

Antimalarial Effects of *Areca catechu* L.

Jing-Hua Jiang, Suk-Yul Jung, Youn Chul Kim¹, Sae Ron Shin², Seung Taek Yu³, Hyun Park*

Department of Infection Biology, Zoonosis Research Center, Wonkwang University School of Medicine,

1: College of Pharmacy, Wonkwang University,

2: Department of Family Medicine, 3: Department of Pediatrics, College of Medicine, Wonkwang University

The emergence and spread of drug-resistant malaria parasites is a serious public health problem in the tropical world. Useful antimalarial drugs such as chloroquine have resistance in the world now. Moreover, other antimalarial drugs such as mefloquine, halofantrine, atovaquone, proguanil, artemether and lumefantrine retain efficacy but have limitations, one of which is their high cost. New antimalarial drugs are clearly needed now. Cytotoxicity assay and susceptibility assay were performed for the selectivity of herb extracts in vitro. On the basis of high selectivity, 4-day suppressive test and survival test were progressed in *Plasmodium berghei*-infected mice. The selectivity of *Areca catechu* L. (ACL) and butanol extract of ACL (ACL-BuOH extract) were 3.4 and 3.0 in vitro, respectively. Moreover in vivo, 4-day suppressive test showed 39.1% inhibition effect after treated with 150 mg/kg/day ACL-BuOH to *P. berghei*-infected mice. Survival test also showed 60% survival rate with ACL-BuOH-treated group while all other group mice died. In this study, ACL and ACL-BuOH were investigated for antimalarial activity in vitro and in vivo and they showed a potent antimalarial activity. In particular, ACL-BuOH could specifically lead higher survival rate of mice in vivo. Therefore ACL-BuOH would be a candidate of antimalarial drugs.

Key words : *Areca catechu* L., *plasmodium berghei*, selectivity, suppressive test

Introduction

Malaria is a disease of global concern. Historically, malaria has killed more people than any other infectious disease and still accounts for massive levels of mortality and morbidity in over 100 countries of the world¹. Malaria parasites spread by successively infecting two types of hosts: humans and female Anopheles mosquitoes. Four species of malaria parasite can infect humans under natural conditions: *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The first two cause the most infections worldwide. The most severe form of malaria is caused by *P. falciparum*². *P. falciparum* has a complicated life-cycle, requiring both a human and a mosquito host, and differentiating multiple times during its transmission or infection process. When an infected mosquito bites a human, sporozoites enter the human circulation. These go to and penetrate the liver cells, where

* To whom correspondence should be addressed at : Hyun Park,

Department of Infection Biology, Zoonosis Research Center, Wonkwang University School of Medicine, 344-2 Shinyong-dong, Iksan, Chonbuk 570-749, Korea

· E-mail : hyunpk@wonkwang.ac.kr, · Tel : 063-850-6769

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they asexually reproduce, via the process of schizogony. When the hepatocytes burst, exoerythrocytic schizonts release merozoites in to the blood, which are capable of infecting erythrocytes. Inside the erythrocytes, the merozoites develop into ring-like trophozoites and which then form the merozoites developing into ring-like trophozoites, which then form the erythrocytic schizonts. Mature erythrocytic schizonts form merozoites again by breaking apart inside the erythrocytes releasing merozoites rapidly infect new red blood cells to complete the erythrocytic cycle^{2,3}.

The major obstacle to control the disease is the emergence of drug-resistance in *P. falciparum*. Chloroquine (CQ) has long been used in the treatment or prevention of malaria. However, the first occurrence of CQ resistant *P. falciparum* happened in 3 to 4 foci in Southeast Asia, Oceania and South America in the late 1950's and early 1960's. Since then, CQ resistance has spread to nearly all areas of the world. *P. falciparum* has also developed resistance to same other available antimalarial drugs, such as sulfadoxine/pyrimethamine, mefloquine, halofantrine and quinine⁴. Although resistance to these drugs tends to be much less widespread geographically, in some areas of the world, the impact of multi-drug resistant malaria can be extensive⁵. Thus

developing new effective antimalarial drugs has become the primary task to control the malaria disease.

One hundred and forty seven kinds of the natural herbs and their relative compounds were compiled in "DongYiBoGam" and dictionary of "Traditional Chinese Medicine". In the present study, the antimalarial effects of ACL were estimated in vitro and in vivo.

Materials and Methods

1. Drug Toxicity against Mammalian cells

Hep G2 cells (ATCC, HB-8065) were cultured in RPMI 1640 medium (Gibco, 22400) supplemented with 10% FBS (Gibco, 16000-044) at 37°C in a 5% CO₂. They were grown with a doubling time of about 12 hrs. Prior to exposure to drugs, the cell density was adjusted to 15000/well. One hundred micro liter of cell suspension were added to 96well test plate and after 2 hrs, compound at various concentrations suspended in RPMI 1640 (10 µl) was added to individual wells. After another 24 hrs, "cell titer 96® Aqueous one solution cell proliferation assay" (Promega, G3580) reagent were applied for 90minute sand then the absorbance at 490 nm were measured. All of the tested compounds were assayed in duplicate at each concentration⁹.

2. Culture of malaria parasites

Human O+ red blood cells (research use, RBC) were kindly gifted from THE REPUBLIC OF KOREA NATIONAL RED CROSS JeonBuk chapter, the RBCs were washed and prepared as a 50% erythrocyte suspension. CQ resistant *P. falciparum* (FCR-3, ATCC, 50005) were cultured in RPMI 1640 (GIBCO) medium supplemented with 5% NaHCO₃, 0.5% Albumax I (Gibco), 25 µg/ml gentamycin (Sigma) under a 5% O₂, 5% CO₂, 90% N₂ gas mixture⁷.

3. Herbs Preparation, Dilutions and Plate Design

Over one hundred herbs and its relative compound have been studied previously in our group before (data not shown). Extract of ACL was obtained by Plant Extract Bank (Korea, Daejeon). According to Plant Extract Bank, ACL herb extract was dissolved in DMSO or media as 200 mg/ml. An initial dilution of 1:40 precludes any solvent effect on growth of the parasite. Subsequent dilutions are determined by the magnitude of dilutions needed to reach the desired starting concentration in the micro titer well. Herbs diluted into stock solutions in either DMSO or media and then stored in the lab until use, aliquot into multiple tubes and frozen at -70°C until use, stored at 4°C for up to one month or fresh dilutions of the

ACL herbare prepared for each assay. Chloroquine diphosphate salt (Sigma, C6628) was used for positive control⁹.

4. Susceptibility testing of ACL herb

Parasitemia, the quantitative relative content of parasites in the blood, is commonly used in malaria diagnosis in patients as well as for in vitro testing of new antimalarial compounds in research laboratories⁹. This measure can be obtained using various approaches; though the preferred and most reliable is microscopic examination of Diff-Quick stained thin blood films as previously described¹⁰ and counted more than 1000 RBCs per every slide.

Triplicate assays were performed in 96 well tissue culture plates (Nunc Brand products, Fisher, Paris, France) containing 200 µl of asynchronous parasite culture at 0.5% parasitemia and 2% hematocrit, and appropriate extract dissolved in DMSO or water. Negative control (untreated group) treated by solvent (DMSO or water) and positive controls (CQ treated group) were added to each set of experiments. After 72 hrs incubation without medium change, every well was stained with Diff-Quick stain for counting. The EC₅₀ (50% effective concentration) values represented the mean value calculated from triplicate experiments. The inhibition ratio of each herb was compared with negative control. The concentration of drug required to effective a 50% decrease of infected erythrocytes (EC₅₀ Plasmodium) was calculated by nonlinear regression analysis processed on dose - response curves, using the Table Curve Program (Sigma-plot scientific graph system, Version 3.0, Jandel Scientific, Corte Madera, CA).

5. Determination of a Selectivity index

A selectivity index (SI), corresponding to the ratio between antiparasitic and cytotoxic activities, was calculated according to the following formula¹¹:

$$SI \text{ Plasmodium} = EC_{50} \text{ Human} / EC_{50} \text{ Plasmodium}$$

6. Mice and *P. berghei*

Female ICR mice, 20-27 g, 5 week old, purchased from ORIENT BIO CO., LRD, South Korea were used. All animal experiments were performed according to the Guidelines for Animal Experimentation, Wonkwang University School of Medicine. Murine malaria parasite, *P. berghei* (NK65) was bought from ATCC (30090), and blood stage parasites were stored in liquid nitrogen¹².

7. Four-day suppressive test in vivo

The anti-malarial suppressive test described by Peters¹³

was modified by Carvalho *et al.*¹⁴. Parasites were maintained by weekly blood passage with ICR mice by the intraperitoneal route, 10⁶ infected red blood cells per mouse. The animals were randomly separated into groups of six for each drug test and treatment was daily carried out by oral route for 4 days. 1.0, 2.5 or 3.5 mg/kg/day of CQ were orally treated to NK65-infected mice.

Two control groups were used in each test, one receiving the standard antimalarial drug CQ in 1.0 mg/kg doses while other group was not treated. Butanol extract of ACL-BuOH extracts were suspended in DMSO, and then diluted with water so that doses of 150 mg/kg were administered in a 0.3 ml volume per animal.

Antimalarial activity was evaluated by counting parasitemia in blood smears taken 4 days after parasite inoculation⁹. Inhibition of parasite growth in the drug-treated groups was calculated in relation to the control (no drug-treated) group as follows: average parasitemia in the control group minus parasitemia in the test group, divided by parasitemia in the control group. Results were expressed as a percentage of parasitemia reduction; extracts that induced the reduction of parasite growth by ≥30%, were considered active¹⁴. Overall mortality was daily monitored for 16 days.

8. Statistical analysis

Mann-Whitney U test was used for in vivo results analysis (SPSS 11.5.1 Software for Windows, standard version).

Results

1. Antimalarial activity of ACL-BuOH in vitro

The EC₅₀ values of ACL and ACL-BuOH extract were 8.17 and 8.87 μg/ml based on microscopy, respectively. And the EC₅₀ values of them in Hep G2 cells were 28.05 and 26.79 μg/ml. Therefore, selectivity of ACL and ACL-BuOH extract were 3.4 and 3.0. They were shown the similar antimalarial effect with CQ (it was 4.3, Table 1).

Table 1. Selectivity of herbal extracts

Drugs	EC ₅₀ on Hep G2 cells	EC ₅₀ on <i>P. falciparum</i>	Selectivity
ACL	28.05 μg/ml	8.17 μg/ml	3.4
ACL-BuOH	26.79 μg/ml	8.87 μg/ml	3.0
Chloroquine	2995.22 nM	699.41 nM	4.3

ACL: *Areca catechu* L. ACL-BuOH: Butanol extract of ACL

2. Determination of antimalarial effect of ACL-BuOH in ICR mice

1) Four-day suppressive test

Four-day suppressive test was used in vivo experiment in

this study(Fig. 1). Firstly, different concentrations of CQ (1.0, 2.5 and 3.5 mg/kg) were treated to mice, and after 4 days, 5.1, 4.3 and 1.5% parasitemia were obtained from tail vein blood, and parasitemia were reduced 50.3%, 57.6% and 85.5% compared with negative control group (10.2% parasitemia; 0% inhibition), respectively. The ED₅₀ (50% effective dose) of CQ was 1.0 mg/kg. Thus the 1.0 mg/kg dose of CQ was used in this study. Furthermore, in order to confirm the antimalarial effect of ACL-BuOH extract on this model, dose of 150 mg/kg were orally treated to parasite infected-ICR mice (Fig. 2). On negative control group, the parasitemia was 6.8%, while on ACL-BuOH extract-treated group, it was 4.1%. Therefore, the parasite was inhibited 39.1% as compared with control group. For 1.0 mg/kg CQ, 3.7% of parasitemia and 45.7% inhibition rate were obtained(Fig. 2).

2) Survival test

In survival test, on the 13th day all mice of the negative control group died, and on the 15th day all mice of CQ-treated group died (Fig. 3). While on the 16th day, mice of ACL-BuOH-treated group remained 60% survival rate after infected parasite. It was longer than antimalarial drug CQ-treated mice

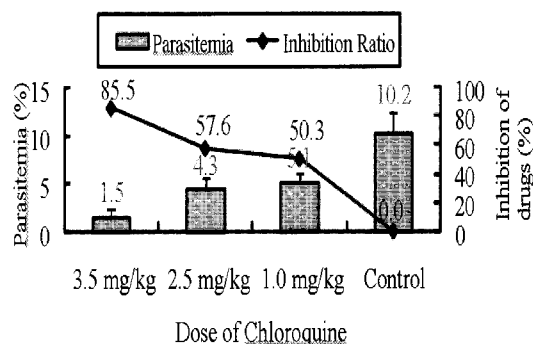


Fig. 1. Four-day suppressive test of Chloroquine on ICR mice. 1.0, 2.5, 3.5 mg/kg chloroquine were treated to *P. berghei* infected mice. Control: D.W. treated to *P. berghei*-infected mice.

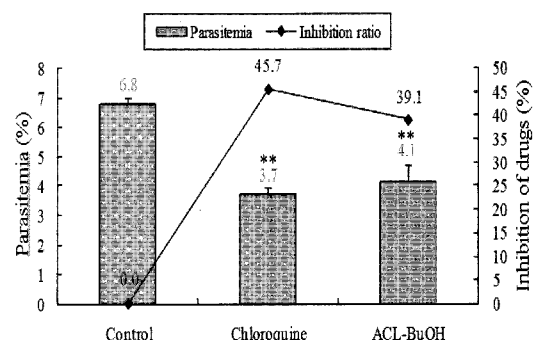


Fig. 2. Four-day suppressive test of ACL-BuOH on ICR mice. Chloroquine (1.0 mg/kg): chloroquine treated to *P. berghei*-infected mice. ACL-BuOH (150 mg/kg): BuOH extract of ACL treated to *P. berghei*-infected mice. Control: D.W. treated to *P. berghei* infected mice. P<0.01

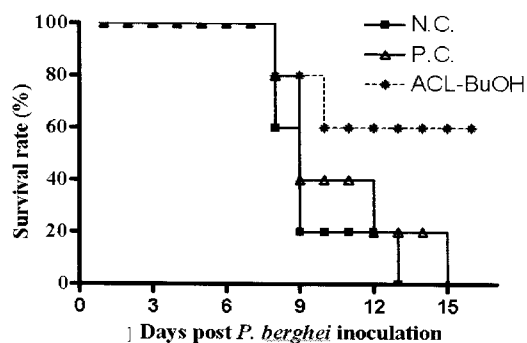


Fig. 3. Survival rate of *P. berghei* infected ICR mice by ACL-BuOH. *P. berghei*-infected RBC were injected into mice tail vein. Drugs were daily treated for 4 days. N.C.: No drug-treated group. P.C.: Chloroquine (1.0 mg/kg) treated group. ACL-BuOH (150 mg/kg): ACL-BuOH treated group.

Discussion

Nowadays, herb extracts have been considered as a desired properties of potency and low toxicities for the identification of new chemotherapeutic agents¹⁵. In our study, ACL herb was estimated for antimalarial effects. The selectivity of ACL was 3.4 high in vitro (Table 1). These results suggested that ACL have antimalarial effects.

It has reported that small doses of ACL generally lead to euphoria and increased flow of energy while large doses often result in sedation¹⁶. Most experts attribute the psychoactive effects to the alkaloids found in ACL¹⁷. The pericarp of ACL was effective in the treatment of flatulence, oedema, dysuria and hyperaemesis of pregnancy; the kernel was used to treated diarrhoea and dysentery¹⁸. As well as anti-aging effects of ACL extract on skin were investigated both in vitro and in vivo¹⁹, ACL extracts have exhibited potent inhibitory activities against pancreatic cholesterol esterase in vitro²⁰. The hepatic and intestinal ACAT (acyl-CoA: cholesterol acyltransferase) activities were significantly decreased in the ACL group compared with the control group²¹. However, the anti-malarial effect of ACL has not been reported yet.

To find out which isolated layer of ACL extract showed the good antimalarial activity, different solvent-extracted ACL was separated. Butanol extract of ACL (ACL-BuOH) was most effective against malaria parasite and showed similar EC₅₀ value of ACL on Hep G2 cells and *P. falciparum* in vitro (Table 1).

To estimate antimalarial effects of ACL-BuOH, 4-day suppressive test and survival test were carried out in vivo. After *P. berghei*-infected mice was treated with 1.0 mg/kg CQ as positive control group, the antimalarial inhibition rate was presented 45.7% compared with no drug-treated control group. However, the antimalarial inhibition ratio of 150 mg/kg ACL-BuOH-treated mice was shown 39.1% (Fig. 2). In addition, mice of ACL-BuOH-treated group remained 60%

survival rate after infected parasite on the 16th day, but all other group mice died at that time (Fig. 3). Here, the antimalarial inhibition effect of ACL-BuOH was not better than the effect of CQ, but survival days was longer than CQ. The reason of this interesting phenomenon may be caused by the different ability of CQ and ACL-BuOH. CQ interferes with parasite haem detoxification^{22,23}, thought to inhibit the FePPIX crystallisation by binding to monomeric or dimeric Fe(III)PPIX, resulting in the build up of toxic free FePPIX which could kill the parasite²⁴. In our study, after *P. falciparum* were treated with CQ or ACL-BuOH, both of them showed a significant efficacy against parasite growth in RBC. Structural analysis and target finding will be helpful to more evaluate ACL-BuOH antimalarial effects.

Acknowledgments

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