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Assessment of *in vivo* antimalarial activity of arteether and garlic oil combination therapy



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ABSTRACT

The study evaluates *in vivo* antimalarial activity of arteether and garlic pearl oil combination in *Plasmodium berghei*-infected mouse model of malaria. 72 h (Day 3) post infection, at 2–4% parasitemia, mice were treated with single dose intramuscular injection of α - β arteether, at 750 μ g, in combination with three 100 μ L oral doses of garlic pearl oil on Day 3, Day 4 and Day 5. Following the treatment, 100% protection and survival of mice were observed. Inhibition of parasitemia in combination treated animals and protection during recrudescence interval of α - β arteether monotherapy was observed in Giemsa-stained blood smears. In addition, a striking increase in anti-parasite antibody IgG contributing protective immunity during the recrudescence phase was observed. These results correlate with western blot analysis, where sera from the recrudescence stage and later period of arteether and garlic oil combination treated animals found to interact with several parasite specific proteins as compared to controls. The present approach shows that arteether and garlic pearl oil combination provides complete protection in *P. berghei*-infected mice. Thus, for the first time, garlic pearl oil appears to be an ideal antimalarial candidate in artemisinin combination therapy.

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1. Introduction

Malaria, a mosquito-borne disease caused by protozoa parasites (a type of unicellular microorganism) of the genus *Plasmodium*, is the main cause of mortality in children, pregnant women and immune depressed people in disease-endemic countries [1]. According to statistics, ~60% of malaria death occurs in underdeveloped countries [2]. In the last decade, several attempts have been made to improve the situation by multifaceted approach, such as discovering and developing new antimalarial drugs, facilitating their delivery to remote areas and distributing them at an affordable price. In spite of these concerted efforts, the success was less than desired; the reason being development of drug resistance to monotherapy, high cost of artemisinin-based therapy, lack of investment for development of anti-parasitic drugs and unawareness among the general public about the malaria prevention and treatment [3]. Thus, need of the hour is improvement in the therapeutic potential of existing antimalarial drugs by using them in combination and by developing suitable drug delivery system. Among these two approaches, to succeed in combination therapy,

search for an ideal combination of drugs that can act synergistically or additively is of prime importance to (a) prolong the development of resistance to antimalarial drugs, (b) reduce the cost of treatment and (c) stop recrudescence.

Among different types of combination therapies, artemisinin- a well known antimalarial drug- based combination therapy is the most preferred one [4]. Artemisinin and its derivatives are essentially sesquiterpene lactone endoperoxides and are potent antimalarials [5]. Mechanism of action of artemisinin basically involve free-radical generation by alkylated heme adducts and proteins [6]. There's also a view that artemisinins have a specific target PfATP6, a SERCA-type calcium-ATPase [7]. Several combinations with artemisinin derivative have been advocated for their antimalarial activity such as artesunate + sulfadoxine-pyrimethamine, artesunate + mefloquine, dihydroartemisinin + piperazine and artemether + lumefantrine [8]. However, the existing artemisinin-based combination therapies have several drawbacks, such as toxicity, high cost, limited availability of artemisinin and low bioavailability of artemisinin and its partner drugs [9]. In this regard, we have made an attempt to find a suitable partner drug for arteether, a semi-synthetic derivative of artemisinin, which is preferred for the treatment of chloroquine-resistant malaria in most parts of the world [10].

Garlic (*Allium sativum*), for years, has been widely used as a medicine to reduce various risk factors associated with several

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diseases. It contains bioactive components that have proven beneficial in treatment of cancer, infection, inflammation etc. [11]. It is also known to exhibit anticoagulant, antioxidant, antibiotic, hypocholesterolaemic, hypoglycaemic and hypotensive activities [12]. The problem, however, is that the biological studies have not gone hand in hand with the chemical studies, with the frequent use of garlic products of undefined composition. Very few studies have used a chemically characterized product [13]. The composition of garlic depends on the source, age, storage conditions, type of processing and method of consumption. Unfortunately, the different forms of garlic are frequently referred to as “garlic” in both the common and scientific literature. The amount of main component of garlic organosulfur compounds vary within the different strains of garlic, and the problem is compounded by the volatile and reactive nature of these compounds [14].

Major volatile organosulfur functional compounds present in garlic oil are alliin, diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS). Fresh garlic contains mainly alliin, upon crushing, converted to thiosulfinate compound, allicin by alliinase. The mechanism of action of garlic oil identified by various studies include (a) suppressed LDL oxidation for anti-atherosclerotic effect, (b) inhibition or inactivation of thrombin for anticoagulant effect (c) arachidonic acid cascade pathway inhibition for anti-platelet activity (d) ROS mediated cytotoxicity for anticancer effects [12]. On the other hand, allicin (diallyl thiosulfinate) effects with a wide range of biological activities and has been used for centuries owing to its therapeutic and health-promoting properties [15]. Garlic when crushed produces allicin by non-proteinogenic amino acid alliin (S-allyl cysteine sulfoxide). Allicin, a reactive sulfur species (RSS) [16] with oxidizing properties, acts via redox-dependent mechanisms [17], and has wide-ranging and promising applications in medicine. Allicin acts as an antioxidant at lower dose in cardiovascular problems at physiological level [18,19]. It also acts at immune-correlated diseases [20]. The antimicrobial properties of garlic have been well studied, with studies being recorded as early as the 1940s [21]. Garlic has also been shown to have role against plant pathogens [22], pathogenic fungi [23] and at molecular level in human pathogens [24].

There is a clear necessity for standardization of garlic preparations, especially for use in biological studies. More recent biological studies have attempted to rectify this problem. Hence, we have used the ayurvedic medicine garlic pearl oil, locally available in India which is already in use for management of cholesterol, hypertension, digestion and to improve immunity. Garlic pearls are made from extract of concentrated source of a special variety of garlic. Garlic pearl oil is known for its activity due to the presence of active compounds like allicin and diallyl sulfide (DAS), which are preserved through a special process by garlic pearls' manufacturers. Garlic pearl oil from Ranbaxy India has been used in several studies such as antioxidant activity in myocardial infarction [25], gastroprotective activity in gastric ulcers [26], as a supplement for hypertension [27] and also when used in combination was effective in the correction of dyslipidemia [28]. Studies have also shown that allicin and ajoene, bioactive components of garlic, could partially protect mice from malaria infection individually or in combination with other antimalarial drugs [29,30]. In addition, use of garlic has several other advantages such as it is non-toxic, easily available in plenty, been used by humans for > 5000 years. Thus, garlic could be an ideal candidate for antimalarial drug. In this study, we have explored the novel arteether and garlic pearl oil combination therapy for malaria treatment.

2. Materials and methods

2.1. Drugs and chemicals

Commercially available antimalarial drug α - β arteether (E MAL™, Themis Medicare Ltd., Mumbai, India) and ayurvedic medicine garlic pearl oil (Ranbaxy Laboratories Ltd., New Delhi, India) were procured locally.

2.2. Animals and treatment in mice

Swiss mice (25 \pm 5 g) were used in the study. They were obtained from the Central Animal Facility, Indian Institute of Science, Bangalore, India. All precautions were undertaken to minimize suffering. Animal experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA). The treatment of mice and all experimental protocols were according to the Institutional Animal Care and Use Committee guidelines.

To study the *in vivo* antimalarial activity of arteether and garlic pearl oil either individually or in combination at various dosage levels, malaria parasite *P. berghei*-infected blood with 60–70% parasitemia was collected and injected intraperitoneally into experimental mice on Day 0 (start of experiment) after appropriate dilution. All the control mice died between Day 4 and Day 6 post infection. After 72 h (Day 3), when the parasitemia was about 2–4%, infected mice were treated with either single dose of arteether (750 μ g or 1.5 mg) intramuscularly and/or three oral doses of garlic pearl oil (50, 100, 150 μ L/mouse on Day 3, 4 and 5) to non-anesthetized mice as shown in Table 1. Blood was drawn from tail vein to check the parasitemia progression or inhibition at regular time intervals and mouse mortality was noted daily by staining blood smears with Giemsa. *In vivo* antimalarial activity was examined in groups of 10 male mice in three independent experiments.

2.3. Serum sample preparation

Blood was collected from mice on different days of post infection and treatment by cardiac puncture and allowed to clot for 2 hours at room temperature. The clotted material was removed by centrifugation at 5000 rpm for 15 minutes. The obtained serum was stored at -80 °C in 100 μ L aliquots until further analysis.

2.4. Analysis of parasite specific Immunoglobulin G (IgG) responses by ELISA

Soluble parasite proteins were prepared as described by Ang [34]. The parasite pellet was suspended in an appropriate volume of PBS followed by sonication (5 min at 4 °C) and centrifugation at

Table 1
Distribution of animals for treatment and treatment protocol ($n=10$).

Group	Treatment (Day 3)	Treatment (Day 4)	Treatment (Day 5)
Control	–	–	–
G50	G	G	G
G100	G	G	G
G150	G	G	G
AE 750 μ g	AE	–	–
AE 1.5 mg	AE	–	–
G50 μ L+AE 750 μ g	AE/G	G	G
G100 μ L+AE 750 μ g	AE/G	G	G
G150 μ L+AE 750 μ g	AE/G	G	G

G; garlic pearl oil; AE; arteether; AE/G; garlic pearl oil and arteether combination treatment.

10,000 g for 10 min. The supernatant was stored at -80°C and used to estimate parasite-specific IgG by ELISA. 10 μL of the supernatant was coated onto 96-well ELISA plates (4°C over night) and reacted with 1:10 diluted serum in a total volume of 100 μL . After carrying out the blocking and washing steps, 50 μL of secondary goat anti-mouse IgG-peroxidase conjugate (Bangalore GeNei™, India) at 1:3000 dilution was added per well and color developed with ABTS substrate was measured at 405 nm using an ELISA plate reader.

2.5. SDS-PAGE and western blot analyses

Parasite proteins (100 μg) were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were cut in vertical strips, blocked and incubated with individual serum samples diluted 1:50 in PBS, pH 7.4. Antibody responses were revealed with monoclonal secondary antibodies in dilution of 1:1000 followed by incubation with AP-conjugated anti-mouse antibodies (Dako, Hamburg, Germany). Western blots were performed with SDS-PAGE gels; all loaded with the same protein preparation (100 μg) and run under identical electrophoretic parameters. The strips shown originate from a single blot.

2.6. Statistical analysis

Log rank test was used for analysis of survival curves. All calculations were performed on the Prism 5.0 statistical program (GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered significant.

3. Results and discussion

As shown in Figs. 1 and 2a, untreated mice died within 4–6 days post infection as the parasitemia increased consistently. In contrast, administration of 50 $\mu\text{L}/\text{mouse}$ of garlic pearl oil resulted in the survival of animals up to Day 8 (20%). However, increase in the administration volume of garlic pearl oil to 100 and 150 $\mu\text{L}/\text{mouse}$ did not result in extension in survival period as compared to group treated with 50 $\mu\text{L}/\text{mouse}$ garlic pearl oil. But percentage of mice survived up to Day 8 increased from 20% to ~60 and 70% in case of 100 and 150 $\mu\text{L}/\text{mouse}$ garlic pearl oil treated mice, respectively (Fig. 2b). Though the data from the garlic monotherapy is encouraging, all animals died of malaria infection with little resistance compared to untreated mice group. Later α - β arteether, a synthetic derivative of artemisinin, was chosen as the partner drug for garlic pearl oil. Initially, the effect of arteether

monotherapy on parasitemia progression and mortality was studied. Treatment of infected mice with arteether at 750 $\mu\text{g}/\text{mouse}$ resulted in the initial clearance of parasitemia, but all animals died of malaria infection between Day 18 and 21 (Fig. 2c and d). However, treatment of mice with 1.5 mg of arteether resulted in complete protection of animals from malaria infection for the study duration in terms of mortality, parasitemia progression and recrudescence (Fig. 2f). Further, to study the effect of combination therapy, infected animals were treated with both arteether (single dose) and garlic pearl oil (three doses). As shown in Fig. 1, treatment with 50 $\mu\text{L}/\text{mouse}$ garlic pearl oil and 750 μg of arteether resulted in enhanced protection. In this combination treated group, 100% of animals survived up to Day 19 and they succumbed to malaria infection between Day 19 and 22.

Interestingly, increasing the garlic pearl oil quantity from 50 $\mu\text{L}/\text{mouse}$ to 100 or 150 $\mu\text{L}/\text{mouse}$ in combination with α - β arteether at 750 μg resulted in complete protection of animals from malaria infection (Fig. 2e). These results clearly indicate that by adding garlic pearl oil to arteether therapy as a partner drug antimalarial activity can be enhanced. Particularly this combination was successful in avoiding the recrudescence problem which is often the major limiting factor in artemisinin and its derivative based monotherapy as shown in our study (Fig. 2d) and earlier clinical trials [31]. Although the exact mechanism of action is not clear yet, from earlier studies it can be presumed that when infected mice were given suboptimal dose of arteether, it will clear the parasite quickly from the blood (erythrocytic stage) and garlic pearl oil, which mainly composes of cysteine protease inhibitors and thio-sulfinate products (Diallyl disulfide, Diallyl trisulfide, Allyl methyl trisulfide etc), may have evaded infection of new blood cells by inhibiting cysteine protease enzyme which is essential for the invasion of malaria parasites to new red blood cells. In addition, garlic pearl oil may have decreased the parasite load from the reservoirs such as liver and spleen leading to reduced sporozoite infectivity which in turn will clear the pre-erythrocytic parasites as observed in earlier studies [32]. Adding to this, garlic pearl oil treatment may elicit innate and adaptive immunity which are shown to be useful in eradicating the parasites and to avoid the recrudescence of malaria parasites [29]. Studies have also shown similar immunomodulatory effect of another well known natural compound curcumin in malaria infected mice [33].

To know the immunomodulatory role of the existing combination therapy, we studied the levels of parasite specific immunoglobulin (IgG) with different drug treatments to parasite infection.

IgG concentrations were measured using ELISA in order to determine the level of humoral immune response provoked in the *P. berghei*-infected mice due to drug treatment [34]. AE and garlic combination therapy resulted in a striking increase in anti-parasite antibody IgG contributing protective immunity during the recrudescence phase (Fig. 3). These results correlate with western blot analysis, where sera from the recrudescence stage and later period of arteether and garlic pearl oil combination treated animals found to interact with several parasite specific proteins as compared to the controls (Fig. 4).

The above observation suggests that there is growing evidence concerning the protective role of IgG in malaria infections and also demonstrates a differential parasite antigen reactivity of IgG response acquired by host against malaria infection. This has been carried out using ELISA and western blot analysis, which are the conventional approaches widely used to study host immune response against malaria antigens. At later period of time (after 90 days), when the antibody titre went down, fresh parasite injection (Figs. 3 and 4) lead to increase in the levels of IgG, showing the previous observation of animals. Our data support studies that showed mice once cured of parasite infection remain completely

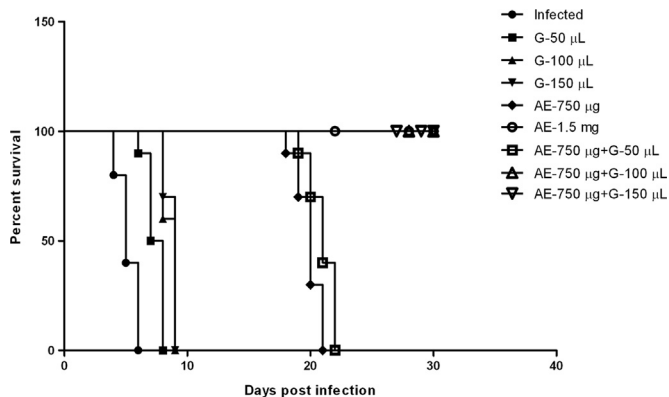


Fig. 1. Survivability of *P.berghei*-infected mice after treatment with garlic oil and/or arteether. Survival curves were generated from three independent experiments with a total of 10 mice per group. AE, arteether; G, garlic pearl oil.

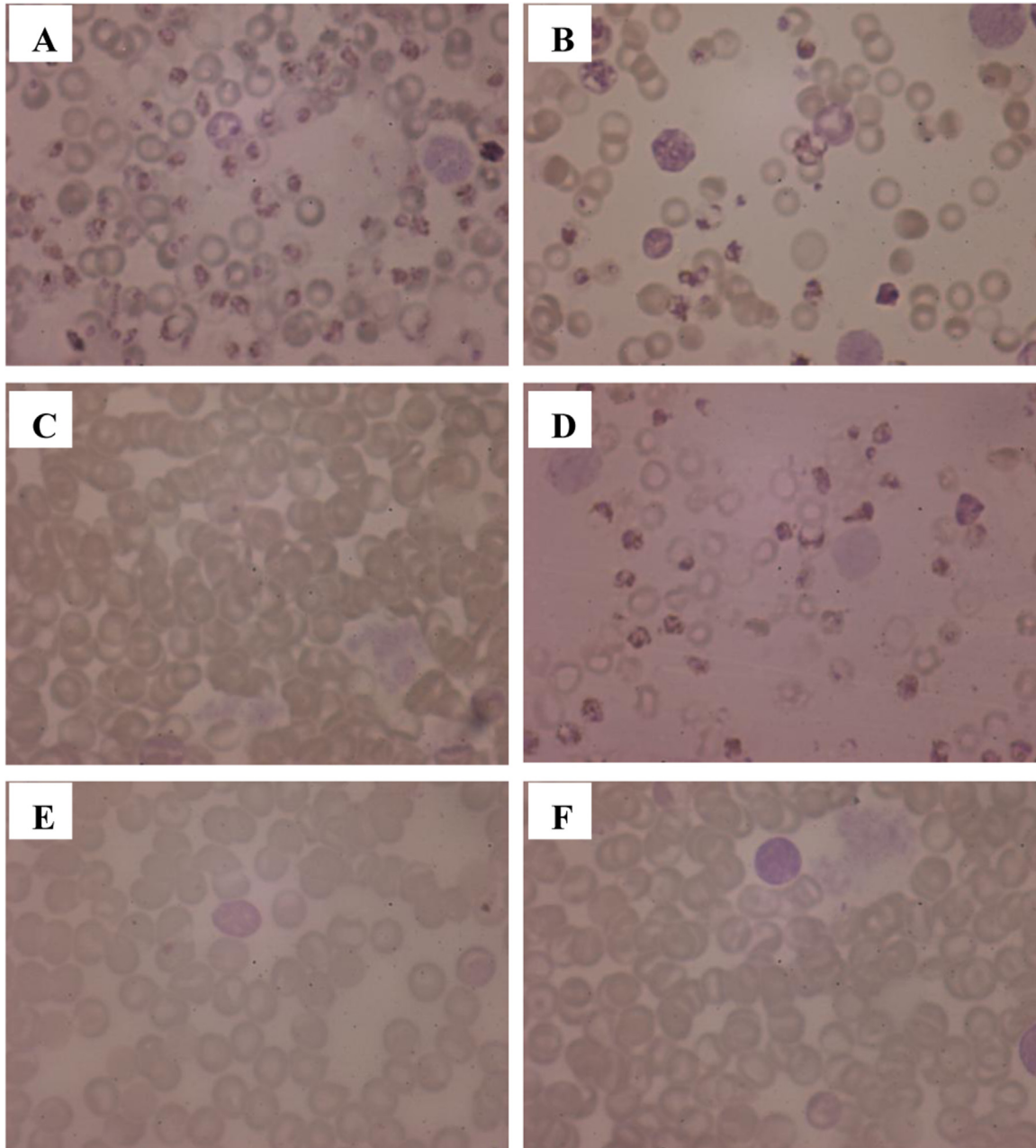


Fig. 2. Photomicrographs of blood smears showing the effect of different treatments on parasite growth with reference to control group. (A) Control on Day 5. (B) Garlic oil 150 $\mu\text{L}/\text{mouse}$ on Day 5. (C) AE 750 μg on Day 5. (D) Recrudescence in AE 750 μg treated on Day 21. (E) AE 750 μg +garlic oil 100 μL on Day 21 (F) AE 1.5 mg on Day 21. Photographs were taken at 100X magnification.

protected by further infections [35].

It is known that for *cystic fibrosis* one garlic capsule has been taken daily for eight weeks; for *Helicobacter pylori* infection, allicin (the main ingredient in garlic) has been taken daily in combination with standard treatment orally for 14 days and is non-toxic. Additionally, Kyolic aged garlic extract plus steam-distilled garlic oil taken orally, daily twice, for seven years have showed no toxic effect [36]. These results have shown that garlic extract has no side effects or toxicity in human.

The exact mechanism(s) of garlic as an antimalarial drug is not clear. However, allicin has proven antimicrobial activity and also active against protozoan parasites including *Plasmodium*, where it is thought to be mediated by inhibiting cysteine proteases [37]. There are several reports of combination of garlic extracts with antibiotics [38] and also known for its antitubercular activity [39].

Protection of *P. berghei*-infected mice from early death by allicin through immunomodulation has also been documented [32,40].

4. Conclusions

The arteether and garlic pearl oil combination may prove superior from several perspectives. Both are herbal medicines of long term use, as such no resistance is known to garlic that's used as a dietary supplement. The results of this study are encouraging, as the most available artemisinin combination therapies suffer from one or the other drawbacks such as toxicity, high cost, resistance etc. The study shows that arteether and garlic pearl oil combination therapy provides complete protection in *P. berghei*-infected mice. This seems promising in terms of addressing some of the

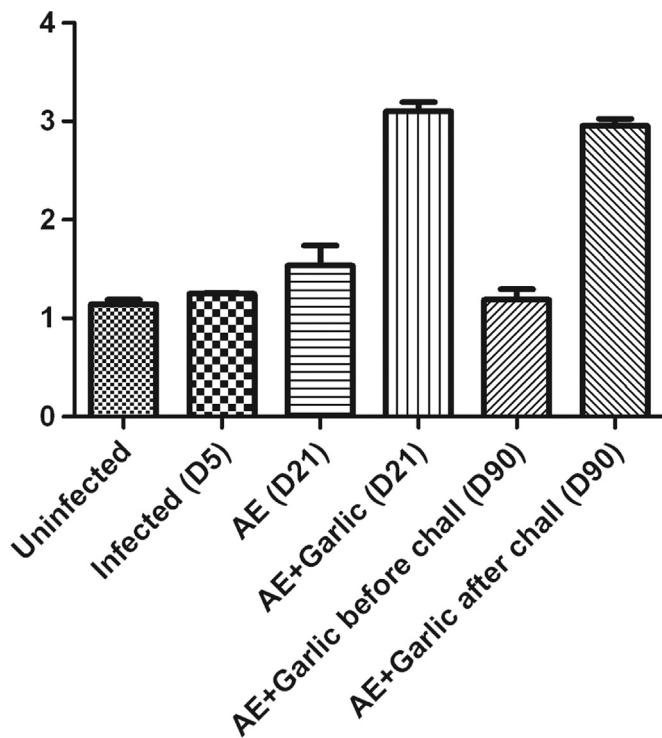


Fig. 3. Parasite specific Immunoglobulin G (IgG) responses from sera of different treatments of *P. berghei*-infected mice by ELISA. The data represent Mean with SEM from three different experiments.

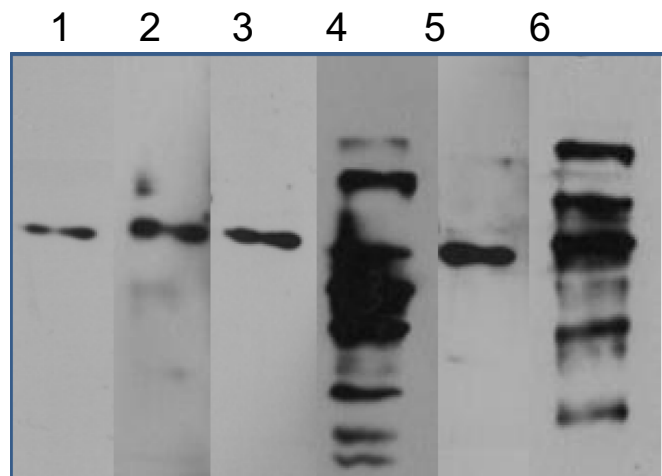


Fig. 4. Western blot analysis of parasite proteins with different treatments of *P. berghei*-infected mice 1, Uninfected; 2, Infected (D5); 3, AE (D21); 4, AE+Garlic (D21); 5, AE+Garlic before challenge (D90); 6, AE+Garlic after challenge (D90).

pertaining issues of current therapy such as dosage of treatment and cost of therapy. In view of the above vision, further studies to identify and characterize specific pathways involved in the mechanism of the action of artemisinin and garlic oil combination therapy would be beneficial in developing low-cost antimalarial drug therapies, especially for drug resistant malaria.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.01.015>.

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