Parasitology International 59 (2010) 278–282

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Short communication

Inhibitory effect of terpene nerolidol on the growth of *Babesia* parasites

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ARTICLE INFO

Article history:
Received 26 July 2009
Received in revised form 25 January 2010
Accepted 16 February 2010
Available online 21 February 2010

Keywords:
Nerolidol
Babesia
In vitro
In vivo

ABSTRACT

Nerolidol is a sesquiterpene present in the essential oils of many plants, approved by the U.S. FDA as a food flavoring agent. Nerolidol interferes with the isoprenoid biosynthetic pathway in the apicoplast of *P. falciparum*. In the present study, the *in vitro* growth of four *Babesia* species was significantly ($P<0.05$) inhibited in the presence of nerolidol (IC_{50}s values = 21 ± 1, 29.6 ± 3, 26.9 ± 2, and 23.1 ± 1 μM for *B. bovis*, *B. bigemina*, *B. ovata*, and *B. caballi*, respectively). Parasites from treated cultures failed to grow in the subsequent viability test at a concentration of 50 μM. Nerolidol significantly ($P<0.05$) inhibited the growth of *B. microti* at the dosage of 10 and 100 mg/kg BW, while the inhibition was low compared with the high doses used. Therefore, nerolidol could not be used as a chemotherapeutic drug for babesiosis.

Babesia, a tick-born protozoan parasite, is one of the major pathogens that infect erythrocytes in a wide range of wild and economically valuable animals, such as cattle and horses. The clinical symptoms of babesiosis include malaise, fever, hemolytic anemia, jaundice, hemoglobinuria, and edema in the infected animals. *Babesia* parasites are prevalent worldwide, mainly in tropical and sub-tropical areas. Serious economic damage in the livestock industry has been caused by *Babesia* infections in such areas [1,2]. Several babesicidal drugs that have been in use for years have proven to be ineffective owing to problems related to their toxicity and the development of resistant parasites [3,4]. Therefore, the development of new drugs that have a chemotherapeutic effect against babesiosis with high specificity to the parasites and low toxicity to the hosts has been desired.

The apicoplast was acquired by horizontal transfer (secondary endosymbiosis) from a eukaryotic alga [5] and has been identified in many apicomplexan parasites, including *Babesia* [6]. The apicoplast is essential for long term parasite viability and has been an attractive target for development of parasiticidal drug therapies as the biosynthetic pathways represented therein are of cyanobacterial origin and differ substantially from corresponding pathways in the mammalian host [7,8]. The apicoplast has synthetic pathways such as fatty acid biosynthesis and isoprenoid biosynthetic pathways. In apicomplexan parasites, synthesis of isopentenyl diphosphate (IPP), the universal isoprenoid precursor, has long been assumed to proceed exclusively via the acetate/mevalonate (MVA) ubiquitous pathway, a pathway that is absent from malaria parasites [9]. However, an alternative MVA-independent pathway for the formation of isopentenyl diphosphate (IPP) occurs in bacteria, algae and chloroplasts of higher plants [9]. The initial step of this MVA-independent pathway is the formation of 1-deoxy-D-xylulose4-phosphate (DOXP) by condensation of pyruvate and glyceraldehyde-3-phosphate, catalyzed by DOXP synthase. In the second step, DOXP reductoisomerase synthesizes 2-C-methyl-D-erythritol-4-phosphate (with 2-C-methyl-D-erythrose-4-phosphate as an intermediate), in a single step, by intramolecular rearrangement followed by a reduction process [9]. IPP is the basic five-carbon building block (C_5) that forms the next member of the series. Two C_5 units condense to form geranyl pyrophosphate (C_{10}), which condenses with another molecule of IPP to form farnesyl pyrophosphate (FPP) (C_{15}). FPP is incorporated in the formation of prenylated proteins, ubiquinones (Coenzyme Q), and dolichols [9]. Isoprenoid biosynthesis in malaria parasites operates by a different mechanism than that in humans; therefore, could be a promising target for drug therapy [9]. While in most eukaryotic cells, the isoprenoid biosynthesis is achieved through a mevalonate dependent pathway which generates essential metabolic products in addition to cholesterol and ergosterol, such as the dolichols, which are present in all membranes in variable amounts and, in a modified phosphorylated form, are required for the asparagine-linked glycosylation of proteins. The pathway also generates the isoprene side chains attached to the benzoquinone ring of ubiquinone, prenyl groups transferred to prenylated proteins, and prenylated transfer RNAs [9]. *Leishmania* species cannot synthesize cholesterol de novo but are able to produce ergosterol through the mevalonate pathway [10]. In addition to ergosterol, other products of the mevalonate pathway have been identified in *Leishmania*: coenzyme Q9 (CoQ9) was detected as the predominant species of ubiquinone in promastigotes and amastigotes of *L. amazonensis*, whereas CoQ7 and CoQ8 were also identified in promastigotes [11]; prenylated proteins were observed in *Leishmania mexicana*

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doi:10.1016/j.parint.2010.02.006

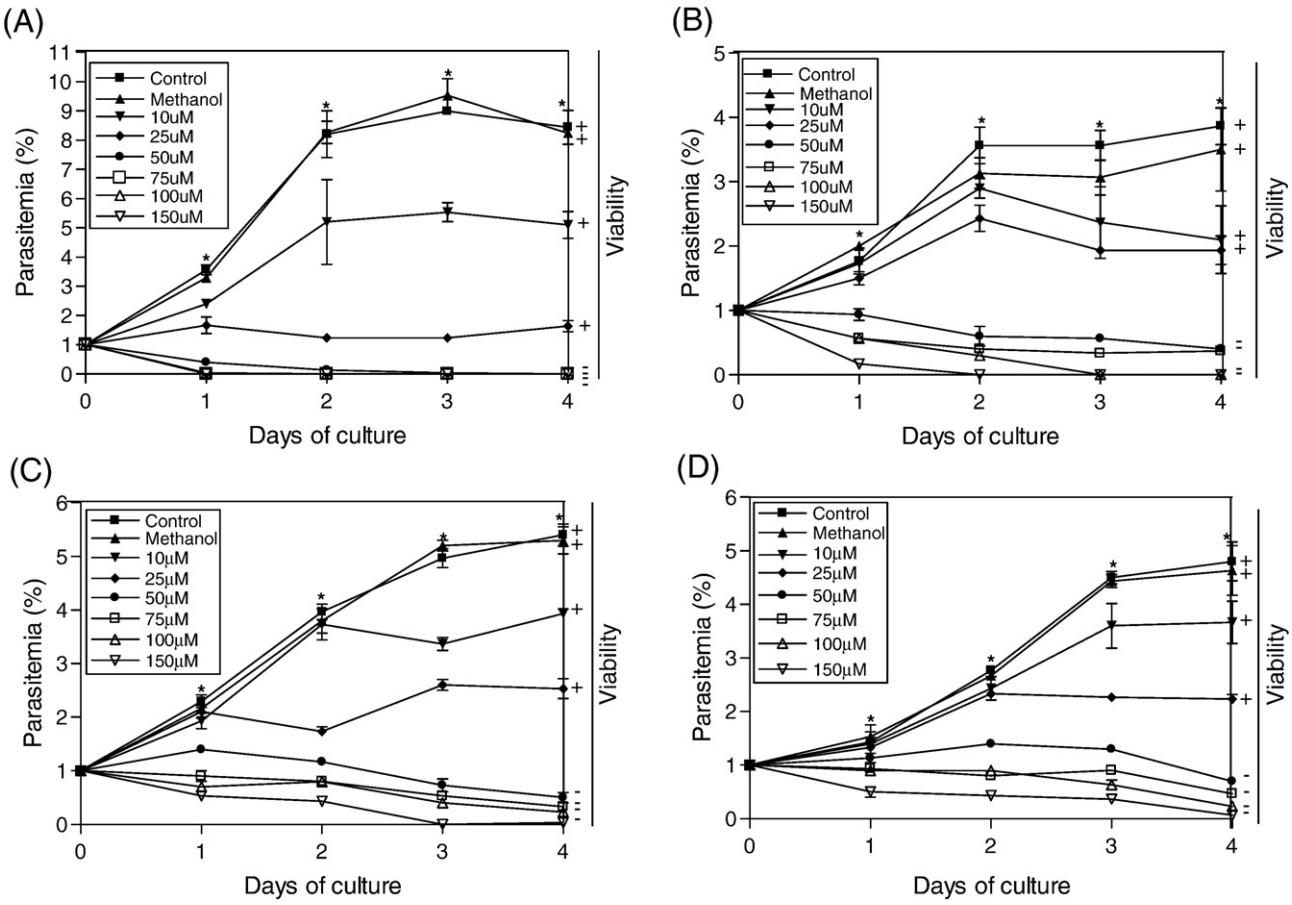

Fig. 1. Inhibitory effect of different concentrations of nerolidol on the *in vitro* growth of *B. bovis* (panel A), *B. bigemina* (panel B), *B. ovata* (panel C), and *B. caballi* (panel D). Each value represents the mean ± standard deviation in triplicate. These curves represent the mean of three experiments carried out in triplicate. *, statistically significant differences (student's *t* test, $P<0.05$) between 10 µM (panels A and B) and 25 µM (panels C and D) treated cultures and the control. Parasite viability was monitored in subcultures without nerolidol for 10 days (±, viable cells and −, dead cells).

after incorporation of labeled mevalonate, and phosphorylated dolichol has been detected as a sugar donor for glycosylation of proteins in *L. mexicana* [12]. The isoprenoid biosynthetic pathway is the only active pathway in *B. bovis* apicoplast, and is similar to that of *P. falciparum* [13]. Therefore, the blockade of the mevalonate independent pathway in the apicoplast could potentially have serious effects for *Babesia* parasites.

Nerolidol is a sesquiterpene present in essential oils of several plants, approved by the U.S. Food and Drug Administration as a food flavoring agent, it has been tested as a skin penetration enhancer for the transdermal delivery of therapeutic drugs [14]. In *Leishmania amazonensis*, nerolidol inhibited the isoprenoid biosynthesis, as shown by reduced incorporation of mevalonic acid (MVA) or acetic acid precursors into dolichol, ergosterol, and ubiquinone, in treated promastigotes due to the blockage of an early step in the mevalonate pathway [14], while in *P. falciparum*, nerolidol interferes with the isoprenoid biosynthetic pathway of the apicoplast leading to the interference with the biosynthesis of the dolichols, with the isoprenic chain of ubiquinones, and with protein isoprenylation of the parasites [15]. Nerolidol have leishmanicidal [14], and antimalarial [15,16] activities. The aim of this study was to evaluate the inhibitory effect of nerolidol on the growth of *Babesia* parasites.

B. bovis Texas strain, *B. bigemina* Argentina strain, *B. ovata* Miyake strain, and *B. caballi* U.S. Department of Agriculture strain were used in this study. Parasites were grown in a micro-aerophilous stationary phase culture system using bovine (for bovine *Babesia*) or equine (for *B. caballi*) erythrocytes (RBC) and sera as described previously [17,18]. GIT medium alone was used to test the effect of nerolidol without serum on *B. bovis* and *B. caballi* [19]. The overlaying medium was replaced daily with a fresh medium, and the infected RBC were passaged every 3 days [20]. Nerolidol (a mixture of *cis*- and *trans*-nerolidol) was purchased from Sigma-Aldrich (St. Louis, MO). A 20 mM stock solution prepared in methanol was used for *in vitro* experiments.

The *in vitro* growth inhibition assay for nerolidol followed the methods previously described [17,21] with some modifications. Parasite-infected RBC were diluted with uninfected RBC to obtain an RBC pack with 1% parasitemia. Twenty microliters of RBC with 1% parasitemia was dispensed to a 96-well microtiter plate together with 200 µl of the culture medium containing the indicated concentration of nerolidol (10, 25, 50, 75, 100, and 150 µM), and then incubated at 37 °C in a humified multi-gas water-jacketed incubator. For the control, cultures without the drug and another containing only methanol similar to the highest concentration

Table 1
The IC_{50} values of nerolidol for *Babesia* parasites and mammalian cells.

Organism	IC_{50} (µM)[a]
B. bovis	21 ± 1
B. bigemina	29.6 ± 3
B. ovata	26.9 ± 2
B. caballi	23.1 ± 1
J774.A1 macrophages[b]	125.69 ± 14.40
Human foreskin fibroblasts[b]	134.94 ± 32.19

[a] IC_{50} values expressed as nerolidol concentration are in micromolar of the growth medium and were determined on day 3 of *in vitro* culture using a curve fitting technique. IC_{50} values represent the mean and standard deviation of 3 separate experiments.

[b] The IC_{50} values for mammalian cells reported by Arruda et al. [14].

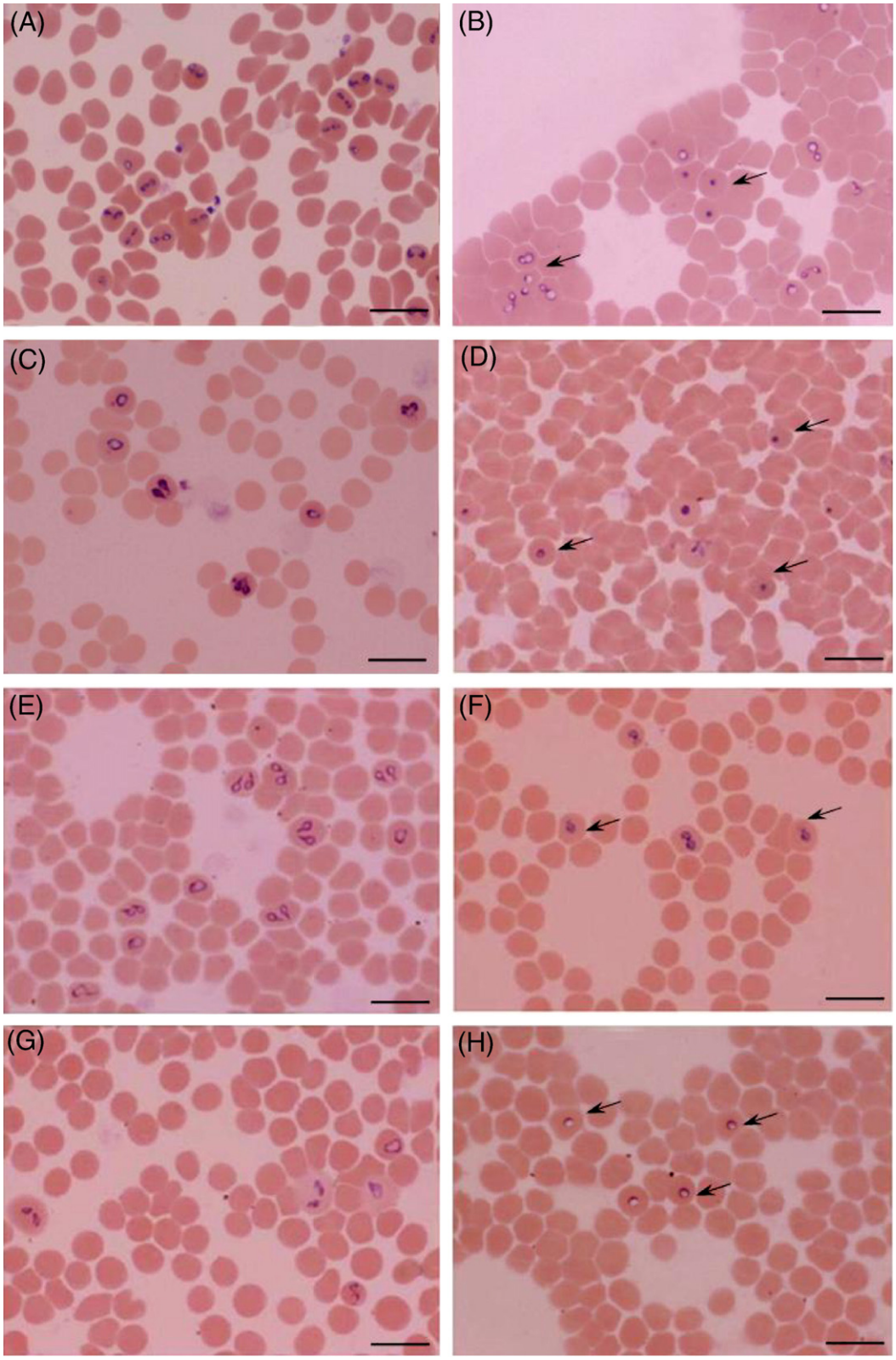

Fig. 2. Light micrographs of nerolidol-treated *Babesia* parasites in an *in vitro* culture. Micrographs were taken on day 3 of the experiments for 10 μ M treated cultures. *B. bovis*: control (panel A), and treated (panel B). *B. bigemina*: control (panel C), and treated (panel D). *B. ovata*: control (panel E), and treated (panel F). *B. caballi*: control (panel G), and treated (panel H). The drug-treated cultures showed a higher number of degenerated and swollen parasites than the control cultures. Scale bars = 10 μm.

used in drug were prepared. The experiments were carried out in triplicate for each drug concentration for 3 separate trails for a period of four days. The culture medium was replaced daily with 200 μl of a fresh medium containing the appropriate concentration of the drug. Parasitemia was monitored daily by counting the parasitized RBC to approximately 1000 RBC in Giemsia-stained thin blood smears. After the fourth day of treatment, 6 μl of each of the control and nerolidol-treated (at the various concentrations) RBC was mixed with 14 μl of

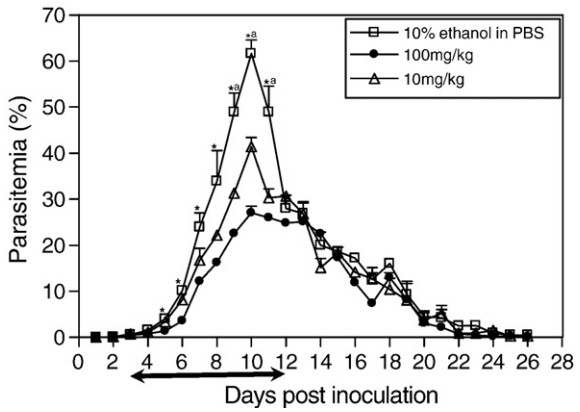

Fig. 3. Inhibitory effect of nerolidol (10 and 100 mg/kg) on the *in vivo* growth of *B. microti* for observations of 5 mice in each group. Each value represents the mean ± standard deviation of two separate experiments.* and [a], statistically significant differences ($P<0.05$) between 100 mg/kg and 10 mg/kg treated groups, and the control group, respectively. The double head arrow (↔) indicates the nerolidol intraperitoneal injection period.

parasite-free RBC and suspended in fresh growth medium without nerolidol supplementation. The plates were incubated for the next 10 days. The culture medium was replaced daily, and parasite recrudescence was determined by light microscopy to evaluate the parasite viability [20]. The values of a 50% inhibitory concentration (IC_{50}) of nerolidol against all parasites were calculat/ed based on parasitemia observed at 3 days after drug treatment by interpolation after curve fitting technique.

The *in vivo* growth inhibition assay for nerolidol was carried out two times according to the method previously described [14,20] with some modifications. Fifteen 8-week-old female BALB/c mice (CLEA Japan, Tokyo, Japan) were divided into 3 groups each contain five mice and intraperitoneally inoculated with 1×10^6 Munich strain *B. microti*-infected RBC that was maintained by passage in the blood of BALB/c mice [22]. Nerolidol was administered at a dose rate of 10 and 100 mg/kg after dissolving in 0.3 ml 10% ethanol in 0.01 M phosphate buffer (pH 7.4) for the first and second groups respectively. The third group was only administered 0.3 ml 10% ethanol in 0.01 M phosphate buffer (pH 7.4) as a control. When the infected mice showed about 1% parasitemia, all groups underwent daily intraperitoneal injections from days 3 to 12 post-infection. The levels of parasitemia in all mice were monitored daily until 26 days post-infection by examination of stained thin blood smears prepared from venous tail blood. All animal experiments were conducted in accordance with the Standard Relating to the Care and Management of Experimental Animals promulgated by the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

JMP statistical software (version 5.1., SAS institute Inc., USA) was used to compare means of parasitemia percentage in the *in vitro* and *in vivo* studies using the independent Student's *t* test and considered to be significantly different when $P<0.05$.

The *in vitro* growth was significantly inhibited ($P<0.05$) at 10 μM (for *B. bovis*, and *B. bigemina*) (Fig. 1A, B), and at 25 μM (for *B. ovata*, and *B. caballi*) (Fig. 1C, D). The parasites' growth was completely suppressed at 50 μM (for *B. bigemina*), and at 75 μM (for *B. bovis*, *B. ovata*, and *B. caballi*). The calculated IC_{50}s values of nerolidol on the third day of culture for the growth of *B. bovis*, *B. bigemina*, *B. ovata*, and *B. caballi* were 21 ± 1, 29.6 ± 3, 26.9 ± 2, and 23.1 ± 1 μM, respectively (Table 1). *B. bovis is* more susceptible to nerolidol compared to other species of *Babesia*. Complete clearance was observed on the third (for *B. bovis*, *B. bigemina*, and *B. ovata*), and the fourth (for *B. caballi*) days of drug treatment. Subsequent cultivation of the parasites without the drug for a 10-day period showed no regrowth of the four species at 50 μM (Fig. 1) as shown by light microscopy. The serum has no effect on the inhibition of nerolidol to *Babesia* species as indicated from the use of GIT medium without serum for *B. bovis* and *B. caballi* (data not shown). The addition of only methanol to the culture had no influence on the growth. The effective doses of nerolidol for the growth inhibition of *Babesia* parasites were lower than the effective doses of heparin [20], and in nearly similar level to those of other drugs that had been tested as babesicidal drugs [21,23–27]. While they have medium level between those reported for *P. falciparum* [15] and *L. amazonensis* [14]. The average IC_{50} values of nerolidol for *Babesia* parasites were low compared with nerolidol IC_{50} values for mammalian cells (125.69 ± 14.40 μM; J774.A1 macrophages and 134.94 ± 32.19 μM; human foreskin fibroblast) [14]. The light microscope study showed that the drug affected the morphology of the parasite in treated cultures of *B. bovis* (Fig. 2B), *B. bigemina* (Fig. 2D), *B. ovata* (Fig. 2F), and *B. caballi* (Fig. 2H). The drug-treated cultures showed high number of degenerated and swollen parasites without cytoplasm compared to the control cultures. Based on light microscopic observations of the changes in the host cell shape, size, and color, nerolidol was non-toxic to erythrocytes.

The effect of nerolidol on the course of *B. microti* in mice was revealed. In the treated groups the level of parasitemia increased significantly (Student's *t* test, $P<0.05$) more slowly than the control, achieving peak parasitemia of 27 and 41.3% in the presence of 100 mg/kg and 10 mg/kg on day 10 post inoculation. In contrast, in the control group, the peak parasitemia was 58.3% (10% ethanol in PBS) on day 10 post inoculation (Fig. 3). The difference in growth inhibition (Student's *t* test, $P<0.05$) between 10 and 100 mg/kg and the control groups was significant on days 9–11 and days 5–11 PI, respectively. There was a significant difference between the 2 used doses of nerolidol ($P<0.05$) from days 5 to 11 PI, but the difference in the growth inhibition between the two doses was low (Fig. 2). The overall inhibition was moderate relative to the high doses used, while the inhibitory effect on *L. amazonensis* was low [14], this may be due to the differences in the life cycle, and pathogenicity of the two parasites. Toxic effects were not observed on mice after intraperitoneal administration of nerolidol; this is in good agreement with what was reported by Arruda et al. [14], where the mice injected with 100 mg/kg nerolidol for 12 days did not show any signs of toxicity. The half life of nerolidol may be very short; therefore, this may lead to its degradation and consequently low effect and no toxic signs even at high doses on the host.

The presence of an active isoprenoid pathway for the biosynthesis of dolichol of 11 and 12 isoprenic units was reported in *L. amazonensis* promastigotes [14] and in *P. falciparum* [28]. Dolichols composed of 11 to 13 isoprene units had been previously characterized in *Trypanosoma brucei* [29], and side chain of the 8 and 9 isoprenic units attached to benzoquinone rings of ubiquinones in *P. falciparum* [30]. While in the mammalian cells, the isoprenic chains of dolichols and ubiquinones comprise 20 to 22 and 10 isoprenic units, respectively [31]. In *Babesia* parasites, the presence of an active isoprenoid pathway was reported [13], while the structure of the dolichol and the side chain attached to the benzoquinone rings of ubiquinones are not known and need further studies.

In *Leishmania amazonensis*, nerolidol inhibited the isoprenoid biosynthesis, as shown by inhibiting incorporation of mevalonic acid (MVA) or acetic acid precursors into dolichol, ergosterol, ubiquinone, and by inhibiting the synthesis of geraniol, farnesol, and the putative hexaprenol intermediate in treated promastigotes due to the blockage of an early step in the mevalonate pathway [14], while in *P. falciparum*, nerolidol interferes with the isoprenoid biosynthetic pathway of the apicoplast leading to the interference with the biosynthesis of the dolichols, with the isoprenic chain of ubiquinones, and with protein isoprenylation of the parasites [15]. *B. bovis* has active isoprenoid pathway which is similar to that of *P. falciparum* [13]; therefore, the mechanism of inhibition for *Babesia* parasites may be similar to that of *P. falciparum* which requires further studies.

In conclusion, the results of the present study showed that nerolidol effectively inhibited the *in vitro* growth of *Babesia* parasites, while its *in vivo* effect on *B. microti* was weak in spite of the high doses

used. Therefore, nerolidol could not be used for the treatment of babesiosis.

Acknowledgements

This study was supported by Grants from the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), the 21st Century COE Program (A-1), Ministry of Education, Culture, Sports, Science, and Technology, Japan, the Japan International Cooperation Agency (JICA), and The Ministry of Higher Education Egypt.

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