

Fig. 3. Bar diagram representative of adapted viable *T. evansi* in *in vitro* culture treated with standard anti-trypanosomal drugs isometamidium chloride. (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns>0.05).

T. evansi density with the absence of parasite at 2500 nM and higher concentration. At 72 h interval on treatment, highly significant (p<0.0001) *T. evansi* growth inhibition was observable in wells treated with 500 nM and above concentrations), with absence of parasites in wells treated with 2500 nM (p<0.0001) and above concentrations (Fig. 3).

Relative gene expression analysis after DA and ISM treatment: All the targeted genes except ESAG8, aurora kinase and ornithine decarboxylase showed a significant down-regulation (p<0.01) of mRNA expression at 12 h exposure to DA. After a period of 12 h, a highly significant decline (p<0.001) in mRNA expression of hexokinase (1.33-folds), trans-sialidase (1.61-folds), trypanothione reductase (1.61-folds), oligopeptidase B (1.09-folds), casein kinase 1 1.38-folds), arginine kinase (1.30-folds), topoisomerase II (1.46-folds), calcium ATPase 1 (1.29-folds), ribonucleotide reductase I (1.31-folds) and ribonucleotide reductase II (1.14-folds) was observed on exposure to DA. Further, the highly significant (p< 0.001) up-regulation of ornithine decarboxylase (1.44-folds) and ESAG8 (1.88-folds) was also observed. At 24 h,

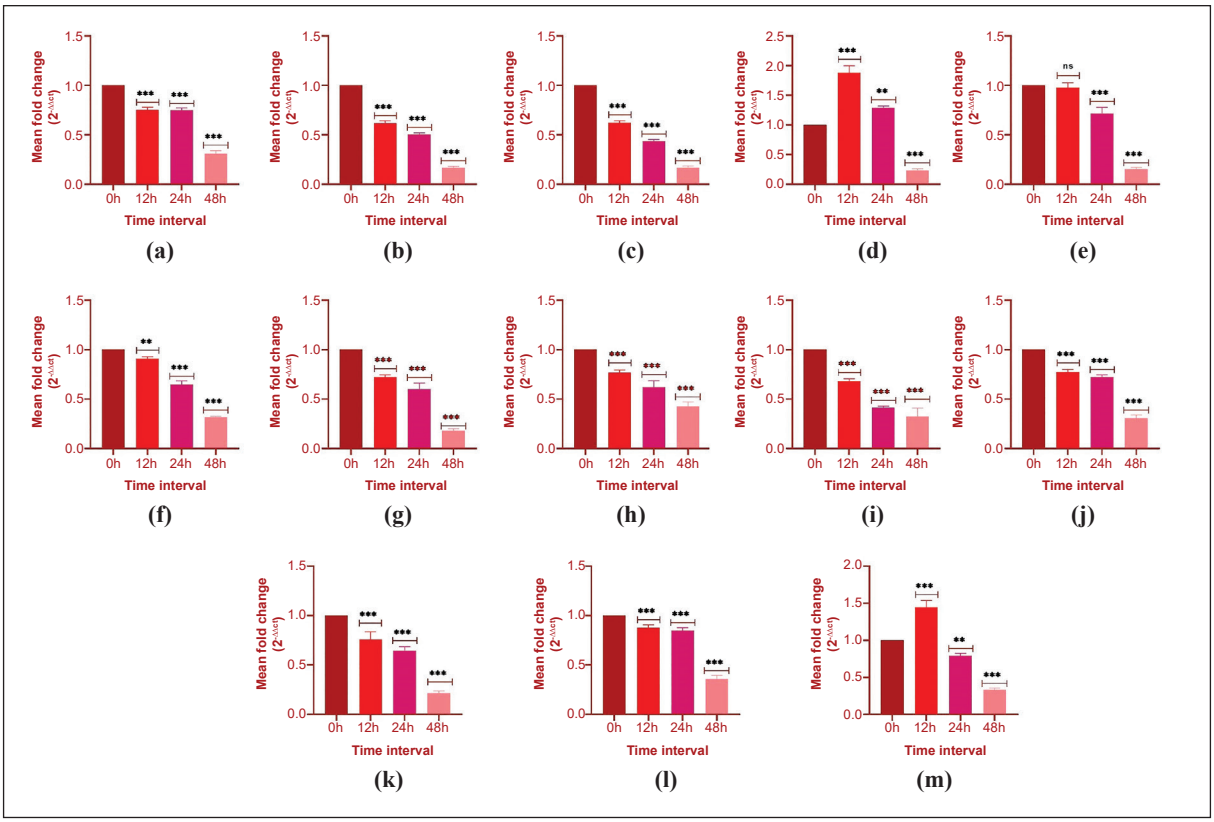

Fig. 4. Relative quantification of mRNA expression of selected metabolic gene of *T. evansi* (a: Hexokinase; b: Trans-sialidase; c: Trypanothione reductase; d: Expression site associated Gene 8; e: Aurora kinase; f: Oligopeptidase B; g: Casein kinase; h: Arginine kinase; i: Topoisomerase II; j: Calcium ATPase 1; k: Ribonucleotide reductase 1; l: Ribonucleotide reductase 2; m: Ornithine decarboxylase) using qPCR after exposure with IC_{50} concentration of diminazine aceturate drug. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns>0.05.

a significant down-regulation (p<0.001) of the mRNA expression of hexokinase (1.33-folds), trans-sialidase (1.98-folds), trypanothione reductase (2.28-folds), oligopeptidase B (1.54-folds), casein kinase 1 (1.66-folds), arginine kinase (1.60-folds), topoisomerase II (p2.41-folds), calcium ATPase 1 (1.38-folds), ribonucleotide reductase I (1.54-folds) and ribonucleotide reductase II (1.18-folds) was observed on exposure to DA. Similarly, up-regulation of ESAG8 continued at 24 h of exposure with DA. However, functional genes such as aurora kinase (1.39-folds) and ornithine decarboxylase (1.26-folds) showed significant down-regulation at 24 h intervals. After 48 h of exposure to DA, ESAG8 gene of *T. evansi* was significantly (p<0.001) down-regulated (4.21-folds). In addition, highly significant (p<0.001) down-regulation of the mRNA expression of hexokinase (3.23-folds), trans-sialidase (5.98-folds), trypanothione reductase (5.98-folds), oligopeptidase B (3.169-folds), casein kinase 1 (5.48-folds), arginine kinase (2.33-folds), topoisomerase II (3.08-folds), aurora kinase (6.55-folds) calcium ATPase 1 (3.23-folds), ribonucleotide reductase I (4.62-folds), ribonucleotide reductase II (2.79-folds) and ornithine decarboxylase (1.44-folds) was also observed after 48 h of exposure with DA (Fig. 4).

Similarly, after 12 h of exposure with ISM, significant (p<0.001) decline in gene expression of hexokinase (4.21-folds), topoisomerase II (4.21-folds) and calcium ATPase 1 (4.21-folds), whereas, rise in mRNA expression of trans-sialidase (4.21-folds), trypanothione reductase (4.21-folds), ESAG8 (4.21-folds), aurora kinase (4.21-folds), ornithine decarboxylase (4.21-folds) and ribonucleotide reductase II (4.21-folds) was recorded. In 24 h period of exposure with ISM, significant down-regulation (p<0.001) of calcium ATPase 1 (1.64-folds) remains continued. Likewise, significant (p<0.01) rise in mRNA expression of aurora kinase (1.40-folds), hexokinase (1.05-folds), trypanothione reductase (1.09-folds), arginine kinase 1 (1.06-fold), trans-sialidase (1.30-folds), ESAG8 (2.93-folds), ornithine decarboxylase (2.84-folds) and ribonucleotide reductase II (1.52-folds) was also observed. In addition, significant (p<0.001) up-regulation of oligopeptidase B (3.19-folds), casein kinase 1 (1.15-folds) and ribonucleotide reductase I (1.34-folds) was also noted. At 48 h, all targeted genes except ornithine decarboxylase I (1.60-folds) showed significant (p<0.001) down-regulation. The various drug target gene such as hexokinase (5.414-folds), topoisomerase II (1.61-folds), trans-sialidase (2.33-folds), aurora kinase (2.54-folds), arginine kinase 1

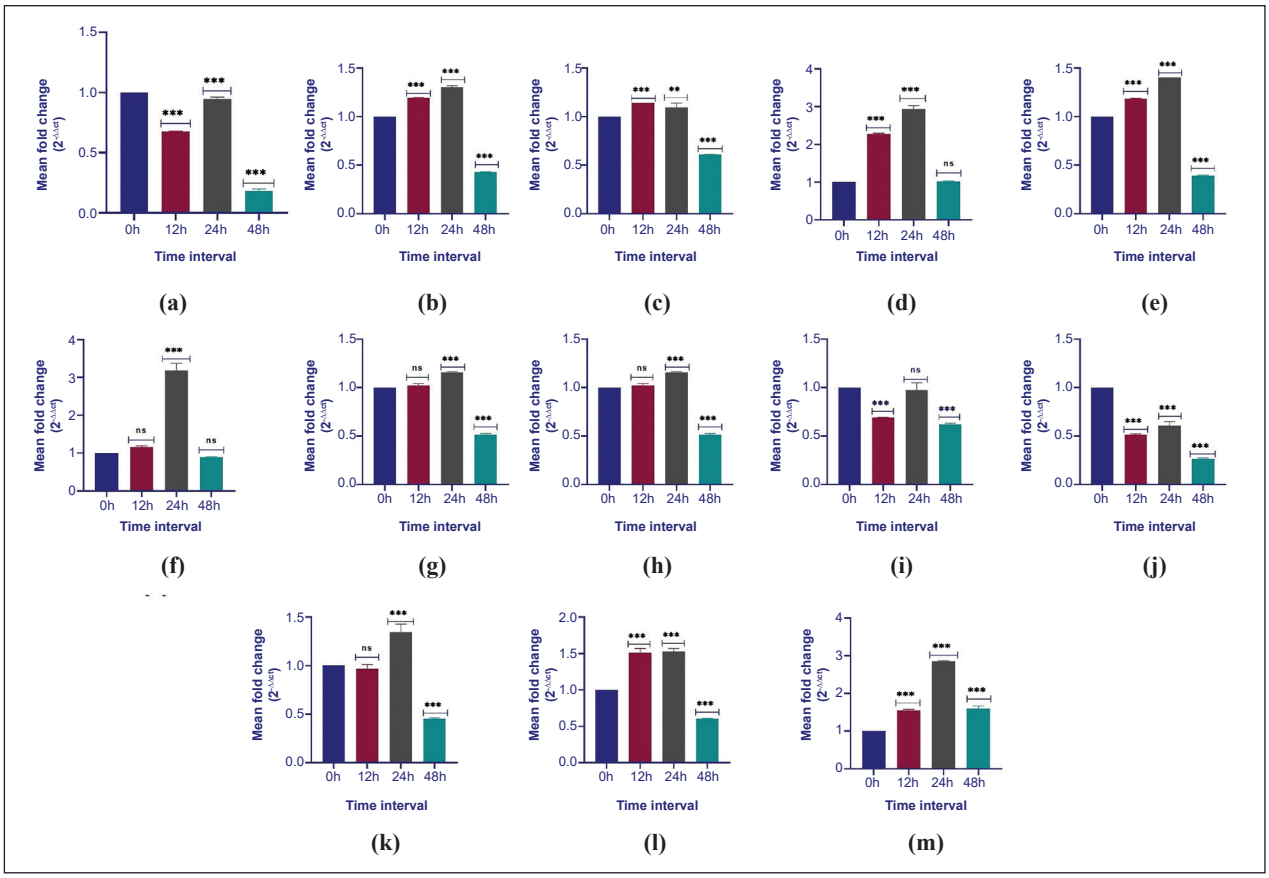

Fig. 5. Relative quantification of mRNA expression of selected metabolic gene of *T. evansi* (a: Hexokinase; b: Trans-sialidase; c: Trypanothione reductase; d: Expression site associated Gene 8; e: Aurora kinase; f: Oligopeptidase B; g: Casein kinase; h: Arginine kinase; i: Topoisomerase II; j: Calcium ATPase 1; k: Ribonucleotide reductase 1; l: Ribonucleotide reductase 2; m: Ornithine decarboxylase) using qPCR after exposure with IC_{50} concentration of isometamedium chloride drug. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns>0.05.

(1.54-folds), calcium ATPase 1 (3.75-folds), trypanothione reductase (1.44-folds), ribonucleotide reductase I (2.21-folds), ribonucleotide reductase II (1.65-folds) and casein kinase 1 (1.94-folds) showed significant ($p<0.001$) decline in mRNA expression after 48 h of exposure with ISM (Fig. 5).

The DA is still used to treat trypanosomiases (*T. congolense, T. brucei, T. evansi* and *T. vivax*), and babesiosis (*B. canis, B. vogeli*, *B. caballi* and *B. gibsoni*) (Tuntasuvan 2003). The specific chemical mechanism of DA trypanocidal action is still unknown, although it has been linked to the aromatic diamidine's linkage to trypanosome kinetoplast DNA (kDNA). DA has a strong affinity for the adenine-thymine base pairs in kDNA due to its chemical structure, which comprises two identical cationic groups (dicationic diamidine), resulting in non-covalent interactions. DA interacts significantly with the minor groove of the double helix in the mitochondrial genome, compromising the crucial replication processes and causing alterations in ribosomes, mitochondrial membranes, and amino acid transport (Kasozi *et al.* 2022, Keneth *et al.* 2022, Venturelli *et al.* 2022). DA can also decrease the activity of trypanosome mitochondrial topoisomerase II, which is responsible for the release of covalently closed minicircles during kDNA replication. As a result, topoisomerase II is unable to interact with the diminazene aceturate-occupied minor groove region in kDNA. Apart from babesiosis and trypanosomosis, DA is implicated in treatment and control of leishmaniasis and cytauxzoonosis (Greene *et al.* 1999). The anti-leishmanial activity is linked with the inhibition of S-adenosylmethionine decarboxylase (AdoMetDC), which is involved in polyamine biosynthesis. DA also alleviates the levels of diamine putrescine and spermidine in the protozoa (Mukhopadhyay *et al.* 1995). Amoebicidal, flukicidal (*Schistosoma mansoni*), anti-pneumocystis, anti-hypertensive, anti-rheumatoid arthritis and immunomodulatory activity of DA is also reported (Hay *et al.* 1994, da Silva Oliveira and de Freitas 2015, Qaradakhi *et al*. 2020, de Brito *et al.* 2020). Berenil's capacity to inhibit critical intracellular signalling pathways that contribute to the generation of pro-inflammatory cytokines implies that it might be utilised to treat illnesses caused by excessive inflammatory cytokine production (Kuriakose and Uzonna 2014). In this study, the IC_{50} of DA was estimated as 335.3 nM at 24 h intervals of HMI-9 cultured pony isolate of *T. evansi*. Transcript analysis of DA showed its multi-modal effects on the metabolic machinery of *T. evansi* by down-regulating the mRNA expression of all the targeted genes, which alters the cellular homeostasis in *T. evansi*. The present study also corroborates with the previous findings that DA inhibits the mitochondrial type II topoisomerases in trypanosomes which subsequently affects the DNA replication (Shapiro and Englund 1990). It is already established that DA inhibits nucleic acid synthesis by binding to the DNA and RNA duplexes by its intercalating and minor groove binding properties (Pilch *et al.* 1995). Likewise, the data generated showed that DA downregulates the synthesis of topoisomerase II, ribonucleotide reductase I and ribonucleotide reductase II, which are indulged in nucleic acid synthesis. The current findings raised the horizon of understanding the mechanism of DA by establishing its potential activity on glycolytic pathway, cell proliferation, redox metabolism, protein synthesis and virulence of the trypanosomes.

In the current study, the IC_{50} of ISM was estimated as 308.6 nM at 24 h intervals for HMI-9 cultured pony isolate of *T. evansi*. Transcript analysis of ISM showed its multi-modal effects on the metabolic machinery of *T. evansi* by down-regulating the mRNA expression of all the targeted genes except ESAG8, oligopeptidase B and ornithine decarboxylase and thereby significantly affecting the DA formation, DNA replication, redox homeostasis and glycolysis in *T. evansi*. Data generated correlates with the earlier finding that ISM inhibits DNA replication, cellular proliferation, and membrane transport in protozoa (Eghianruwa and Oridupa 2018). On the other hand, ISM was found to significantly enhance the process of polyamine biosynthesis by upregulating the production of ornithine decarboxylase. Likewise, it can increase the virulence of the *T. evansi* by upregulating the production of oligopeptidase B enzyme. Expression site associated genes aid the parasite in adaptability to the host (Gupta *et al*. 2022a). ISM also upregulate the production of *T. evansi* ESAG 8, which help the parasite to maintain the surface coat by acquiring host molecules. In spite of all these parasite beneficial activity, the ISM was found lethal for parasite by affecting glycolysis (down-regulating hexokinase), sialic acid uptake (down-regulating trans-sialidase), redox homeostasis (down-regulating trypanothione reductase), calcium homeostasis (down-regulating calcium ATPase 1), cellular proliferation and signalling pathway (down-regulating aurora kinase and casein kinase), energy homeostasis (down-regulating arginine kinase) and nucleic acid synthesis (down-regulating topoisomerase II, ribonucleotide reductase I and ribonucleotide reductase II).

DA and ISM showed high anti-trypanosomal activity with IC_{50} value in the nanomolar range. Trypanocidal activity of DA against *T. evansi* results from quick down-regulation of metabolic genes implicated in glycolysis, redox homeostasis, cellular trafficking, nucleic acid formation, cell division, signal regulation and calcium homeostasis. In comparison, the gradual effect of ISM was observed in mRNA regulation of genes involved in cell division, immune evasion, redox homeostasis, cell signal regulation and nucleic acid synthesis in *T. evansi*. IMM initially targeted the glycolysis, DNA replication and calcium homeostasis of *T. evansi*. The data generated will be of immense use to understand the mechanism of action of DA and ISM on metabolic machinery of *T. evansi*. Therapeutic compounds that similarly target these pathways might be studied further to develop innovative and less toxic trypanocidal agents. To further understand the precise molecular mechanism of action of DA and ISM against *T. evansi*, a complete transcriptomic and proteomic

analysis is required.

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