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Effect of Nelumbo nucifera Stamen Extract on Phagocytosis and Malaria Parasite Growth Against *Plasmodium Berghei* Infected Mice

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Abstract

Antimalarial drug resistant malaria parasites are causing not only the spread of malaria to new areas but also its re-emergence in areas where it had previously been eradicated. In addition, malaria-associated impairment of phagocytosis has been reported during malaria parasite infection. The present study has been carried out to investigate the effect of *Nelumbo nucifera* stamen extract on phagocytosis and malaria parasite growth against *Plasmodium berghei* infected mice. Groups of ICR mice were treated orally by gavage with *N. nucifera* stamen extract (500, 1,000 and 2,000 mg/kg) after infection with *P. berghei* ANKA. Parasitemia, percent phagocytosis and phagocytic index were determined. At these doses, *N. nucifera* stamen extract inhibited parasitemia in dose-dependent manner, with similar level of antimalarial activity to chloroquine (5 mg/kg). In addition, increasing of phagocytosis and phagocytic index has also been observed in dose-dependent in infected mice treated with the extracts. In particularly, the highest activities of *N. nucifera* stamen extract were found at dose 2,000 mg/kg. These results indicated that aqueous crude extract of *N. nucifera* stamens have antimalarial and improve phagocytic activity against *P. berghei* ANKA infected mice.

Keywords: *Nelumbo nucifera*; Phagocytosis; Malaria; *Plasmodium berghei*

Introduction

Malaria remains one of the world's largest burdens of disease. With an estimated 2.5 billion people at risk, it causes 300-500 million infections and 1-3 million deaths annually, the greatest part of the latter in children under five years of age [1]. Malaria is caused by protozoa parasite in genus *Plasmodium* that transmitted by female *Anopheles* mosquito. Five species of malaria parasites including *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* are responsible for human infection, although the majority of fatal cases are caused by *P. falciparum* [2,3]. Although an effective vaccine is the best long term control for malaria, current research on vaccine development is still in the laboratory. Therefore, the strategy for malaria mainly focuses on drug treatment. However, antimalarial drug resistant malaria parasites are causing not only the spread of malaria to new areas but also its re-emergence in areas where it had previously been eradicated [4,5]. In addition, malaria-associated impairment of phagocytosis has been reported during malaria parasite infection [6-9]. This has prompted research towards the discovery of new antimalarial drugs with phagocytosis recruitment properties. In this respect, plant extracts are potential targets for research and development of the alternative drugs.

Nelumbo nucifera Gaertn, commonly known as lotus, is a large aquatic plant and has long been used as a medicinal herb in China, Japan, India, Korea and Thailand. Nearly every part of *N. nucifera* including buds, flowers, anthers, stamens, fruits, leaves, stalks, rhizomes and roots have been used as medicinal plant for treatment of cancer, heart problems, hypertension and insomnia [10]. Catechin, quercetin, quercetin-3-O-glycoside, kaempferol-3-O-glycoside and myricetin-3-O-glucoside have been reported as part of its major components [11,12]. Pharmacological and physiological activities including anti-diabetic, cytoprotective, hepatoprotective, anti-bacterial, antioxidant, anti-hypertensive, anti-hyperlipidemia, hematopoietic system, and anti-obesity effects have been described from the extracts of *N. nucifera* leaves, seed and rhizome [13-16]. In particular, *N. nucifera* stamens are flavonoid-rich and have a variety properties including antioxidant, anti-inflammation, anti-microbial and anti-cancer [11,12,17,18]. Flavonoids are its important constituents that belong to a group of natural substances with variable phenolic structures found in fruits, vegetables, grains, flowers, tea and wine. Moreover, flavonoids have been described to increase phagocytic activity and exerted antimalarial effect *in vitro* against *P. falciparum* [19-21]. Although biological and therapeutic efficacies of *N. nucifera* have been reported to a certain extent, the studies on the antimalarial activity and phagocytosis during malaria infection have not yet been performed. Hence, in the present study, we investigated the effect of *N. nucifera* stamen extract on phagocytosis and malaria parasite growth *in vivo* using *P. berghei* infected mouse model.

Materials and Methods

Plant material

The stamens of lotus (*N. nucifera*) were obtained from Rangsit Science Centre for Education, and authenticated by Dr. Saowanee Buatone.

Preparation of crude extract

Aqueous crude extract of *N. nucifera* stamens was prepared as previously described [22]. In brief, the dried powdered sample was mixed with distilled water in a ratio of 1:5 (w:v), and heated using microwave at 360 W for 5 min. Incubation at room temperature for 30 min was subsequently performed with continuously stirring to complete

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extraction. Then, it was filtered through gauze and centrifuged at 10,000 g, for 10 min. The aqueous crude extract of *N. nucifera* stamens was stored at 4˚C.

Experimental mice

Healthy ICR mice (female, 6-8 weeks old weighting between 30-35 g) obtained from National Laboratory Animal Center, Mahidol University, Thailand were used. The mice were conveniently housed under standard environmental condition at 22-25˚C with a 50% relative humidity and a 12hr light/dark cycle. All mice had ad libitum access to commercial feed pellets and clean water throughout the study. All animal experiments were approved and ratified by the Animal Ethic Committee, Western University.

Rodent malaria parasite

Plasmodium berghei ANKA strain (PbANKA) was used in this study. The parasite was maintained in our laboratory by weekly serial passage of 1×10^7 infected erythrocytes in naïve mice. Parasitemia was daily monitored by microscopic examination of Giemsa stained thin blood smear with 100× oil immersion lens.

Acute toxicity test

Acute toxicity of *N. nucifera* stamen extract was carried out as previously described [23]. Groups of mice (5 mice of each) were given 500, 1,000, 2,000 and 3,000 mg/kg body weight of the extract orally. The mice were then observed for signs of toxicity which include but not limited to paw licking, salivation, stretching of the entire body, weakness, sleep, respiratory distress, coma and death in first 4hr and subsequently daily for 14 days.

Evaluation of phagocytosis

The phagocytosis was performed a method previously described with some modification [24]. Briefly, Blood (10 µl) was collected from tail vein and gently smeared into a square of 1 cm^2 on a clean microscopic slide. The slide was subsequently incubated in a moist chamber at 37˚C for 2 hours and rinsed with 0.9% normal saline solution (NSS) to remove non-adherent cells. Adherent cells were incubated with 100µl of heat-killed *Saccharomyces cerevisiae* (3 mg/ml) for 1 hour in a moist chamber at 37˚C and rinsed 3 times with 0.9% NSS. Subsequently, the slide was fixed with absolute methanol and stained with Giemsa solution. The slide was air-dried and examined under light microscope. The sample was analyzed under an oil-immersion objective, counting at least 100 cells in different field. The percentage phagocytosis and the phagocytic index (PI) were calculated following the formula below.

%Phagocytosis = (number of phagocytic cells/total number of white blood cells) x 100

Phagocytic index (PI) = (number of engulfed yeast cells/total number of phagocytic cells) x 100

Antimalarial drug

Chloroquine (CQ) was used in this study as standard antimalarial drug. The drug was freshly prepared in distilled water and administered orally by gavage. Drug dose, expressed in mg/kg of body weight, was adjusted at the time of administration according to the weight of each mouse. The dose was based on the ED90 (5.0 mg/kg) on PbANKA infected mice [25].

Efficacy test *in vivo*

The standard 4 day test was employed in this study [26]. Randomly groups of naïve ICR mice (5 mice of each) were inoculated by intraperitoneal injection with 1×10^7 infected erythrocytes of PbANKA, and treated for 4 consecutive days with 500, 1,000 and 2,000 mg/kg of *N. nucifera* stamen extracts orally by gavage twice a day (day 0-3). Three control groups were used; the healthy control was given either with distilled water or the extract (2,000 mg/kg); the untreated control was given distilled water; the drug treatment control was given 5.0 mg/kg of CQ. On day 4 of the experiment, tail blood was collected to determine parasitemia, percent phagocytosis and phagocytic index.

Statistics

Statistical analysis of the data was carried out using GraphPad Prism Software (GraphPad software, Inc., CA, USA). The one way ANOVA was used to analyze and compare the results at a 95% confidence level. Values of $p<0.05$ were considered significant. Results were expressed as mean + standard error of mean (SEM).

Results

Acute toxicity test

Behavioral signs of toxicity observed in mice given 3000 mg/kg of *N. nucifera* stamen extract include; paw licking, salivation, stretching and reduce activity. There was however no mortality at all doses used. Therefore, 500, 1000, and 2000 mg/kg were suitable doses for using in this study.

Malaria-associated phagocytic suppression during PbANKA infection

There was a progressive increase in level of parasitemia as the days progressed from day 2 to 12 in the PbANKA infected mice (Figure 1A), and survival time was 12 days (Figure 1B). Interestingly, determination of phagocytosis showed a progressive decrease in the response to the presence of the parasites, which reached significant values on 4 after infection (Figure 1C). Moreover, decreasing of phagocytic index was also observed in the correlation with decreasing of phagocytosis (Figure 1D).

Antimalarial activity of *N. nucifera* stamen extract against PbANKA infected mice

During early malaria infection, *N. nucifera* stamen extract produced a dose-dependent antimalarial effect against PbANKA. The extract caused a significant ($p<0.05$) antimalarial when compared to the untreated control, especially at dose of 2,000 mg/kg showed the highest activity (Figure 2). The standard drug, CQ caused chemosuppression, which was similar to those of the extract treated groups.

Anti-phagocytic suppression of *N. nucifera* stamen extract during PbANKA infection

As showed in Figure 3, significant ($p<0.01$) suppression of phagocytosis was observed in untreated group. Interestingly, *N. nucifera* stamen extract exerted dose-dependent anti-phagocytic suppression in the extract treated groups, especially at a dose of 2,000 mg/kg showed the highest activity. In addition, no effect on phagocytosis was observed in normal mice treated with this extract and CQ treated group.

Discussion

There was a progressive increase in level of parasitemia at the days progressed from day 2 to 12 in the PbANKA infected mice (Figure

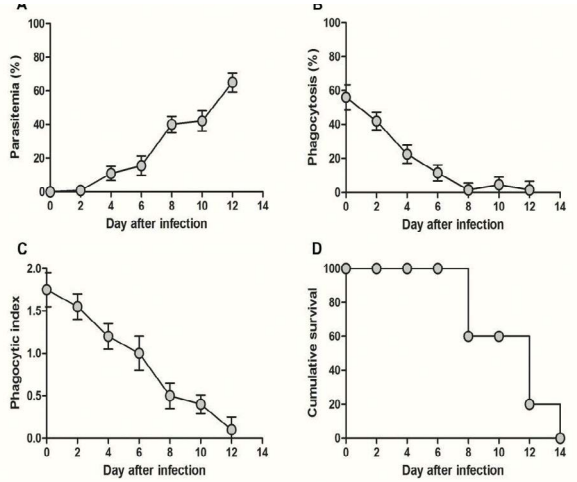

Figure 1: Malaria-associated phagocytic suppression during *Plasmodium berghei* ANKA infection. ICR mice (5 mice of each) were intra-peritoneally infected with 1×10^7 infected erythrocytes of PbANKA. (A) Parasitemia (B) percent phagocytosis and (C) phagocytic index were daily monitored. (D) Percentage survival of infected mice was also observed. Results were expressed as mean± SEM.

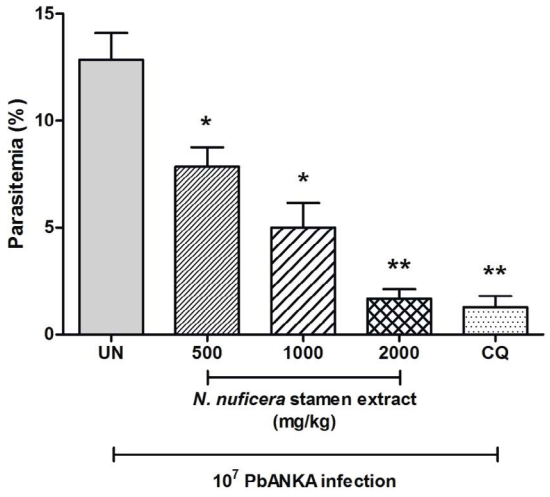

Figure 2: Antimalarial activity of *Nelumbo nucifera* stamen extract against *Plasmodium berghei* ANKA. ICR mice (5 mice of each) were intraperitoneally infected with 1×10^7 infected erythrocytes of PbANKA, and given orally 500, 1,000 and 2,000 mg/kg of the extracts twice a day for 4-consecutive days. Parasitemia was subsequently measured. The results were expressed as mean± SEM. UN; untreated, CQ; 5 mg/kg of chloroquine. * p<0.05 and ** p<0.01 compared to untreated control.

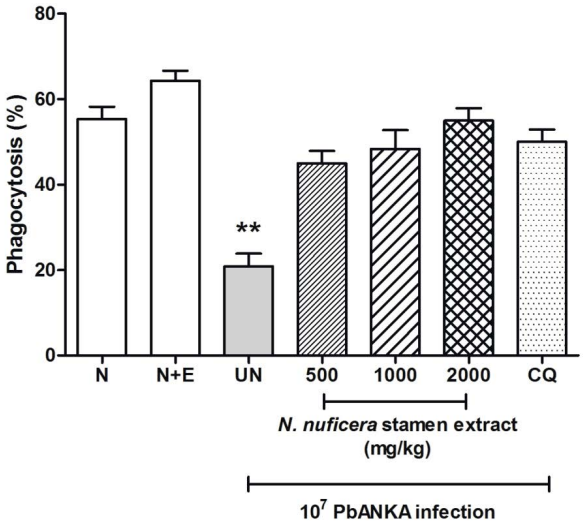

Figure 3: Anti-phagocytic suppression of *Nelumbo nucifera* stamen extract during *Plasmodium berghei* ANKA infection in mice. ICR mice (5 mice of each) were intraperitoneally infected with 1×10^7 infected erythrocytes of PbANKA, and given orally 500, 1,000 and 2000 mg/kg of the extracts twice a day for 4-consecutive days. Percent phagocytosis was then investigated. The results were expressed as mean± SEM. N; normal, N+E; normal mice treated with 2,000 mg/kg of extract, UN; untreated, CQ; 5 mg/kg of chloroquine. *p<0.05 compared to normal control.

1A), and survival time was 12 days (Figure 1B). This is in line with the view that parasitemia increases progressively after inoculation or infection until the point of death in the absence of suitable treatment. Interestingly, determination of phagocytosis showed a progressive decrease in the response to the presence of the parasites, which reached significant values on 4 after infection (Figure 1C). Moreover, decreasing of phagocytic index was also observed in the correlation with decreasing of phagocytosis (Figure 1D). This could be due in part to the fact that during malaria infection, infected erythrocytes and hemozoin (malarial pigment) non-enzymatically generated large amounts of hydroxyl fatty acids that inhibit monocyte function followed by decreasing phagocytosis [27]. Phagocytosis of hemozoin or hemozoin-containing trophozoites alters functionality of monocytes and macrophages. Monocyte ability to perform oxidative burst is compromised, bacterial killing abolished, antigen presentation altered and ability to differentiate to functional dendritic cells disturbed. Moreover, hemozoin-laden monocytes produce increased amounts of peroxidation products of polyunsaturated fatty acids and stimulate generation of several cytokines, such as TNF, IL-1beta, MIP-1alpha and MIP-1beta [28,29]. These appears to be causally related to decreasing phagocytosis and phagocytic index in our finding during PbANKA infection in mice.

During early malaria infection, the extract of *N. nucifera* stamens produced a dose-dependent antimalarial effect against PbANKA. The extract caused a significant (p<0.05) antimalarial when compared to the untreated control, especially at dose of 2,000 mg/kg showed the highest activity (Figure 2). The standard drug, CQ caused chemosuppression, which was similar to those of the extract treated groups. It has been reported the antioxidant potential was related to antimalarial activity in several plant extracts. Moreover, flavonoids, quercetin and kaempferol have been reported to have potent antimalarial activity against *P. berghei* infected mice [19,30,31]. Hence, these compounds in *N. nucifera* stamen extract, and its potent antioxidant activity might play a central role to inhibit PbANKA growth *in vivo*. Moreover, oxidative damage in order to inhibit malaria parasite of artemisinin has also been described [32], and might related to antimalarial activity of *N. nucifera* stamen extract. However, the modes of action and other mechanisms should be searched for.

As showed in Figure 3, significant (p<0.01) suppression of phagocytosis was observed in untreated group. Interestingly, *N. nucifera* stamen extract exerted dose-dependent anti-phagocytic suppression in the extract treated groups, especially at a dose of 2,000 mg/kg showed the highest activity. Several studies have been reported the activity of *N. nucifera* extract to activate innate immune response and phagocytic

activity. Knowledge of properties of flavonols and polyphenolic compounds in order to protect white blood cells and macrophage from oxidative stress and activate phagocytic activity have been described [20]. Moreover, protective effect of flavonoid-rich extract on macrophage from oxidative stress-induced apoptosis has also been reported [33]. Hence, significant antioxidant potential and flavonoid-rich stamen extract might be properties on increasing of phagocytosis during malaria infection.

It is interesting to note that *N. nucifera* stamen extract was found the antimalarial and anti-phagocytic suppression against *P. berghei* infected mice. Although the bioactive components and mechanism are yet to be identified, the results of this study provide the basis for further studies.

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