

In vivo and *in vitro* antimalarial activity of bergenin

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Abstract. Malaria is a potentially life-threatening protozoal parasitic disease transmitted by female *Anopheles* mosquitoes. Drug therapy is currently the most widely used method for the control and treatment of this disease. Several plants were found to contain substances possessing antimalarial properties. In this study, we investigated the antimalarial activity of bergenin, a sesquiterpene lactone compound derived from *Rodgersia aesculifolia* Batal. The results indicated that bergenin effectively inhibited *Plasmodium falciparum* growth *in vitro* (IC₅₀, 14.1 µg/ml, with ~100% inhibition at 50 µg/ml), without apparent cytotoxicity to erythrocytes or to mammalian HeLa and HepG2 cells. Bergenin exhibited less cytotoxic activity and the selectivity index (SI) was 887 and 1,355 for HeLa and HepG2 cells, respectively. The administration of bergenin to *Plasmodium berghei*-infected mice for 6 days significantly inhibited the growth of the parasites. Taken together, these findings provide evidence that bergenin may be a promising novel drug for antimalarial treatment.

Introduction

Malaria is a protozoal parasitic disease transmitted by female *Anopheles* mosquitoes that threatens the health of approximately one third of the world's population and kills ~655,000 individuals in 106 malaria-endemic countries and regions worldwide annually (1). Drug therapy is currently the most widely used method for the control and treatment of this disease. Artemisinin-based combination therapies were recommended by the World Health Organization as first-line treatment for *Plasmodium falciparum* (*P. falciparum*) infection in 2006 (2,3). However, emerging resistance of the parasites to artemisinin, which is characterized by delayed parasite clearance time after treatment, has been observed in Southeast

Asia. It was demonstrated that the half-life of the parasites, which normally declines after artesunate treatment, increased from a mean of 2.6 h in 2001 to 3.7 h in 2010 on the north-western border of Thailand, compared to 5.5 h in 116 patients from Western Cambodia between 2007 and 2010 (4), where artemisinin resistance has been confirmed (5,6). Therefore, there is an urgent need for the development of novel drugs for malaria therapy, particularly in regions where resistant *Plasmodium* strains are present.

The use of natural products has provided a prospective strategy for identifying novel antimalarial drugs. It is estimated that >1,200 plant species from 160 families have been used to treat malaria and fever, including the widely used antimalarial drugs artemisinin and quinine derivatives (7). Since the discovery of artemisinin, the research of sesquiterpene lactone compounds has become the focus of antimalarial drug development (8,9).

Bergenin is a sesquiterpene lactone compound isolated from *Rodgersia aesculifolia* (*R. aesculifolia*) Batal, which is an effective and broad-spectrum antifungal and antiviral Chinese medicine. Bergenin was reported to possess antimicrobial properties against filamentous fungi, yeast and HIV, but not against bacteria (10-13). Bergenin also exhibits anti-inflammatory activity through the inhibition of cyclooxygenase-2 or by means of affecting the Th1- or Th2-skewed cytokine production (14,15). Bergenin also exerts an antioxidant effect by scavenging free radicals, such as ·H, ·OH and ·CH₃ (16). In addition, bergenin was reported to possess hepatoprotective, neuroprotective and gastroprotective properties (17-19). *R. aesculifolia* Batal containing bergenin was used to treat protozoal infection and fever in rural China; however, whether bergenin possesses antimalarial properties requires further elucidation. In this study, we evaluated the antimalarial activity of bergenin *in vitro* and *in vivo* trials.

Materials and methods

Mice and *P. falciparum*. Female BALB/c mice (aged 6-8 weeks) obtained from the Experimental Animal Center of The Fourth Military Medical University, Xi'an, China) were selected for the experiments. The mice were kept in a 12 h light/12 h dark cycle and were given commercial rodent solid diets and water *ad libitum*.

P. falciparum strain 3D7 (conserved in our laboratory) was maintained in human erythrocytes in RPMI-1640 medium supplemented with 10% human serum at 37°C in a humidified

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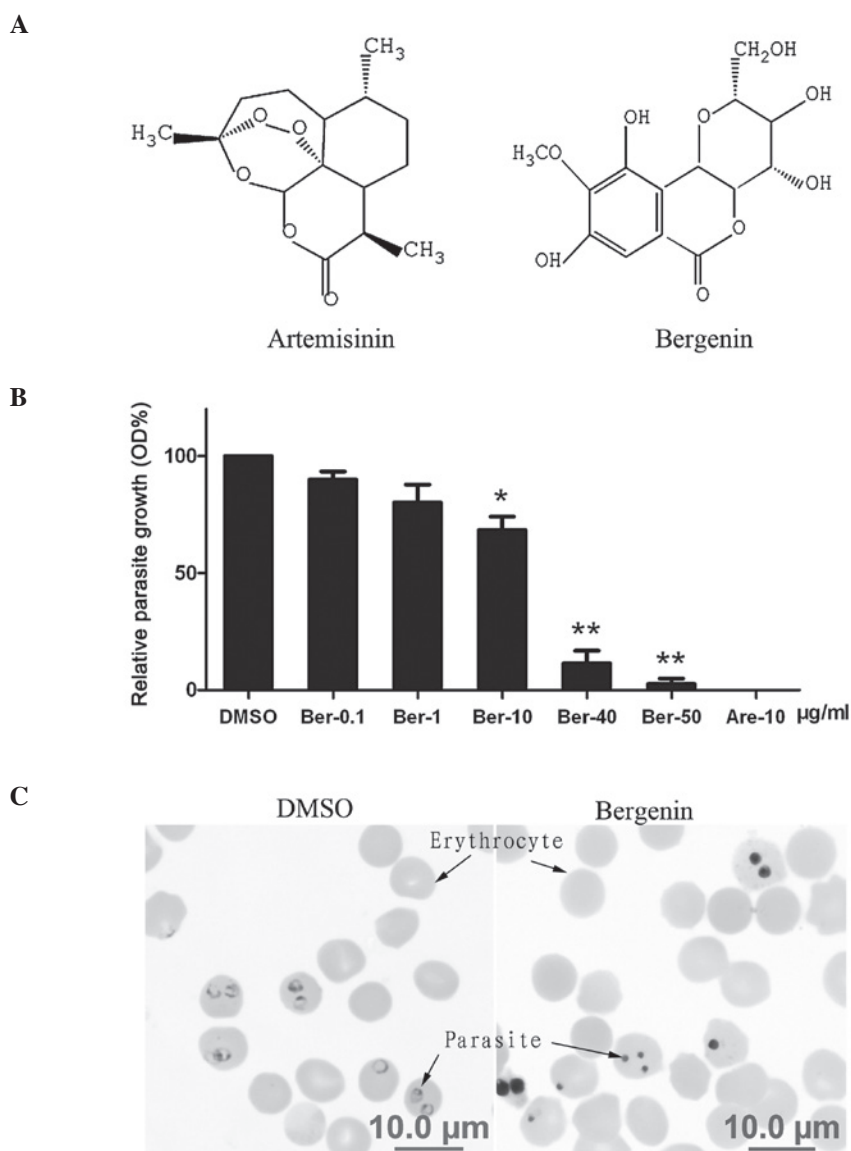


Figure 1. Antiplasmodial activity of bergenin *in vitro*. (A) Molecular structures of artemisinin and bergenin (Ber). (B) Growth inhibition of *Plasmodium falciparum* (*P. falciparum*) by bergenin. *P. falciparum* samples were maintained in erythrocytes and treated with different concentrations of bergenin (0.1–50 µg/ml) for 72 h. The viability of the parasites was determined by the lactate dehydrogenase assay. Data are expressed as means ± SEM based on three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. dimethyl sulfoxide (DMSO) control. (C) Microscopic examination of the morphological change in *P. falciparum* induced by bergenin. Left panel, cell shape of red blood cells infected with *P. falciparum* treated with DMSO; right panel, *P. falciparum*-infected red blood cells treated with bergenin at 50 µg/ml for 72 h. Morphological changes in the uninfected erythrocytes were not observed during the trials, even when exposed to 50 µg/ml bergenin for 72 h. The data demonstrated that bergenin was not cytotoxic to erythrocytes at concentrations effective for inhibiting parasite growth. OD, optical density.

fied incubator with 5% CO₂. The parasites were continuously cultured as previously described (20).

In vitro assessment of antiplasmodial activity. For the *in vitro* experiment, *P. falciparum* was synchronized at ring stage as previously described (21), bergenin (provided by the ShaanXi Institute for Food and Drug Control, Xi'an, China) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and dispensed as 0.2 µl with various concentrations of bergenin stock solution in a 100-µl aliquot of cell suspension with 1% parasitemia and 2% haematocrit, inoculated in a 96-well plate. The positive control was treated with 0.2 µl of DMSO and the negative control was treated with 0.2 µl of artemether stock solution (Sigma-Aldrich) also dissolved in DMSO. The parasites were cultured for 72 h to

ensure they went through all the stages of the cell cycle and parasite growth was evaluated using the lactate dehydrogenase (LDH) activity assay, as described below.

Evaluation of parasite growth using the LDH activity assay. After 72 h of incubation, the plate was frozen at -70°C overnight and thawed at room temperature to hemolyze red blood cells. A 25-µl aliquot was then dispensed into a new well. To evaluate LDH activity, 120 µl of freshly made reaction mix [143 mM 3-acetyl pyridine adenine dinucleotide (APAD, Sigma-Aldrich), 286 mg/ml diaphorase (2.83 U/ml, Worthington Biochemical Corporation, Lakewood, NJ, USA), 143 mM sodium L-lactate (Bio Basic Canada Inc., Ontario, Canada), 178.75 mM Nitro Blue tetrazolium chloride (NBT, Bio Basic Canada Inc.), 0.7% Tween 20, 100 mM Tris-HCl

pH 8.0) (22) was dispensed and incubated at room temperature for 30 min. The plate was shaken for 10 sec and the absorbance was monitored at 650 nm (Model 680 Microplate Reader, Bio-Rad, Hercules, CA, USA). Data were normalized to percent parasite growth using negative and positive controls using the following equation: % parasite growth = $(A_{\text{well}} - A_{\text{neg}} / A_{\text{pos}} - A_{\text{neg}}) \times 100$.

In vivo assessment of antimalarial activity. Bergenin powder was suspended in 1% sodium carboxymethyl cellulose (CMC; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and intragastrically administered to the mice. *Plasmodium berghei* (conserved in our laboratory; *P. berghei*)-infected BALB/c mice with parasitemia levels of ~1% were randomly divided into three groups, the phosphate-buffered saline (PBS), CMC and bergenin groups. Each group included 13 mice housed in separate cages. The mice from the bergenin group were intragastrically administered 800 mg/kg/day of bergenin for 4 or 6 days (100 μ l twice daily). The mice in the PBS and CMC groups, which served as control, were treated with 100 μ l of PBS and CMC, respectively. Thin blood smears were performed on days 4 and 6 after drug treatment and stained with Giemsa. The parasitemia was assessed by microscopic (Olympus Corp., Tokyo, Japan) examination.

In vitro assessment of cytotoxic activity. The HepG2 and HeLa cells were conserved in our laboratory and were cultured in DMEM (high glucose) supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO₂. The cells were plated in a 96-well plate with 10⁴ cells/well and the medium was replaced the following day by a 100- μ l aliquot containing 0.5 μ l of bergenin stock solution of various concentrations. The positive control was treated with 0.5 μ l of DMSO. The cells were cultured for 72 h and cell viability was evaluated using the MTT assay (Bio Basic Canada Inc.) (23). The absorbance of each well was read on a microplate reader at 492 nm after 30 sec of shaking.

Statistical analysis. The half-maximal inhibitory concentration (IC₅₀) of bergenin represented a 50% parasite LDH activity inhibition compared to the positive control, referred to as 100% parasite LDH activity. The half-maximal cytotoxic concentration (CC₅₀) of bergenin represented a 50% cell growth inhibition compared to the positive control, referred to as 100% cell growth. IC₅₀ and CC₅₀ were estimated using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA) (24).

Statistical analysis was performed using Statistics Toolbox of Matlab 7.1 (The MathWorks, Inc., Natick, MA, USA) and the differences among the groups were calculated using one-way ANOVA. Data are expressed as means \pm SEM. P<0.05 was considered to indicate a statistically significant difference.

Results

Bergenin suppresses *P. falciparum* growth in vitro. Bergenin and artemisinin belong to the family of sesquiterpene lactones in structure (Fig. 1A). To demonstrate the antiplasmodial effect of bergenin, we evaluated the parasite growth after treatment with different concentrations of bergenin for 72 h with the

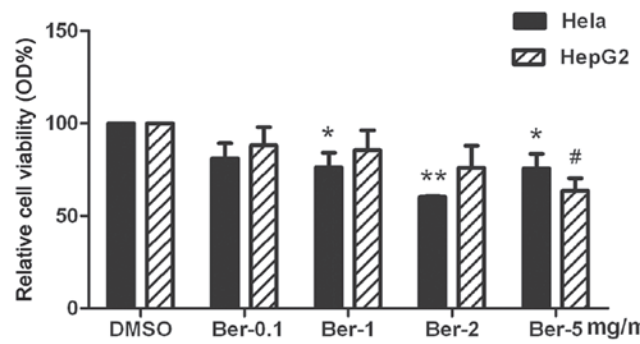


Figure 2. Cytotoxic activity of bergenin on HeLa and HepG2 cells. The cells were treated with different concentrations of bergenin (0.1-5 mg/ml) for 72 h. The cytotoxic activity was assessed with the MTT assay. Data are expressed as means \pm SEM and the results represent three independent experiments. *P<0.05 and **P<0.01 vs. dimethyl sulfoxide (DMSO) control of HeLa cells. #P<0.05 vs. DMSO control of HepG2 cells. OD, optical density.

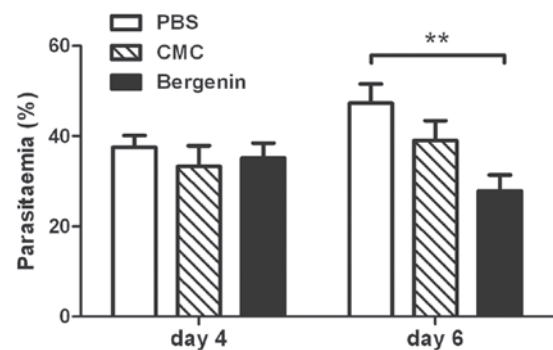


Figure 3. Growth inhibition of *Plasmodium berghei* (*P. berghei*) by bergenin in mice. *P. berghei*-infected mice were treated with phosphate-buffered saline (PBS), carboxymethyl cellulose (CMC), or bergenin for 4 and 6 days, followed by parasitemia assessment. Data are expressed as means \pm SEM, generated from three independent experiments. **P<0.01.

LDH activity assay. As shown in Fig. 1B, bergenin at 10 μ g/ml significantly inhibited the parasite growth compared to the DMSO control (P<0.05). The inhibitory effect of bergenin on parasite growth was dose-dependent in a range of 0.1-50 μ g/ml and parasite growth was almost completely suppressed by bergenin at 50 μ g/ml. The IC₅₀ of bergenin was 14.1 μ g/ml, which was in the range of moderate antiplasmodial activity (10<IC₅₀<25 μ g/ml) (25). Morphological changes in the uninfected erythrocytes were not observed, even following exposure to 50 μ g/ml bergenin for 72 h (Fig. 1C). Furthermore, the group treated with an equal volume of DMSO (0.2%, v/v) exhibited no effect on parasite growth compared to normally cultured parasites (data not shown). Taken together, these results indicated that bergenin effectively suppresses parasite growth *in vitro*.

Bergenin is less cytotoxic to mammalian cells. To determine whether bergenin is cytotoxic to mammalian cells, we evaluated the viability of HeLa and HepG2 cells following treatment with serial concentrations of bergenin for 72 h with the MTT assay. As shown in Fig. 2, bergenin exhibited no detectable cytotoxicity against HeLa and HepG2 cells at concentrations of 1-5 mg/ml. The CC₅₀ of bergenin was ~12.5 on HeLa cells and 19.1 mg/ml on HepG2 cells, which may be defined as non-toxic (CC₅₀>30 μ g/ml) (26).

Bergenin inhibits parasite growth in vivo. To determine whether bergenin suppresses plasmodium growth *in vivo*, we treated *P. berghei*-infected mice with bergenin by oral administration at 800 mg/kg/day for 6 days. The mouse parasitemia was assessed on days 4 and 6 during the trial. As shown in Fig. 3, there was no significant difference in parasitemia among the PBS, CMC and bergenin groups on day 4. However, on day 6, the parasitemia in bergenin-treated mice decreased from 35 to 27%, with a statistically significant difference compared to the PBS or CMC control groups ($P < 0.01$). These data indicated that bergenin suppresses the growth of *P. berghei* *in vivo*.

The body appearance, ingestion and defecation of bergenin-treated mice appeared to be normal during the trials, with no detectable differences between the bergenin-treated, CMC or PBS control group mice, which indicated that bergenin exhibited good tolerability even at considerably high dosage (e.g., 800 mg/kg/day).

Discussion

Plants have been used as the basis of traditional medicine throughout history and they continue to serve as sources of numerous pharmaceuticals widely used today (27). Plant-derived antimalarial drugs, such as quinine, artemisinin, atovaquone and azithromycin, are currently the primary choice for malaria treatment. Although several plant-derived compounds cannot be used directly as therapeutic agents due to their low bioavailability or poor solubility, their chemical structure may be further improved, in terms of increased efficacy or target specificity, in order to develop new antimalarial drugs (28). Bergenin was isolated from *R. aesculifolia* Batal, which is one of the most common plants in China and has been reported to possess multiple pharmacological properties, including antifungal, anti-inflammatory and antioxidant (29). As bergenin was used in folk medicine to treat protozoal infection and fever, we hypothesized that it may also exhibit antimalarial activity.

Through parasite LDH activity assay and morphologic examination, we demonstrated that bergenin was able to kill cultured *P. falciparum* effectively with a considerably low dosage, whereas it exerted no toxic effect on normal erythrocytes and other mammalian cells, such as HepG2 or HeLa cells. Furthermore, the parasitemia in *P. berghei*-infected mice was significantly decreased following treatment with bergenin for 6 days. These data indicated that bergenin possesses antimalarial properties. However, compared to its high potency *in vitro*, bergenin exhibited limited antimalarial effectiveness *in vivo*, which may be explained as follows: i) the water solubility of bergenin is poor, which may significantly reduce the bioavailability of orally administered bergenin and poor water solubility may hinder drug development in ~40% of lead candidates (30); ii) it is well-known that there is currently no suitable small animal model for *P. falciparum* research; therefore, although we assessed the anti-*P. falciparum* activity *in vitro* and anti-*P. berghei* activity *in vivo*, the possibility that *P. berghei* exhibits a different response to bergenin compared to *P. falciparum* cannot be excluded; and iii) chemical modification may be necessary to enhance the bioavailability of bergenin. For example, the bioavailability of the bergenin-phospholipid complex was significantly increased to 439% compared to that

of bergenin (31). In addition, bergenin pentaacetate was shown to exhibit considerable antibacterial activity, while bergenin was inactive (11). Those findings indicate that changing the physicochemical properties of bergenin to enhance its antimalarial activity may be feasible.

In addition, we assessed the possible cytotoxic effect of bergenin on normal erythrocytes and two other mammalian types of cells (HeLa and HepG2 cells). According to the bergenin maximal solubility in DMSO and the maximal tolerance of the cells for DMSO [the cells will die when DMSO reaches 1% (v/v)], the maximal dosage of bergenin was set to 5 mg/ml in our trials. The calculated CC_{50} of bergenin was >10 mg/ml (HeLa cells, 12.5 mg/ml and HepG2 cells, 19.1 mg/ml). Although the antiplasmodial IC_{50} of bergenin was found to be >10 μ g/ml (14.1 μ g/ml), the selectivity index (SI), defined as $SI = CC_{50}/IC_{50}$, was considerably high (HeLa cells, 887; HepG2 cells, 1,355), due to its good tolerability, which suggests that bergenin may be a promising novel antimalarial compound ($SI > 60$) (32).

In summary, this study demonstrated the antimalarial activity of bergenin, a sesquiterpene lactone compound isolated from *R. aesculifolia* Batal, against *P. falciparum* *in vitro* and *P. berghei* *in vivo*. However, further investigation is required to establish bergenin as a novel lead compound for antimalarial drug research and development.

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