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Original Article

Efficacy of enoxacin and levofloxacin against *Theileria annulata* schizont-infected cell line

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Abstract

Background: Theileriosis is a tick-borne disease that significantly hampers livestock industries in developing nations. Buparvaquone is the only available option for the management of theileriosis. On the other hand, drug use or organism evolution has led to the evolution of resistance over past twenty years. Treatment for theileriosis requires alternative therapeutic approaches. **Aims:** This work examined the in-vitro effectiveness of enoxacin and levofloxacin compounds against *Theileria annulata* cultures, targeting the topoisomerase II pathway of organism. **Methods:** The schizont infected cell line was established from infected cattle blood. The inhibitory effects of both drugs were assessed on *T. annulata* along with their cytotoxicity and hemolytic activity. **Results:** Enoxacin and levofloxacin efficiently inhibited 50% of the infection at 1,71,723 µM and 10,084 µM, respectively. Levofloxacin showed encouraging results during the experiment. For enoxacin and levofloxacin, the 50% cytotoxic effects were also measured at 10,529 µM and 10,116 µM, respectively. Enoxacin was safe on bovine red blood cells with an HC_{50} value of 26,49,031 µM compared to levofloxacin with 19,828 µM value. **Conclusion:** Levofloxacin showed satisfactory results compared to enoxacin. Further study is required along with other parameters to evaluate and assess the inhibitory activity of enoxacin and levofloxacin.

Key words: Cytotoxicity, Enoxacin, In-vitro, Levofloxacin, *Theileria annulata*

Introduction

Tropical theileriosis due to *Theileria annulata* represents in North Africa and parts of Asia an important disease of cattle of high economic importance. Anti-parasitic chemotherapy drugs are crucial for both treatment and attempts to destroy or eradicate parasites, as treating the effects of these organisms is crucial for human health and prosperity (Keroack *et al*., 2019). *Theileria annulata* is transmitted by *Hyalomma* ticks. In India, theileriosis has been reported from many states, causing huge economic loss (Edith *et al*., 2018; Selim *et al*., 2020; Sinha *et al*., 2021; Velusamy *et al*., 2023). Anemia, enlarged lymph node, presence of ticks, exophthalmos and pseudo-pericarditis are the symptoms of the deadly disease theileriosis, with chronic cases carrying a higher risk (Singh *et al*., 2015; Lempereur *et al*., 2017; Prajapati *et al*., 2019).

The conditions of geographic location have favoured tick multiplication, which has gradually increased the incidence of tick-borne diseases (Kohli *et al*., 2014). Theileriosis susceptibility has increased due to population growth and the introduction of exotic and crossbred cattle, particularly in endemic areas. The adverse effects of theileriosis have been exacerbated by stress-related predisposing factors, including high production, low nutrition, substandard housing, unclean indoor conditions, and the emergence of drug resistance

to acaricidal agents (Sahoo *et al*., 2017).

One of the new plagues impacting the veterinary and public health fields is drug resistance. Anti-protozoal drug depletion is a result of several factors, including growing drug resistance in organisms, inadequate medication usage, cross-resistance, and a failure to replace older treatments (Koning, 2017). The most effective way to treat bovine tropical theileriosis is with buparvaquone. Many researchers and field veterinarians have reported resistance to buparvaquone during the past two decades (Mhadhbi *et al*., 2010; Sharifiyazdi *et al*., 2012; Hostettler *et al*., 2014; Mhadhbi *et al*., 2015; Chatanga *et al*., 2019; Salim *et al*., 2019; Yousef *et al*., 2020; Ali *et al*., 2022). Antiprotozoal medication overuse or underdosage has created selection pressure that has aided in the emergence of resistance (Hyde, 2007). Buparvaquone, the only available and effective medication in livestock, is a an important factor for theileriosis control. According to reports of clinical studies, buparvaquone resistance poses a major threat to productive cattle production (Mhadhbi *et al*., 2010; Cui *et al*., 2015). As a result, novel medications that can be used for the therapeutic care of cattle with *Theileria annulata* are required.

Enoxacin and levofloxacin have been tested against the apicomplexan organisms, namely *Plasmodium* and *Toxoplasma* (Gozalbes *et al*., 2000; Mahmoudi *et al*., 2003). However, the efficacy of enoxacin and levofloxacin against *T. annulata* has not been established. Therefore, the present study aims to assess the efficacy of enoxacin and levofloxacin against *T. annulata* in-vitro.

Materials and Methods

Ethical approval

The present work has been approved by the college IAEC committee (IAEC No. VETCOLL/IAEC/2022/20/ PROTOCOL-16).

Culture source

T. annulata was cultured from infected blood of a bovine at Veterinary Clinical Complex, Sardarkrushinagar, Gujarat. A microscopic examination and PCR evaluation were carried out for confirmation. The DNA was extracted using a QIamp Blood Mini Kit. PCR assay for *T. annulata* detection was performed using the forward primer 5´-CCA GGA CCA CCC TCA AGT TC-3´ and the reverse primer 5´-GCA TCT AGT TCC TTG GCG GA-3´ and resulted with the final amplicon of 430 bp (Kundave *et al*., 2015).

Anti-theilerial activity (resazurin based cell viability assay)

T. annulata culture was established using infected bovine mononuclear cells, separated by histopaque method, and maintained in RPMI-1640 medium with 20% fetal bovine serum. 100 µL of infected bovine lymphocytes (2.5×10^5 cells/ml) were seeded into a 96 well culture plates. Cells were incubated at 37°C, with 95% relative humidity and 5% CO_2. Enoxacin and levofloxacin from 500-32000 µM (Merk, Germany) were added into the wells. The in-vitro growth inhibition assay was carried out in accordance with Hayat *et al*. (2012) with a modification in incubation period. Resazurin was then added as 10% of the total volume and incubated for 4 h to measure the cell survival following the 96 h medication trial. Absorbance was measured at 570 and 650 nm after 4 h (O'Brien *et al*., 2000). A standard curve fitting technique was used to compute the IC_{50} (50% inhibitory concentration) values for the enoxacin and levofloxacin (Bork *et al*., 2004). The experiment was performed three times. The following formula was used to calculate the effect of medication molecules in terms of viability percentage (Suthar *et al*., 2021).

$$\text{Viability (\%)} = \frac{\text{OD of test sample} - \text{OD of positive control}}{\text{OD of negative control} - \text{OD of positive control}} \times 100$$

Cytotoxicity assay with bovine PBMC

Bovine blood peripheral mononuclear cells (PBMC) were isolated from cattle located at the Livestock Research Unit, Sardarkrushinagar, Gujarat. Histopaque-1077 was used to collect bovine PBMCs, which were then suspended in 1 ml of complete medium that contained 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and RPMI-1640 supplemented with L-glutamine. The hemocytometer technique was used to calculate the final concentration of PBMC, which was maintained at approximately 2×10^5 cells/ml. The assay for PBMC cytotoxicity in-vitro was carried out using 96-well culture plates. A 96-well culture plate containing 100 µL of cells was used in the assay (Xie and Wang, 1994). The well culture plates were incubated at 37°C with 5% CO_2 for 48 h. 100 µL of 62.5-8000 µM doses of enoxacin and levofloxacin were added to the wells and incubated for 24 h. Resazurin dye (25 µL, 150 µg/ml) was added to the wells and incubated for 4 h. Optical density (OD) at 570 and 650 nm of coloured wells was measured and CC_{50} (cytotoxic concentration 50) was obtained as described by Suthar *et al*. (2021).

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

Hemolytic assay on bovine red blood cells

A blood sample was collected from healthy bovine from Livestock Research Station, Sardarkrushunagar, Gujarat and centrifuged for 10 min at 161 g. White blood cells and plasma in the topmost layer were discarded. The leftover red blood cell were washed with PBS (phosphate buffer saline) for 5 min and centrifuged at 161 g. The last RBC pellet was suspended in PBS following three washing processes. In a 96-well culture plate, 20 µL of RBC suspension was added to each well. Additionally, each well received 180 µL of 62.5-8000 µM drug concentrations in a solubilizing buffer (10% dimethylformamide in PBS). A positive control consisted of RBC suspension mixed with 180 µL of distilled water,

whereas a negative control consisted of RBC suspension in the absence of any medication. The 96-well plate was incubated for 90 min at 37°C. The content of each well was subsequently transferred into a 2 ml tube, and the mixture was centrifuged for 5 min at 1000 g. The supernatants were transferred to a new 96-well plate, the OD was measured at 543 nm, and HC_{50} (50% hemolytic concentration) was calculated as described by Suthar *et al*. (2021).

$$\text{Percentage hemolysis} = \frac{\text{OD of different drug concentrat ion} - \text{OD of negative control}}{\text{OD of positive control - OD of nagative control}}$$

Statistical analysis

The effectiveness of enoxacin and levofloxacin compounds against *T. annulata* was determined by regression analysis and correlation. 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

Anti-theilerial activity (resazurin based cell viability assay)

Enoxacin and levofloxacin had dose dependent effect on the cell viability of *T. annulata* infected lymphocyte. Negative correlation between cell viability and drug concentration was found for enoxacin (r=-0.888) and levofloxacin (r=-0.9535) (Table 1). The IC_{50} values for enoxacin and levofloxacin compounds were 1,71,723 and 10,084 µM, respectively (Fig. 1, A1-A2). No vialbility was obtained from the positive control treated with triton.

Cytotoxicity assay with bovine PBMC

The values of cytotoxicity percentage along with standard deviation for different concentrations of both drugs were shown in Table 2. Positive correlation was observed between the cytotoxicity and concentrations of enoxacin (r=-0.950) and levofloxacin (r=-0.899). Levofloxacin showed almost same CC_{50} value i.e., 10,116 µM compared to CC_{50} value of enoxacin (Fig. 1, B1-B2). Positive control cells treated with triton showed maximum cytotoxicity whereas negative control cells in PBS showed no cytotoxicity.

Hemolytic assay on bovine red blood cells

Enoxacin and levofloxacin showed dose dependent positive correlation with hemolytic activity on bovine red blood cells as shown in Table 3. HC_{50} of enoxacin was 26,49,031 µM (r=0.940), while HC_{50} of levofloxacin was surprisingly 19,828 µM (r=0.891) which was several

Table 1: Effect of different concentrations of enoxacin and levofloxacin on monomuclear cell viability (in percent) of a *Theileria annulata* cell line

Drug concentration (µM)	Enoxacin		Levofloxacin	
	Mean % viability ± SE	IC_{50} value and correlation coefficient (r)	Mean % viability ± SE	IC_{50} value and correlation coefficient (r)
32000	64.44 ± 0.02	173,723 µM; -0.888	19.31 ± 0.06	10,084 µM; -0.9535
16000	70.48 ± 0.08		37.61 ± 0.04	
8000	77.99 ± 0.01		57.07 ± 0.01	
4000	92.92 ± 0.08		75.89 ± 0.10	
2000	88.57 ± 0.02		85.31 ± 0.05	
1000	86.81 ± 0.05		94.91 ± 0.07	
500	82.55 ± 0.02		88.56 ± 0.03	
DC (cells + DMSO)	48.06 ± 0.08		38.20 ± 0.03	
PC (cells + triton)	0.02 ± 0.02		0 ± 0.03	
NC	76.26 ± 0.02		82.94 ± 0.11	

DC: Dilution control, DMSO: Dimethyl sulfoxide, PC: Positive control, and NC: Negative control

Table 2: Cytotoxic activity of enoxacin and levofloxacin on PBMCs

Drug concentration (µM)	Enoxacin		Levofloxacin	
	Mean % cytotoxicity ± SE	CC_{50} value and correlation coefficient (r)	Mean % cytotoxicity ± SE	CC_{50} value and correlation coefficient (r)
8000	32.55 ± 0.02	10,529 µM; 0.950	25.53 ± 0.02	10,116 µM; 0.899
4000	5.50 ± 0.04		1.32 ± 0.02	
2000	5.21 ± 0.11		1.24 ± 0.07	
1000	0.00 ± 0.00		1.18 ± 0.01	
500	0.00 ± 0.00		1.10 ± 0.02	
250	0.00 ± 0.00		0.00 ± 0.00	
125	0.00 ± 0.00		0.00 ± 0.00	
62.5	0.00 ± 0.00		0.00 ± 0.00	
PC (cells + triton X-100)	100.00 ± 0.00		100.00 ± 0.04	
DC (cells + DMSO)	31.98 ± 0.02		25.08 ± 0.01	
NC (cells + PBS)	0.00 ± 0.03		0.00 ± 0.01	

DC: Dilution control, DMSO: Dimethyl sulfoxide, PC: Positive control, and NC: Negative control

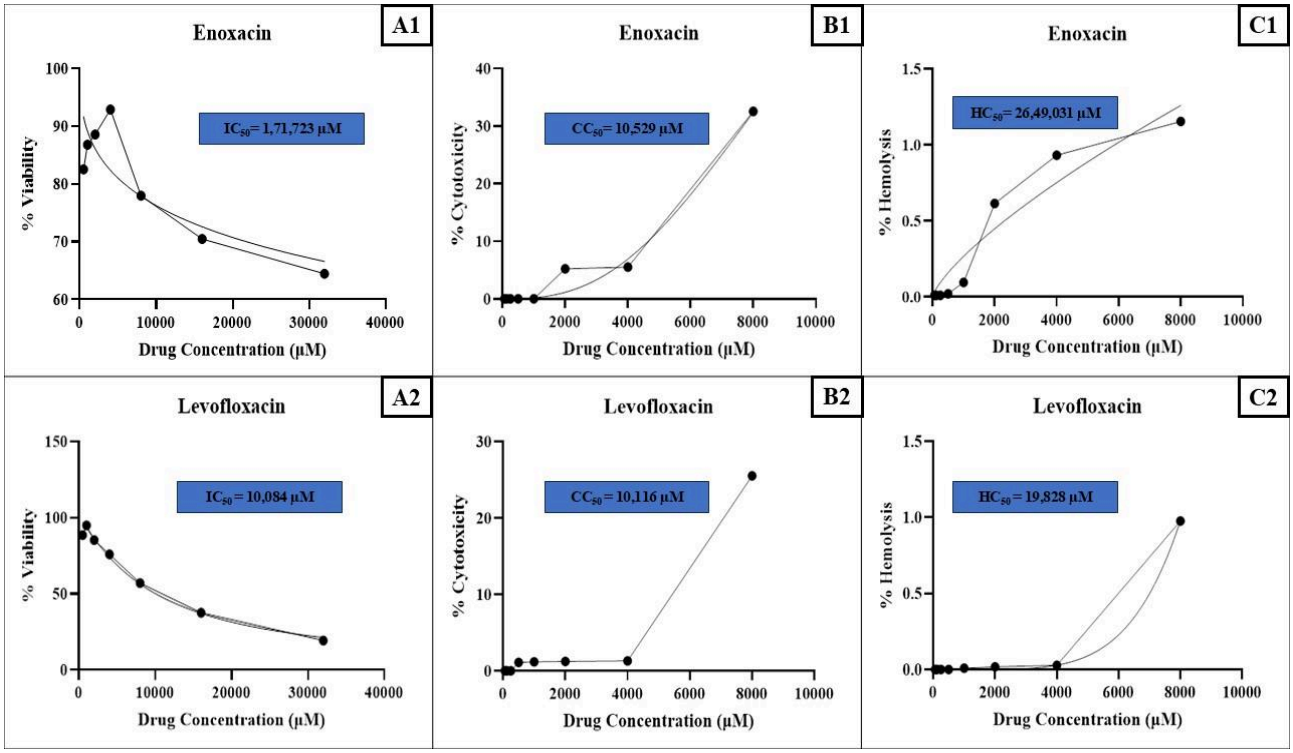

Fig. 1: Graphs showing IC_{50} value of (**A1**) Enoxacin and (**A2**) Levofloxacin, cytotoxicity CC_{50} of (**B1**) Enoxacin, (**B2**) Levofloxacin, and hemolytic activity HC_{50} of (**C1**) Enoxacin, and (**C2**) Levofloxacin

Table 3: Hemolytic activity of enoxacin and levofloxacin on red blood cells

Concentration (µM)	Enoxacin		Levofloxacin	
	Mean % hemolysis ± SE	HC_{50} value and correlation coefficient (R)	Mean % cemolysis ± SE	HC_{50} value and correlation coefficient (R)
8000	1.15 ± 0.00	2,649,031 µM; 0.940	0.98 ± 0.01	19,828 µM; 0.891
4000	0.93 ± 0.00		0.03 ± 0.00	
2000	0.61 ± 0.00		0.02 ± 0.00	
1000	0.09 ± 0.00		0.01 ± 0.00	
500	0.02 ± 0.00		0	
250	0.01 ± 0.00		0	
125	0.01 ± 0.00		0	
62.5	0.01 ± 0.00		0	
PC (cells + water)	100 ± 0.01		100 ± 0.05	
NC (cells + PBS)	0.00		0.00	

PC: Positive control, and NC: Negative control

times lower than enoxacin (Fig. 1, C1-C2). Positive control red blood cells treated with distilled water showed 100% hemolysis. Negative control red blood cells showed 100% viability.

Discussion

Buparvaquone is commercially used for the therapeutic management of theileriosis in cattle. However, its resistance has been reported globally against *T. annulata* (Salim *et al*., 2019; Yousef *et al*., 2020; Ali *et al*., 2022). Point mutations in various genes of *T. annulata* have been documented (Chatanga *et al*., 2019). New drug development for the management of such conditions is a need of the hour in the new era.

Various drugs were tested against *T. annulate*, which were not primarily indicated for the management of *Theileriosis* (Batiha *et al*., 2019; Buvanesvaragurunathan *et al*., 2022; Prasanna *et al*., 2022). According to numerous, studies enoxacin and levofloxacin are effective against a variety of organisms, including apicomplexan organisms (Gozalbes *et al*., 2000; Mahmoudi *et al*., 2003; Tunitskaya *et al*., 2011; Omar *et al*., 2016; Song *et al*., 2016; Ptaszynska *et al*., 2020; Jalbrzykowska *et al*., 2022). Quinolone antibiotics specifically target DNA gyrase and topoisomerase IV, two essential type II topoisomerases, to effectively impede DNA synthesis. Both targets allow a double-stranded DNA molecule to pass through another, rejoining the original strand in the process.

Enoxacin and levofloxacin topoisomerase II pathways have been evaluated and documented for their anti-bacterial activity and new drug development against *Theileria* spp. (Heifetz *et al*., 1988; Levine *et al*., 1998;

Fabrega *et al*., 2009; Lizundia *et al*., 2009; Garcia-Estrada *et al*., 2010; Hooper and Jacoby, 2016; Idowu and Schweizer, 2017; Fief *et al*., 2019). Enoxacin and levofloxacin were in-vitro evaluated for anti cancer activity by Song *et al*. (2016), Valianatos *et al*. (2017), and Jalbrzykowska *et al*. (2022). Gozalbes *et al*. (2000) investigated in-vitro activity of 24 quinolone compounds against *Toxoplasma gondii*, and Mahmoudi *et al*. (2003) investigated activity of 25 quinolones against erythrocytic stages of *Plasmodium falciparum*. The fluoroquinolones showed appreciable results against apicomplexan organisms. On the other hand, Omar *et al*. (2016) documented in-vitro activity of enoxacin on *Babesia* and *Theileria* organisms and observed a promising outcome.

Enoxacin and levofloxacin are primarily used as antibacterial agents. But, researcher has tested these drugs against apicomplexan organisms (Gozalbes *et al*., 2000; Mahmoudi *et al*., 2003) as well as *Babesia* and *Theileria* (Omar *et al*., 2016). In the present study both of these drugs were tested against *T. annulata* in in-vitro. The current investigation found that levofloxacin (10,084 µM) was more effective against *T. annulata* than enoxacin (1,71,723 µM) in terms of IC_{50} value. The higher IC_{50} value of enoxacin may be due to stimulating cells rather than inhibiting the growth of organisms. The uncommon findings were due to the transformation of host cells by *T. annulata*, which induces cell division and produces a neoplastic phenotype, the most significant mechanisms of the pathogen (Liu *et al*., 2020). Tayebwa *et al*. (2018) investigated nitidine chloride and camptothecin, targeting topoisomerase enzyme, on the growth of *Babesia* and *Theileria* parasites. They reported IC_{50} values of 2.05 ± 0.4 µM and 0.33 ± 0.02 µM for nitidine chloride and camptothecin, respectively. The findings of their study showed an IC_{50} value much lower than that of enoxacin and levofloxacin values. Prasanna *et al*. (2022) selected MMV000062 and MMV560185 compounds out of 400 based on their IC_{50} value of 2.97 µM and 3.07 µM against *T. annulata*. They also reported that the compounds were non toxic to BoMac cells with CC_{50} of 34 µM and >100 µM, respectively, and suggested further development of the drug for chemotherapeutic use. The findings of the present study did not agree with the results of Prasanna *et al*. (2022). Omar *et al*. (2016) who studied enoxacin against *T. equii* with the IC_{50} value of 24.2 µM which is many fold lower than the result of the present study.

Titus *et al*. (2023) evaluated in-vitro efficacy of two phytochemical compounds i.e., plumbagin and thymol against *T. annulata* and found IC_{50} values of 0.019 µM and 0.009 µM, respectively which were too low compared to results of the present study. In this study, topoisomerase II pathway has been targeted for evaluating the in-vitro drug efficacy. The topoisomerase II in *T. annulata* is different from other apicomplexan parasites like *Plasmodium*. Fluoroquinolones, although has delayed effect, can efficiently inhibit the topoisomerase II (Lizundia *et al*., 2009). The experiment was conducted for short time. This is the probable reason for low effectiveness of the selected drugs.

In the cytotoxicity assay, enoxacin (10,529 µM) had a greater 50 lethal dosage (CC_{50}) than levofloxacin (10,116 µM), indicating that enoxacin was less harmful to healthy cells than levofloxacin. However, according to the HC_{50} value, the hemolytic assay showed that enoxacin was less detrimental to red blood cells than levofloxacin. The antimalarial activities of enoxacin and levofloxacin, with IC_{50} values of 38.8 ± 0.3 µg/ml and 111.6 ± 21.5 µg/ml, respectively, were studied by Mahmoudi *et al*. (2003). According to Valianatos *et al*. (2017), normal cells were cytotoxically affected by enoxacin at doses between 78 and 312 µM. The results of this study do not match the findings of Valianatos *et al*. (2017). The results of using levofloxacin suggest that the active concentrations of the compound fall within the range of its toxic effects, thereby limiting its potential value for treating tropical theileriosis.

The effectiveness of these drugs may vary depending on factors like the species strains of *Theileria annulata*, concentration, and in-vitro exposure time. Additional studies are required to determine their efficacy and safety profile, as well as their potentials in clinical or veterinary settings.

The present study investigated the inhibitory, cytotoxic, and hemolytic activity of enoxacin and levofloxacin against *T. annulata*. Both compounds exhibited very low potential against *T. annulata* infecting mononuclear cells; levofloxacin showed lower activivity than enoxacin. Both drugs had relatively similar cytotoxicity on PBMCs. Enoxacin was safe on red blood cells compared to levofloxacin. Topoisomerase II can be further investigated for new drug discovery against *T. annulata*.

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Conflict of interest

The authors declare no competing interests.

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