

# *In vitro* antiparasitic effects of six beverages on the growth of *Babesia* and *Theileria* parasites

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## Introduction

Blood parasites, of the red blood cells of genera *Babesia* and *Theileria*, hinder the livestock industry and trade of horses worldwide. The losses in the profit are due to the treatment costs or the death of the animals. Several species are implicated as the cause of diseases such as *Babesia bovis*, *Babesia bigemina*, *Babesia divergens*, *Babesia caballi*, and *Theileria equi* [1, 2]. Hard tick is the transmitter of the parasites either by trans-ovarial or trans-stadial methods [2]. The infection has clinical signs including hemoglobinuria, anemia, icterus, fever, and death [1, 2]. There is no existing vaccine [3] and the commercially available drugs are toxic [4]. Therefore, new natural products should be evaluated as a substitution of the chemical drugs.

Natural products from green tea [5] and other plants [6, 7] were tested for babesiosis *in vitro* and *in vivo*. Human drank natural beverages for centuries. Their beneficial effects were attributed to their content of antioxidant polyphenolic compounds. Green tea was known as a beneficial beverage for health. It contains catechins and polyphenols such as (-)-Epigallocatechin-3-gallate (EGCG).

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## ABSTRACT

**Introduction:** To evaluate the inhibitory effects of the decoction of green tea, black tea, instant coffee, hibiscus, cinnamon, and peppermint on the *in vitro* growth of *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, *Babesia divergens*, and *Theileria equi*.

**Method:** *In vitro* inhibition assay of the decoction of green tea, black tea, instant coffee, hibiscus, cinnamon, and peppermint was used for *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, *Babesia divergens*, and *Theileria equi*. Viability test was applied after treatment of the cultures with beverages.

**Results:** In the present study, the six beverages significantly inhibit the *in vitro* growth of the four *Babesia* species and *T. equi*. The IC<sub>50</sub> values of green tea were 3.83, 6.25, 2.2, 5.3, and 1.8 % (v/v) for *Babesia bovis*, *Babesia bigemina*, *Babesia divergens*, *Babesia caballi*, and *Theileria equi*, respectively. The IC<sub>50</sub> values of black tea were 3.8, 10, 2.92, 12.6, and 1.9 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. The IC<sub>50</sub> values of instant coffee were 0.9, 0.5, 0.42, 4.5, and 0.75 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. The IC<sub>50</sub> values of hibiscus were 16.5, 26.5, 5, 7.9, and 14.1 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. The IC<sub>50</sub> values of cinnamon were 7.83, 19, 5.9, 12.1, and 6% (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. The IC<sub>50</sub> values of peppermint were 26.4, 27.5, 7.6, 26.8, and 8.54 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively.

**Conclusion:** The present study presented the health benefits of these natural beverages as antiprotozoal agents *in vitro*. Further study for their *in vivo* evaluation is needed.

## KEY WORDS:

Beverages  
*In vitro*  
*Babesia*,  
*Theileria equi*

Green tea has antioxidant [8], anticoccidial [9], anti-toxoplasma [10], antileishmanial [11], and anti-molluscan [12] activities. Black tea also has similar contents of polyphenols and has antibacterial [13], anti-inflammatory [14, 15] and antitrypanosomal [14] activities. Cinnamon contains

the active compounds such as cinnamaldehyde and cinnamic acid. It has antitumor [16], antidiabetic [17], insecticidal and fumigant [18], antimicrobial [19, 20], anthelmintic [21], and antiprotozoal [22] effects. Hibiscus rosa-sinensis has active compounds for instance quercetin, cyanidin, calcium oxalate, hentriacontane, thiamine, niacin, riboflavin, and ascorbic acids. Hibiscus has hypotensive [23], antioxidant [24], hepatoprotective [25], anti-atherosclerotic [26], anti-inflammatory [27], anti-filarial [28, 29] activities. Peppermint contains vitamin B complex, vitamin C, beta carotene, potassium, flavonoids, volatile oils (menthol, menthene, methyl acetate, limonene, pulegone) menthone, tannins, resin, and rosmarinic acid [30]. It has repellent [31, 32], insecticidal [33, 34], antimicrobial [35], anthelmintic [36, 37], and anti-giardia [38] activities. Instant coffee contains the active principals such as caffeic acid and caffeine. It has anticancer [39], antidiabetic [39], antioxidant [40], antitussive [41], anti-inflammatory [42], immunomodulatory [41, 43], and antiviral [44] effects. Although these beverages are beneficial for health their direct effect on *Babesia* species and *T. equi* is unknown. Therefore, the study aimed to evaluate the inhibitory effects of these beverages on the *in vitro* growth of four *Babesia* species and *T. equi*.

## Materials and methods

### Chemical reagents

The chemicals used in this study were Japanese green tea (ITO EN Ltd., Tokyo, Japan), Sri Lankan black tea (Ahmed Tea Pvt Ltd., Colombo, Sri Lanka), instant coffee (Nescafe classic, Nestlé Japan Ltd., Kobe, Japan), Egyptian hibiscus (*Hibiscus rosa*) (Family Pharmacia, Cairo, Egypt), Egyptian cinnamon (Family Pharmacia, Cairo, Egypt), and Egyptian peppermint (Family Pharmacia, Cairo, Egypt). The stock was prepared by pouring 132 ml<sup>3</sup>, the volume of a drinking mug, of boiling water at 100 ° C on the 2-gram packet of each herb in a beaker and left for 5 minutes. Then sieved through filter paper and followed by filtration through 0.22 µm syringe filter (Merk Millipore Corp., Darmstadt, Germany). Then cooled to 37 ° C and immediately added to the cultures. SYBR Green I (SGI) nucleic acid stain (Lonza, NJ, USA; 10,000 x) was stored at -20°C and thawed before use. A red blood cells (RBCs) lysis

buffer was prepared and stored at 4°C as previously reported [45, 46]. Diminazene aceturate (Novartis, Tokyo, Japan) was prepared at 100 mM stock solution.

### Parasites

Tea decoction of Japanese green tea, Sri Lankan black tea, instant coffee, Egyptian hibiscus, Egyptian cinnamon, and Egyptian peppermint were evaluated for their chemotherapeutic effect against *B. bovis* (Texas strain) [47], *B. bigemina* (Argentina strain) [45], *B. divergens* (German strain) [46], and *B. caballi* [48] and *T. equi* [49](U.S. Department of Agriculture).

### *In vitro* culture of *Babesia* parasites

Bovine or equine RBCs were used in parasite cultures using a continuous micro-aerophilous stationary phase culture system [45]. The culture medium, M199 was used for *B. bovis*, *B. bigemina*, and *T. equi* (obtained from Sigma-Aldrich, Tokyo, Japan) and was supplemented with 40 % bovine or equine serum and 60 µg/ml of streptomycin, 60 U/ml of penicillin G, and 0.15 µg/ml of amphotericin B (Sigma-Aldrich). Hypoxanthine (ICN Biomedicals, Inc., Aurora, OH) is a vital supplement for *T. equi* was added at 13.6 mg/ml to the culture. RPMI 1640 medium was supplemented with antibiotics, amphotericin B, and either with 40 % horse serum, for *B. caballi* [5] or 10 % fetal calf serum for *B. divergens*.

### *In vitro* growth inhibition assay

The *in vitro* growth inhibition assay was done as previously reported [45, 46]. *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* were gained from cultures with a parasitemia of 10 %, which was diluted with appropriate fresh erythrocytes to a parasitemia of 1 % for the assay. The growth inhibition assay was done in 96-well plates containing HCT values of 2.5 % for *B. bovis* and *B. bigemina* and 5 % of *B. caballi*, *B. divergens*, and *T. equi* packed erythrocyte inoculum and 100 µL of M199 or RPMI640 supplemented with either 40 % horse or bovine or 10 % fetal calf sera. Culture media contained 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 30, 40 µL of Japanese green tea, Sri Lankan black tea, instant coffee, Egyptian cinnamon, and Egyptian peppermint and 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 25, 30, 40 µL of Egyptian hibiscus for *B. bovis*, *B. bigemina*, *B. divergens*,

*B. caballi*, and *T. equi*, respectively. Wells containing only medium either with fresh or infected RBCs were used as negative control. Furthermore, a negative DDW control was prepared with the concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 30, 40  $\mu$ L per well to determine the influence of water on the growth of the parasites. Diminazene aceturate was used at concentrations of 1, 5, 10, 25, 50, 100, 260, 500, 1000, and 2000 nM [7]. The experiments were carried out three times in triplicate. Cultures were incubated at 37 °C in an atmosphere of 5 % CO<sub>2</sub>, 5 % O<sub>2</sub>, and 90 % N<sub>2</sub> for a period of four days without change of the media. Parasitemia was examined on the fourth day by measuring parasites' DNA concentration by a fluorescence assay [45]. The 50 % inhibitory concentration (IC<sub>50</sub>) values were calculated as previously shown [45] by using Graph Pad Prism 5 (Graph Pad Software, CA).

### Viability test

Plates were prepared as for *in vitro* inhibition assay and incubated for 4 days without changing media. The medium was removed and infected RBCs were transferred to a new plate containing 100  $\mu$ l of the culture medium alone. The percentages of infected and fresh RBCs were 42.8 % and 57.2 % of total RBCs concentration, respectively. Plates were incubated for 5 days without changing media instead of 4 days used in the previous study [46].

### Statistical analysis

The differences in the percentage of parasitemia for the *in vitro* cultures were analyzed with JMP statistical software (SAS Institute, Inc., USA) using the student's *t*-test. A *P* value of < 0.05 was considered statistically significant.

## Results

### *In vitro* inhibition assay

Green tea significantly inhibited the growth (*P* < 0.05) at concentrations of 1, 2.5, 1, 2.5, and 1 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. Black tea significantly inhibited the growth at concentrations of 2.5, 5, 0.5, 10, and 2.5 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. Cinnamon tea significantly inhibited the growth at concentrations of 10, 5, 2.5, 10, and 5 % (v/v) for *B. bovis*,

*B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. Peppermint tea significantly inhibited the growth at concentrations of 0.5, 0.5, 5, 10, and 10 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. Hibiscus tea significantly inhibited the growth at concentrations of 10, 25, 2.5, 5, and 5 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. Instant coffee tea significantly inhibited the growth at concentrations of 0.5, 0.5, 0.1, 5, and 1 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively.

The IC<sub>50</sub> values of green tea were 3.83, 6.25, 2.2, 5.3, and 1.8 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively (Table. 1). The IC<sub>50</sub> values of black tea were 3.8, 10, 2.92, 12.6, and 1.9 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively (Table 1). The IC<sub>50</sub> values of instant coffee were 0.9, 0.5, 0.42, 4.5, and 0.75 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively (Table. 1). The IC<sub>50</sub> values of hibiscus were 16.5, 26.5, 5, 7.9, and 14.1 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively (Table. 1). The IC<sub>50</sub> values of cinnamon were 7.83, 19, 5.9, 12.1, and 6 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively (Table. 1). The IC<sub>50</sub> values of peppermint were 26.4, 27.5, 7.6, 26.8, and 8.54 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively (Table. 1). Diminazene aceturate IC<sub>50</sub> values were 14, 270, 190, 8, and 750 nM for *B. divergens*, *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*, respectively (Table. 1).

### Viability test

Parasites could not regrow at green tea concentrations of 10, 10, 10, 10, 20 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively (Table. 2). Parasites could not regrow at black tea concentrations of 20, 20, 10, 20, 10 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively (Table. 2). Parasites could not regrow at hibiscus tea concentrations of 30, 40, 20, 20, 20 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively (Table. 2).

**Table 1.** IC<sub>50</sub> values of green tea, black tea, peppermint tea, hibiscus rosa tea, cinnamon tea, and instant coffee for *B. divergens*, *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*

Parasite	IC <sub>50</sub> (% V/V)*						
	Green tea	Black tea	Peppermint tea	Hibiscus rosa tea	Cinnamon tea	Instant coffee	Diminazene <sup>‡</sup>
<i>B. divergens</i>	2.2	2.92	7.6	5	5.93	0.42	140
<i>B. bovis</i>	3.83	3.8	26.4	16.47	7.83	0.9	270
<i>B. bigemina</i>	6.25	10	27.5	26.47	19	0.5	190
<i>B. caballi</i>	5.3	12.6	26.8	7.9	12.17	4.5	8
<i>T. equi</i>	1.8	1.9	8.54	14.1	6	0.75	750

\*IC<sub>50</sub> values are in percent volume per volume and measured on day four of the experiment. Three experiment were conducted in triplicate for each tea.

<sup>‡</sup>IC<sub>50</sub> values of diminazene acetate are in nanomolar

Parasites could not regrow at peppermint tea concentrations of 40, 30, 30, 30, 20 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively (Table 2) Parasites could not regrow at cinnamon tea concentrations of 40, 30, 20, 40, 30 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. Parasites

could not regrow at instant coffee concentrations of 5, 2.5, 10, 10, 2.5 % (v/v) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively (Table. 2). In the DDW negative control plates, parasites' growth in DDW-treated wells was similar to the growth in the untreated wells.

**Table 2.** Viability of green tea, black tea, peppermint tea, hibiscus rosa tea, cinnamon tea, and instant coffee for *B. divergens*, *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*

Parasite	Viability Concentration (% v/v)*					
	Green tea	Black tea	Peppermint tea	Hibiscus tea	Cinnamon tea	Instant coffee
<i>B. divergens</i>	10	10	20	20	20	10
<i>B. bovis</i>	10	20	40	30	40	5
<i>B. bigemina</i>	10	20	30	40	30	2.5
<i>B. caballi</i>	10	20	30	20	40	10
<i>T. equi</i>	20	10	40	20	30	2.5

\*Viability after five days

## Discussion

The beverages repressed the growth of the parasites *in vitro* cultures. The double-distilled water had no influence on the growth of the parasites; therefore, the observed inhibition was due to the beverages. *T. equi* was the most susceptible for green tea and black tea. *B. divergens* was the most susceptible to instant coffee, hibiscus tea, and peppermint tea. In contrast, *T. equi* and *B. divergens* are similarly susceptible to cinnamon tea. Instant coffee had the highest inhibitory effect on the five parasites followed by green tea and black tea. The influence of green tea and black tea was nearly similar and this agreed with their results on *Trypanosoma brucei* [14]. Peppermint tea has the least outcome on the five parasites. Hibiscus inhibited the growth of the *Babesia* species and *T. equi* similar to its filaricidal effect

on *Setaria cervi* [28] although the preparation methods were different. Therefore, drinking these beverages might be of benefit as supportive treatment for parasitic infections.

The beverages inhibitory effects might be due to their content of active principles. Green tea and black tea contain catechins and polyphenols such as (-)-Epigallocatechin-3-gallate (EGCG). Cinnamon contains the active compounds such as cinnamaldehyde and cinnamic acid. Hibiscus *rosa-sinensis* contains quercetin, cyanidin, calcium oxalate, hentriacontane, thiamine, niacin, riboflavin, and ascorbic acids. Peppermint contains flavonoids, volatile oils (menthol, menthene, methyl acetate, limonene, pulegone) menthone, tannins, resin, and rosmarinic acid [30]. Instant coffee

contains the active principals such as caffeic acid and caffeine. Additional studies are necessary to evaluate extracts prepared by different methods, their active principles, and bioavailability for *Babesia* and *Theileria* parasites.

In conclusion, natural beverages inhibited the *in vitro* growth of four *Babesia* species and *T. equi*. Natural beverages had antiparasitic as well as beneficial antioxidants activities. Further research is necessary to evaluate the outcome of these plant extracts on the *in vivo* growth of *Babesia* and *Theileria* parasites.

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### Conflict of Interest

Authors declared that no conflict of interest.

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