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Antagonistic antimalarial properties of pawpaw leaf aqueous extract in combination with artesunic acid in *Plasmodium berghei*-infected mice

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ABSTRACT

Background & objectives: Artemisinins, the main stay in the treatment of malaria are used in combinations with other antimalarials to forestall resistance, as artemisinin-combination therapies (ACTs). However, ACTs are expensive and some of the non-artemisinin components are not well-tolerated by patients. There are several folkloric and scientific proofs of the efficacy of herbal remedies for malaria. Mature leaves of *Carica papaya* is widely used to treat malaria in several African countries. An ACT involving a medicinal herb extract or its active constituent(s) will provide an indigenous alternative/herbal ACT.

Methods: Mature fresh leaves of *Carica papaya* were grounded and macerated in cold distilled water for 24 h and the extract (PCE) was stored in the refrigerator for seven days. Fresh extracts were made as needed. The antiplasmodial activity of PCE and/or artesunic acid were determined by using the Peter's 4-day suppressive test in *Plasmodium berghei*-infected mice. The ED_{50} and ED_{90} were calculated from the dose-response relationships.

Results: The combination of 50 mg/kg of PCE and 15 mg/kg of artesunic acid produced a significant reduction of parasitemia (81.25%), compared to 50 mg/kg PCE alone (37.7%). The mean survival time of the combinations of PCE and 15 mg/kg of artesunic acid, and PCE alone followed a dose-dependent manner. The ED_{50} of PCE showed that it has a very good activity. The isobolar equivalent (IE) calculated from the ED_{90} of PCE in combination with artesunic acid showed that the interaction was antagonistic.

Interpretation & conclusion: Although pawpaw alone was found to have a very good activity, its combination with artesunic acid is antagonistic. Combinations of artemisinins and pawpaw show little promise for combination therapy development.

Key words Aqueous pawpaw leaf extract; artemisinin-based combination therapy; artesunic acid; *Plasmodium berghei*

INTRODUCTION

Malaria is one of the world's leading killers, having a greater morbidity and mortality than any other infectious diseases of the world[1,2], with greater mortality in children and pregnant women[3]. The malaria parasite has developed resistance to drugs used in the therapy of malaria except the artemisinins. However, the artemisinins produce fast recrudescence when used alone due to their short half-lives. Due to this and to forestall resistance they are used in combinations with other antimalarials, a combination known as Artemisinin-combination therapies (ACTs).

Mature leaves of *Carica papaya* (Caricaceae; Common name: pawpaw) is widely used to treat malaria and splenomegaly[3]. The anti-plasmodial activity of *Carica papaya* is weak as it has an IC_{50} of 60 mg/ml[4]. However, a recent study has shown it to reduce parasitemia at an activity second to that of SP. Also, aqueous extract of its leaves had been shown to reduce parasite count from 9.2± 0.06 to 2.60 ± 0.06%[5] in *Plasmodium berghei*-infected mice. In Nigeria, a weak decoction of the leaves is taken for the treatment of malaria. Its antimalarial activity has been attributed to its ability to increase total antioxidant status in patients and thus, inhibiting the development of anemia in malaria[6]. This study provides a basis for the combination of artemisinins and medicinal herbs or their active constituents to make an indigenous cost-effective ACT.

MATERIAL & METHODS

Animals

The mice used in this study were 8–14 weeks old non-pregnant Swiss albino females of 25 ± 2 g and were obtained from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka (UNN). These were left to acclimatize in the Experimental Animal House Unit of the Department of Biochemistry, UNN, for 5 days, and were given standard mice feed supplemented with p-aminoben-

zoic acid (45 mg/kg) and water *ad libitum.*

Parasites

The parasite used was a chloroquine-sensitive strain of *P. berghei* NK 65, maintained in mice, from the National Institute of Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria. The parasite was then subsequently passaged into fresh mice, which served as donor mice in this study.

Herbal extract and drug

The plant used in this study was *Carica papaya,* identified by Mr. Ozioko, Bioresources Development Centre, BDC, Nigeria, and a voucher specimen with No, INTERCEDD (International Centre for Ethnomedicine and Drug Developmnet) 918 was deposited there. Eighty grams of mature fresh leaves of *Carica papaya* were allowed to drip dry. The leaves were grounded in a clean porcelain mortar and then macerated in cold distilled water for 24 h. The fluid was recovered by passing through a fine mesh of muslin cloth and allowed to sediment. The supernatant, called pawpaw crude extract (PCE) was stored in a refrigerator at 2–4^{o}C until used. A measured aliquot of NCE was evaporated to dryness in order to determine the concentration. PCE was diluted with a mixture of Tween 80 and ethanol in sterile distilled water, so as to enable the administration of doses of 100, 500 and 1000 mg/kg of the extract. The control and test groups were given equal volumes of the placebo and drug.

The pure artesunic acid powder used in this study was a generous gift from M/s. Emzor Pharmaceutical Industries, Lagos, Nigeria. It was dissolved in a mixture of 7% Tween 80 and 3% ethanol to provide doses of 6, 15 and 20 mg/kg. These doses were based on the ED_{50} of artesunate on *P. berghei*-infected mice[7].

In vivo schizontocidal activity

This was carried out according to standard protocol following the Peter's 4-day suppressive test[8]. On Day 0 of this test, the percentage parasitemia and red blood cell count of the donor mice were determined by using a Giemsa-stained thin blood smear of the donor mice and improved Neubauer Counting Chamber, respectively. Then, the blood of the donor mice was collected by cardiac puncture and from the retro-orbital plexus vein, and diluted with physiological saline (Normal saline) to give a concentration of 10^8 parasitized erythrocytes per ml. 2×10^7 parasitized erythrocytes (i.e. 0.2 ml of 10^8 parasitized erythrocytes/ml) was injected intraperitoneally into each of the experimental mice. The mice were randomly shared into 7 groups of five mice each. The negative control group was given 7% Tween 80 in sterile distilled water. Equal volumes of the drug, extract and placebo were administered orally at 4, 24, 48 and 72 h post-infection. On Day 4 and 7 post-inoculation, thin blood smears of the test mice stained with 10% Giemsa solution, were used to determine the percentage parasitemia microscopically, by counting 4 fields of approximately 100 erythrocytes per field. The antimalarial activity was determined by using the equation:

$$\text{Activity} = 100 - \frac{\text{Mean parasitaemia in treated group}}{\text{Mean parasitaemia in control group}} \times 100$$

The mice were monitored for 30 days post-infection and time of death (in days) was recorded. Each mouse still alive on Day 30 was checked for parasitemia. The lowest dose of artesunic acid that gave a significant reduction in parasitemia both on Days 4 and 7, i.e. 15 mg/kg, was chosen as the dose of artesunic acid to be combined with different doses of PCE. The antimalarial activity of these combinations was also determined using the above method.

Determination of ED_{50} and ED_{90}

The linear equations of the dose-response relationship of the crude drug and/or artesunic acid were used to calculate their ED_{50} and ED_{90} on Day 7. The ED_{90} was used to calculate the isobolar equivalent (IE) of the crude drug[7], as shown below:

$$\text{IE} = \frac{\text{ED}_{90} \text{ of drug in combination}}{\text{ED}_{90} \text{ of drug alone}}$$

Data analysis

All the results were analyzed using ANOVA with multiple comparison test (Games-Howell's and Tukey HSD Test).

RESULTS

In vivo schizontocidal activity

Table 1 shows the antimalarial activity, i.e. the mean percentage reduction in parasitemia caused by the drugs alone or in combination, compared to the control, on Days 4 and 7. The mean parasitemia values on Days 4 and 7 are shown in Figs. 1 and 2.

Survival time and percentage cure

Tables 2 and 3 show the survival time based on a 30 days observation period, percentage survival at the end of this period and percentage of the infected mice that are cured, i.e. do not have any parasite in their blood on Day 30.

Table 1. Effect of Artesunic acid and/or PCE on the growth of *P. berghei* in mice on Day 4 and Day 7

Treatment	Reduction in parasitemia (%) ± SD	
	Day 4	Day 7
Arta 6 mg/kg	63.72 ± 16.96*	37.27 ± 18.78
Arta 15 mg/kg	62.83 ± 17.31*	70.26 ± 11.66*
Arta 20 mg/kg	68.14 ± 8.51*	72.12 ± 13.55*
PCE 50 mg/kg	37.70 ± 11.42	73.05 ± 10.07*
PCE 100 mg/kg	34.73 ± 24.34	47.03 ± 19.33
PCE 200 mg/kg	59.29 ± 20.37*	48.96 ± 12.73
Arta 15 mg/kg + PCE 50 mg/kg	79.17 ± 20.50*	57.59 ± 16.69*
Arta 15 mg/kg + PCE 100 mg/kg	83.33 ± 11.88*	72.57 ± 17.11*
Arta 15 mg/kg + PCE 200 mg/kg	96.87 ± 2.85†	81.40 ± 6.66*

*Significantly greater than the control at $p < 0.05$; †Significantly greater than the control and 50 mg/kg, all at $p < 0.05$; Arta = Artesunic acid; PCE: Pawpaw crude extract.

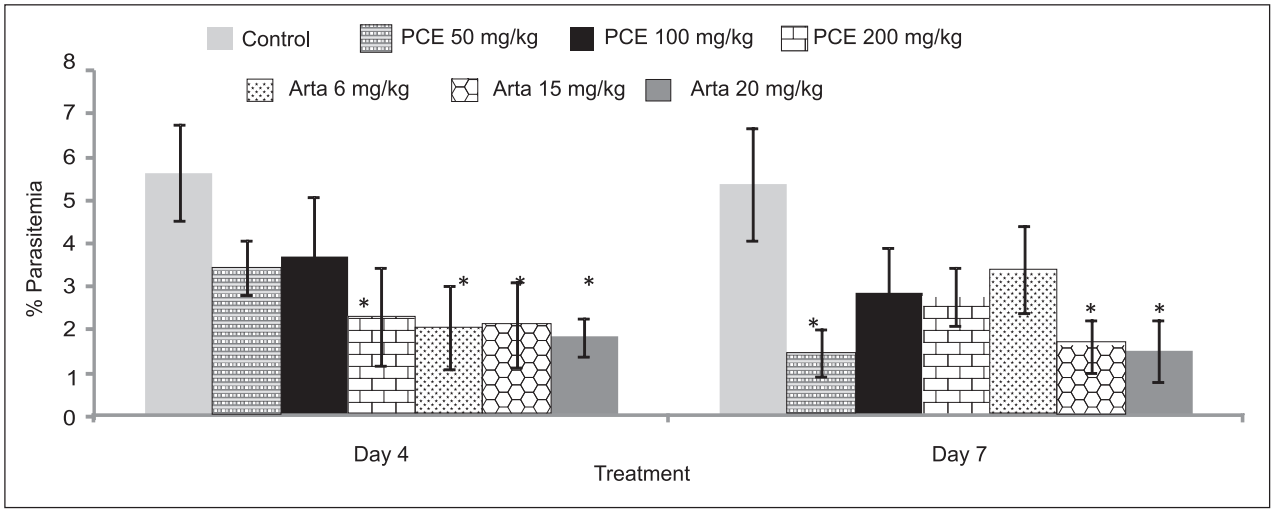

Fig. 1: Percentage parasitemia of *P. berghei*-infected mice given single treatment.

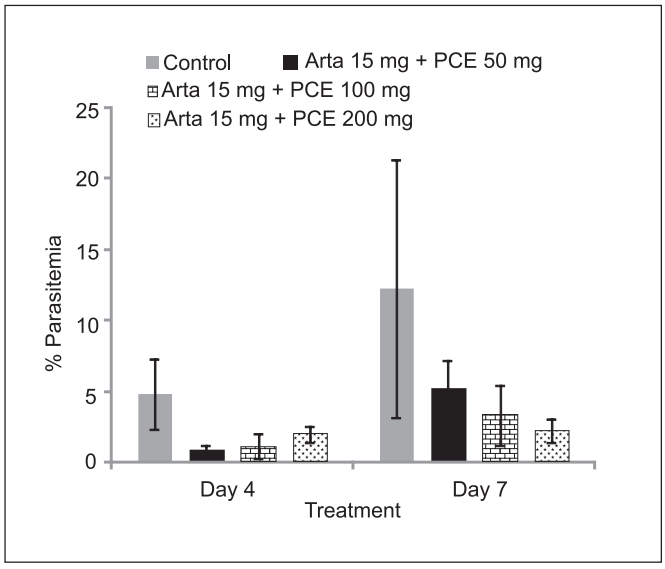

Fig. 2: Percentage parasitemia of *P. berghei*-infected mice given combination treatment.

ED_{50} of the crude extract and drug

Due to the plasmodiastatic effect brought about by *Eperythrozoon coccoides*, the ED_{50} of artesunic acid and PCE was calculated based on Day 7 and not Day 4. The ED_{50} of the drugs was calculated by using the linear equation of the dose-response relationship of the drugs. Per-

Table 2. Effect of single treatment (PCE or Artesunic acid) on *P. berghei*-infected mice survival and percentage cure

Experimental condition	Mean survival time (days)	Survival (%)	Cure (%)
Control group	19.80 ± 4.44	0	0
Arta 6 mg/kg	21.00 ± 3.74	0	0
Arta 15 mg/kg	21.80 ± 3.34	0	0
Arta 20 mg/kg	22.80 ± 2.28	0	0
PCE 50 mg/kg	21.00 ± 3.74	0	0
PCE 100 mg/kg	17.40 ± 4.93	0	0
PCE 200 mg/kg	17.60 ± 5.27	0	0

Table 3. Effect of combination treatment (PCE and artesunic acid) to *P. berghei*-infected mice survival and percentage cure

Experimental condition	Mean survival time (days)	Survival (%)	Cure (%)
Control Group	11.2 ± 4.55	0	0
Arta 15 mg + PCE 50 mg	23.0 ± 6.60*	40	20
Arta 15 mg + PCE 100 mg	22.6 ± 8.62*	40	20
Arta 15 mg + PCE 200 mg	19.6 ± 5.94	20	20

* Significant when compared to control at $p < 0.05$.

centage reduction of parasitaemia and log dose of artesunic acid yielded the regression equation y = 70.91x–17.17; $R^2 = 0.969$. Whereas percentage reduction of parasitaemia and log dose of PCE yielded the regression equation y = –40.01x + 136.3; $R^2 = 0.690$. The ED_{50} of artesunic acid and PCE are 8.814 and 143.53 mg/kg respectively.

Determination of the kind of pharmacodynamic interaction between the pure drug and plant extract

The ED_{90} of the drug alone and in combination was calculated from the linear equation of the dose-response relationship of the drugs alone (on Day 7) and in combination (on Day 7). Percentage reduction of parasitaemia and log dose of PCE + artesunic acid yielded the regression equation y = 37.8x – 4.701; $R^2 = 0.985$. The ED_{90} was then used to calculate the isobolar equivalent (IE) of PCE. The IE of the combination of PCE and artesunic acid was 22.29.

DISCUSSION

The development of an affordable ACT or an alternative cost-effective antimalarial drug is imperative in the rural areas where majority of the people are poor. Many scientists are now even turning towards herbs to seek for answers to drug resistance.

On the average the percentage yield of pawpaw aqueous crude leaf extract (PCE) was 5.42 ± 3.16%. At the initial stage of this study, a lag in the replication of the parasite was observed in the single mice treatment groups. Such plasmodiastatic effect has been attributed to possible blood contamination by *Eperythrozoon coccoides*[9]. *E. coccoides* inhibits the multiplication of the plasmodium, and enables the mice to survive longer after infection by *P. berghei*. It interferes with the course of the malaria[10]. *E. coccoides* causes parasitemia that peaks on Day 2 to 5 (acute infection stage) and subsequently declines rapidly (latent infection stage), such that by Day 6 or 7, the number of organisms in the peripheral blood is very low, even beyond detection. So, for the first five days, the growth of *P. berghei* was inhibited by both the drugs and *E. coccoides*. Death from infections with *E. coccoides* is very rare[11]. The combination treatment depicts clearly the behavior of *P. berghei* in both treated and untreated groups, with the parasitemia levels increasing from Day 4 to Day 7 for the untreated and treated groups.

On Day 7 the parasitemia level decreased and increased, for artesunic acid and PCE in a dose dependent manner, respectively. The activity of artesunic acid increased in a dose dependent manner, with artesunic acid 6 mg/kg losing its activity on Day 7, probably due to recrudescence. *P. berghei* infected mice treated with 200 mg/kg of pawpaw experienced a significant reduction in its parasitemia level (59.29%), while those treated with 50 and 100 mg/kg of PCE experienced a mild reduction in their parasitemia levels compared to the untreated group on Day 4. On Day 7, the reduction in parasitemia of the single treatment group decreased for the treatment groups that previously showed a significant reduction in parasitemia, (artesunic acid 6 mg/kg and pawpaw 200 mg/kg), probably due to recrudescence as a result of *E. coccoides* plasmodiastatic effect. But, artesunic acid 15 and 20 mg/kg still retained their activities.

Paradoxically, PCE 50 mg/kg, significantly suppressed the parasitemia of the infected mice on Day 7 (69.09%), compared to the control, but not on Day 4. This may be due to its slow activity. Increasing the dose of PCE produced a mild reduction in parasitemia on Day 7, probably due to ingredients of the complex mixture of the pawpaw crude extract antagonizing the activity of one another at higher dose levels.

The combination treatment yielded a more significant reduction in parasitemia compared to the control on Day 4. The combination of artesunic acid 15 mg/kg and pawpaw 50 mg/kg, produced a significant reduction in parasitemia compared to pawpaw 50 mg/kg, alone (Table 1). The artesunic acid thus enhanced the antimalarial activity of PCE via a pharmacodynamic interaction. On Day 7, all the combinations of artesunic acid 15 mg/kg and the crude extract still retained their antimalarial activities.

The survival times of PCE 100 and 200 mg/kg are lower than that of the control group, but not significantly lower. This may be due to their toxicity and increased presence of antagonistic constituents of the PCE at higher dose levels. On the contrary, the survival time of PCE 50 mg/kg is not lower than that of the control. Their percentage survival and cure rate are 0%. For the combination treatment, all the treatment groups survived significantly compared to the control (11.2 days, 0%), except for the combination of artesunic acid 15 mg/kg and papaw 200 mg/kg as shown in Table 3. This further justifies its least percentage

in reduction in parasitemia observed for this dose.

Only one of the mice in the entire combination treatment group was cured, the cure rate being 20% for all groups. The toxicity of pawpaw at this dose may also play a role in the reduction of the survival time, percentage survival and cure rate of this combination. The percentage survival and cure rates are higher for all combinations than the extract or pure drug given alone.

Due to the plasmodiastatic effect brought about by *E. coccoides*, the ED_{50} of artesunic acid, and PCE was calculated based on Day 7 and not on Day 4. The ED_{50} of artesunic acid agrees with the ED_{50} of artesunate from literature, and is 8 mg/kg against *P. berghei*, ANKA strain infected mice[11]. The *in vivo* anti-plasmodial activity of PCE is very good[12]. Based on the IE, the kind of pharmacodynamic interaction between the crude extracts and artesunic acid was determined from the criteria; synergistic effect (IE <1), additive effect (IE = 1), antagonistic effect (IE >1)[7]. The combinations of artesunic acid and PCE are antagonistic. Pawpaw is known to have antioxidant effect, it being a free radical scavenger, and helping with splenomegaly, while artesunic acid is known to act by being converted to a free radical. Thus, a pharmacological antagonism may be occurring at some minute level, thus inhibiting to an extent the activity of artesunic acid.

CONCLUSION

This study combines two antimalarial drugs PCE (a herbal drug) and artesunic acid (an orthodox drug). Artesunic acid enhanced the antimalarial activity of pawpaw. The combinations of artesunic acid and pawpaw also prolonged the survival time of the infected mice and increased the cure rate, compared to artesunic acid alone. However, the combinations of artesunic acid and pawpaw are antagonistic, despite the fact that artesunic acid enhances its activity, because its IE is 22.29. Combinations of artesunic acid and pawpaw show little promise for development as antimalarial combination therapy.

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