In vivo ANTIMALARIAL ASSESSMENT AND TOXICITY EVALUATION OF GARLIC (Allium sativum) IN Plasmodium berghei NK65-INDUCED MICE

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ABSTRACT
Garlic or Allium sativum is widely applied as alternative medicine and in ethnopharmacological studies. This study was done to evaluate the antimalarial properties of aqueous extract of garlic against Plasmodium berghei NK65. The groups of male ICR mice were intraperitoneally (i.p) infected with 0.1 mL of 1 × 10^7 parasitised red blood cells (RBC) before being orally given pre- and post-infection treatments with 0.2 mL of 100 mg/kg body weight (bw) of freeze-dried aqueous garlic extract. Parasitemia was microscopically examined and measured by Giemsa stained thin blood smear. There was a positive correlation (p<0.05, n = 6) for all assessed parameters; parasitemia density (%), survival time (day) and the ability to inhibit the parasite growth (%) between pre-treated infected mice with the other groups. However, the value recorded was still lower compared to the mice treated with commercial antimalarial drug primaquine and chloroquine. However, biochemical parameters of treated animals were in the normal range indicative of no toxicity. Histological examination showed no abnormalities and injuries on the selected vital organs. This study proved garlic has potential as alternative antimalarial drug.

Key words: Garlic, antimalarial, in vivo, Plasmodium berghei NK65, parasitemia

INTRODUCTION
Malaria is a deadly vector-borne disease in which most of the epidemics are being reported in tropical and subtropical regions of the world. The control against this disease was actively undertaken after Second World War (Carnevale & Mouchet, 1987). However until today, malaria is considered as neglected disease in the tropical regions in which hundreds of millions of population are at risk (Kilama & Ntoumi, 2009). Approximately in 2014, out of 3.3 billion people are at risk, 1.2 billion are at high risk of malaria. Surprisingly in high risk regions which involved 97 countries and territories, more than one case of malaria occurs in 1000 population where 712,000 deaths were being reported in African region alone (World Health Organization’s World Malaria Report, 2014). According to Vector-Borne Disease Unit, Ministry of Health Malaysia (MOH) Annual Report 2014, of 4,725 reported cases in 2014, 61.6% were human malaria infection and a proportion of 38.4% were significantly zoonotic. There are about more than 100 species of Plasmodium were found worldwide but significantly, only five distinct Plasmodium species that infect humans: P. falciparum, P. vivax, P. malariae, P. knowlesi and P. ovale (Sinden & Gilles, 2002).

Antimalarial treatment is dependent on chemical synthetic drugs which are facing development of resistance. Allium sativum or garlic has long been known and applied in daily life for various purposes as it contains various medical properties and elements that can be utilised to treat various diseases among many communities in the world (Gurusubrammaniam et al., 1997, Oommem et al., 2004). Somehow, very limited information was available regarding its properties towards antimalarial activities. On top of exhibiting antibacterial, antifungal and anticancer properties (Moazeni & Nazer, 2010, Oommem et al., 2004), scientifically documented, garlic extracts are also outstanding inhibitor towards the growth of some important blood flagellated protozoa such as
Trypanosoma brucei and Leishmania donovani (Ramos et al., 2006), as well as Entamoeba histolytica and Trichomonas vaginalis (Saleehen et al., 2004).

Herbs and plants are utilised by the world population as their daily alternative medicine to treat many vector borne diseases including malaria (Baba et al., 2015). This study was aimed to evaluate the in-vivo antimalarial activities of freeze-dried aqueous garlic extract against P. berghei NK65 infected mice. The toxicity assessment by means of enzyme activities and total protein in mice blood, the histopathology assessment of mice liver and kidney were investigated in this study.

MATERIALS AND METHODS

Preparation of garlic extract

The method documented by Varmaghany et al. (2015) with modification was followed. The skin from garlic cloves were removed. The aqueous extract was prepared by mixing 1.5 kg of ground garlic with 2.0 L of sterile distilled water (sdH2O). The mixture was allowed to stand for over night at 28°C before it was filtered through 11 µm qualitative cellulose filter paper. Filtration process was repeated for twice with the final filtered solution was transferred into several containers to undergo freeze drying process. The final product of 250 g powder form of freeze dried-garlic was stored in 4°C freezer.

Experimental animal

All animal experiments were conducted following approval by the Institutional Animal Care and Use Committee (IACUC-IIUM) coded IUUM/IACUC Approval/2016(9)(31). Male mice (The Philadelphia Institute of Cancer Research, ICR strain) with body weight (bw) between 25 – 30 g and age (6 – 8 weeks old) were obtained from the Animal House, Universiti Kebangsaan Malaysia (UKM). The handling and management of the mice were organised based on the guidelines provided by IACUC-IIUM where every mice group (n = 6 per group) were placed in stainless steel cages at room temperature and fed ad-libitum in 12 hours with and without light periods.

Parasite inoculum

The in vivo experiment in mice was performed by using non-pathogenic human parasite, P. berghei NK65. To maintain this rodent malarial parasite, ICR male mice were intraperitoneally (i.p) administered with the inoculum consisted of 1.0 × 10⁷ P. berghei parasitised red blood cells (RBC) from the donor mouse before the parasitised RBC were serial diluted with Alsever’s solution. Every mouse was i.p administered on Day-0 (D0) with lethal dose of 0.1 mL blood solution containing 1 × 10⁷ P. berghei-infected RBC (Abdulaleh & Zainal-Abidin, 2007).

Treatment regime and schemes

The mice in each tested group were daily treated with 0.2 mL 100 mg/kg bw of sdH2O-garlic extract. A single dose of 0.1 mL 15 mg/kg bw of primaquine was given to the mice in CP group on Day-3 (D3) pre-infection as the preventive positive control group. This was in parallel with the normal practice where prophylactically, primaquine should be taken three days before entering any malarial-endemic areas (Hill et al., 2006). As the signs and symptoms of malaria were practically appeared within three to four days after infection (Okokon et al., 2005), a single dose of 0.1 mL 10 mg/kg bw of chloroquine was given to the mice in CC group on D3 post-infection (Okokon et al., 2005) as the curative positive control group. Starting on D7 pre-infection, a daily dose of 0.1 mL 0.9% normal saline solution was given to the negative control group (CN). Two groups treated under preventive regime were P7 (daily treated from D7 pre-infection) and P3 (daily treated from D3 days pre-infection). Two groups treated under curative regime were C3 (daily treated from D3 post-infection) and C7 (daily treated from D7 post-infection). All of these treatments were orally force-fed to each mouse.

Parameters for antimalarial screening

Observations on the parasitised RBC were done under light microscope (Leica ICC50 HD) using Giemsa stained thin blood smear. The parasitaemia density (%) and inhibition rate (%) of each group of the mice were calculated. Four days (4D) suppression test method (Abdulaleh & Zainal-Abidin, 2007) with modification was used to evaluate the garlic extract activity and determination of inhibition rate against the parasite’s growth. The following formulae were used:

\[
\text{Parasitemia density} = \frac{\text{The mean of total of infected RBCs observed}}{\text{The mean of total RBCs observed}} \times 100
\]

\[
\text{Inhibition rate} = \frac{\text{Parasitemia of negative control} - \text{Parasitemia of treatment}}{\text{Parasitemia of negative control}} \times 100
\]

The mice were observed daily in order to assess their survival period (days) from the infection day (D0). Inhibition rate percentage at more than 65% was set as having in-vivo antimalarial activity and the mice group receiving the best treatment regime was
considered when they recorded the longest survival period (Abdulelah & Zainal-Abidin, 2007).

Parameters for in-vivo toxicity assessment
Toxicity assessment of treated mice was also done by using biochemical analyses. Blood samples of the mice treated with 0.2 mL 100 mg/kg bw of sdH2O-garlic extract were taken from mice that were treated in acute toxicity exposure (daily treatment for seven days) and sub-acute toxicity exposure (daily treatment for 28 days). Every toxicity exposure was divided into two groups of treatment; treatment without infection and treatment immediately within 2 hours post-infection. The mice groups were respectively labelled as AA for acute toxicity exposure without infection, AB (acute toxicity exposure within 2 hours post-infection), SA (sub-acute toxicity exposure without infection) and SB (sub-acute toxicity exposure within 2 hours post-infection).

For comparison, the data from two control groups; CA (normal mice with no infection and treatment) and CB (mice injected with lethal dose of blood solution containing 1 × 10^7 P. berghei-infected RBC) were also recorded. Under diethyl ether anesthesia, these mice were sacrificed and 0.8–1.0 mL of the blood was collected from each mouse by cardiac puncture on D8 (for acute toxicity exposure group) and D29 (for sub-acute toxicity exposure group). Comparing with the data from Research Animal Resources, University of Minnesota, USA, the blood was tested for alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and serum total protein (STP) levels. Two vital organs, kidney and liver, from these anaesthetised mice were extracted for organ histopathology studies. Using a computerised light microscopic camera (Zeiss Primo Star, New York Microscope Co., New York, NY, USA, and Dell, Dell Global Business Sdn. Bhd., Selangor, Malaysia), the hematoxylin-and-eosin (H&E) stained slides (Zin et al., 2017) were observed.

Statistical analysis
Results for antimalarial screening and biochemical toxicity assessment were expressed as the mean ± standard deviation (s.d.). The Shapiro-Wilk test was used (sample size, n=6) to analyse the significance of the antimalarial activity. All the tested groups were normally distributed and the statistically significant when P value was equal to or less than 0.05 (P < 0.05).

RESULTS AND DISCUSSION
Parasitemia density
Recorded at less than 2.0%, there was a significant difference (P<0.05, n=6) for parasitemia density between the mice in P7 group and the other three groups (P3, C3 and C7). Nevertheless, the parasitemia density recorded for P3 group was still far lower than that in C3 and C7 groups (Table 1). Outstandingly for all tested groups, the treatment at 0.2 mL 100 mg/kg bw of sdH2O-garlic extract was giving lower parasitemia as compared with the mice in CN group. As for CP, primaquine (0.1 mL of 15 mg/kg bw) treated and CC, chloroquine (10 mg/kg bw) administered groups, no parasitised RBCs were observed in any mice on D4 post-infection.

Inhibition rate
Since the value obtained for C3, C7 and CN was lower than 65%, the inhibition rate percentage for P7 and P3 groups was the only values where antimalarial activities could be best evaluated (Table 1). For sdH2O-garlic extract (0.2 mL 100 mg/kg bw) treated group, a significant difference was recorded (P<0.05, n=6) for this parameter between

<table>
<thead>
<tr>
<th>Regime</th>
<th>Group</th>
<th>Parasitemia density (%)</th>
<th>Inhibition rate (%)</th>
<th>Survival time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preventive</td>
<td>P7</td>
<td>1.13 ± 0.21*</td>
<td>83.43 ± 2.02*</td>
<td>149.36 ± 2.22*</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>2.19 ± 2.36*</td>
<td>67.89 ± 1.76*</td>
<td>53.77 ± 0.57*</td>
</tr>
<tr>
<td>Curative</td>
<td>C3</td>
<td>4.38 ± 1.10*</td>
<td>35.78 ± 1.54*</td>
<td>29.36 ± 3.42*</td>
</tr>
<tr>
<td></td>
<td>C7</td>
<td>5.09 ± 3.07*</td>
<td>25.37 ± 3.33*</td>
<td>18.09 ± 1.98*</td>
</tr>
<tr>
<td>Control</td>
<td>CP</td>
<td>0</td>
<td>100</td>
<td>&gt;360</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>0</td>
<td>100</td>
<td>&gt;360</td>
</tr>
<tr>
<td></td>
<td>CN</td>
<td>6.82 ± 1.61*</td>
<td>0</td>
<td>10.13 ± 2.22*</td>
</tr>
</tbody>
</table>

Notes: (*) Value in mean ± standard deviation (SD), (g) Value with significant different (P<0.05, n=6). P7: daily preventive treatment starting from seven days pre-infection, P3: daily preventive treatment starting from three days pre-infection, C3: daily curative treatment starting from 3 days post-infection, C7: daily curative treatment starting from seven days post-infection, CP: positive preventive control treatment with single dose of 0.1 mL of 15 mg/kg bw of primaquine on D3 pre-infection, CC: positive curative control treatment with single dose of 0.1 mL of 10 mg/kg bw of chloroquine on D3 post-infection, CN: negative treatment with daily dose of 0.1 mL 0.9% normal saline. bw: body weight.
the mice in P7 group and the rest of the tested groups except for positive control groups of CP and CC. This was in parallel with the earlier statements that the percentage for parasitemia density was directly reflects with the value of inhibition percentage. Abdulelah and Zainal-Abidin (2007) claimed that the lower the parasitemia density, the higher the inhibition percentage. More treatment that is effective will be observed if lower percentage of parasitemia density was recorded similar to observation by Baba et al. (2015) and Zin et al. (2017). Besides the inhibition rate at ≥ 65% was chosen as a benchmark value for in-vivo antimalarial activity according to Ata-ur-Rehman et al. (1985), the dosage of 15 mg/kg bw of primaquine (CP group) and 10 mg/kg bw of chloroquine (CC group) in positive control mice were effective in 100% inhibition against P. berghei NK65 on D4 post-infection. There may also be a tendency to obtain more significant and positive values of inhibition rate in specific stages of P. berghei NK65 life cycle (Rajendra et al., 2004) which however this factor was not considered in this study. Other than the mechanism and progression of the erythrocytic cycle in Plasmodium-infected host is yet still actively being debated (Reininger et al., 2011; Marhalim et al., 2014). Some researchers also believed that the immune status and development in the malarial-infected host incombating the Plasmodium infectionmay influence the outcomes of antimalarial activity (Kurosawa et al., 2000; Ali et al., 2014).

Mice survival time

In parallel with the inhibition percentage, the mice in P7 group was recorded as the most prolonged survival period as compared with the other regime groups. Inhibition percentage steadily extended and rose up almost three fold longer than the mice of P3 group which recorded as the second longest survival time (Table 1). At 149.36 ± 2.22 days, there was a significant difference (P≤0.05, n=6) of the mice survival between P7 group and the rest of the groups except for positive control; CP and CC groups. In previous in-vivo and in-vitro studies, it had been demonstrated that the survival times of the treated mice would be longer at a higher inhibition rate (Kurosawa et al., 2000; Jochen et al., 2002; Abdulelah & Zainal-Abidin, 2007; Prudhomme et al., 2008; Baba & Zainal-Abidin, 2011). This observation was also observed in this study.

At 53.77 ± 0.57 days, there was also a significant difference (P≤0.05, n=6) of the survival time for the mice of P3 group as compared with two mice groups of curative regime (C3 and C7). This indicated that the infected mice received prophylactic or preventive regime of garlic extract which recorded a longer survival time than those with curative regime. This is in parallel with the real human malarial prophylactic regime where primaquine are advised to be taken by travellers for at least five days before travelling to any malarial endemic regions (Hill et al., 2006). Outstandingly however, all of the tested mice treated with garlic extract were able to survive far longer that the mice in CN group. Due to perfect inhibition rate at 100% against P. berghei NK65 on D4 post-infection, the mice of CP and CC groups survived up to 18 months individually, which this period was exceeding the life span period of the normal mice (Sage et al., 1993; Szenczi et al., 2012).

Toxicity evaluation

Using the guideline issued by the Department of Research Animal Resources, University of Minnesota, USA (2012) as the reference, the results for all biochemical toxicity tests and serum total proteins were within the normal range (NR) (Table 2). In this study, ALT, AST, ALP and STP were all the enzymes and proteins investigated for the blood biochemical test on the mice treated with sdH2O-

Table 2. The level of ALT, AST, ALP (IU/L) and STP (g/dL) of the mice group treated with 0.2 mL 100 mg/kg bw of sdH2O-garlic extract as compared with control groups

<table>
<thead>
<tr>
<th>Regime</th>
<th>Group</th>
<th>ALT(IU/L)</th>
<th>AST(IU/L)</th>
<th>ALP(IU/L)</th>
<th>STP(g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute exposure</td>
<td>AA</td>
<td>43.25 ± 2.01*</td>
<td>142.20 ± 2.91*</td>
<td>72.16 ± 4.20*</td>
<td>6.62 ± 3.10*</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>47.79 ± 3.70*</td>
<td>150.98 ± 3.38*</td>
<td>78.43 ± 3.13*</td>
<td>7.21 ± 1.07*</td>
</tr>
<tr>
<td>Sub-acute exposure</td>
<td>SA</td>
<td>69.06 ± 3.04*</td>
<td>162.22 ± 3.09*</td>
<td>66.03 ± 3.01*</td>
<td>8.09 ± 2.76*</td>
</tr>
<tr>
<td></td>
<td>SB</td>
<td>72.12 ± 1.19*</td>
<td>177.01 ± 2.10*</td>
<td>70.70 ± 3.82*</td>
<td>8.76 ± 2.83*</td>
</tr>
<tr>
<td>Control</td>
<td>CA</td>
<td>49.25 ± 2.73*</td>
<td>119.88 ± 2.17*</td>
<td>60.09 ± 2.68*</td>
<td>6.95 ± 3.06*</td>
</tr>
<tr>
<td></td>
<td>CB</td>
<td>48.18 ± 1.93*</td>
<td>130.13 ± 2.84*</td>
<td>68.77 ± 2.94*</td>
<td>6.82 ± 3.81*</td>
</tr>
</tbody>
</table>

Indicator (NR)

|                   | 40 – 93 | 92 – 206 | 54 – 115 | 5.8 – 9.5 |

Notes: (*) Value in mean ± standard deviation (SD). AA: acute exposure for 7 days without infection, AB: acute exposure for 7 days started within 2 hours after infection, SA: sub-acute exposure for 28 days without infection, SB: sub-acute for 28 days started within 2 hours after infection, CA: normal control mice without any infection and treatment, CB: single lethal-dose of P. berghei NK65 infected mice. bw: body weight, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, STP: serum total protein, NR: normal range data for ICR strain male mice aged 8 weeks old for the indicator as supplied by Research Animal Resources, University of Minnesota, MN, USA, IU/L: international unit per litre, g/dL: gram per decilitre.
garlic extract. Enzyme levels are often associated with infectious diseases, immune-mediated disease and some types of cancer (Ganheim et al., 2007). However, in clinical practice, both ALT and AST level is the most reliable indicator for liver damage and malfunction (Craig et al., 2012).

Assessment of organ histology

Microscopically, all selected H&E stained kidney and liver tissues from the mice showed no abnormalities and injuries. Figure 1 and Figure 2 showed the H&E stained slides for kidney and liver tissues from mice treated with sdH2O-garlic. No sign of cellular damage and necrosis were observed. There were also normal intact of tubules and glomeruli in hepatocellular structure similar to the observation by Finn and Portr (2008). No Kupfer cells presented, hepatocytes degeneration structural and elevated number of mitotic figures in the liver indicative of normal conditions (Sturm et al., 2006).

Fig. 1. The microscopic H&E histology slides (200× magnification) of the kidney for toxicity assessment of selected preventive treated mice from group AA (A), AB (B), SA (C) and SB (D) as compared with normal control mice from group CA(E). Normal glomeruli (GML), Bowman’s space (BMS), proximal convoluted tubule (PCT) and distal convoluted tubule (DCT).
Besides, the sinusoids were uncongested and the hepatocytes were seemly intact with each other.

These two organs were also observed having no physical injuries or swellings, no morphological abnormalities and no changes of colour on the targeted tissues due to the exposures to garlic extract. Thanaporn et al. (2010) stated that, theoretically, any suspicious abnormalities observed on the five vital organs; kidney, liver, spleen, brain and lungs during the process of organ isolation, histologically, it will be expected to be observed in H&E staining.

CONCLUSION
The aqueous extract of garlic has promising antimalarial properties which can be further
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REFERENCES


