Inhibitory effect of allicin on the growth of Babesia and Theileria equi parasites

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Inhibitory effect of allicin on the growth of *Babesia* and *Theileria equi* parasites

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**Abstract** Allicin is an active ingredient of garlic that has antibacterial, antifungal, antiviral, and antiprotozoal activity. However, the inhibitory effects of allicin on *Babesia* parasites have not yet been examined. In the present study, allicin was tested as a potent inhibitor against the in vitro growth of bovine and equine *Babesia* parasites and the in vivo growth of *Babesia microti* in a mouse model. The in vitro growth of *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, or *Theileria equi* was inhibited by allicin in a dose-dependent manner and had IC$_{50}$ values of 818, 675, 470, and 742 μM, respectively. Moreover, allicin significantly inhibited ($P<0.001$) invasion of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* into the host erythrocyte. Furthermore, mice treated with 30 mg/kg of allicin for 5 days significantly ($P<0.05$) reduced the parasitemia of *B. microti* over the period of the study. To further examine the potential synergism of allicin with diminazene aceturate, growth inhibitory assays were performed in vitro and in vivo. Interestingly, combinations of diminazene aceturate with allicin synergistically potentiated its inhibitory effects in vitro and in vivo. These results indicate that allicin might be beneficial for the treatment of babesiosis, particularly when used in combination with diminazene aceturate.

**Introduction**

Babesiosis is a parasitic infection caused by a hemotropic protozoa of the genus *Babesia* belonging to phylum Apicomplexa that infects wide range of warm-blooded mammals (Mehlhorn and Schein 1984). These parasites cause symptoms including fever, hemolysis, and hemoglobinuria and sometimes may lead to systemic shock ending in death (Homer et al. 2000). The infection is associated with economic losses for the livestock industry and has recently emerged as a zoonotic disease problem in some *Babesia* infections (Schuster 2002). *Babesia bovis* and *Babesia bigemina* are serious bovine *Babesia* parasites affecting cattle health and productivity (Uilenberg 1995). Additionally, *Babesia caballi* and *Babesia equi* [recently re-classified as *Theileria equi* “T. equi” (Mehlhorn and Schein 1998)] are the causative agents of equine piroplasmosis, which has gained importance by affecting racehorses (Schein 1988). *Babesia microti* mainly causes an infection in rodents as well as a relatively mild but persistent human babesiosis that has been served as a useful experimental model for analyzing animal babesiosis (AbouLaila et al. 2010a; Vannier et al. 2008). Although intensive efforts have been focused on vaccine development, to date, no vaccine has been commercialized, and chemotherapy is the main measure for control. However, the emergence of drug resistance and the toxicity of these drugs to the host (Bork et al. 2005; Vial and Gorenflo 2006) make promising the search for a new potent antibabesial chemotherapeutic agent. So far, several drugs have been tested in vitro and in experimental animals with promising results, but they are still not available for field use. For instance, epoxomicin, ciprofloxacin, thiostrepton, rifampicin.

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(AbouLaila et al. 2010a, 2012), triclosan, and clodinafop-propargyl (Bork et al. 2003a, b) suppressed the growth of parasites and exhibited potential for application. Natural plant products have been used to treat many diseases; of these, garlic’s active ingredients or allicin extracted from garlic showed inhibitory effects against protozoan diseases such as Entamoeba histolytica, Leishmania spp., Blastocystis hominis, and Entamoeba histolytica (Al-Quraishy et al. 2011; Sharma et al. 2009; Yakoob et al. 2011; Degerli et al. 2012). Allicin is the most abundant thiosulfinate in garlic; it is generated when the enzyme alliinase reacts with its substrate alliin in crushed garlic (Lawson 1996). Accumulating evidence indicates that thiosulfinates have the antibacterial, antifungal, and antiprotozoal properties of garlic (Reuter et al. 1996). Within this context, allicin possesses antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria; antifungal activity, particularly against Candida albicans; antiparasitic activity, including against Plasmodium species, Entamoeba histolytica, and Giardia lamblia; and antiviral activity (Ankri and Mirelman 1999).

Moreover, allicin significantly inhibits the sporozoite infectivity of Plasmodium berghei and Plasmodium yoelii in vivo and, consequently, decreases the parasite burden in mice by disrupting the invasion of parasites into the host hepatic cell (Coppi et al. 2006).

In the present study, the growth inhibitory effect of allicin was evaluated against B. bovis, B. bigemina, B. caballi, and T. equi in vitro as well as against B. microti in a mouse model. Results revealed that allicin has a potent effect on the growth of Babesia, most likely disturbing invasion into host erythrocytes. Combining the drug with diminazene aceturate might offer new strategies for babesiosis treatment.

Materials and methods

Parasites

The Texas strain of B. bovis (Hines et al. 1992), the Argentine strain of B. bigemina (Hotzel et al. 1997), the USDA strain of B. caballi (Avarzed et al. 1997), Theileria equi (Bork et al. 2004), and the Munich strain of B. microti (AbouLaila et al. 2012) were used in this study.

Culture conditions

The Babesia parasites used in this study were maintained in bovine or equine red blood cells (RBCs) using a microaerophilic stationary-phase culture system (Igarashi et al. 1994; AbouLaila et al. 2010a). Briefly, Medium 199 was used for B. bovis, B. bigemina, and T. equi, while RPMI-1640 was used for B. caballi (both from Sigma-Aldrich, Tokyo, Japan). Media were supplemented with 40 % normal bovine serum for bovine Babesia or 40 % normal equine serum for equine Babesia, and 60 U/ml of penicillin G, 60 μg/ml of streptomycin, and 0.15 μg/ml of amphotericin B (all three drugs from Sigma-Aldrich) were prepared and used in the culture media. Additionally, 13.6 μg of hypoxanthine (ICN Biomedicals Inc., Aurora, OH) per milliliter was added to the T. equi culture as a vital supplement.

Chemical reagents

Allicin was purchased from iHerb.com and used for examining babesicidal effects. A stock solution of 100 mM was dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industrial, Ltd., Osaka, Japan) and then stored at 4 °C until use. Ten millimolars of stock solution of diminazene aceturate (Ciba-Geigy Japan Limited, Tokyo, Japan) was prepared in distilled water and stored at −30 °C until use; it was used as a comparator drug in vitro and used in combination with allicin either in vivo or in vitro.

In vitro growth inhibition assay

The inhibitory effect of allicin on the growth of Babesia parasites was tested using an assay previously described (Bork et al. 2003a; AbouLaila et al. 2010a) with slight modification. Briefly, parasite-infected RBCs were diluted with uninfected RBCs to obtain 1 % parasitemia. From this stock, 20 μl was dispensed into a 96-well microtiter plate (Nunc, Roskilde, Denmark) with 200 μl of the culture medium containing the indicated concentration of allicin (5, 50, 200, 500, 1,000, and 4,000 μM) for B. bovis, (1, 100, 200, 500, 2,000, and 4,000 μM) for B. bigemina, (1, 10, 100, 500, 1,000, 4,000, and 5,000 μM) for B. caballi, and (5, 50, 100, 200, 500, 2,000, and 5,000 μM) for T. equi. Diminazene aceturate was used at concentrations of 5, 25, 50, 100, 500, 1,000, and 2,000 nM for all four parasites from the same cultures used for the allicin experiment and then incubated at 37 °C in a humidified multi-gas water-jacketed incubator. For experimental control, cultures without the drug and cultures containing only DMSO (0.06 %, for allicin) or distilled water (0.02 %, for diminazene aceturate) were prepared. Parasitemia was monitored daily by counting the parasitized RBCs to approximately 1,000 RBCs in Giemsa-stained thin blood smears. The IC_{50} values (50 % inhibitory concentration) for the two drugs upon growth of all parasites tested were calculated based on the parasitemia recorded on day 3 in the in vitro cell culture system using interpolation after the curve fitting technique (AbouLaila et al. 2010a).

In vitro drug combination test

Combination therapies of allicin and diminazene aceturate were tested in the in vitro cultures of B. bovis and B. caballi. Allicin/diminazene aceturate combinations (M1, M2, M3,
M4, M5, M6, M7, and M8) were prepared as previously described (AbouLaila et al. 2010a) with some modifications. Combinations were based on the calculated IC_{50} values obtained from the in vitro inhibition assay (Table 1). For experimental control, drug-free cultures were used. Cultures containing only diminazene aceturate IC_{50} of each parasite were used as positive drug controls. Three separate trials were performed, consisting of triplicate experiments for individual drug concentrations over a period of 4 days. During the incubation period, the overlaying culture medium was replaced daily with 200 μl of fresh medium containing the indicated concentrations of allicin. Parasitemias were monitored daily by counting the parasitized RBCs to approximately 1,000 RBCs in Giemsa-stained thin blood smears. The growth of all parasites was calculated based on the parasitemia observations recorded on 4 days of the in vitro cell culture system (AbouLaila et al. 2010a).

Viability test for in vitro growth inhibition assays

After 4 days of treatment, 6 μl of each of the control and drug-treated (at the various indicated concentrations) infected RBCs was mixed with 14 μl of parasite-free RBCs and suspended in fresh growth medium without allicin supplementation. The plates were incubated at 37 °C for the next 10 days. The culture medium was replaced daily, and parasite recrudescence was determined by light microscopy in order to assess the parasite viability (Bork et al. 2004).

Invasion inhibition assay

The assay was performed as described earlier (Gaffar et al. 2004), but with modification. Briefly, B. bovis-parasitized erythrocytes harvested at the peak parasitemia were pelleted, washed once, and resuspended in an equal volume of GIT (Wako). Merozoites were liberated by five intermittent high voltage pulses (1.25 kV, 200 Ω, 25 μF) with 10 s in ice between pulses in a 4-mm cuvette (Bio-Rad) using a Bio-Rad Gene Pulser with a pulse controller, and then they were resuspended in GIT. A similar method was used for B. bigemina, B. caballi, and T. equi. Merozoites of these infected RBCs were liberated by ten intermittent high voltage pulses (1.20 kV, 300 Ω, 25 μF) with 5 s in ice, and then they were resuspended in M199 for B. bigemina and T. equi and in GIT for B. caballi.

The liberated parasites were incubated at 37 °C in a humidified multi-gas water-jacketed incubator (90 % N₂, 5 % CO₂, and 5 % O₂) with 100 μM and 200 μM of allicin for 30 min. Bovine or equine erythrocytes were then added to final PCV of 10 % and incubated again at 37 °C in a humidified multi-gas water-jacketed incubator (90 % N₂, 5 % CO₂, and 5 % O₂) for 24 h. Giemsa-stained smears were prepared after 1, 3, and 6 h. Invaded and freed parasites were counted on the basis of approximately 3,000 observed erythrocytes. The incubation of merozoites and RBCs without drugs was used as a control.

| Table 1 | Concentrations of allicin combined with diminazene aceturate applied to the cultures of B. bovis and B. caballi |
|---------|--------------------------------------------------|--------------------------------------------------|
|         | B. bovis^{a}                                   | B. caballi^{a}                                   |
|         | Allicin (μM) Diminazene aceturate (nM)          | Allicin (μM) Diminazene aceturate (nM)          |
| M1      | 100 315                                        | 50 14.3                                         |
| M2      | 100 210                                        | 50 9.5                                         |
| M3      | 200 315                                        | 100 14.3                                        |
| M4      | 200 210                                        | 100 9.5                                         |
| M5      | 300 315                                        | 200 14.3                                        |
| M6      | 300 210                                        | 200 9.5                                         |
| M7      | 400 315                                        | 250 14.3                                        |
| M8      | 400 210                                        | 250 9.5                                         |

^{a} Combinations were based on the calculated IC_{50} values obtained from the in vitro inhibition assay
administered s/c and diminazene aceturate at a dosage of 6.75 mg/kg was administered s/c to all three previous groups as a combination drug for the same inoculation period; the fourth group was administrated 15 mg/kg of allicin combined with s/c administration of 12.5 mg/kg diminazene aceturate. The fifth and sixth groups were administrated diminazene aceturate at dosages of 6.75 and 25 mg/kg as control groups, and the last group was the DMSO control.

By checking, when the infected mouse parasitemia reached approximately 1% in all groups, mice were administrated daily injections of the specified drugs for 5 days from days 1 to 5 post-inoculation (p.i.). The levels of parasitemia in all mice were monitored daily until 21 days post-inoculation or cessation of parasitemia by examination of the Giemsa-stained thin blood smears prepared from venous tail blood. Chemicals were prepared by dissolving allicin in 0.06% DMSO and resuspended in 0.1 ml double-distilled water. Diminazene aceturate dissolved in 0.1 ml double-distilled water and DMSO was administrated to the control groups in 0.1 ml PBS in all experiments (AbouLaila et al. 2012). All animal experiments were conducted in accordance with the Standard Relating to the Care and Management of Experimental Animals set by the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

Statistical analysis

The differences in the percentage of parasitemia for the in vitro cultures, drug combination test, and in vivo inhibition assay were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA) using the independent Student’s t test and one-way ANOVA (AbouLaila et al. 2012).

Results

The in vitro growth inhibitory effect of allicin on Babesia and T. equi

Growth inhibition assays were performed on B. bovis, B. bigemina, B. caballi, and T. equi with different concentrations of allicin and diminazene aceturate as a reference drug. Notably, significant inhibition of the growth of parasites (P < 0.05) was observed at allicin concentrations of 50 μM for B. bovis (Fig. 1a), 1 μM for B. bigemina (Fig. 1b), 10 μM for B. caballi (Fig. 1c), and 100 μM for T. equi (Fig. 1d) on the third day of culture. However, high concentrations of allicin did not completely suppress the growth of parasites. No morphological changes were noted in allicin-treated Babesia parasites. On the other hand, diminazene aceturate showed significant inhibition at 10 nM for B. bigemina and B. caballi and at 50 nM for B. bovis and T. equi. Complete suppression of diminazene aceturate-treated parasites was observed at concentrations of 2 μM for B. bovis, 1 μM for B. bigemina, 0.5 μM for B. caballi, and 3 μM for T. equi (data not shown). The IC_{50} values of allicin were 818, 675, 470, and 742 μM, while the IC_{50} values of diminazene aceturate were 420, 65, 19, and 951 nM for B. bovis, B. bigemina, B. caballi, and T. equi, respectively. The low IC_{50} values observed on B. bigemina and B. caballi as compared to those of B. bovis and T. equi for allicin revealed that B. bigemina and B. caballi were sensitive to allicin treatment in vitro. Subsequent viability tests showed no regrowth of parasites at allicin concentrations of 4,000 μM for B. bovis and B. caballi, 2,000 μM for B. bigemina, and 5,000 μM for T. equi. There was no regrowth of diminazene aceturate-treated parasites in the subsequent viability test at concentrations of 0.5 μM for B. bovis and B. bigemina, 0.025 μM for B. caballi, and 0.1 μM for T. equi (data not shown). Allicin-treated Babesia showed the morphological changes including degenerative or dot shapes in contrast to untreated control parasites (Figs. 2 and 3). Pretreatment of high concentrations of allicin with bovine and equine RBCs showed no difference in the growth pattern of allicin-pretreated RBCs when compared to DMSO– or non-pretreated RBCs. This indicates that allicin has no hemolytic effect on bovine or equine RBCs (data not shown).

Allicin/diminazene aceturate combination test in vitro

To examine whether allicin with diminazene aceturate had a synergistic or an antagonistic effect, combination therapies at different concentrations (M1–M8) were assessed on B. bovis and B. caballi in vitro as bovine and equine Babesia models, respectively. The IC_{50} of the diminazene aceturate dose of each parasite was used as a control. The combination of allicin/diminazene aceturate had significantly higher inhibitory effects at all concentrations used (M1–M8) for both B. bovis and B. caballi as compared to the untreated controls during treatment except a combination of M1 for B. bovis on day 1 (Table 2). Furthermore, treatments of B. bovis or B. caballi parasites with allicin/diminazene aceturate combination significantly enhanced growth inhibition as compared to the diminazene aceturate control but not in combinations of M1 and M2 for B. bovis on days 2 and 3 of culture. Remarkably, complete eradication of B. bovis and B. caballi was observed for combinations M8 and M6–M8, respectively, on day four. Subsequent viability tests showed no regrowth of the parasites at M8 and M4–M8 for B. bovis and B. caballi, respectively. These results suggest that combinations of allicin with diminazene aceturate synergistically potentiate their inhibitory effects in vitro on B. bovis and B. caballi.

The effect of allicin on the invasion of Babesia and T. equi into RBCs

To evaluate inhibition efficacy of allicin on the invasion of Babesia parasites, merozoites were liberated from RBCs and
then preincubated with allicin (100 and 200 μM) for 30 min before being mixed with fresh RBCs (Table 3). At 100 μM of allicin, the invasion was significantly inhibited\(^*\) (\(P < 0.001\)) for \(B.\ bovis\) at 3 and 6 h, for \(B.\ bigemina\) at 1 and 6 h, for \(B.\ caballi\) at 6 h, and for \(T.\ equi\) at 3 h after culture. Correspondingly, 200 μM of allicin significantly inhibited invasion of \(B.\ bovis\) at 1, 3, and 6 h; of \(B.\ bigemina\) at 1 and 6 h; of \(B.\ caballi\) at 3 and 6 h; and of \(T.\ equi\) at 3 and 6 h after culture. Furthermore, the inhibition percentages noted after 3 h of culturing were 67 % and 72 % for \(B.\ bovis\), 72.5 % and 75 % for \(B.\ bigemina\), 65 % and 69 % for \(B.\ caballi\), and 59 % and 63 % for \(T.\ equi\) at concentrations of 100 and

![Fig. 1](image1.png) **Fig. 1** In vitro inhibitory effects of different concentrations of allicin on the growth of Babesia: \(a\) \(B.\ bovis\), \(b\) \(B.\ bigemina\), \(c\) \(B.\ caballi\), and \(d\) \(T.\ equi\). Each value represents the mean±standard deviation for experiments performed in triplicate. Curves represent the result of one representative experiment out of three separate replicates. Statistically significant differences are indicated by asterisks \((*P < 0.05)\) between the drug-treated cultures and the control cultures.

![Fig. 2](image2.png) **Fig. 2** Light micrographs of allicin treated bovine Babesia parasites in an in vitro culture. Micrographs were taken on day 3 of the experiment. \(B.\ bovis\): \(a\) control and \(b\) 50 μM allicin. \(B.\ bigemina\): \(c\) control and \(d\) 50 μM allicin. The drug-treated cultures showed a higher number of degenerated and dot shape parasites than the control cultures. Scale bars 10 μm.
200 μM, respectively. These results suggest that allicin might interfere with the invasion process of Babesia spp. and T. equi parasites into erythrocytes.

The in vivo inhibitory effect of allicin on B. microti growth

To further validate allicin as an antibabesial drug, an in vivo study was performed using B. microti in a mouse model in three separate trials. Of note, parasitemias significantly decreased in allicin-treated mice ($P<0.01$) from days 4 to 14 post-inoculation (p.i.) as compared to those of controls (Fig. 4). Control mice treated with DMSO showed peak parasitemia of 52.01 % at 8 days p.i., while treatment with 25 mg/kg of diminazene aceturate (reference drug) resulted in significant reduction of parasitemia as compared to the controls and allicin-treated mice, showing peak parasitemia

Table 2 Growth inhibition effect of allicin/diminazene aceturate combinations on B. bovis and B. caballi cultures for 4 days

<table>
<thead>
<tr>
<th>Parasitemia (%±SD)</th>
<th>B. bovis</th>
<th>B. caballi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>2.5±0.4</td>
<td>4.5±0.4</td>
</tr>
<tr>
<td>Diminazene aceturate IC$_{50}$</td>
<td>2.4±0.2</td>
<td>2.9±0.1</td>
</tr>
<tr>
<td>M1</td>
<td>1.9±0.3</td>
<td>2.4±0.67*</td>
</tr>
<tr>
<td>M2</td>
<td>1.7±0.21**</td>
<td>2.5±0.6*</td>
</tr>
<tr>
<td>M3</td>
<td>1.7±0.3**</td>
<td>2.3±0.4*</td>
</tr>
<tr>
<td>M4$^a$</td>
<td>1.4±0.12**</td>
<td>2.1±0.3*</td>
</tr>
<tr>
<td>M5$^b$</td>
<td>1.3±0.3**</td>
<td>1.6±0.7*</td>
</tr>
<tr>
<td>M6$^b$</td>
<td>1.2±0.2**</td>
<td>1.6±0.2*</td>
</tr>
<tr>
<td>M7$^b$</td>
<td>0.97±0.1**</td>
<td>1.5±0.35**</td>
</tr>
<tr>
<td>M8$^{ab}$</td>
<td>1±0.1**</td>
<td>1.6±0.35**</td>
</tr>
</tbody>
</table>

$^*$P<0.05 significant differences between the combined-drug-treated and control groups

$^{**}$P<0.05 significant differences between the combined-drug-treated group and both the diminazene aceturate and control groups

$^a$Subsequent viability test for 10 days showed no regrowth of B. caballi

$^b$Subsequent viability test for 10 days showed no regrowth of B. bovis
Remarkably, the peak parasitemia of the treated mice with 30 and 100 mg/kg allicin reached an average parasitemia of 21.76 % at the eighth day p.i. and 14.4 % at the sixth day p.i., respectively.

The inhibitory effect of allicin combined with diminazene aceturate on *B. microti* growth in mice

To ascertain the potent synergistic effect of allicin with diminazene aceturate, in vivo experiments were performed using *B. microti* in mice with two separate trials. Diminazene aceturate at concentrations of 25 and 6.75 mg/kg was used as reference drug controls. Control mice treated with DMSO exhibited rapid growth of parasitemia that reached 51.33 % at day 7 p.i., while mice treated with diminazene aceturate alone either 25 and 6.75 mg/kg showed peak parasitemia of 7 % at day 5 p.i. and 8.73 % at day 6 p.i., respectively. On the other hand, all combinations were effective against the growth of *B. microti*, and mice significantly decreased their parasitemias (*P* < 0.01) as compared to DMSO control groups on days 4–15 p.i. (Fig. 5). Peak parasitemias in these treated mice reached an average of 6.6, 5.26, and 4.14 % at day 6 p.i. and of 6.02 % at day 4 p.i. in the presence of 6.75 mg/kg of diminazene aceturate combined with 15, 30, and 50 mg/kg allicin and 12.5 mg/kg of diminazene aceturate combined with 15 mg/kg allicin, respectively. Notably, mice treated with 6.75 mg/kg of diminazene aceturate combined with 15, 30, and 50 mg/kg of allicin exhibited significantly lower parasitemias as compared to those treated with 6.75 mg/kg of diminazene aceturate alone from days 7–14.

**Discussion**

Garlic’s active ingredients have been known to be potent inhibitors of many protozoan parasites such as *Eimeria papillata*, *Leishmania* spp., *Blastocystis hominis*, and *Entamoeba histolytica* (Al-Quraishy et al. 2011; Sharma et al. 2009; Yakoob et al. 2011; Degerli et al. 2012). Of these garlic’s active ingredients, allicin is known to have inhibitory

### Table 3 Significantly inhibitory effect of allicin on invasion of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* into host erythrocytes

<table>
<thead>
<tr>
<th>Organism</th>
<th>Inhibition of invasion %±SD</th>
<th>1 h</th>
<th>3 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>100 µM</td>
<td>60.6±4</td>
<td>29±6.1</td>
<td>25±3.3</td>
</tr>
<tr>
<td><em>B. bovis</em></td>
<td>100 µM</td>
<td>61.5±5.5*</td>
<td>61.8±2.3*</td>
<td>67±4.8*</td>
</tr>
<tr>
<td><em>B. bigemina</em></td>
<td>100 µM</td>
<td>67.7±4.2*</td>
<td>58.7±10.8</td>
<td>72.8±2.5*</td>
</tr>
<tr>
<td><em>B. caballi</em></td>
<td>100 µM</td>
<td>61.8±2.2</td>
<td>65.3±2.6</td>
<td>69.3±6.17*</td>
</tr>
<tr>
<td><em>T. equi</em></td>
<td>100 µM</td>
<td>59.5±7.5</td>
<td>65.3±2.6</td>
<td>69.7±7.6*</td>
</tr>
</tbody>
</table>

Inhibition is represented by the average inhibition of three separate experiments (%)+SD

*P*-0.001 significant differences

### Fig. 4 Inhibitory effect of allicin and diminazene aceturate on the growth of *Babesia microti*. Each value represents the mean±standard deviation of 5 mice per experimental group. Asterisks indicate significant differences (*P*-0.01) from days 4 to 15 post-inoculation between the allicin-treated and control groups

### Fig. 5 Inhibitory effect of the combination of diminazene aceturate and allicin with different concentrations on the growth of *Babesia microti*. Each value represents the mean±standard deviation of five mice per experimental group. Asterisks indicate significant differences (*P*-0.01) from days 4 to 13 post-inoculation between the allicin-treated and control groups
effects on a wide range of bacteria, fungi, and protozoan parasites (Ankri and Mirelman 1999; Coppi et al. 2006); however, its potential as an antibabesial drug has not been examined. In the present study, allicin was tested against the in vitro growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* as well as against the in vivo growth of *B. microti* in a mouse model. The growth of these parasites was modestly inhibited in the presence of high concentrations of allicin. Moreover, the higher concentration of the drug did not completely suppress the in vitro growth of the parasites. The IC$_{50}$ values of allicin for *Babesia* parasites were greater than those for fusidic acid (Salama et al. 2013), ciprofloxacin, clindamycin, thiostrepton, rifampicin, tetracycline, and epoxomicin (AbouLaila et al. 2012; 2010a), triclosan (Bork et al. 2003a), imidocarb dipropionate and clindamycin phosphate (Brasseur et al. 1998), and pyrimethamine (Nagai et al. 2003). On the other hand, IC$_{50}$ values of allicin for *Babesia* parasites were lower than those for metronidazole against *B. gibsoni* (Matsuu et al. 2008) and clodinafop-propargyl against *B. bovis* and *B. bigemina* (Bork et al. 2003b). Therefore, the potential inhibitory effects of allicin on free merozoites were evaluated in an invasion assay. Interestingly, allicin significantly inhibited the host erythrocyte invasion of parasites by rates of 59 to 75 %. Consistently, allicin at a concentration of 50 μM inhibited the invasion of *Plasmodium* sporozoites into host hepatocytes (Coppi et al. 2006).

Diminazene aceturate is widely used as an antibabesial drug for the treatment of infected animals; however, the emergence of drug resistance and the toxicity of these drugs, especially in equines, suggest that a search for a new therapeutic agent is needed. Therefore, we have attempted to examine the inhibitory effects of allicin combined with low doses of diminazene aceturate to reduce its side effects. Notably, allicin synergistically potentiated the inhibitory effects of diminazene aceturate on *B. bovis* and *B. caballi* in vitro. The greater inhibitory effects caused by combined drugs might be due to the capability of allicin to inhibit parasite invasion, allowing diminazene aceturate direct interaction with extracellular parasites.

Next, different concentrations of allicin were examined alone or in combination with diminazene aceturate in mouse models. Treatment of mice with allicin 30 and 100 mg/kg resulted in 58.3 and 72.4 % inhibition at days 7 and 6 p.i., respectively. The inhibitory effects of allicin on *B. microti* growth were higher than those caused by 100 mg/kg nerolidol treatment, showing 53.7 % inhibition on day 10 p.i. (AbouLaila et al. 2012). Allicin had marked inhibitory effects of 59 to 75 % on day 10 p.i. (Marley et al. 1997). The reason behind the synergistic inhibitory effect of diminazene aceturate with allicin in mice infected with *B. microti* is not fully understood. However, this synergistic inhibitory effect might be explained based on the fact that allicin has a potent immune-modulatory effect on host lymphocytes (Patya et al. 2004) and macrophages (Kang et al. 2001). Allicin could be used alternatively in the field to reduce the toxic effect of diminazene aceturate alone, although further study is required to investigate the precise role of allicin in *Babesia* parasite growth inhibition.

In conclusion, allicin has a potent inhibitory effect on the growth of *Babesia* parasites in vitro and in vivo, and the inhibition may occur at the invasion step. The combination of diminazene aceturate with allicin was more toxic to *Babesia* in vitro and in vivo. This may offer a novel strategy for treatment of babesiosis.

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References


AbouLaila M, Yokoyama N, Igarashi I (2010b) Inhibitory effects of (−)-epigallocatechin-3-gallate from green tea on the growth of *Babesia* parasites. Parasitology 137:785–791
