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# Combinations of *Chrysophyllum albidum* and *Citrus aurantifolia* as Antimalarial Agents and their Effects on Orthodox Antimalarial Drugs in Mice

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## Abstract

**Objective:** The decoctions of *Chrysophyllum Albidum* Leaf (CAL) and stem bark (CAB), and *Citrus Aurantifolia* Leaf (LCA) and fruit (FCA) used in Africa for malaria and fevers were evaluated for antimalarial activities.

**Methods:** Their prophylactic, chemosuppresive and curative antiplasmodial activities were assayed individually and variously combined with each other and standard drugs of Pyrimethamine (PYR) and Chloroquine (CQ), using *Plasmodium berghei* infected mice.

**Results:** Using their ED50 values, standard drugs were significantly ($p<0.05$) more active than the individual plant extracts, while extracts of *C. albidum* leaf, *C. aurantifolia* fruit and *C. albidum* stem bark had the highest prophylactic, chemosuppresive and curative activities, respectively. Co-administrations of individual plant extracts with PYR gave prophylactic activity comparable ($p>0.05$) to pyrimethamine, except combination with CAB that gave significantly ($p<0.05$) lower activity. The CAL+CAB+LCA+FCA combination doubled the mice survival time, similar to PYR. Combinations with CQ significantly lowered suppressive and curative activities of chloroquine and gave no better survival time in mice. No plant-plant extract combinations gave comparable prophylactic and suppressive activities as the standard drugs. Only CAL+FCA and LCA+FCA combinations gave comparable and significantly higher curative activity than CQ, respectively. A 73% of the plant-plant combinations gave a survival time comparable to that of CQ, indicating contributory factor of immune booster. This, rather than higher antiplasmodial activity, may justify the practice of combination of plants in ethnomedicinal malarial therapy.

**Conclusion:** Care should be taken in the concomitant administration of orthodox and herbal drugs and as well in the choice of plants to be combined in herbal drug formulations or decoctions.

**Keywords: *Chrysophyllum albidum*; *Citrus aurantifolia*; Plant-plant combinations; Plant orthodox drug combinations; *Plasmodium berghei*; Antimalarial assays**

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## Introduction

The plasmodium parasites, *P. vivax, P. ovale, P. malariae and P. falciparum* cause malaria in Africa. The evasive response of their resistant strains has made combating this life threatening and 'neglected' disease difficult [1]. Therefore, WHO had abolished reliance on single drug therapy for malaria treatment and adopted the Artemisinins Combination Therapy (ACT) policy that involves combination of antimalarial drugs [2]. Combination of drugs is also used effectively in treating/managing tuberculosis, HIV-AIDS, stomach and peptic ulcers, etc [3-5]. This has encouraged scientists to continue their search for a good combination of drugs that can effectively combat parasites responsible for the resistant malaria [6,7]. Since plants remain great reservoir of compounds that could be used to manage all kinds of diseases, it is natural that such search should include plants and their various combinations [8]. Hence, plant constituents have been suggested to work synergistically in killing the malaria parasites, especially their resistant strains [2,9]. Therefore, ethno-botanical surveys, ethno-medicinal and folkloric claims of usage of plants and their parts should be in the front line of providing creditable information on such plant combinations for greater effectiveness in treating malaria [10,11]. Unfortunately, such herbal remedies, which contain two

or more plants or their parts, have not been thoroughly investigated [8,12-14]. *Chrysophyllum albidum* G Don Holl (*Sapotaceae*) is common throughout the tropical Central, East and West Africa regions for its sweet and edible fruits and various ethno-medical uses, including traditional rituals and medico-magical [15]. Different parts of the plant are used to treat varied ailments, including malaria and yellow fever [16]. Its bark and leaves are used in treating malaria, skin eruption occasioned by infections and inflammatory reactions [17,18]. Its antiplasmodial [19], antimicrobial [18], hypoglycemic [20], anti-inflammatory and antioxidant [17], etc activities have also been reported. However, little is known about the plant chemistry [21]. *Citrus aurantifolia* (Christm). Swingle (*Rutaceae)*, a *citrus* hybrid of *C. micrantha* and *C. medica* commonly known as "Lime", is used in traditional medicine as an antiseptic, antiviral, antifungal, anthelmintic, mosquito repellent, and also for a plethora of diseases, including arthritis, colds, coughs, etc [22]. Lime and lemon juice are also widely used for douches among women at high risk of HIV transmission in Central Nigeria and as a barrier contraceptive due to its anti-fertility potential [23,24]. Its antibacterial, anti-mycobacterial, especially against isoniazid-resistant strains, antihelminthic activities and cytotoxic activity against colon and other cancers have been demonstrated [25-30]. This promises its use in resistant malarial parasites. Its potential for the protection of food and feeds from toxigenic fungal growth as well as their aflatoxin contamination is suggested due to its antifungal and anti-aflatoxigenic activities [23,31]. Flavonoids and vitamin-C present in its fruit juice and peels were responsible for its antioxidant and immuno-modulatory activities, while its anti-obesity properties may be because it promotes anorexia [32-38]. Monoterpenes, sesquiterpenes, limonoids, furo and pyrano-coumarins, fatty acids and flavonoids have been reported [28,39-42]. Since antimalarial activity has been confirmed for *C. albidum* leaf [19], and there is a common herbal remedy in Oke Igbo Community, Ondo State, Nigeria that contains different parts of *C. albidum* and *C. aurantifolia* [8], extracts made from four parts of these two plants were tested individually, in combinations with chloroquine and pyrimethamine, the standard drugs for *in vivo* prophylactic, chemosuppresive and curative antimalarial models, and with each other. This was to establish antimalarial activities for these plants parts, determine the plant and part with best prophylactic, chemosuppresive and curative activities, effects of these extracts on orthodox drugs [13], and any beneficial antimalarial effects of these plant combinations, hence this study.

## Methods and Materials

### Plant materials

The leaves and barks of *C. albidum* were collected in September, 2014 from Erefe village near Ile-Ife while the fresh unripe fruits and leaves of *C. aurantifolia* were collected in October, 2014 from Amoloja village near Obafemi Awolowo University (OAU) Investment Farm, Ile-Ife and the Medicinal Plant Garden of Department of Pharmacognosy, Faculty of Pharmacy, OAU, Ile Ife. They were identified and authenticated by Mr. I.I. Ogunlowo of the Department of Pharmacognosy, OAU, Ile-Ife and voucher specimens FPI 2030 and FPI 2138 were deposited in the Faculty of Pharmacy Herbarium, OAU, Ile-Ife. The leaves and bark (0.5 kg, each) were separately air dried for 7 days at room temperature and thereafter in a hot-air oven at 40ºC, and powdered. The fruits were air dried for several days to reduce their water content and thereafter cut into smaller pieces. Each plant part (0.5 kg, each) was macerated in 70% ethanol for 72 h; the resulting menstrum was filtered in each case and re-extracted twice. They were separately concentrated using rotary evaporator at 40ºC, and freeze-dried to obtain extracts weighing 76.2, 84.1, 72.75 and 86.05 g and percentages yields of 15.24%, 16.82%, 14.55% and 17.01%, respectively.

### Animals

Swiss albino mice of both sexes, weighing 18 g to 22 g, were purchased from the animal house of the Faculty of Basic Medical Sciences, College of Health Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria. Based on their sexes, they were separately housed in cages, under a 12 h light/dark cycle with free access to water and fed with commercial food pellets purchased from Dangote Feed Store, Lagere, Osun State, Nigeria. They were acclimatized for two weeks prior to use and divided into groups of five animals for each assay.

### Parasite

Chloroquine-sensitive *Plasmodium berghei* NK65 strain was obtained from Professor O.G. Ademowo of the Institute of Advanced Medical Research and Training (IMRAT), University of Ibadan, Nigeria. The parasites were maintained by continuous blood passaging in mice. A standard inoculums of $1 \times 10^7$ parasitized erythrocytes was prepared by dilution of blood collected through cardiac puncture from a donor mouse (>30% parasitemia) with normal saline and administered intraperitoneally (200 µL) to each test mouse [13,14].

### *In vivo* antimalarial assays of individual extracts of parts of the two plants

The prophylactic, chemosuppresive and the curative activities of the aqueous-ethanolic extracts of *C. albidum* leaf and bark (CAL, CAB), fresh unripe fruit and leaf (FCA, LCA) of *C. aurantifolia*, and standard drugs of chloroquine (CQ, 10 mg/kg) and pyrimethamine (PYR, 1.2 mg/kg) were determined by previously reported methods [43-45]. A 0.2 ml of the extracts (100 mg/kg, 200 mg/kg, 400 mg/kg, 800 mg/kg), standard drugs (positive controls) and normal saline (negative control) were administered per oral using oral cannula to groups of five mice each, daily for 3 days before infection in the prophylactic model. Same extracts and drugs were also given per oral two hours after infection and thereafter daily for 3 days in the chemosuppresive model, while in the curative model, administration was done daily for 5 days starting from the third day after infection. The temperature of each mouse was taken using a digital clinical thermometer inserted into the rectum before the administration of the extracts or drugs. The level of parasitemia was determined for each mouse at 3 ($D_3$) and 4 ($D_4$) days and daily after infection for these 3 models by cell counting of 10 fields in a view of the microscope of a thin blood smear, fixed with methanol and stained with Giemsa, obtained from the tail of each mouse [43-45]. The average percentage parasitemia for each group of 5 animals was calculated using the formula: $100 \times \{N_p/N_t\}$, where $N_p$ is the number of parasitized red blood cells and $N_t$ is the total number (parasitized+unparasitised) of red blood cells per view of count. The percentage reduction in parasitemia, chemosuppression and percentage clearance was calculated using the formula: $100 \times \{(A-B)/A\}$, where A is the average percentage parasitemia in the negative control group and B is the average parasitemia in the test group. The median effective doses, $ED_{50}$ and $ED_{90}$, as measurements of the antimalarial activities of individual plant extracts or standard drugs, were estimated from a graph of percentage reduction, chemosuppression and clearance against doses, using the Microsoft office excel 2010 [13,14].

**Table 1:** *In vivo* anti malarial activities of *Chrysophyllum albidum* leaf and stem bark and *Citrus aurantifolia* leaf and fruit extracts.

| Extract/Drugs | Antimalarial Activity (mg/kg) | | | | | |
|---|---|---|---|---|---|---|
| | $ED_{50}$ (mg/kg) | | | $ED_{90}$ (mg/kg) | | |
| | PRO | SUP | CUR | PRO | SUP | CUR |
| *C. albidum* leaf (CAL) | 281.9 ± 2.9$^{b}$ | 367.0 ± 2.9$^{c}$ | 225.5 ± 1.3$^{c}$ | 577.2 ± 5.1$^{b}$ | 662.0 ± 4.5$^{d}$ | 464.8 ± 1.7$^{c}$ |
| *C. albidum* stem bark (CAB) | 305.5 ± 1.0$^{c}$ | 372.2 ± 0.3$^{c}$ | 203.5 ± 2.1$^{b}$ | 613.6 ± 1.8$^{c}$ | 449.9 ± 0.5$^{b}$ | 362.6 ± 3.8$^{b}$ |
| *C. aurantifolia* leaf (LCA) | 717.0 ± 9.0$^{e}$ | 543.5 ± 9.1$^{d}$ | 527.3 ± 3.9$^{e}$ | 1367.1 ± 16.2$^{d}$ | 1258.6 ±16.2$^{e}$ | 874.2 ± 10.3$^{e}$ |
| *C. aurantifolia* fruit (FCA) | 379.6 ± 4.6$^{d}$ | 274.4 ± 2.2$^{b}$ | 468.8 ± 4.7$^{d}$ | 637.2 ± 9.6$^{c}$ | 553.5 ± 3.0$^{c}$ | 838.7 ± 6.7$^{d}$ |
| PC (Positive Controls) | 0.5 ± 0.1$^{a}$ | 2.2 ± 0.1$^{a}$ | 2.2 ± 0.0$^{a}$ | 0.9 ± 0.2$^{a}$ | 4.3 ± 0.2$^{a}$ | 4.1 ± 0.0$^{a}$ |

**Keys:** Data show the mean ± SEM n=5, $ED_{50}$: Doses that produced 50% and 90% activities, PRO: Prophylactic, SUP: Chemo suppressive and CUR: Curative models, PC (Positive Controls): Pyrimethamine (1.2 mg/kg) for prophylactic, and Chloroquine (10 mg/kg) for chemosuppresive and curative models. Only values with different superscripts within columns are significantly different ($p<0.05$, one-way analysis of variance followed by the Student-Newman-Keuls' test).

### *In vivo* antimalarial assays of combinations of plant extracts with standard drugs

The *in vivo* antimalarial assays of combinations of plant extracts and standard drugs, represented as CAL+CQ, CAB+CQ, LCA+CQ, FCA+CQ, were carried out using the doses of the individual plant extracts that were equal to their respective median effective dose ($ED_{50}$) in mg/kg for each model of prophylactic, chemosuppresive and curative (Table 1). These together with the therapeutic dose of pyrimethamine (1.2 mg/kg) were given daily for 72 h before infection for prophylactic model, or with chloroquine (10 mg/kg) were either given 2 h after infection and subsequently daily for 3 days or given for 5 days, starting from 72 h after infection, for chemosuppresive and curative models, respectively. Antimalarial activities of combinations of these plant extracts and standard drugs, expressed as average percentage parasitemia, percentage reduction in parasitemia, chemosuppression and percentage clearance were similarly calculated for the 3 antimalarial models [13,14].

### *In vivo* antimalarial assays for the varied combinations of extracts of plant parts

Varied combinations were made from the extracts as CAL+CAB, CAL+LCA, CAL+FCA, CAB+LCA, CAB+FCA, LCA+FCA, CAL+CAB+LCA, CAL+CAB+FCA, CAL+LCA+FCA, CAB+LCA+FCA, CAL+CAB+LCA+FCA. The antimalarial activities of these eleven combinations of plant extracts were similarly determined under the 3 antimalarial models. They were combined based on the estimated ($ED_{50}$) for each extract, and their average percentage parasitemia, percentage reduction in parasitemia, chemosuppression and percentage clearance were similarly calculated [13,14,43,44,46].

### Determination of survival times of the mice

The mice in each test group were monitored for survival from the day of drug administration for a period of 28 days, and the survival time was expressed as mean in days [43]. The values obtained for the negative control, expressed as percentage survival time ± SEM, were taken as 100% and the values of other test groups were expressed as percentages of these values [13,14].

### Statistical analysis

One way analysis of variance (ANOVA) followed by Student Newman Keuls post-hoc test was used for comparison to determine the source of significant differences for all the values. Values of $p<0.05$ were considered to be statistically significant.

## Results

The results of this study were shown in Table 1-5.

**Table 2:** *In vivo* anti malarial activities of combinations of standard drug with *Chrysophyllum albidum* leaf or stem bark and *Citrus aurantifolia* leaf and fruit extracts.

| Extract/Drug* | Percentage Reduction in Parasitemia | | |
|---|---|---|---|
| | Prophylactic | Chemosuppresive | Curative |
| NC (Negative Control) | 0.0$^{a}$ | 0.0$^{a}$ | 0.0$^{a}$ |
| *C. albidum leaf* (CAL) | 67.0 ± 4.$^{7c,d}$ | 16.0 ± 1.1$^{b}$ | 76.3 ± 0.5$^{f}$ |
| *C. albidum leaf* (CAL) PC | 64.5 ± 4.1$^{c,d}$ | 70.8 ± 7.4$^{d}$ | 55.6 ± 4.3$^{c,d}$ |
| *C. albidum* stem bark (CAB) | 68.7 ± 0.7$^{d}$ | 65.3 ± 0.3$^{d}$ | 93.8 ± 0.1$^{g}$ |
| *C. albidum* stem bark (CAB)+PC | 20.0 ± 4.2$^{b}$ | 59.2 ± 4.4$^{d}$ | 26.9 ± 5.9$^{b}$ |
| *C. aurantifolia* leaf (LCA) | 53.3 ± 5.5$^{c}$ | 31.1 ± 1.1$^{c}$ | 38.5 ± 9.7$^{b,c}$ |
| *C. aurantifolia* leaf (LCA)+PC | 70.2 ± 1.3$^{d}$ | 84.1 ± 1.3$^{e}$ | 45.7 ± 6.3$^{c,d}$ |
| *C. aurantifolia* fruit (FCA) | 49.3 ± 13.3$^{c}$ | 92.9 ± 1.2$^{f}$ | 60.5 ± 3.4$^{d,e}$ |
| *C. aurantifolia* fruit (FCA)+PC | 55.6 ± 9.2$^{c,d}$ | 51.0 ± 17.2$^{c,d}$ | 61.9 ± 5.7$^{d,e}$ |
| PC (positive controls) | 73.2 ± 5.4$^{d}$ | 97.0 ± 0.1$^{g}$ | 75.1 ± 3.1$^{f}$ |

**Keys:** Data show the mean ± SEM, n=5; *: Doses of the extracts given were those that gave 50% activity; NC (Negative Control): Tween 80 in normal saline; PC (positive controls): Pyrimethamine (1.2 mg/kg) for prophylactic, and Chloroquine (10 mg/kg) for chemo suppressive and curative models. Only values different superscripts within columns are significantly different ($p<0.05$, one-way analysis of variance followed by the Student-Newman-Keuls' test).

## Discussion

How the traditional medical practitioner became aware of the possible synergistic effects of the medicinal plants in herbal combination therapies is unknown [47]. However, the pharmacognosists and other natural product scientists, as a matter of duty, ought to investigate these plant combination therapies. Hence, in this study, four extracts from *C. albidum* leaf and stem bark (CAL, CAB) and *C. aurantifolia* leaf and fruit (LCA, FCA) were tested individually, in combinations with standard drugs of Chloroquine (CQ) and Pyrimethamine (PYR), and with each other, using the *in vivo* antimalarial prophylactic, chemosuppresive and curative models. This was with the view of establishing antimalarial activities for these plants' parts and determining which plant part is best suited for prophylactic, chemosuppresive and curative actions. Also, it was to evaluate the common African practice of co-administration of herbal drugs with orthodox antimalarial drugs [48], especially with regards to the effectiveness, safety or toxicity of such practice [13], and determining if truly plant combinations, as practiced in African ethnomedicinal, have any beneficial effect in malarial therapy.

### *In vivo* antimalarial activity of individual plant extracts

In all the test models used, the median effective doses ($ED_{50}$) of the standard drugs, CQ and PYR, was significantly ($p<0.05$) lower

**Table 3:** Survival times of *Chrysophyllum albidum* leaf or stem bark or *Citrus aurantifolia* leaf or fruit extracts or combinations with standard drugs.

| Extract/Drug* | Survival time as percentage of negative control per model type | | |
|---|---|---|---|
| | Prophylactic | Chemosuppresive | Curative |
| NC (Negative Control) | 100.0 ± 0.0$^{c}$ | 100.0 ± 0.0$^{b}$ | 100.0 ± 0.0$^{b}$ |
| *Chrysophyllum albidum* leaf (CAL) | 165.4 ± 43.0$^{d,e}$ | 120.3 ± 8.9$^{c}$ | 106.4 ± 9.5$^{b,c}$ |
| *Chrysophyllum albidum* leaf (CAL)+PC | 134.9 ± 7.4$^{d}$ | 217.7 ± 51.1$^{d,e}$ | 158.9 ± 49.0$^{b,c,d}$ |
| *Chrysophyllum albidum* stem bark (CAB) | 56.7 ± 31.1$^{a}$ | 91.0 ± 9.2$^{b}$ | 80.1 ± 21.9$^{a,b}$ |
| *Chrysophyllum albidum* stem bark (CAB)+ PC | 134.9 ± 7.4$^{d}$ | 254.0 ± 23.1$^{d,e}$ | 182.7 ± 96.6$^{a,b,c,d}$ |
| *Citrus aurantifolia* leaf (LCA) | 83.1 ± 3.2$^{a,b}$ | 83.6 ± 16.7$^{a,b}$ | 115.6 ± 10.0$^{b,c}$ |
| *Citrus aurantifolia* leaf (LCA)+PC | 217.7 ± 46.1$^{e}$ | 68.1 ± 6.4$^{a}$ | 207.3 ± 53.6$^{d,e}$ |
| *Citrus aurantifolia* fruit (FCA) | 98.4 ± 6.9$^{c}$ | 199.3 ± 76.0$^{c,d,e}$ | 125.0 ± 5.01$^{c}$ |
| *Citrus aurantifolia* fruit (FCA) + PC | 124.9 ± 7.2$^{d}$ | 254.0 ± 23.$^{1d,e}$ | 101.1 ± 30.2$^{a,b,c}$ |
| PC (Positive Controls) | 217.1 ± 44.7$^{e}$ | 412.6 ± 77.3$^{f}$ | 196.7 ± 5.7$^{d}$ |

**Keys**: Data show the mean ± SEM, n=5, *: Doses of the extract given were those that gave 50% activity; NC (Negative Control): Tween 80 in normal saline; CAL: *Chrysophyllum albidum* leaf; CAB: *Chrysophyllum albidum* stem bark; LCA: *Citrus aurantifolia* leaf; FCA: *Citrus aurantifolia* fruit; PC (positive controls): Pyrimethamine (1.2 mg/kg) for prophylactic, and Chloroquine (10 mg/kg) for chemosuppressive and curative models. Values with different superscripts within columns are significantly different ($p$<0.05, one-way analysis of variance followed by the Student-Newman-Keuls' test.

**Table 4:** Antimalarial activities of varied combinations of extracts of different parts of *Chrysophyllum albidum* or *Citrus aurantifolia.*

| Extract/Drug* | Percentage reduction in parasitaemia per model type | | |
|---|---|---|---|
| | Prophylatic | Chemosuppressive | Curative |
| NC (negative control) | 0.0 ± 2.4$^{a}$ | 0.0 ± 1.3$^{a}$ | 0.0 ± 2.0$^{a}$ |
| CAL+CAB | 55.0 ± 4.0$^{e}$ | 64.8 ± 6.2$^{c,d}$ | 36.4 ± 4.9$^{c}$ |
| CAL+LCA | 31.4 ± 0.1$^{c}$ | 49.0 ± 4.7$^{b,c}$ | 36.7 ± 6.8$^{c,d}$ |
| CAL+FCA | 87.4 ± 1.4$^{f}$ | 70.8 ± 4.9$^{d,e}$ | 71.7 ± 1.5$^{f}$ |
| CAB+LCA | 18.7 ± 1.2$^{b}$ | 56.5 ± 9.5$^{c,d}$ | 38.2 ± 4.7$^{c,d}$ |
| CAB+FCA | 34.6 ± 7.3$^{c,d}$ | 88.7 ± 1.9$^{f}$ | 64.2 ± 0.5$^{e}$ |
| LCA+FCA | 27.9 ± 1.6$^{c}$ | 61.4 ± 1.4$^{d}$ | 87.1 ± 1.6$^{g}$ |
| CAL+CAB+LCA | 28.5 ± 2.0$^{c}$ | 41.8 ± 2.3$^{b}$ | 49.8 ± 4.4$^{d}$ |
| CAL+CAB+FCA | 53.7 ± 5.4$^{e}$ | 66.5 ± 4.2$^{d}$ | 35.9 ± 1.3$^{c}$ |
| CAL+LCA+FCA | 53.5 ± 2.8$^{e}$ | 75.1 ± 2.8$^{e}$ | 69.9 ± 0.9$^{e}$ |
| CAB+LCA+FCA | 43.6 ± 6.9$^{d,e}$ | 40.7 ± 12.$^{9b,c}$ | 27.5 ± 1.3$^{b}$ |
| CAL+CAB+LCA+FCA | 53.4 ± 1.1$^{e}$ | 51.7 ± 2.5$^{c}$ | 66.1 ± 0.4$^{e}$ |
| PC (positive controls) | 96.4 ± 0.1$^{g}$ | 97.0 ± 0.1$^{g}$ | 75.1 ± 0.8$^{f}$ |

**Keys:** Data show the mean ± SEM, n=5, *: Doses of the extract given were those that gave 50% activity; NC (Negative Control): Tween 80 in normal saline; CAL: *Chrysophyllum albidum* leaf; CAB: *Chrysophyllum albidum* stem bark; LCA: *Citrus aurantifolia* leaf; FCA: *Citrus aurantifolia* fruit; PC (Positive Controls): Pyrimethamine (1.2 mg/kg) for prophylactic, and Chloroquine (10 mg/kg) for chemosuppressive and curative models. Values with different superscripts within columns are significantly different ($p$<0.05, one-way analysis of variance followed by the Student-Newman-Keuls' test.

**Table 5:** Survival times of mice given varied combinations of extracts of different parts of *Chrysophyllum albidum* and *Citrus aurantifolia.*

| Extract/Drug* | Survival time as percentage of negative control | | |
|---|---|---|---|
| | Prophylactic | Chemosuppressive | Curative |
| NC (negative control) | 100.0 ± 0.0$^{c}$ | 100.0 ± 0.0$^{a}$ | 100.0 ± 0.0$^{c}$ |
| CAL+CAB | 83.2 ± 12.9$^{a,b}$ | 119.0 ± 29.8$^{b-d}$ | 150.0 ± 36.5$^{c,d}$ |
| CAL+LCA | 157.6 ± 26.8$^{d,e}$ | 71.0 ± 10.9$^{a,b}$ | 184.5 ± 41.2$^{d,e}$ |
| CAL+FCA | 96.9 ± 19.0$^{a-c}$ | 151.0 ± 17.4$^{d}$ | 89.1 ± 5.6$^{a,b}$ |
| CAB+LCA | 161.8 ± 25.6$^{e}$ | 65.5 ± 11.5$^{a,b}$ | 151.3 ± 42.8$^{c,d}$ |
| CAB+FCA | 113.3 ± 14.0$^{c,d}$ | 254.6 ± 23.1e | 171.7 ± 90.7$^{a-e}$ |
| LCA+FCA | 156.6 ± 33.9$^{d,e}$ | 69.3 ± 15.1$^{a,b}$ | 183.4 ± 33.2$^{d,e}$ |
| CAL+CAB+LCA | 168.1 ± 19.1$^{e}$ | 78.7 ± 12.2$^{a,b}$ | 95.6 ± 18.9$^{a-c}$ |
| CAL+CAB+FCA | 66.9 ± 9.2$^{a}$ | 194.6 ± 49.4$^{d,e}$ | 67.4 ± 20.4$^{a}$ |
| CAL+LCA+FCA | 179.6 ± 22.6$^{e,f}$ | 103.8 ± 10.3$^{b,c}$ | 187.0 ± 16.6$^{d,e}$ |
| CAB+LCA+FCA | 91.6 ± 31.3$^{a-d}$ | 106.8 ± 15.3$^{b,c}$ | 149.7 ± 73.2$^{a-e}$ |
| CAL+CAB+LCA+FCA | 222.8 ± 49.4$^{e,f}$ | 53.8 ± 13.7$^{a}$ | 169.6 ± 83.3$^{a-e}$ |
| PC (positive controls) | 217.1 ± 44.7$^{e,f}$ | 412.6 ± 77.3$^{f}$ | 196.7 ± 5.7$^{d}$ |

**Keys**: Data show the mean ± SEM, n=5, *: Doses of the extract given were those that gave 50% activity; NC (Negative Control): Tween 80 in normal saline; CAL: *Chrysophyllum albidum* leaf; CAB: *Chrysophyllum albidum* stem bark; LCA: *Citrus aurantifolia* leaf; FCA: *Citrus aurantifolia* fruit; PC (positive controls): Pyrimethamine (1.2 mg/kg) for prophylactic, and Chloroquine (10 mg/kg) for chemo suppressive and curative models. Only values with different superscripts within columns are significantly different ($p$<0.05, one-way analysis of variance followed by the Student-Newman-Keuls' test.

than those of the four plant extracts (Table 1), indicating that the standard drugs were more effective as antimalarial agents [13,14]. Also, the ($ED_{50}$) values of the tested extracts, showed that extracts of *C. albidum* leaf, *C. aurantifolia* fruit and *C. albidum* stem bark were significantly the most active in the prophylactic, chemosuppresive and curative models, respectively (Table 1), and should be noted as such in ethnomedicine. Earlier in folk medicines, it was thought that "bad air" causes infections and infestations [49]. Since the years of this ignorance are gone forever, users of Nigerian and African medicinal plants based on their folkloric claims should allow themselves to be guided by results of scientific investigations of these claims. The traditional medical practitioners should therefore be the first to embrace this philosophy to the benefits of their numerous clients and patients. For example, it should be a natural occurrence for the herbalists and traditional medical practitioners to demand for results of malaria test during consultations with their clients. Therefore, increased enlightenment of the traditional medical practitioners and herbalists through workshops on the relevance of consulting literatures on evaluations of folkloric and ethnomedicinal claims to their practice is imperative [49]. Hence, governments, non-governmental organizations, professional and academic societies in African countries should not continue with the dangerous apathy of tolerating the outlandish claims in posts/videos of the social media while charlatans send many to early graves. Hence, similar to the use of different orthodox drugs for these treatment regimens, Table 1 showed that different plants or plant parts should be used

in ethnomedicine for these treatment regimens [5]. Furthermore, the orders of percentage parasitemia reductions demonstrated by the extracts in the respective prophylactic, chemosuppresive and curative models were PYR=CAL=CAB>LCA=FCA; CQ>FCA>CAB>LC >CAL; and CAB > CQ=CAL>FCA>LCA (Table 2). These results confirmed pyrimethamine as the standard drug (positive control) for prophylactic model and chloroquine for chemosuppresive and curative models [13,14]. They also affirmed that CAL, FCA and CAB were the most active plant extracts for the prophylactic, suppressive and curative models, respectively, since they gave the highest percentage reductions in parasitemia in the mice (Table 1 and 2). A doubled survival time in the curative test had been suggested as an indication of a good antimalarial activity [50]. However, there is no literature that could guide interpretation of this in prophylactic and chemosuppresive models. Therefore, the authors are of the opinion that similarly, plant extracts that demonstrated high antiplasmodial activity in the prophylactic and chemosuppresive models should also significantly doubled/increased the survival times of mice that were administered with these plant extracts. Thus, *C. albidum* leaf extract with the significantly lowest ($ED_{50}$) value (Table 1), gave the highest percentage reduction in parasitemia (Table 2) and significantly longest surviving time of the mice (Table 3) may be confirmed as a creditable prophylactic drug, similar to pyrimethamine, the orthodox drug used for prophylaxis of malaria. Also, the 67% reduction in parasitemia given by CAL was comparable ($p>0.05$) to the 69% and 73% given by its stem bark extract and pyrimethamine, respectively. However, it was worrisome that the equally prophylactic and curative active *C. albidum* stem bark (Tables 1 and 2) gave the significantly lowest survival times in the mice (Table 3). This may be an indication of the toxicity of CAB and calls for caution in its use and demands full investigation of its toxicity profile. Also, the poor prophylactic activity and low survival times given by *C. aurantifolia* leaf and fruits (Table 1-3) may suggest that they should not be used in prophylaxis of malaria. These parts may be more beneficial in their traditional use of repelling/killing mosquitoes among others [51]. Furthermore, the doubling of survival time of the mice given by *C. aurantifolia* fruit (Table 3) agreed with the fact that it gave the highest (93%) suppression of parasitemia (Table 2), thereby confirming it as a good malarial suppressive plant drug. On the other hand, that none of the plant parts gave doubled the survival time of the negative control mice (Table 3) cast doubt on credibility of the significantly high curative antimalarial activity of *C. albidum* leaf or stem bark (Table 2). The *C. albidum* leaf and stem bark that gave 76% and 94% reductions in parasitemia, respectively only gave 106% and 80% survival times, respectively that was comparable to the 100% found in the negative control group of mice (Table 2 and 3). Therefore, for foreign visitors to the malarial endemic tropical African or Asian countries, *C. albidum* leaf should offer the needed protection throughout their stay, similar to other orthodox medications, such as atovaquone/proguanil, doxycycline, mefloquine and primaquine [52]. Also, the chemosuppresive *C. aurantifolia* fruit would only be appropriate for citizens residing in malaria endemic countries, as they are always exposed to these mosquitoes and parasites. Hence, similar to microbial infections, where antibiotics significantly reduce the microbial load to the level that the human immune system could effectively get rid of them [53,54], antiplasmodial suppressive plants would only sufficiently keep down the malarial parasites' load, thereby preventing sickness or manifestation of malaria symptoms. Therefore, antimalarial assays of African medicinal plants or evaluation of their antimalarial ethnomedicinal claims should appropriately be only for suppression of malaria. Nevertheless, their full antimalarial profiles, making use of these three models, may be needed in drug discovery of other hidden therapeutic potentials of these plants.

### *In vivo* antimalarial activity of combinations of plant extracts with standard drugs

The methodology of co-administration employed in this study was that of giving the therapeutic doses of the standard drugs and the median doses of the extracts. Since the antiplasmodial activity of such combination of orthodox drugs and herbs should naturally be expected to be better than that of the composite plant, this methodology should therefore test effects of the extracts on the antiplasmodial activity of standard or orthodox drugs. It is a common knowledge that some home patients or those admitted in Nigerian hospitals, especially those with resistant malarial or other parasitic infections or suffering from chronic diseases, such as hypertension, cancer and diabetes, concomitantly use orthodox drugs and herbal preparations [48,55]. A 74% of subjects with parasitemia confessed to pre-hospital medication, with a 52% of analgesic use being the most prevalent [56]. Also, some traditional medical practitioners incorporate orthodox drugs, such as sedatives and other anxiolytics, into their herbal preparations for competitive advantage in the management of psychiatric patients and other patients earlier enumerated [49]. This therefore makes the investigation of combination of orthodox drugs with herbal drugs very imperative and urgent to reduce the number of casualties of this practice. Generally, co-administrations of plant extracts with the standard drugs produced comparable or significantly lowered activities, when compared with the activities of standard drugs (Table 2). In the prophylactic model, CAL+PYR and LCA+PYR combinations gave comparable activity as pyrimethamine while CAB and FCA extracts significantly reduced the activity of this orthodox drug. However, that the CAB+PYR combination also gave an activity that was significantly lower than that of the extract is worrisome. The 20% reduction in parasitemia that this combination elicited in mice was significantly the least produced by all the extracts or extract-pyrimethamine combinations (Table 2). With the exception of the LCA+PYR combination, all the extract-PYR combinations gave survival times in mice that were significantly higher than the untreated mice but were not doubled its value (Table 3). This may indicate that there will be no beneficial effect of the co-administration of these extracts with pyrimethamine in the prophylactic treatment of malaria and that the CAB+PYR combination is contraindicated in this treatment [57]. Furthermore, the 70% reduction in parasitemia given by LCA+PYR combination was comparable to the 73% elicited by PYR alone and significantly higher than the 53% given by LCA (Table 2). Also, only this combination gave the doubled survival time of untreated mice, similar to pyrimethamine (Table 3), indicating that although *C. aurantifolia* leaf extract did not hinder the antiplasmodial activity of pyrimethamine, it did not have any beneficial effect on its treatment of malaria. Hence, these results (Table 2 and 3) showed there is no justification for the co-administration of any of these four plants with pyrimethamine in the prophylaxis of malaria. Moreover, only FCA-CQ combination significantly reduced the chemosuppresive activity of chloroquine and gave a percentage reduction in parasitemia that was significantly lower than that of *C. aurantifolia* fruit extract (Table 2). Mice survival time given by this combination was doubled that of the untreated mice, comparable to that of FCA extract alone and significantly lower than that given by chloroquine (Table 3), indicating that concurrent administration of chloroquine and *C. aurantifolia* fruit extract is also contraindicated

[57]. In addition, the CAL-CQ and CAB-CQ combinations significantly reduced the suppressive activity of chloroquine and gave an activity that was significantly higher and comparable to those of their extracts, respectively. They also doubled the survival times of the untreated mice, but these values were significantly lower than that of chloroquine (Table 2 and 3), suggesting that co-administering of these plants with chloroquine would not benefit the suppressive effect of chloroquine. On the other hand, LCA-CQ combination gave significantly lower and higher suppressive activity than CQ and LCA extract, respectively. It also elicited 68% of the survival time of untreated mice, a value that was comparable to that of the extract. Since this combination was not able to improve the extremely low survival time observed with the LCA extract, co-administration of *C. aurantifolia* leaf extract with chloroquine was also not be beneficial in CQ suppressive treatment of malaria (Table 2 and 3). The results therefore confirmed the toxicity of *C. aurantifolia* leaf extract alone or when combined with chloroquine. Hence, the use of *C. aurantifolia* leaf extract alone in malarial ethnomedicine, and more especially the combinations of *C. aurantifolia* leaf or fruit extract with chloroquine as a chemosuppresive herbal drug, should be discouraged. All combinations of the plant extracts with chloroquine gave significantly lowered values of percentage clearance of parasitemia in the curative model, when compared with that of chloroquine (Table 2), indicating that they all inhibited the curative activity of this orthodox drug. These values for CAL-CQ and CAB-CQ combinations were also significantly lower than those of the individual extracts, suggesting that the co-administration of *C. albidum* leaf or stem bark with chloroquine is contraindicated. Perhaps worthy of special mentioning is the 27% reduction in parasitemia of co-administered *C. albidum* stem bark and chloroquine as opposed to 94% and 75% reductions for *C. albidum* stem bark extract and chloroquine, respectively. The LCA-CQ and FCA-CQ combinations gave curative activity that was significantly lower than that of CQ but was also comparable with their individual extracts (Table 2). With the exception of FCA-CQ combination, all the other combinations gave survival times in mice that were comparable to that given by chloroquine and significantly higher than those elicited by their individual extracts (Table 3). Furthermore, the survival times of the mice given FCA-CQ combination were comparable with those of the untreated mice and those given the extract only, but were significantly lower than those given CQ. These results did not support the moderate curative activities of the *C. aurantifolia* fruit extract or its combination with chloroquine (Table 2 and 3). Hence, neither *C. aurantifolia* fruit extract nor its combination with chloroquine is advised when the curative activity is desired in malaria treatment [13,14]. The results of this study showed that in some cases, co-administrations of plant extracts with the orthodox prophylactic, suppressive and curative drugs significantly hindered the antiplasmodial activities of pyrimethamine and chloroquine. They also indicated possible toxicity of *C. albidum* stem bark when used alone in prophylaxis, suppression and curing of malaria, of *C. aurantifolia* leaf when used alone in prophylaxis and suppression of malaria, and of *C. aurantifolia* leaf in combination with chloroquine in malaria suppression. Therefore, concurrent administration of chloroquine and *C. aurantifolia* fruit extract, pyrimethamine and *C. albidum* stem bark extract, chloroquine and *C. albidum* leaf or stem bark extract in the suppressive, prophylactic and curative models, respectively is contraindicated. This because these co-administrations may lead to increased resistance of the plasmodium parasites [13], loss of man hours due to terrible sickness or death as a result of compromised immunity [58]. Hence, there is no justification for the co-administration of any of these plant extracts with the orthodox drugs of pyrimethamine and chloroquine.

### *In vivo* antimalarial activity of combinations of plant-plant extracts

Drug combination is one of the effective means of combating parasite resistance in anti-malarial chemotherapy [59-62]. The ACT drugs used in malaria treatment in Nigerian hospitals contain two or more antimalarial drugs with different mechanisms of action that are combined in single formulations [5]. Similar combinational therapy is employed in the treatment of HIV/AIDS, tuberculosis, peptic ulcers [3-5]. It is therefore interesting that in African ethnomedicine, medicinal plants are combined together in various proportions to manage different disease conditions. Such combinations are believed to work synergistically to give the desired effect. For example, co-administration of Ephedra, Cinnamon twig, Bitter apricot seed and Liquorice root as multiple ingredients in the Chinese decoction of Ephedra is believed to result in complementary interactions that will reduce headache, general aching, symptoms of cold, cough, asthma, and also act as a diaphoretic (increased sweating) [63,64]. This is an example of the holistic nature of herbs or herbal drugs [49]. In this study, the plant combinations generally gave percentage reductions in parasitemia that were significantly lower than those of the standard drugs under the three antimalarial models used, showing that the respective prophylactic and chemosuppresive activities of pyrimethamine and chloroquine were significantly better than all the plant-plant combinations (Table 4). Order of the prophylactic activity for the top five plant combinations was PYR>CAL+FCA>CAL+CAB=CAL+FCA+CAB= CAL+FCA+LCA=CAL+FCA+CAB+LCA, showing that combinations that had either CAL or FCA or both had significantly higher prophylactic activity. Therefore, CAL and FCA may have potentiated the prophylactic activity of the plant combinations tested. On the other hand, CAB and LCA inhibited the prophylactic activity of the plant combinations that had them as composite plants, because combinations that had either CAB or LCA had significantly lower prophylactic activity. Hence, the fact that combinations CAL+FCA and CAB+LCA gave 87 and 19% reductions in parasitemia that were the highest and lowest values for the combinations tested; respectively (Table 4) may confirm this assertion. Furthermore, CAL+FCA with the highest prophylactic activity gave survival time that was comparable with that of the untreated mice. The CAL+FCA+CAB+LCA combination gave a 223% survival time that was comparable to 217% given by PYR. These were the only two agents that significantly doubled the 100% survival time given by the untreated mice. The 180% survival time given by CAL+FCA+LCA was also comparable to PYR and combination of the four plant parts (Table 5). Therefore, combinations that gave the highest percentage reductions in parasitemia and consequently had the highest prophylactic antiplasmodial activity did not necessarily give the highest survival times in the mice (Table 4 and 5). This may indicate that other properties in these extracts, such as antipyretic, immune enhancement, etc, in addition to their moderate antiplasmodial activity, may be responsible for the significantly increased survival times of mice that were administered with these combinations [13,14]. The CAL+FCA were the only combination that gave a significantly higher prophylactic activity over their component extracts (Table 2 and 4), thereby justifying ethnomedicinal combinations of plants for enhanced antimalarial activity. Its prophylactic activity was next in the order of activity after PYR and was therefore the most active plant-plant combination tested.

Furthermore, prophylactic activity of the other binary, triple and four plants' combinations were significantly lower than their component plants, especially CAB+LCA, LCA+FCA and CAL+CAB+LCA that gave 19%, 28% and 29% reductions in parasitemia, respectively (Table 4). These further confirmed the assumption that when *C. albidum* leaf and *C. aurantifolia* fruit are present in plant combinations, they significantly enhanced prophylactic activities of such plant-plant combinations, while presence of *C. albidum* stem bark and *C. aurantifolia* leaf inhibited this activity. Similar reports had earlier been made [13,14]. The order of activity of the combinations with percentage chemosuppression values of ≥ 50% was CQ>CAB+FCA >CAL+LCA+FCA=CAL+FCA>CAL+CAB+FCA=LCA+FCA>CA B+LCA= CAL+CAB+LCA+FCA=CAL+LCA. The values of 88.7% ± 1.9%, 75.1% ± 2.8% and 70.8% ± 4.9% for the binary and triple plant combinations of CAB+FCA, CAL+LCA+FCA and CAL+FCA, respectively make them the top three plant combinations active as suppressive (Table 4). Possibly, the *C. aurantifolia* fruit extract with the highest suppressive activity and common to these three combinations significantly enhanced their suppressive activities. Conversely, the lowest 16% and 31% reductions in parasitemia given by CAL and LCA, respectively may have significantly contributed to the reduced suppressive activities of CAL+LCA and CAL+CAB+LCA combinations (Table 2 and 4). Furthermore, the survival time in mice given by chloroquine was the highest and significantly higher than those given by the various combinations. Only the most active combination CAB+FCA doubled the survival time given by untreated mice, while CAL+FCA and CAL+CAB+FCA combinations that also had high suppressive activity gave survival times that were significantly higher than the untreated mice (Table 5), thereby confirming CAB+FCA, CAL+FCA and CAL+CAB+FCA combinations as good malaria suppressive herbal drugs. This may be due to that fact that FCA had the highest suppressive activity and survival time among the component plants. Also, combinations that had moderate activity gave 54% to 100% survival times, probably due to the toxicity of CAB and LCA (Table 2-5).

Also, the order of curative activity for the combinations that had percentage reduction of parasitemia values of ≥ 50% was LCA+FCA> CQ=CAL+FCA>CAL+LCA+FCA=CAL+CAB+LCA+FCA=CAB+F CA>CAL+CAB+LCA (Table 4). The results showed that combination of CAB, which had the highest individual curative activity (Table 2) and was, reported a good curative drug [19], with other plant parts led to the loss of its significantly better curative activity, while LCA with the lowest individual curative activity was a component of 67% of the combinations with ≥ 50% curative activity. However, CAL or FCA with curative activity that was significantly better or comparable to that of CQ, respectively was a component of 83.3% of these combinations (Table 4). Hence, FCA potentiated the curative activity of LCA+FCA combination, agreeing with the suggestion that synergism of activities may be responsible for higher activities of combinations over their component plants [13]. Furthermore, FCA contributed significantly in enhancing antiplasmodial effects of plant combinations across all the models, especially suppressive and curative models. The fruit of *C. aurantifolia* is a common component of some herbal antimalarial remedy [8]. The CQ with the highest curative activity gave almost double the survival time of the untreated mice. Similarly, high percentage reductions in parasitemia and survival times that were comparable to that given by CQ, may confirm that CAB+FCA, LCA+FCA, CAL+LCA+FCA and CAL+CAB+LCA+FCA (Table 4 and 5), were good curative herbal drugs [50]. Other combinations, such as CAB+LCA, CAL+CAB, CAL+LCA, CAB+FCA, LCA+FCA, CAL+LCA+FCA, CAB+LCA+FCA and CAL+CAB+LCA+FCA also gave survival times that were comparable to CQ. That these survival times were significantly better than those given by their composite plants (Table 3 and 5) may confirm one of the acclaimed beauties of herbal combinations. Similar synergistic effects have recently been reported for some decoctions of Nigerian herbs [14]. Therefore, the plant combinations of CAL+CAB, CAL+LCA, CAB+LCA and CAB+LCA+FCA that had low antiplasmodial activity and yet had survival times that were significantly higher than the untreated mice (Table 4 and 5) may indicate that these plants have other properties that elongated the lives of these mice [13]. Multicomponent herbal preparations or remedies are claimed to be holistic in nature [49], as apart from the main antimalarial activity in this case, other properties that are inherent in the plants, such as immune enhancement or immune booster, hepatoprotection, anti-inflammatory, etc also contribute to their management or treatment of disease [65]. However, CAL+CAB+LCA and CAL+CAB+FCA with both low antiplasmodial activity and poor survival times do not qualify as herbal curative drugs and these triple combinations should never be used as curative herbal drugs. Lastly, CAL+FCA with high percentage clearance of parasitemia but had survival times that were lower than those of the untreated mice (Table 4 and 5) would suggest toxicity of such combination. Hence, care should be taken when choosing plants that would be used in combinational antimalarial herbal drug formulations. However, it should be noted that though scientific reports of antimalarial studies on individual plants may be helpful in this consideration, this information should not be held sacrosanct because antimalarial property of composite plant drug may be different in combinational herbal drug formulations. Some combinations may produce the desired potentiation of activity, but others that produce inhibitions or even present with toxicity necessitate increased biological and pharmacological assays of these herbal combinations. For example, although combinations of *Murraya koenigi* with *Nauclea latifolia*, *Enantia chloranta* and *Artocarpus altilis* gave significantly enhanced suppressive activities over their individual plants, combinations of these with *A. altilis* gave significantly reduced curative activities over their individual extracts. Also, combining *M. koenigii* with *E. chloranta* resulted in reduced prophylactic and curative activities [13]. Furthermore, *Turraea robusta* and *Boscia salicifolia* exhibited 64% and 44% suppressive activity in vivo when tested singly while their combination elicited 21.5% [62].

## Conclusion

Extreme care should be taken in concomitant administration of orthodox and herbal drugs and as well in the choice of plants to be combined in herbal formulations or decoctions as some may leads to inhibition of activity of the latter, be toxic and may encourage development of parasite resistance to these orthodox drugs.

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