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Efficacy of Lophira alata Leaf Extract and its Combination with Artesunate in Mice Prior Exposed to Plasmodium berghei

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ABSTRACT

Enhanced antimalarial activity of plant extracts used for treatment of malaria in endemic areas is attributed to partial immunity gained by prior infection. This suggests synergy between immunity and extract activity in treatment. Testing this hypothesis, rodent malaria was used to determine efficacy of Lophira alata leaf extracts in treating malaria in prior infected mice. One round of P. berghei infection and Pyrimethamine drug-cure was used to establish partial immunity in mice. Previously Exposed Mice (PEM) and Previously Unexposed Mice (PUM) mice challenged with P. berghei were used to determine influence of partial antimalarial immunity on efficacy of L. alata leaf extracts, administered alone or in combination with Artesunate (ART) in malaria treatment. There was a significant reduction in parasitemia in PEM when compared to PUM animals ($P < 0.001$) irrespective of treatment regimen. Administration of L. alata combined with ART significantly reduced parasitemia ($P < 0.0032$) and prolonged ($P = 0.0109$) survival than when L. alata was administered alone in infected mice. These findings suggest that the action of L. alata in treating malaria infections in a murine model is enhanced by prior exposure to the malaria parasite. Thus the requirements of using plants in treating malaria in endemic populations may differ for those used in western systems, where trials are carried out with non-immune cohorts. Combining artemisinin derivatives and medicinal plants in malaria exposed populations may provide an alternative control measure in endemic regions and may justify the continued use of these plants by indigenous populations in treating malaria.

Introduction

Malaria control requires concerted efforts to reduce the number of deaths brought about primarily because of drug resistance in Plasmodium falciparum [1]. With the deployment of an effective vaccine still years away and vector resistance to insecticides a growing problem, there is a greater burden on chemotherapy, which has proved to be the most viable option for current eradication efforts [2]. Recent attempts directed at the treatment of resistant infections have been to use Artemisinin and its derivatives administered in combination therapies (ACTs) [3]. However, the high cost of these combinations and cases of increased in vitro and in vivo tolerance of artemisinin and some combinations have been reported in Southeast Asia there is a fear that sub-Saharan Africa may not be immune [4–6]. Thus, the search for newer drugs and combinations are needed to improve treatment options. As a result, further improvement of the methods available for control of malaria in endemic areas may include the use of indigenous medicinal plants as partners for the artemisinins [7–9].

Artemisinin and quinine are two drugs used to treat malaria that were derived from plants [10]. Ethnobotanical surveys have established the folkloric use of medicinal plants in the treatment of febrile illnesses in endemic areas and in recent years, there has been an increase in the number of plant species that have been assessed for in vitro and in vivo antiplasmodial activity [11–14]. Although these studies point to the possibilities of developing novel drugs from medicinal plants, few new antimalarial drugs have actually

been found [9, 15]. Part of the reasons ascribed for the high attrition of most natural compounds from plants is toxicity and lack of parasiticidal activity [16–15]. Nevertheless, in many endemic areas plant extracts are still popular for medication of parasitic diseases, particularly in remote areas without access to standard treatment [9, 17–19].

In areas where malaria is endemic, individuals acquire partial immunity against malaria only after repeated exposure to the parasite [20]. In these malaria endemic areas, infected individuals may recover from infection in spite of harbouring in some cases drug-resistant P. falciparum. This was also observed in patients with neurosyphilis receiving malaria therapy, who were first shown to respond better to treatment upon experiencing several paroxysms of fever, which then allowed them to mount an immune response to infection [21]. In addition, the ability to remove chloroquine-resistant parasites in infected individuals has also been attributed to the acquisition of immunity due to repeated exposure to mosquito infection [22]. P. falciparum parasites bearing drug resistant mutations in genes such as Pfcrt and Pfmdr1 have been cleared from infected individuals in endemic populations after drug treatment substantiating an important role of prior exposure to parasite infection in the outcomes of drug treatment [23–25]. This ability of immunity, brought on by prior parasite exposure, to enhance treatment might also explain the efficacy of quinine in regions of high transmission than in areas of low transmission [26]. Furthermore, animal model experiments have also pointed to the fact that antimalarial drugs clear resistant parasites from partially immune hosts and that antimalarial treatments during primary Plasmodium berghei infection influenced the acquisition of protective immunity against reinfection [27–29]. This phenomenon suggests that a synergy exists between drug treatment and acquired immunity; implying that the natural acquired immunity to malaria possessed by people living in endemic areas may be responsible for the antiplasmodial effect shown by plants used in those regions. The possibility therefore exists that many medicinal plants used in traditional medicines may prove effective in people who have been earlier exposed to the disease and possess some level of immunity to malaria [19].

The plant, L. alata (Ochnaceae), had previously been identified as being used for the treatment of malaria infection in traditional medicine through an ethnobotanical survey in Nigeria [30] and its leaf extract has also been shown to possess in vitro and in vivo antiplasmodial activity in addition to treating febrile conditions, cough, jaundice, and gastrointestinal disorders [14]. Using the rodent parasite P. berghei, which is a good laboratory model for understanding the biology of P. falciparum infections, we have studied the relationship between prior parasite infection and the capacity of plant extracts to clear malaria parasites in a bid to determine whether partial immunity enhances the activity of L. alata in reducing the growth of parasites in partially immune animals. The ability to limit parasitemia measured by estimating parasite growth kinetics has been employed as a surrogate in assessing the levels of host immunity in human hosts and infected mice [27–29, 31, 32]. The effects of partial immunity estimated via parasite kinetics and the ability of plant extracts to treat infection have not been thoroughly examined. Various studies have examined the contribution of plant extract in enhancing antimalarial immunity during or prior to infection [33]. In this work we evaluated the effect of L. alata leaf extracts administered alone or in combination with ART on parasitaemia and survival of Previously Exposed Mice (PEM) and Previously Unexposed Mice (PUM) animals. We envisage that our results will help give some informed suggestions about alternative therapies utilizing plant products for malaria treatment in endemic populations.

Materials and Methods

Drug and Extract preparation

The methods for plant collection, authentication and extraction have been previously described [14]. Briefly, grounded fresh leaves of L. alata (LA) were extracted into redistilled methanol by maceration at room temperature (29–33°C) for 72 h. The resultant mixture was filtered and concentrated to dryness under reduced pressure using a rotary evaporator (Heidolph Laborata 4001, Germany). ART (Mekophar Chemical Pharmaceutical, Vietnam) and dried LA extracts were dissolved in 100 % DMSO. The crude extract of LA was evaluated for its toxicity in non-infected Swiss albino mice aged 2 months weighing 18–20 g. Eighteen mice randomized into six groups of three mice each were orally administered, 50, 100, 200, 400, 700 or 1000 mg/kg body weight of extract. The mice were observed for changes in physical appearance and gross behavioural changes (such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhoea, mortality) for three days. The concentrations of drug/extracts were adjusted so that the final dose in mg/kg body weight was administered in a volume 0.2 ml. All animals were treated with drugs/extracts through oral gavage once daily starting from Day 3 post-infection.

Mice and parasite

Inbred Swiss albino mice weighing 20 ± 2 g and housed at the animal facilities of the Department of Zoology, University of Ibadan were used for all experimental studies. The animals were kept in cages at room temperature and moisture. They were fed on standard diet and given drinking water ad libitum. P. berghei ANKA used for all experiments was obtained from the Malaria Research and Reference Reagent Resource (MR4) and maintained in mice via serial passage. In vivo animal experiments were approved by the University of Ibadan Ethical Committee on the use of laboratory animals for research.

Induction of partial immunity

In order to induce partial immunity and primary infection, mice were inoculated intraperitoneally (IP) with 1×10^6- parasitized red blood cells (PRBC). Five to six days post-infection (PI), when patent parasitemia was at about 5 %, mice were treated with pyrimethamine (10 mg/kg orally) (Sigma, St. Louis, MO, USA) administered over four days a modification of Evans et al., 2006 [34]. Previous studies have shown that this protocol results in partial-immunity in infected animals [34]. This infection and drug cure produced the PEM animals. PEM animals confirmed to have undetectable patent parasitemia after one week were considered partially immune [34]. Further verification of parasite clearance was made via sub-inoculation of blood from immune animals into naïve recipient mice.

Thin blood films from these mice were analysed after 7 days post-infection, with negative slides confirming total parasite clearance [35]. Parallel control group experiments, were conducted with animals sham infected and administered water. This group served as PUM animals.

Challenge infection and treatment

Two weeks after primary infection and drug cure described for PEM and PUM animals, a challenge infection (secondary) was initiated in both PEM and PUM animals. Both groups of animals were inoculated IP with 1×10^6 PRBC. Seventy-two hours after infection, the curative action of extract, extract/drug combinations in PEM and PUM animals were then treated with extracts (LA) alone or drug plus extracts (ART + LA) to assess curative action of drug regimens according to described methods [36, 37]. Plant extract doses used were based on previous published effective concentrations [14]. Infected untreated (placebo) groups received drug diluents. The six treatment groups for both PEM and PUM animals were as follows: 2 mg/kg ART (Positive Control); 250 mg/kg LA; 500 mg/kg LA; 2 mg/kg ART + 250 mg/kg LA; 2 mg/kg ART + 500 mg/kg LA; Placebo (untreated).

Monitoring of parasitemia and survival

For all treatment groups, drugs and extracts were administered for four consecutive days while parasitemia was monitored by daily microscopic examination of Giemsa-stained blood films taken from tail snips of study animals for 60 days. The percentage parasitemia was calculated as parasitemia (%) = [(number of infected erythrocytes)/(total erythrocytes)] X 100. Mortality and survival was also recorded for animals over the study duration.

Statistical analysis

Analyses of all data were performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc, La Jolla, CA, USA). Data are presented as the means ± S.E.M. The statistical significance of the differences was analyzed by one-way ANOVA. Survival data were analysed using the Kaplan-Meier statistical method with Log-Rank significance test. $P < 0.05$ was considered significant.

Results

Effect of prior infection on course of infection

Establishment of parasitemia after challenge (secondary) infection took a longer time (day 4 post-infection) to be observed in peripheral blood obtained from tail snips of all PEM animals. Analysis of parasitemia data (▶ **Fig. 1a**) for the course of infection showed that the pre-patent period for observation of parasitemia was extended by 3 days in PEM placebo group, this group experienced parasitemias, which were statistically significantly lower than PUM placebo group for both treated and untreated animals. Furthermore, the level of parasitemia in PEM animals (both treated and untreated) were much lower than corresponding PUM animals ($P < 0.0001$). Artemisinin administered at a concentration of 2 mg/kg significantly reduced parasitemia ($P < 0.01$) in the PEM animals when compared to the PUM animals with parasites appearing in peripheral blood after 10 days PI. It was obvious from comparison of parasitemias between the PEM and PUM mice that partial immunity as a result of prior infection was successful in reducing parasite growth with or without treatment, with treated controls in the PEM, having the most reduced patent parasitemia.

Effect of prior infection and treatment on course of infection during secondary parasite challenge

Following the establishment of immunity in the PEM, a challenge (secondary) infection was initiated in the PEM and PUM animals. The results show the parasitemia profiles of PEM (▶ **Fig. 1b**) and PUM (▶ **Fig. 1c**) animals treated with LA alone or ART + LA leaf extracts seventy-two hours after the secondary infection. It was observed that the level of parasitemia in PEM group, irrespective of treatment protocol, were significantly lower in all groups when compared with the corresponding PUM groups (▶ **Fig. 1b and c**). Although LA leaf extracts administered alone reduced parasitemia in both the PEM and PUM animals, the combination of ART + LA reduced parasitemia significantly more in the PEM animals (▶ **Fig. 1d**) and the combination of 2 mg/kg ART + 500 mg/kg LA was the most effective of all treatment regimes with animals in this group completely resolving patent parasitemias (▶ **Fig. 1d**).

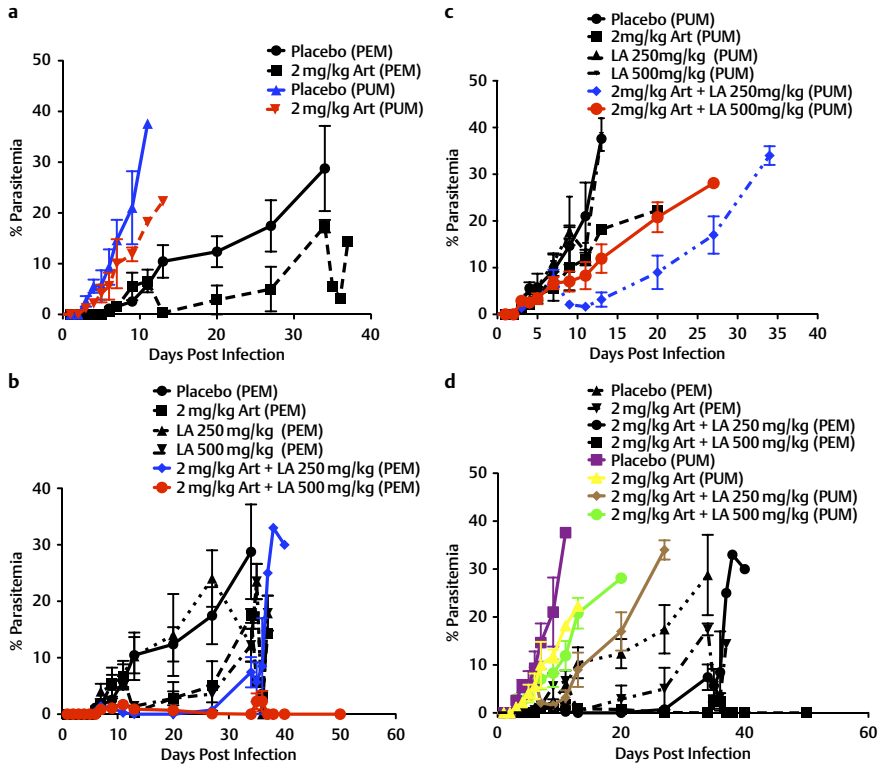
Effect of prior infection and treatment on survival

Survival was significantly prolonged in the PEM animals compared with the PUM animals irrespective of treatment regimen. Although the LA leaf extracts administered alone improved the survival of animals in both the PEM and PUM animals. Survival curves show that after treatment, the combination of ART + LA resulted in a significant increase in survival of the PEM animals, and the combination of 2 mg/kg ART + 500 mg/kg LA was the most effective of all combinations, significantly extending survival in this treatment group after the death of animals in all groups (▶ **Fig. 2a and b**).

Discussion

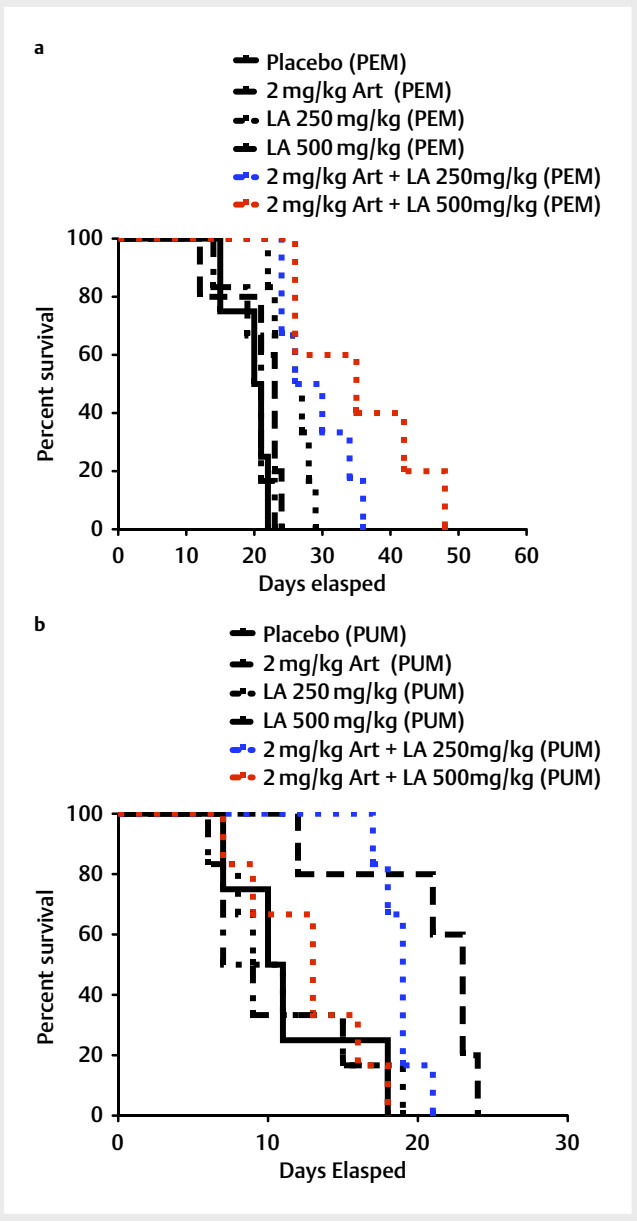
Numerous studies have described host immunity as an important determinant of antimalarial efficacy [38, 20, 22]. One of the factors upon which the efficacy of antimalarial chemotherapy is deemed to depend on is the host's immunity [22]. However, in spite of the prevalence of acquired immunity in sub-Saharan Africa, the interaction between plant therapies and immune response has not been fully explored [9]. In this study, we have used parasite kinetics as a surrogate to evaluate the effect of prior infection in enhancing the chemotherapeutic potential of LA in mice infected with P. berghei. The plant LA selected for this study in ethnomedicine is used in treating malaria infections [30], with a recent report demonstrating its in vivo activity in rodent malaria [14]. We sought to assess the in vivo antimalarial activity of LA in monotherapy or in combination with ART in non-immune and partial immune animals. Our intentions were with a view to suggesting alternative therapies for malaria treatment in endemic populations.

Our experiments revealed a significant in vivo antimalarial activity for leaf extracts of LA in PEM animals than in PUM animals. Studies in both humans and animals have shown that immunity initiated by prior infection influences the action of drugs [27–29, 32]. In studies involving patients with malaria, partially immune patients respond better to chemotherapy than non-immune patients



▶ **Fig. 1** Course of infection (A-D) of Previously Exposed Mice (PEM) and Previously Unexposed Mice (PUM) mice (n = 5) i.p. Infected with 1×10^6 P. berghei and treated with either: 250 mg/kg, 500 mg/kg LA, 2 mg/kg ART, ART 2 mg/kg + LA 250 mg/kg or ART 2 mg/kg + LA 500 mg/kg. Placebo (Untreated), Positive control administered Artesunate (2 mg/kg). All PUM mice had been previously infected with 1×10^6 P. berghei. All animal experiments were averages from three experiments. LA: Lophira alata; ART: Artesunate.

[38]. Several animal studies have implicated immunity via reduced patent parasitemia in immune animals when compared to non-immune animals, as being responsible for enhanced parasite clearance [27–29]. Hence the ability of PEM animals treated with LA to have significantly reduced parasitemias when compared with PUM animals may be attributed to immunity; and we can suggest that LA was able to reduce patent parasitemia significantly better than when immunity was absent as observed in the PUM animals. In vivo response to antimalarial treatment is determined by numerous factors, including parasite load, innate host resistance and naturally acquired immunity [38]. Malaria manifests itself with symptoms including fever and pains and though some plants may lack direct antiplasmodial activity, many have been shown to possess antipyretic, analgesic and immune stimulatory effects that may work in concert with antimalarials [39]. This hypothesis may lend itself to our observations on LA activity in PEM animals where LA, was more effective in animals with partial immunity, thereby justifying its use in the traditional treatment of malaria [30]. However, our intentions were to observe if any, improved activity of LA when combined with ART. In the drug combination experiments, there was a greater reduction in parasitaemia when ART was combined with LA in all treatment groups; this parasite decrease was more significant than when ART or LA were administered alone. Parasite reduction was more significant in the PEM than PUM animals. The group of mice treated with the ART + LA combination also had a longer survival time when compared with mice treated with ART or LA alone. Thus, in our study, extract efficacy was further enhanced by the presence of a partner drug ART and partial immunity. ACTs are now accepted as the best treatments for uncomplicated falciparum malaria, replacing other parasite resistant antimalarials [40]. However, the cost, or lack of availability of ACTs precludes their use in the poorest of communities, particularly in remote endemic areas where indigenous medicinal plants are still used for the treatment of febrile infections. Even where they are available, reduced sensitivities of artimisinin and its derivatives is a growing problem. Therefore, partnering an artemisinin derivative with an efficacious medicinal plant may improve its efficacy or the therapeutically useful lifetime of the drug before parasite resistance emerges in malaria endemic populations. Consequently, the prospect of combining established antimalarials with bioactive compounds derived from medicinal plants e. g. the combination of chloroquine and febrifugine and isofebrifugine compounds from Hydrangea macrophylla, has been demonstrated [41]. Curcumin from turmeric was found to be effective when combined with artemisinin in preventing parasite recrudescence in mice infected with P. berghei [42]. In their study, Nanadakumar and colleagues discovered that in the mouse model, Artemether treatment alone resulted in parasite recrudescence, which was prevented by an Artemether-cur-



▶ **Fig. 2** Survival curves (A and B) of Previously Exposed Mice (PEM) and Previously Unexposed Mice (PUM) mice (n = 5) i.p. Infected with 1×10^6 P. berghei and treated with either: 250 mg/kg, 500 mg/kg LA, 2 mg/kg ART, ART 2 mg/kg + LA 250 mg/kg or ART 2 mg/kg + LA 500 mg/kg. Placebo (Untreated), Positive control administered Artesunate (2 mg/kg). All PEM mice had been previously infected with 1×10^6 P. berghei. All animal experiments were averages from three experiments. LA: *Lophira alata*; ART: Artesunate

cumin combination treatment. A combination therapy of ART with extracts from Cinchona officinalis in treating P. berghei was shown to be highly efficacious as it completely cleared blood stage infection [43]. Whereas, the aforementioned medicinal plant combination studies demonstrate the improved activity of artemisinin derivatives with medicinal plants, our study highlights the importance of immunity brought about by prior infection in enhancing the effects of the drug/plant combinations.

Identifying new drug combinations is high on the antimalarial control agenda and the discovery of naturally derived compounds may yield new options for drug combinations. The development of an affordable ACT or an alternative cost-effective antimalarial drug is imperative in rural areas where the majority of people are poor. With the problems of increasing levels of drug resistance and difficulties of drug affordability, medicinal plants could be an important and sustainable source of identifying new molecules that can serve as adjuncts to ACTs [44]. Thus, we hope to identify in subsequent studies the particular immune molecules responsible for the enhanced antimalarial activity observed. Our study may lead the way in guiding more extensive animal and human studies. It is also envisaged that bioassay guided fractionation of LA may provide compounds that may be useful for drug combination regimes as noted by Guantai and Chibale 2011 [15]. Although many obstacles still remain before these plant extracts can be approved for use, our data provide a foundation, upon which further exploration into the use of medicinal plants for combination therapy with artemisinin derivatives can be established in sub-Saharan Africa.

Conclusion

In conclusion, we sought out to determine whether LA leaf extract and its combination with ART could improve treatment in animals with partial immunity. Mice with prior infection treated with LA significantly reduced parasitemia and prolonged survival, which was marked in animals, treated with a combination of ART and LA. We have shown that an enhanced effect of partial immunity manifested in significantly reduced parasitemia and increased survival is the most likely explanation for the improved antimalarial effect in PEM animals. Malaria control programs at present emphasize the use of ACTs whilst encouraging discovery and identification of new and efficacious drug combinations. The identification of new, active, naturally derived compounds from medicinal plants could provide additional possibilities in the development of ACTs. The potential of combining established antimalarial drugs with other bioactive compounds derived from natural sources, which has been demonstrated experimentally, may provide alternative sources of treatment for malaria in endemic populations. This study also highlights the importance of taking into consideration the existence of partial immune populations of individuals naturally exposed to malaria during drug trials.

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Conflict of Interest

The authors declare no conflict of interest

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