

Article

Effects of Methanolic Extract from Turmeric (*Curcuma longa*) against the In Vitro Multiplication of Several *Babesia* Species and *Theileria equi*

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Abstract: Anti-piroplasm drugs currently on the market have proven toxicity to the host and parasite resistance. Plants are possible sources of novel drugs. Subsequently, a novel strategy should be used to find new anti-piroplasm agents that are both effective and safe. In the present study, we have evaluated the effect of turmeric (*Curcuma longa*) methanolic extract on the in vitro growth of *Babesia* (*B.*) *bovis*, *B. divergens*, *B. caballi*, and *Theileria* (*T.*) *equi*. The in vitro inhibitory effectiveness of turmeric was assessed using a fluorescence test. The enhancement in the in vitro inhibitory efficacy of turmeric when administrated in combination with diminazene aceturate (DA) was investigated using in vitro cultures of different piroplasm parasites. Turmeric reduced the in vitro growth of *B. bovis*, *B. divergens*, *T. equi*, and *B. caballi* with IC₅₀ values of 0.830 0.078, 0.375 0.055, 1.405 0.575, and 0.720 0.090 mg/mL, respectively. An amount of 1 mg/mL turmeric for *B. bovis*, 0.5 mg/mL turmeric for *B. divergens*, 1 mg/mL turmeric for *T. equi*, and 0.5 mg/mL turmeric for *B. caballi* exhibited 73.43%, 80.065%, 73.47%, and 47.375% inhibitions in the growth of the parasites, respectively. When turmeric was combined with DA, its in vitro inhibitory impact on bovine *Babesia* and equine *Babesia/Theileria* parasites was amplified. These findings show that a methanolic extract of turmeric could be a promising medicinal plant for the treatment of babesiosis, especially when administered in conjunction with DA.

Keywords: *Babesia*; *Theileria*; turmeric; *Curcuma longa*; in vitro; combination therapy



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1. Introduction

Babesia and *Theileria* are tick-transmitted parasites, which result in considerable economic losses in the livestock industry and worldwide animal commerce. Symptoms of the disorder include fever, malaise, jaundice, hemoglobinuria, and mortality [1]. The most prevalent causative agents of the infection in cattle are *Babesia* (*B.*) *bovis*, *B. bigemina*, and *B. divergens*, which result in a significant reduction in animal productivity [1]. The most common causes of equine disease are *Theileria equi* and *B. caballi* [2].

To date, newly developed anti-piroplasm drugs such as endocrine-like quinolones (ELQ)-300 and ELQ-316 [3], tulathromycin [4], fluoroquinolone [5], clofazimine [6], and Medicines for Malaria Venture compounds from the malaria box [1,7] are not available for use in the veterinary market [8]. Moreover, anti-piroplasm drugs currently on the market have a proven toxic effect to the host associated with imidocarb dipropionate and parasite resistance associated with diminazene aceturate (DA) in the treated parasite [9]. Therefore, improving the efficacy and safety of anti-piroplasm medicines has become a primary priority. Natural phytochemicals may be a potential alternative in this scenario.

Turmeric (*Curcuma longa*) is a bright yellow-colored spice that has been used for many years in cooking, cosmetics, dye, and medicinal cures all over the world [10]. Turmeric contains anti-inflammatory, antibacterial, antioxidant, and anti-neoplastic effects according to [10]. The major active ingredient of turmeric is curcumin, which has exhibited a potent and wide range of anti-parasitic effects including anti-malarial [11], anti-schistosomal [12], and anti-cryptosporidial [13]. There has been no previous research on the anti-piroplasm properties of turmeric extracts. Therefore, in the current study, we examined the use of turmeric as an anti-piroplasm alternative against the in vitro development of *Babesia/Theileria* species in both bovine and equine animals.

2. Results

2.1. Turmeric Inhibits the Development of *Babesia* and *Theileria* In Vitro

According to the computed IC_{50} s, the best inhibitory efficacy of turmeric was determined against the development of *B. divergens*, *B. caballi*, *B. bovis*, and *T. equi* parasitemia in vitro (Table 1). In a dose-dependent manner, the anti-babesial medication DA inhibited the growth of *B. bovis*, *B. divergens*, *T. equi*, and *B. caballi* (Table A1). An amount of 1 mg/mL turmeric inhibited the development of *B. bovis* and *T. equi* ($p < 0.05$) (Figures 1A and 2A). Furthermore, the parasitemia of *B. divergens* and *B. caballi* was considerably decreased ($p < 0.05$) by 0.5 mg/mL of turmeric (Figures 1B and 2B).

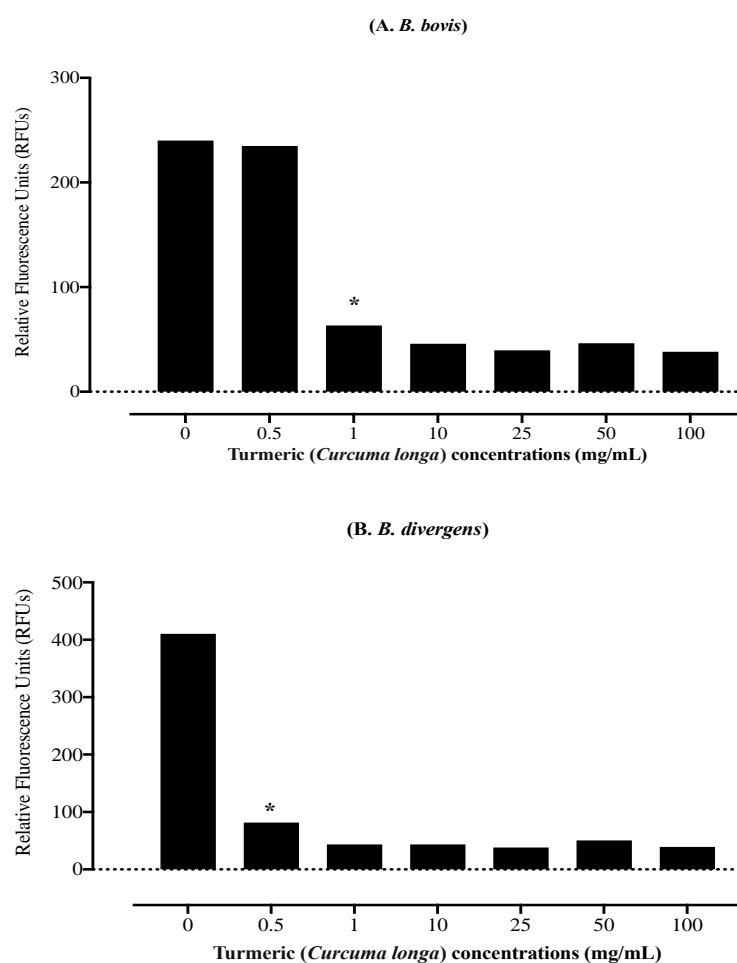


Figure 1. Inhibitory effect of turmeric (*Curcuma longa*) on bovine babesiosis after four days of treatment. (A) *B. bovis*. (B) *B. bigemina*. Each value represents the mean \pm standard deviation of three independent trials. Asterisks show a significant difference ($p < 0.05$) between the treated and the control cultures. Standard deviations were calculated based on biological replicates.

Table 1. IC₅₀ values of turmeric (*Curcuma longa*) against different piroplasm parasites.

Organism	IC ₅₀ (mg/mL) ^a
<i>B. bovis</i>	0.830 ± 0.078
<i>B. divergens</i>	0.375 ± 0.055
<i>T. equi</i>	1.405 ± 0.575
<i>B. caballim</i>	0.720 ± 0.090

^a IC₅₀ represent the mean ± standard deviation of three different experiments.

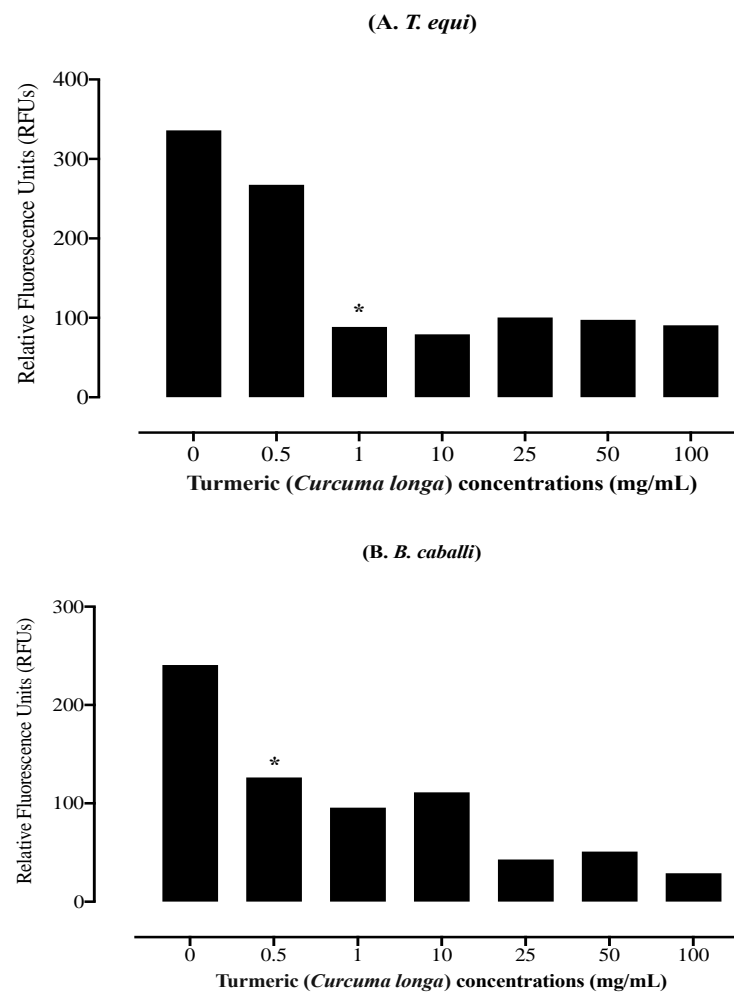


Figure 2. Inhibitory effect of turmeric (*Curcuma longa*) on equine piroplasm parasites after four days of treatment. (A) *T. equi*. (B) *B. caballi*. Each value represents the mean ± standard deviation of three independent trials. Asterisks show a significant difference ($p < 0.05$) between the treated and the control cultures. Standard deviations were calculated based on biological replicates.

In the resultant viability test at a dosage of 10 mg/mL turmeric, the regrowth of *B. bovis*, *B. caballi*, and *T. equi* was suppressed (Table 2). The treatment of *B. divergens* in vitro with 1 mg/mL turmeric reduced parasite regrowth (Table 2). At 0.25 mg/mL, DA stopped the regrowth of the screened piroplasm parasites (Table 3). The lack of a significant difference ($p > 0.05$) between the DMSO-treated positive control well and the untreated wells demonstrated that DMSO had no effect on the efficacy of the turmeric methanolic extract. The pretreatment of erythrocytes with a high concentration of turmeric methanolic extract (100 mg/mL) showed no influence on both parasite growth and the morphology of red blood cells (RBCs) when compared with non-treated erythrocytes using a light microscope (data not shown).

Table 2. Turmeric (*Curcuma longa*) viability test results for piroplasm parasites.

Parasite	Drug Concentrations (mg/mL) ^a					
	0.5	1.0	10	25	50	100
<i>B. bovis</i>	+	+	-	-	-	-
<i>B. divergens</i>	+	-	-	-	-	-
<i>T. equi</i>	+	+	-	-	-	-
<i>B. caballi</i>	+	+	-	-	-	-

^a The obtained values were determined depending on the emitted fluorescence signals in three distinct experiments with triplicate trials of each drug concentration. + indicates the parasite was alive; - indicates the parasite was dead.

Table 3. Effect of diminazene aceturate on *Babesia* and *Theileria* viability in vitro.

Drug	Drug Concentrations (μM) ^a				
	10	5	1	0.5	0.25
<i>B. bovis</i>	-	-	-	-	-
<i>B. divergens</i>	-	-	-	-	-
<i>T. equi</i>	-	-	-	-	-
<i>B. caballi</i>	-	-	-	-	-

^a Distinct experiments were used to calculate each value. In each experiment, each drug concentration was made in triplicate. - indicates the parasite was dead.

2.2. DA Improves the In Vitro Efficacy of Turmeric Methanolic Extract

On piroplasm parasites, different combinations of turmeric and DA were tested. At M5 (1/4 turmeric:3/4 DA) and M6 (1/4 turmeric:1/2 DA), respectively, the turmeric/DA combination inhibited the development of *B. bovis* and *T. equi* more effectively than DA alone (Table 4). Even at M8, which contained 1/8 IC₅₀ turmeric and 1/2 IC₅₀ DA, turmeric/DA induced a substantial suppression ($p < 0.05$) in the in vitro development of *B. caballi* compared with DA alone (Table 4). In the *B. divergens* culture, turmeric/DA had the lowest in vitro inhibitory effectiveness (Table 4). Such findings validated the anti-piroplasm capability of turmeric, especially when used with DA in lower doses.

Table 4. In vitro inhibitory effect of turmeric (*Curcuma longa*)/diminazene aceturate combinations on piroplasm parasites.

Group	The Emitted Fluorescence Signal (Mean ± SD)			
	<i>B. bovis</i>	<i>B. divergens</i>	<i>T. equi</i>	<i>B. caballi</i>
Control	271.11 ± 8.22	235.13 ± 10.23	331.15 ± 14.18	288.11 ± 6.75
DA IC ₅₀	123.01 ± 3.20	121.11 ± 8.09	153.21 ± 6.70	120.03 ± 4.98
M1 ($\frac{3}{4}:\frac{3}{4}$)	20.33 ± 3.11 **	15.71 ± 3.11 **	11.11 ± 2.02 **	51.11 ± 5.76 **
M2 ($\frac{3}{4}:\frac{1}{2}$)	37.22 ± 5.33 **	20.98 ± 5.34 **	17.14 ± 5.35 **	45.81 ± 6.11 **
M3 ($\frac{1}{2}:\frac{3}{4}$)	44.33 ± 3.21 **	143.66 ± 10.13 *	15.66 ± 5.32 **	51.02 ± 4.32 **
M4 ($\frac{1}{2}:\frac{1}{2}$)	42.28 ± 3.87 **	155.17 ± 11.34 *	19.18 ± 3.76 **	76.12 ± 7.35 **
M5 ($\frac{1}{4}:\frac{3}{4}$)	80.51 ± 4.99 **	141.33 ± 7.43 *	25.22 ± 5.88 **	81.97 ± 8.61 **
M6 ($\frac{1}{4}:\frac{1}{2}$)	185.48 ± 11.85 *	144.13 ± 6.43 *	31.33 ± 6.45 **	87.14 ± 4.32 **
M7 (1/8: $\frac{3}{4}$)	172.33 ± 8.09 *	159.12 ± 12.35 *	156.29 ± 8.21 *	94.11 ± 8.24 **
M8 (1/8: $\frac{1}{2}$)	198.32 ± 11.02 *	162.15 ± 11.29 *	166.55 ± 9.91 *	95.09 ± 4.51 **

* Statistically significant differences between the turmeric/DA-treated and control groups when $p < 0.05$ was used.

** Differences between the turmeric/DA-treated group and both the DA and control groups were statistically significant at $p < 0.05$. The turmeric/DA combinations are referred to as M1–8. DA: diminazene aceturate.

3. Discussion

The effect of turmeric on the growth of *B. bovis*, *B. divergens*, *T. equi*, and *B. caballi* in vitro was investigated in this study. Turmeric has a higher IC₅₀ for *Babesia* and *Theileria* parasites than allicin [14], fusidic acid [15], and thymoquinone [16], which were recently tested herbal therapies. Turmeric was less effective against *T. equi* than against *Babesia*. In general, several characteristics associated with the screening parasite such as the parasite species, strain, and size have an impact on the therapeutic efficacy of the tested drug [1,8]. In vitro culture parameters such as the utilized medium, hematocrit (HCT), and the presence or absence of serum also influence the calculated IC₅₀s of the tested medication [17,18]. As a result, differences in parasite species or culture conditions between different *Babesia* species might explain the high IC₅₀ value of turmeric against the in vitro growth of *T. equi*. A very high dose of turmeric had no effect on bovine or horse RBCs in the current study. This evidence reinforces the non-toxic qualities of turmeric. The problem reveals the safety of turmeric in in vitro cultures and recommends that more research into the inhibitory action of this prospective piroplasm candidate in vivo is needed.

The inhibitory effects of turmeric were amplified when coupled with DA, results similar to those previously observed in myrrh oil/DA [19], allicin/DA [14], and TQ/DA [16] combinations. The potential of turmeric methanolic extract to overcome toxicity and parasite resistance to the regularly used anti-babesial medication DA is highlighted by these studies. Future research is needed to validate the anti-babesial efficacy of a turmeric/DA combination in mice infected with *B. microti*.

Although this study evaluated the inhibitory efficacy of turmeric against the in vitro growth of several piroplasm parasites, the evaluation was performed at day 4 of the treatment only with a neglect evaluation of the inhibitory effect on other days of cultures (day 0 to day 3). We were not able to evaluate the kinetics of the drug effect. Therefore, additional future experiments are required to evaluate the used extract kinetics with a determination of the pharmacokinetics to predict the temporal pattern of the selected drug pharmacologic effect(s), including the maximum intensity and duration of action.

Before clinical studies are conducted in the field, potential new anti-babesial medicines are frequently tested on mice. In this regard, the route of the administration of a drug affects its in vivo inhibitory efficacy [16]. Generally, intraperitoneal drug administration exhibits the highest absorption rate followed by intramuscular, subcutaneous, and oral methods [20]. As a result, more research is needed to prove the anti-babesial efficacy of turmeric in an experimental animal model.

In conclusion, *B. divergens*, *B. caballi*, and *B. bovis* were the parasites most susceptible to the in vitro inhibitory activity of turmeric methanolic extract. In vitro treatment of *B. divergens* cultures with 1 mg/mL turmeric prevented parasite regrowth. Turmeric/DA exhibited a significant inhibition ($p < 0.05$) in the in vitro growth of *B. caballi* compared with those caused by DA monotherapy even at very low concentrations consisting of 1/8 turmeric and $\frac{1}{2}$ DA. These data suggest that a methanolic extract of turmeric could be a promising phytochemical agent for the treatment of piroplasmosis, particularly when given at a low dose of DA.

4. Materials and Methods

4.1. Chemical Reagents

SYBR Green I (SGI) (Lonza, Rockland, ME, USA; 10,000×) was used. A lysis buffer consisting of tris (130 mM; pH 7.5), ethylenediaminetetraacetic acid (EDTA) (10 mM), saponin (0.016%; w/v), and Triton X-100 (1.6%; v/v) was produced in advance and stored at 4 °C. Turmeric powder was purchased from iherb.com. The methanol was 99.8% pure (Wako Pure Chemical Industries, Ltd., Osaka, Japan). We made a stock methanolic extract of turmeric solution by dissolving 100 mg (crude extract) in 1 mL of DMSO (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The DA was purchased from Ganaseg (Ciba-Geigy Japan Ltd., Tokyo, Japan).

4.2. Turmeric Methanolic Extract Preparation

Turmeric powder was prepared in methanol (10 g/50 mL) and incubated at 30 °C for 3 days. After that, a Whatman filter paper No. 1 was used to filter the final product. The resultant extract was concentrated and lyophilized as previously described [21–23].

4.3. Determination of the Toxic Effect of Turmeric Methanolic Extract on Host Erythrocytes

As previously reported [19], the toxicity of turmeric to host RBCs was studied. Bovine and equine RBCs were pretreated for 3 h at 37 °C with either the media alone or media containing 25 mg/mL turmeric methanolic extract. Using drug-free media, a triple wash cycle of RBCs was then performed followed by the cultivation of *Babesia* parasites in the washed RBCs for 72 h. The growth of the parasite was evaluated in both pretreated and control untreated cells. Each medicine concentration was tested in three wells and three different trials for each parasite species.

4.4. In Vitro Growth Inhibition Assay

The inhibitory efficacy of turmeric against *B. bovis* (Texas strain) [17,24], *B. divergens* (German strain) [18], *B. caballi* [2,25], and *T. equi* (US Department of Agriculture) [2,26] (Figure 3) was investigated. Species-specific red blood cells were used to cultivate the parasites [17,19]. A fluorescence assay and SGI stain were used to investigate the efficacy of turmeric to prevent *Babesia/Theileria* proliferation [8,17]. In two 96-well plates (Nunc, Roskilde, Denmark), pRBCs were grown with different *Babesia* species using either the medium only or the medium mixed with different turmeric doses (0.5 to 100 mg/mL). To determine the concentrations, a preliminary examination was conducted. The positive control cultures had DA concentrations ranging from 0.25 to 10 g/mL. Cultures containing the solvent (0.3% DMSO for turmeric or 0.02% DDW for DA) mixed with the medium were used as negative control. RBCs parasitized with bovine and equine *Babesia/Theileria* parasites were cultured in 96-well plates at 1% parasitemia using 2.5% HCT for *B. bovis* and 5% HCT for other *Babesia* and *Theileria* parasites for 4 days. After that, the IC₅₀ values were calculated after adding the SGI lysis buffer to each drug concentration.

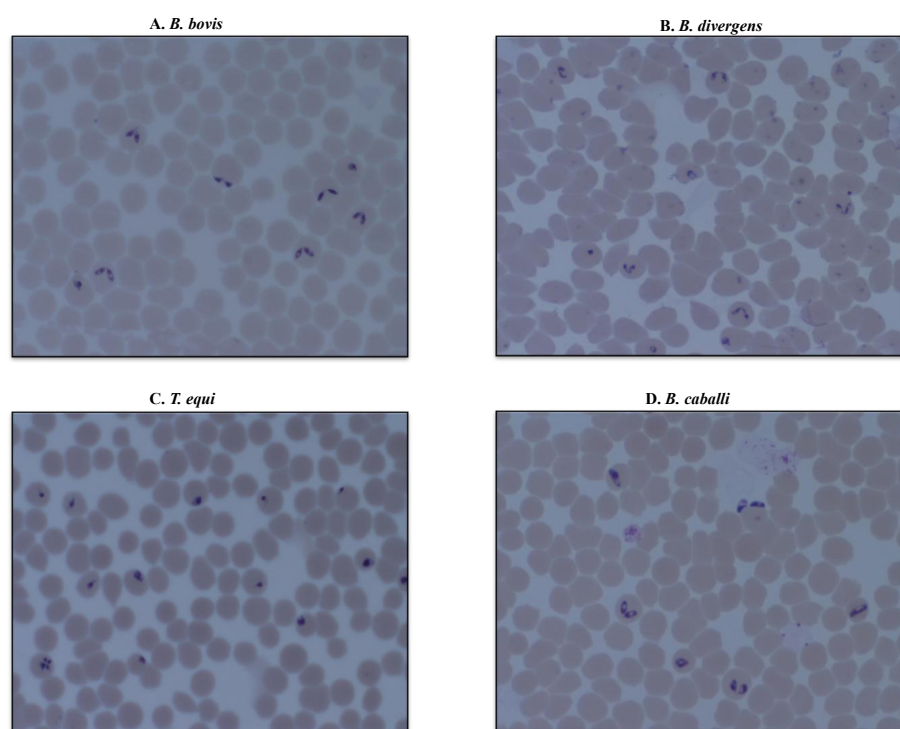


Figure 3. Microscopy of the control cultures for the screened piroplasm parasites. (A). *Babesia bovis*. (B). *Babesia divergens*. (C). *Theileria equi*. (D). *Babesia caballi*.

4.5. Viability Test

After four days of treatment, the vitality of bovine *Babesia* and equine *Babesia* and *Theileria* parasites was tested on the second plate as previously described [18]. In the culture with 2.5% and 5% HCTs, fresh RBCs were mixed with parasitized RBCs (pRBCs) from the control or drug-treated well on day 4 of the culture therapy and pRBCs were suspended in drug-free growth media. All plates were then maintained at 37 °C for 4 days (Figure 4). Each experiment was carried out three times.

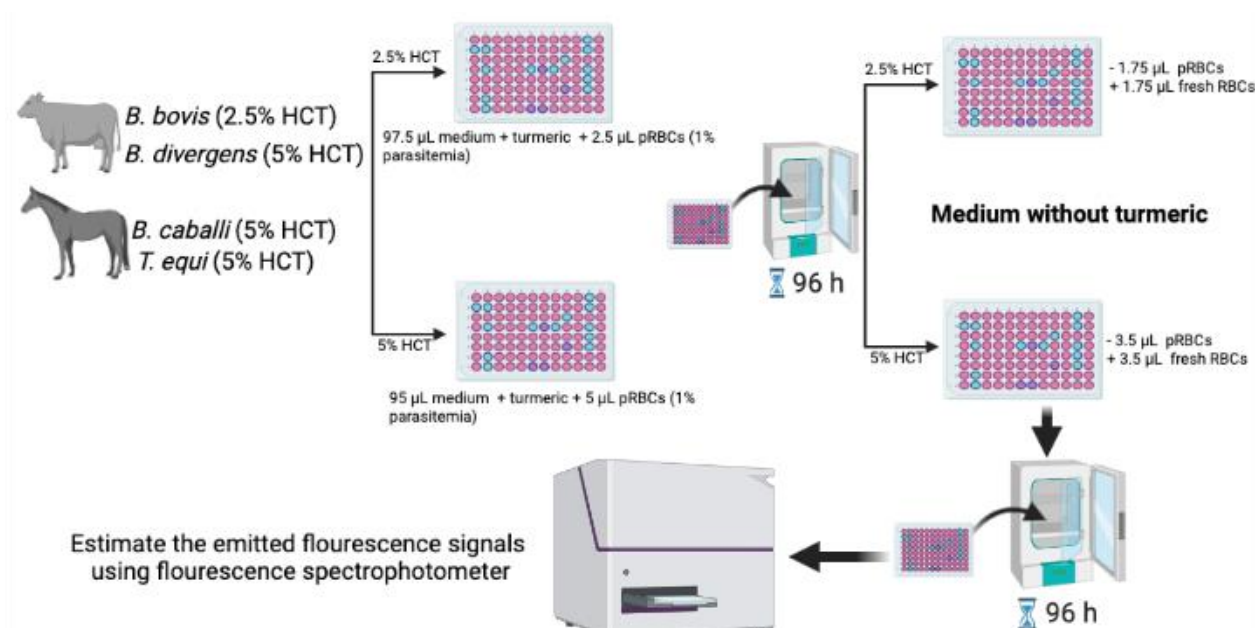


Figure 4. Schematic representation of the viability assay for anti-piroplasm drug screening in vitro. After four days of treatment, the control and drug-containing medium were removed from all wells. In a 2.5% HCT culture, 1.75 µL of the control or drug-treated infected RBCs were replaced with 1.75 µL fresh RBCs. In 5% HCT, 3.5 µL each of the control and drug-treated infected RBCs were replaced with 3.5 µL of fresh RBCs. After that, infected RBCs were suspended in drug-free growth media. The plates were then maintained for 4 days. pRBCs: parasitized RBCs. The Figure was generated using BioRender (for the interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article).

4.6. The Combination of Turmeric and Anti-babesial Drugs In Vitro

In vitro cultures of *B. bovis*, *B. divergens*, *T. equi*, and *B. caballi* were treated with a combination of turmeric and the commonly used anti-piroplasm medication DA. The turmeric/DA (M1–M8) combination proportions were built up as recently described [16,19]. The IC₅₀ was used to determine the combination ratios. As a negative control, drug-free cultures were used. Cultures harboring solely the DA IC₅₀ of the parasite served as positive pharmacological controls. Three separate trials were carried out, each with three triplicate experiments using a four-day drug combination containing 5% HCT. After applying a lysis buffer to each medicine combination on the 96-well plate on day 4 of the growth, the fluorescence levels were calculated.

4.7. Statistical Analysis

GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) was used to determine the significant differences ($p < 0.05$) between the investigated groups using a one-way ANOVA test.

Author Contributions: Conceptualization: M.A.R., S.A.E.-S.E.-S., I.I. Data curation: M.A.R. Formal analysis: M.A.R., S.A.E.-S.E.-S. Funding acquisition: M.A.R., I.I. Investigation: M.A.R., I.I. Methodology: M.A.R., S.A.E.-S.E.-S. Project administration: I.I. Resources: M.A.R., I.I. Software:

M.A.R. Supervision: I.I. Validation: M.A.R., I.I. Visualization: M.A.R., S.A.E.-S.E.-S., I.I. Writing—original draft: M.A.R., S.A.E.-S.E.-S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The Animal Care and Use Committee at the Obihiro University of Agriculture and Veterinary Medicine approved all of the study's experimental protocols (Approval No. 27-65). The pathogen experiment's IDs were as follows: bovine *Babesia*: 201708-4; equine piroplasma parasites: 201910-2.

Informed Consent Statement: Not applicable.

Data Availability Statement: On reasonable request, the corresponding author will provide the datasets created and/or analyzed during the current work.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. IC₅₀ values of diminazene aceturate against different piroplasm parasites.

Drug	IC ₅₀ Values (µg/mL) ^a
	DA
<i>B. bovis</i>	0.16 ± 0.02
<i>B. divergens</i>	0.08 ± 0.003
<i>T. equi</i>	0.28 ± 0.01
<i>B. caballi</i>	0.012 ± 0.003

^a The final obtained IC₅₀s were the mean ± SD of three independent experiments.

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