



In-vitro Activity of Moxifloxacin against *Theileria annulata* - A Preliminary Study

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10.18805/IJAR.B-5753

ABSTRACT

Background: Theileriosis is a tick-borne disease that has a maximum impact on the health and economy of developing countries. A number of clinical manifestations have been found in animals affected with *Theileria annulata*. A similar trend has been observed in the therapeutic management of theileriosis, especially in cattle. Resistance to available treatment options is one of the significant concerns with theileriosis.

Methods: The present study evaluated the *in-vitro* activity of moxifloxacin, the fluoroquinolone compound, against *T. annulata* in 96 well culture plates using resazurin dye. The histopaque method was used for the separation of infected and healthy PMBCs (Polymorph Blood Mononuclear Cells) for growth inhibition assay of *T. annulata* and cytotoxicity assay measurement, respectively. The hemolytic assay was also evaluated against normal bovine RBCs (red blood cells). The 50% inhibitory concentration (IC₅₀) and 50% cytotoxic concentration (CC₅₀) values were measured and analyzed using a correlation and regression method.

Result: The IC₅₀ value of moxifloxacin was observed at 5,559 µM against *in-vitro* *T. annulata*. The CC₅₀ value of cytotoxicity on PMBCs was found to be 3,984 µM. However, the HC₅₀ value of hemolytic assay on bovine red blood cells was observed at 2,59,202 µM. The moxifloxacin showed a wide safety margin on red blood cells. Moxifloxacin can be further investigated *in-vivo* to determine its actual effect on *T. annulata*.

Key words: *In-vitro*, Moxifloxacin, Resazurin, *T. Annulata*, Topoisomerase II.

INTRODUCTION

Theileria annulata in livestock is one of the major health concerns. As *T. annulata* is a vector-borne disease, it covers large groups of animals, including sheep and goats (Verma *et al.*, 2016; Shahedi *et al.*, 2022; Prajapati *et al.*, 2023; Ntesang *et al.*, 2024; Sinha *et al.*, 2024; Alnahari *et al.*, 2026). According to estimates, bovine tropical theileriosis causes an annual loss of about US\$ 384.3 million to the Indian livestock sector (Seeber and Steinfelder, 2016; Keroack *et al.*, 2019). The clinical signs exhibited by the animals also vary (Prajapati *et al.*, 2019). *T. annulata* outbreaks have been reported from India (Sinha *et al.*, 2021; Velusamy *et al.*, 2023). Buparvaquone is the veterinarians' choice of drug. However, resistance against buparvaquone has been reported for a while (Salim *et al.*, 2019; Yousef *et al.*, 2020).

Fluoroquinolones are drugs indicated primarily as anti-bacterial agents (Pathania and Sharma, 2010). They work on microorganisms by inhibiting the topoisomerase II pathway (Pietrusinski and Staczek, 2006; Fabrega *et al.*, 2009). DNA topoisomerases are essential to the survival of a large number of deadly protozoan parasites. Since the topoisomerase structures of protozoan parasites differ from those of humans, DNA topoisomerase serves as a crucial target for the creation of effective medications for parasitic illnesses in animals (Lamba and Roy, 2022). The exact pathway was evaluated in *T. annulata* and it was suggested that it could be used for new drug discovery (Lizundia *et al.*, 2009).

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How to cite this article: Prajapati, A.S., Suthar, A.N., Panchasara, H.H., Das, B. and Patel, P.A. (2026). *In-Vitro* Activity of Moxifloxacin against *Theileria annulata* - A Preliminary Study. *Indian Journal of Animal Research*. 1-5. doi: 10.18805/IJAR.B-5753.

Submitted: 09-01-2026 **Accepted:** 25-02-2026 **Online:** 02-03-2026

Fluoroquinolone compounds have been tested against apicomplexan organisms (Batiha *et al.*, 2020; Fontinha *et al.*, 2020). Among them, moxifloxacin was highly effective

against apicomplexan organisms such as *Toxoplasma gondii* and *Plasmodium falciparum* (Gozalbes *et al.*, 2000; Mahmoudi *et al.*, 2003). The present study was executed to assess the *in-vitro* activity of moxifloxacin, a fluoroquinolone compound, against *T. annulata*. Further, cytotoxicity and hemolytic activity were also evaluated on healthy PBMCs and red blood cells.

MATERIALS AND METHODS

Ethical approval

The Institutional Animal Ethics Committee of the College approved the current study under protocol number IAEC No. VETCOLL/IAEC/2022/20/PROTOCOL-16.

Study period and location

The study was conducted from December 2023 to August 2024 at the Department of Veterinary Medicine, Kamdhenu University, Sardarkrushinagar, Gujarat, India, in collaboration with the Department of Veterinary Biotechnology, Veterinary Microbiology and Veterinary Physiology.

Selection of positive animal

The cattle showed clinical signs of theileriosis and were used for further *in-vitro* evaluation. *T. annulata* organism in the blood was confirmed by Giemsa-stained blood smear examination and polymerase chain reaction. *T. annulata* was explicitly detected in cows using PCR with a 430 bp amplicon using the forward primer CCAGGACCACCTCA AGTTC and the reverse primer GCATCTAGTTCCTTGG CGGA (Kundave *et al.*, 2015).

In-vitro growth inhibition assay

In-vitro activity of moxifloxacin against *T. annulata* was evaluated in 96 well culture plate (Gopalakrishnan *et al.*, 2016). Histopaque was used to separate the infected lymphocytes from blood collected from the infected animals with *Theileria*. Three times, PBS wash was given to lymphocytes and 100 μ L (equivalent to 2.5×10^5 cells/ml) seeded onto the plates. A complete medium consisting of RPMI-1640 supplemented with L-glutamine, 100 IU/ml penicillin and 10% fetal bovine serum was used. Incubation conditions for the culture plates were 37°C, 95% relative humidity and 5% CO₂. Different concentrations of moxifloxacin from 16000 μ M to 250 μ M were evaluated against *T. annulata*. The *in-vitro* growth inhibition assay was conducted, with resazurin added as 10% of the total volume. After four hours, the optical density (OD) was measured at 570 and 650 nm. Moxifloxacin was purchased from Merck (Merck KGaA, Darmstadt, Germany) company. Triton X-100 (T×100) was used as a positive control agent and DMSO was used for dilution control. The IC₅₀ value of moxifloxacin was measured using the formula mentioned (Suthar *et al.*, 2021).

In-vitro cytotoxicity assay on PBMCs

The cytotoxicity assay of moxifloxacin was evaluated from healthy PBMCs to assess the detrimental effect of the drug on cells described earlier (Araveti and Srivastava, 2019).

Healthy PBMCs were separated by the Histopaque method and mixed with complete media. 100 μ L PBMCs were seeded into the 96 well plate. The well culture plates were incubated at 37°C in 5 % CO₂ for the next 48 hours. Moxifloxacin concentration was prepared from 8000 μ M to 62.5 μ M and added into wells. Again, incubation was done for another 24 hours, followed by the addition of 25 μ L of resazurin dye (150 μ g/ml) was done. The culture plate was again incubated for another 4 hours. 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 the OD₅₇₀ value from its respective OD₆₅₀ value. The CC₅₀ (50% Cytotoxic Concentration) value of moxifloxacin was measured using the mentioned formula (Suthar *et al.*, 2021).

In-Vitro hemolytic assay

The hemolytic activity of moxifloxacin on bovine RBCs was evaluated and calculated using the method described earlier (Suthar *et al.*, 2021). The study involved adding 20 μ L of RBC suspension to 96 well culture plates, followed by 180 μ L of different drug concentrations. The culture plates were kept at 37°C for 90 minutes, then transferred to a microcentrifuge tube and centrifuged for five minutes. The optical density was determined to be 543 nm. DMSO was used in the dilution control well and water was used in the positive control well. HC₅₀ (50% Hemolytic Concentration) value was calculated using the formula mentioned (Suthar *et al.*, 2021).

Statistical analysis

Regression analysis and correlation were used to calculate the efficacy of moxifloxacin compounds against *T. annulata*. The differences between the drug-treated wells and the control wells were considered to be statistically significant when the P values were less than 0.05. GraphPad Prism version 10.0 software (GraphPad Software, San Diego, California, USA) was used to facilitate regression analysis and correlation between the drug concentration and its cytotoxicity and hemolytic activity.

RESULTS AND DISCUSSION

In-vitro growth inhibition assay

The *in-vitro* growth inhibition assay of different concentrations of moxifloxacin was mentioned and shown in Table 1. A negative correlation was observed (R=-0.9262; P<0.004) between cell viability and drug concentration. IC₅₀ was calculated and observed at 5559 μ M. Meanwhile, dilution control (DC), positive control (PC) and negative control (NC) showed 94.47%, 0.77% and 100% viability, respectively.

In-vitro cytotoxicity assay on PBMCs

The percent PBMC cytotoxicity of moxifloxacin concentration is shown in Table 2. A positive correlation (R = 0.9673; P<0.001) was observed between moxifloxacin concentration and cell cytotoxicity. The CC₅₀ value for moxifloxacin was calculated and found to be 3,984 μ M. At the same time,

100% cytotoxicity was found in well with positive control. However, 30.79% cytotoxicity was observed in well-labelled with dilution control.

***In-vitro* hemolytic assay**

The per cent hemolysis of moxifloxacin concentration on bovine red blood cells is shown in Table 3. A positive correlation ($R = 0.9900$; $P < 0.001$) was observed between moxifloxacin concentration and hemolysis. On the other hand, 100% hemolysis was observed in a positive control well with water. The HC_{50} value of moxifloxacin hemolytic activity was estimated at 2,59,202 μM .

Fluoroquinolones (FQs) are very well known to inhibit replication and transcription of bacterial DNA either by targeting DNA gyrase or by targeting topoisomerase-II. The imperative presence of both enzymes precedes bacterial growth (Yadav and Talwar, 2019). Topoisomerases are ubiquitous and found in all living organisms (Deweese and Osheroff, 2009). Many eukaryotic organisms, including the protozoan parasite *T. annulata*, which causes tropical theileriosis in cattle, depend on the topoisomerase II pathway. The enzyme Topoisomerase II is in charge of controlling DNA topology during transcription, replication and chromosome segregation. It accomplishes this by causing brief double-strand breaks in DNA, which permit one strand of DNA to cross through another and release supercoils produced during these processes. It is essential for the distinct life cycle of *T. annulata*, especially during the schizont stage when the parasite infects host

leukocytes and changes them into cancerous, proliferating cells. Since *Theileria* species must divide their hosts in order to reproduce, DNA replication and chromosome segregation by Topoisomerase II is essential for their intracellular survival and growth.

Resazurin, a blue dye, is converted into resorufin by cells, releasing a pink compound. Microbiologists use intracellular diaphorase enzymes to reduce resazurin, producing a fluorescent signal that can be measured using a spectrophotometer. This method is cost-effective and cell-friendly, allowing researchers to measure cell proliferation kinetics indirectly. It is safe for cells when used at low concentrations and for short incubation periods, making it valuable for studying cell growth. The resazurin reduction assay is a useful tool in studying cell growth (Zalata *et al.*, 1998; O'Brien *et al.*, 2000; Gloeckner *et al.*, 2001).

The IC_{50} value (5559 μM) of moxifloxacin observed in this study was high compared to the CC_{50} (3984 μM) value on PBMCs. Moxifloxacin can damage normal cells and infected cells in more or less the same manner. However, based on the selective index (CC_{50}/IC_{50}), further exclusive focused trials on moxifloxacin can be suggested to obtain more accurate data against *T. annulata*. Limited research work has been documented for moxifloxacin against apicomplexan organisms. Gozalbes *et al.* (2000) assessed the *in-vitro* activity of moxifloxacin against *T. gondii* and reported that moxifloxacin was the potent drug against *T. gondii*, with IC_{50} of 5.1 mg/litre. Mahmoudi *et al.* (2003) evaluated the antimalarial activity of moxifloxacin and

Table 1: Per cent cell viability in different concentrations of Moxifloxacin against *in-vitro* infected lymphocytes.

Concentration (μM)	Mean % viability $\pm$ SE	IC_{50} value and correlation coefficient (R)
16000	26.67 $\pm$ 0.03	5,559 μM ; -0.9262
4000	54.92 $\pm$ 0.03s	
2000	74.31 $\pm$ 0.04	
1000	93.59 $\pm$ 0.06	
500	92.41 $\pm$ 0.03	
250	96.98 $\pm$ 0.02	
DC (Cells + DMSO)	94.47 $\pm$ 0.08	
PC (Cells + Triton)	0.77 $\pm$ 0.01	
NC (Cells)	100 $\pm$ 0.05	

Table 2: Per cent cytotoxicity in different concentrations of moxifloxacin on peripheral blood mononuclear cell (PBMC).

Concentration (μM)	Mean % cytotoxicity $\pm$ SE	CC_{50} value and correlation coefficient (R)
8000	73.32 $\pm$ 0.03	3.984 μM ; 0.9673
4000	57.20 $\pm$ 0.03	
2000	16.44 $\pm$ 0.03	
1000	7.92 $\pm$ 0.03	
500	0.39 $\pm$ 0.02	
250	0.38 $\pm$ 0.02	
125	0.35 $\pm$ 0.04	
62.5	0.25 $\pm$ 0.08	
PC (Cells + Triton)	100.00 $\pm$ 0.04	
DC (Cells + DMSO)	30.79 $\pm$ 0.4	
NC (Cells)	0.00	

Table 3: Per cent hemolysis in different concentrations of Moxifloxacin on red blood cells (RBCs).

Concentration (μM)	Mean % hemolysis $\pm$ SD	HC ₅₀ value and correlation coefficient (R)
8000	1.89 $\pm$ 0.01	2,59,202 μM ; 0.9900
4000	0.79 $\pm$ 0.02	
2000	0.36 $\pm$ 0.00	
1000	0.19 $\pm$ 0.00	
500	0.19 $\pm$ 0.00	
250	0.18 $\pm$ 0.00	
125	0.07 $\pm$ 0.00	
62.5	0.01 $\pm$ 0.00	
PC (RBC + Water)	100 $\pm$ 0.02	
NC (RBCs)	0.00	

observed an IC₅₀ value of 44.8 $\pm$ 1.7 $\mu\text{g/ml}$. The study data can be further used as a baseline value for evaluating moxifloxacin against apicomplexan organisms.

CONCLUSION

The *in-vitro* *T. annulata* growth inhibitory efficacy of a Topoisomerase II inhibitor drug, moxifloxacin, was investigated using resazurin. Moxifloxacin inhibited the *in-vitro* growth of *T. annulata* with a respective IC₅₀ value of 5,559 μM . The drug was found toxic on the host PBMCs with a CC₅₀ value of 3,984 μM . Moxifloxacin was safe on RBCs with HC₅₀ of 2,59,202 μM . It can be inferred that the *Theileria* topoisomerase II family includes potential drug targets worthy of further investigation.

ACKNOWLEDGEMENT

The authors acknowledge the Department of Microbiology, the Department of Biotechnology and the Department of Physiology, Sardarkrushinagar, Gujarat, India, for providing the necessary facilities for the study. The author also acknowledges the Principal Veterinary College, Sardarkrushinagar, for giving the required funds for the study.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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