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Research Article

Tithonia diversifolia (Hemsl) A. Gray Fractions Enhanced Anti-Plasmodial Efficacy of Chloroquine in Resistant *Plasmodium yoelii* Infected Mice

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Abstract

The evolution of drug resistant strains of *Plasmodium* has presented global health challenges despite the development of chloroquine and other anti-malarial drugs. In this regard, the possibility of *Tithonia diversifolia* reversing the efficacy of CQ was studied. Curative anti-plasmodial potential of *T. diversifolia* ethanolic leaves extract and fraction in combination with CQ were studied in CQ-resistant *P. yoelii* (*P. yoelii*^{R}) infected albino mice. The effects on haematological parameters and possible mechanisms of action were examined using standard procedures. The percentage parasitaemia of *P. yoelii*^{R} infected mice treated with 5 mg/kg b. w of arthemeter, varying doses of extract and chloroform 70%:methanol 30% (C70:M30) fraction administered with 10 mg/kg b. w of CQ from day 7 to 28 showed significant ($p < 0.05$) decrease compared to infected mice administered 0.2 ml of distilled water. Plasma Ca^{2+} and free fatty acid (FFA) level showed significant ($P < 0.05$) reduction in the *P. yoelii*^{R} infected animals administered different doses of the extract with CQ compared to infected untreated animals. The antimalarial drugs and the extract elevated the percentage inhibition of phospholipase A_2. The crude extract and fraction effectively inhibit hypotonicity-induced haemolysis. The present study reveals that ethanolic leaf extract of *T. diversifolia* dose-dependently enhance the activity of CQ against *P. yoelii*^{R} infected mice resulting from rapid rate of recovery from plasmodial infections with synergistic administration.

Keywords: *Artemether; Chloroquine; Haematological; Phospholipase A2; Tithonia diversifolia*

Introduction

Malaria has been reported as one of the most devastating diseases not only to the poverty and underdeveloped countries but remains global health challenge [1]. It is an endemic disease, curable and preventable yet remain challenging with high infection and mortality

[2] presenting about 198 million cases and 0.6 million mortalities yearly [3,4]. Despite effort by the World Health Organization and other agencies to eradicate malaria, it continues to top the list of life threatening diseases globally. This is due to the spread of drug resistant parasites, insecticide-resistant vector, absence of a successful malarial vaccine and rapidly raising distribution of counterfeit drugs [2]. Chloroquine (CQ), an efficacious drug for the treatment of malaria has gradually failed as a preventive and curative malarial drug in many countries due to a corresponding spread of CQ resistant *Plasmodium falciparum* strains [5]. Consequently, alternative antimalarial drugs such as sulphadoxine-pyrimethamine, mefloquine etc. have been developed and their efficacy compromised by development of *P. falciparum* resistant to the drugs [6].

Calcium (Ca) is essential for the correct functioning of the cell and requires regulation to avoid high concentration causing death. Calcium ions (Ca^{2+}) is a ubiquitous intracellular messenger in malaria parasites with important functions in asexual blood stages responsible for malarial symptoms, the hepatic stage of the infection and transmission through the mosquito [7]. Invasion of the erythrocytes by *plasmodium falciparum* channels the pumping of Ca^{2+} by the Ca^{2+}ATPase from the erythrocyte membrane of the host cell to the parasitophorous vacuole formed [8].

Phospholipase A2 (PLA_2) is a protein that catalyzes the hydrolysis of glycerophospholipids at the sn-2 position to release free fatty acids (FFA) and lysophospholipids [9]. Elevated level of circulating PLA_2 activity has been reported in severe malaria infection [10,11]. This enzyme has been reported to play critical role in the cleavage of erythrocyte membrane phospholipids of *Trypanosoma congolense* resulting in lysis of the erythrocytes which might lead to anemia [12,13]. Anemia has been reported has one of the complications of malaria infection [13]. Therefore, the therapeutic target of this protein by medicinal plants might reduce the burden of malaria complications.

In malaria endemic nations, herbal medicines are commonly used for malarial therapy. Over 1200 species of herbs have been reported through various studies for action against malaria [14]. *Tithonia diversifolia* (Hemsl) A. Gray, is a perennial plant belonging to family Asteraceae, tribe Heliantheae, which is native to central America and Mexico, popularly known as Mexican sunflower [15]. This plant is a source of bioactive compounds majorly in the leaves belonging to sesquiterpene lactones, flavonoids classes and derivatives of *trans*-cinnamic acid [16]. Phytochemicals such as phenolics, flavonoids, tannins, alkaloids, saponnins and cardiac glycosides have been reported in *T. diversifolia* leaves [17]. *T. diversifolia* leaves has been found to contain sesquiterpene lactones, taginin C an active molecule against *plasmodium*, diversifolin methyl ether and tirotundin as anti-inflammatory agents, three new sesquiterpenoids; 2-α-hydroxytirotundin, tithofolinolide and 3-α-acetohydroxy diversifolol were also evaluated for their potential anti-tumorigenic activity [18]. The findings of Oyewole., *et al.* [18] indicated that *T. diversifolia* extract shows promising antimalarial property despite its inability to clear parasitaemia completely. Hence, the potential of *T. diversifolia* leaves enhancing the activity of CQ needs to be investigated. In this present study, various doses of ethanolic extract and active fraction of *T. diversifolia* leaves were combined with CQ to study the effect of the plant on the chemotherapeutic efficacy of CQ in mice infected with CQ-resistant *Plasmodium yeolii* strains.

Experimental Study

Plant material

The *T. diversifolia* leaves used in the study were collected within the International Institute of Tropical Agriculture (IITA) Ibadan, Oyo State, Nigeria. The identification and authentication were conducted by Mr. Esimekhuai Donatus of the Department of Botany, University of Ibadan. The (UIH-16679) voucher number was deposited at their herbarium.

Reagents and chemicals

The chemicals used in this study were of analytical grade and supplied by Sigma Inc, USA. The therapeutic drugs for the control and treatment groups were chloroquine (CLARION, Nigeria) and the Artemisinin-based combination therapy (ACT) drug administered was Lokmal® a brand of artemether lumefantrin (EMZOR, Nigeria). These were dissolved in normal saline to obtain fixed dose of 10 mg/kg and 5 mg/kg respectively according to Iwalokun [19].

Extraction and fractionation

The plant extraction and fractionation were carried out as reported by Omoboyowa., *et al.* [20]. Briefly, One thousand, nine hundred and seventy-eight grams (1,978g) of dried ground sample of *T. diversifolia* leaves were macerated with 3,000 ml of analytical grade ethanol for 72 hours with occasional stirring using a stirring rod. The extract was then filtered with Whatman No. 1 filter paper and concentrated by gentle warming in a water bath at 40^0C.

A sintered glass Buckner funnel (6 × 150 cm) attached to a vacuum line was packed with silica gel 60 (0.04 - 0.063 mm, 230 - 400 mesh) to a height of 13 cm of its length. The silica gel was compressed under vacuum to achieve a uniform layer for better separation. The ethanol extract (20.7g) was dissolved in methanol and sonnicated for 5 minutes to obtain a uniform solution. This solution was mixed with silica

gel (10.0g) and the solvent evaporated. The dry fraction was then triturated to get a uniform powder. This was transferred to the column under vacuum to ensure a uniform layer. The column was eluted with a gradient of *n*-hexane in ethylacetate (100:0, 70:30, 50:50, 30:70, 0:100 each 500 ml) and of chloroform in methanol (100:0, 70:30, 50:50, 30:70, 0:100 each 500 ml) to afford 10 sub- fractions. The fractions were collected, concentrated, dried and used for the study. The activity guided study reported in our previous study on the ten sub-fractions [20] revealed that, fraction Chloroform 70% and methanol 30% (C70: M30) has the highest significant percentage parasitemia chemo-suppression. The extract and active fraction of the plant were co-administered with chloroquine for this investigation.

Experimental animals

Male albino mice of 3 - 4 weeks old with average weight of 20 - 34g were obtained from the Faculty of Veterinary Medicine Animal House, University of Nigeria, Nsukka. Acclimatization of the animals under standard environmental conditions was carried out for 14 days, with a regular feed and water *ad libitum*. All the experimental protocols were in compliance with our Institutional Animal Ethics Committee guidelines. The experimental protocol was approved by the faculty of science animal ethics committee of University of Nigeria with reference number PG/12/64667.

Experimental grouping

Total of 96 mice divided into 12 groups of 8 mice each (n = 8) were treated as follows:

- Group A: Uninfected control administered normal saline
- Group B: Infected control administered normal saline
- Group C: Infected mice administered 10 mg/kg b. w CQ
- Group D: Infected mice administered 5 mg/kg b. w. artemether lumefantrine
- Group E: Infected mice administered 100 mg/kg b. w. crude extract
- Group F: Infected mice administered 200 mg/kg b. w. crude extract
- Group G: Infected mice administered 100 mg/kg b. w. crude extract with 10 mg/kg b. w. CQ
- Group H: Infected mice administered 200 mg/kg b. w. crude extract with 10 mg/kg b. w. CQ
- Group I: Infected mice administered 100 mg/kg b. w. C70:M30 fraction
- Group J: Infected mice administered 200 mg/kg b. w. C70:M30 fraction
- Group K: Infected mice administered 100 mg/kg b. w. C70:M30 fraction with 10 mg/kg b. w. CQ
- Group L: Infected mice administered 200 mg/kg b. w. C70:M30 fraction with 10 mg/kg b. w. CQ.

Parasitaemia procurement

The parasite used for this study (*P. yoelii*^{it}) was donated by the MR4/American Type Culture Collection (ATCC), Manassas, USA maintained by serial passage in mice. The preparation of inoculum was from donor mouse with *P. yoelii* established by microscopic examination of stained thin blood film under oil immersion at x100 magnification and measured as percentage erythrocytes [19]. Each mouse was infected intraperitoneally (I.P) with standard inoculums of approximately 10^7 parasitized erythrocytes suspension in normal saline (0.2 ml) from donor mouse.

In vivo Anti-malarial assay of the bioactive fraction and crude extract against CQ resistant *Plasmodium yeolii*

The 28 days curative study (Rane's test) was performed according to modified method of Iwalokun, [19]. Each mouse was intraperitoneally infected with 1.0 x 10^7 parasitized red blood cells in 0.2 ml physiological saline suspension. Administration commenced on 4^{th} day post-inoculation and continued for 7 days via oral route using a canula. Giemsa-stained thin blood films were prepared from tail blood of each animal and examined under X100 objective lens of a light microscope to monitor parasitemia on days 7, 14, 21, and 28. On day 7 before treatment commenced, four mice were sacrificed in each experimental group for serum biochemical assay and the parasite level for the remaining mice in each group were examined on day 14, 21 and 28 post inoculations. Parasite density and Chemo-suppression (percentage reduction in parasitemia relative to infected control) were calculated:

$$Percentage\ parasitemia = \frac{infected\ RBC}{total\ RBC} \times 100$$

$$\%\ chemosuppression = \frac{\%\ parasitemia\ of\ ICR - \%\ parasitemia\ of\ ITR}{\%\ parasitemia\ of\ IRT} \times 100$$

RBC: Red Blood Cell Count; ICR: Infected Control Rats; ITR: Infected Treated Rats.

Determination of haematological parameters

Determination of packed Cell volume (PCV)

PCV was estimated as described by Dacie and Lewis [21]. Blood sample was taken with a heparinised capillary tube, cleaned and sealed with plasticine. The filled tubes were placed in the microhaematocrit centrifuge and spun at 12,000g for 5 minutes. The value was determined using Hawksley microhaematocrit reader.

Determination of haemoglobin (Hb) concentration

HB count was carried out using cyanomethaglobin method according to Ochei and Kolhatkar (2008).

Whole blood from the mice (0.02 ml) was diluted to 4 ml with modified Drabkin's solution. This was allowed to stand for 10 minutes and the optical density was read at 540 nm against a Drabkin's solution (as a blank). Similarly, 57.2 mg/100 ml of the standard cyanomethaemoglobin was treated as the sample. The final haemoglobin result is calculated as follows:

$$Hb.Concentration = \frac{T \times C \times D}{A \times 1000}\ (g/100\ ml)$$

Where T = test absorbance as 540 nm;

A = Standard absorbance at 540 nm;

C = Cyanomethaemoglobin standard concentration (57.2 mg/100 ml); and

D = Dilution factor; 1000 converts from mg/100 ml to g/dl.

Determination of red blood cell Count by haemocytometry

The method of Ochei and Kolhatkar (2008) was used. An aliquot, 0.02 ml of blood was added to 3.98 ml of sodium citrate and mixed well. After 5 minutes, the first few drops were discarded by holding the pipette vertical and the counting chamber was charged with the fluid. It was allowed to settle for 3 minutes. By Switching to low power (10x) objective the centre large square with 25 small squares were adjusted to light and then adjusted to high power (40x) objective. The red blood cells in the four corner squares and one central square were counted.

Calculation

Total RBC/cu mm = number of cells counted x dilution factor.

Determination of white blood cell count

The total leucocyte count was determined by haemocytometry following the method described by Ochei and Kolhatkar (2008).

An aliquot, 0.02 ml of blood was added to 0.38 ml of diluting fluid (acetic acid, tinged with gentian violet) and mixed. The counting chamber was charged with the well-mixed diluted blood (after discarding the first five drops) with the aid of a pipette. Cells were allowed to settle in a moist chamber for 3 minutes. The four corners of the chamber was visualised under a low power (10×) objective and the cells were counted in all the four marked corner squares.

Calculation

Total WBC/cu mm = Number of cells counted x dilution factor.

Biochemical assays

Determination of haemozoin concentration of *P. yoelii* infected mice treated with *T. diversifolia* leaf extract fraction

The haemozoin concentration of *P. yoelii* infected mice treated with ethanol leaf extract and fraction of *T. diversifolia* were determined using the method described by Dibua., *et al* [22]. A 2 ml EDTA blood samples were centrifuged for 5 minutes using bucket centrifuge. The supernatant was discarded and the pellets suspended in normal saline (NaCl) and further centrifuged for 5 minutes and the supernatant discarded. A known quality, 0.5 ml of phosphate solution, pH 7.6 was added to each tube and vigorously shaken mechanically for 2 seconds to haemolyse the erythrocytes. The tubes were then kept on ice for 10 minutes to avoid excess haemolysis and then centrifuged for 5 minutes before discarding the supernatant Approximately 1 ml of Tris buffered solution of pH 7.2 was dispensed into the pellets in the tubes, centrifuged for 10 minutes, and the supernatant discarded. The insoluble pellets were re-suspended in 0.5 ml of 2.5% Sodium dodecyl sulphate solution (SDS), buffer with Tris buffer solution, pH 7.8 and kept at room temperature for 1 hour before centrifuging for 10 minutes. The supernatant was again discarded and the pellets once more resuspended in 0.5 ml of 2.5% Sodium dodecyl sulphate (SDS) solution buffered with Tris buffer solution pH 7.8 and kept at room temperature for 1 hour. The suspension was then centrifuged for 10 minutes, and the supernatant discarded before harvesting the SDS insoluble pellets (haemozoin). The concentration of haemozoin was calculated by completely dissolving the haemozoin in 0.5 ml of diluted sodium hydroxide, and the solution of haemozoin analyzed spectrophotometrically at 400 nm wavelengths [22].

Determination of Ca^{2+} concentration of *P. yoelii* infected mice treated with *T. diversifolia* leaf extract and fraction

Plasma calcium concentration was estimated using the colorimetric procedure according to Faulker and Meites [23]. Test tubes: blank, standard, control, and test, were labeled, working reagent (1.0 ml) was added to each test tube followed by the addition of 0.02 ml (10 µl) of samples to the respective tubes, the content was thoroughly mixed and allowed to stand for 60seconds at room temperature. The spectrophotometer was zeroed with blank at 570 nm and the absorbance of all tubes were read and recorded.

$$\text{Calcium concentration(mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ x Concentration of standard}$$

Determination of free fatty acid concentration of *P. yoelii* infected mice treated with *T. diversifolia* leaf extract and fraction

Plasma free fatty acid concentration was determined according to colorimetric procedure as according to Nwodo., *et al* [24].

Erythrocyte membrane stabilization

Fresh blood samples (5 ml) were collected from healthy volunteers into bottles containing EDTA. The blood samples were centrifuged at 3000 rpm for 15 minutes and the supernatant was discarded. Then a volume of normal saline equivalent to 2-volume of the supernatant was used to re-suspend the red blood cell pellet for use. A total of twenty-eight (28) test tubes were used; fourteen (14) for the main test and the remaining fourteen (14) as the blank; for each main test.

The first test tube contained 0.1 ml of re-suspended blood sample (RBS) and 2.4 ml of normal saline. Test tube two contained 0.1 ml of RBS, 1.9 ml of normal saline and 0.5 ml of distilled water. Test tube three to fourteen contained the same volume of RBS and distilled water but varying volumes of extract, antimalarial drug and normal saline. The volume of each test tube was 2.5 ml. The blank contained everything in the test solution except the RBS [24].

Percentage inhibition of haemolysis was calculated as follows:

$$\% \text{ inhibition} = \frac{OD2 - OD1}{OD3 - OD2} \times 100$$

Where

OD1 = Absorbance of isotonic solution (Test tube one without H_2O).

OD2 = Absorbance of test samples.

OD3 = Absorbance of hypotonic solution (Test tube two with H_2O).

Assay of phospholipase A_2 activity

Venous blood samples were drawn from adult male Negroes free from any drug treatment for at least two weeks into plastic tubes each containing 0.1 volume 3.8% trisodium citrate. Human erythrocytes were obtained as pellets after centrifuging samples of the venous blood at 3000 xg for 10 minutes. The pellet was re-suspended in a volume of normal saline equal to that of plasma and used as source of the substrate. Aliquots (0.1 ml) of red cell suspension were added to 1.8 ml normal saline containing 2 mM $CaCl_2$ in the absence or presence of antimalarials. After incubation at 37^0C for 1 hr, incubates were centrifuged at 3000x g for 10 min. The absorbance of each supernatant solution at 418 nm was read against a blank treated as the sample but not containing red cells. The rate of change of absorption at 418 nm was correlated with the enzyme activity [24].

$$\% \, inhibition = \frac{Abs \, of \, sample - Abs \, of \, blank}{Abs \, of \, sample} \times 100$$

Statistical analysis

The data obtained from laboratory experiment were analyzed using One Way Analysis of Variance (ANOVA) followed by *Post hoc* test to compare the mean values. The results were represented in mean ± SD.

Results and Discussion

Anti-plasmodial activity of ethanol extract and bioactive fraction of *T. diversifolia* leaves against *P. yoelii*^{R}

Table 1 and 2 showed the mean percentage parasitaemia count and percentage chemo-suppression of *P. yoelii*^{R} infected mice administered ethanolic extract and bioactive fraction of *T. diversifolia* leaves co-administered with CQ. In the 28-day curative assay of CQ resistant *P. yoelii* infected mice, co-administration of 200 mg/kg b.w of ethanol extract of *T. diversifolia* with 10 mg/kg b.w of CQ resulted in total clearance of parasitemia after 21 and 28 day post-treatment. The mice infected with CQ resistant *P. yoelii* treated with the bioactive fraction (C70:M30) of the extract combined with CQ showed dose-dependent suppression of parasitaemia at 28th day post-treatment higher than the effect of arthemeter or different doses of the crude extract administered. The combinations of crude plant extract and active fraction with CQ significantly ($P < 0.05$) suppress parasitaemia due to *P. yoelii*^{R} compared with the efficacy of CQ when administered alone. The result obtained with CQ was expected since the parasite had a resistant phenotype. Similar work by Iwalokun [19] also revealed the efficacy of *Vernonia amygdalina* but at lower dose.

Treatment	Percentage Parasitemia (%)			
	Day 7	Day 14	Day 21	Day 28
P. yeolii^{R} infected without Treatment	30.00 ± 5.77	35.00 ± 2.89	38.00 ± 2.52	40.00 ± 1.76^{b}
P. yeolii^{R} infected + 10 mg/kg b.w of CQ	23.33 ± 3.33	28.33 ± 4.41^{a}	30.00 ± 2.52^{b}	34.00 ± 3.33^{a}
P. yeolii^{R} infected + 5 mg/kg b.w of Artemeter	3.33 ± 0.33^{a}	3.33 ± 0.33^{ab}	5.00 ± 1.89^{ab}	6.67 ± 0.34^{ab}
P. yeolii^{R} infected + 100 mg/kg b.w of crude extract	13.33 ± 0.33^{a}	10.00 ± 0.00^{ab}	15.33 ± 2.43^{ab}	23.00 ± 1.45^{ab}
P. yeolii^{R} infected +200 mg/kg b.w of crude extract	6.67 ± 0.67^{ab}	6.67 ± 0.33^{ab}	3.33 ± 0.32^{ab}	3.33 ± 0.31^{ab}
P. yeolii^{R} infected + 100 mg/kg b.w of crude extract and 10 mg/kg b.w of CQ	3.33 ± 0.33^{ab}	3.33 ± 0.33^{ab}	6.67 ± 0.23^{ab}	10.00 ± 0.10^{ab}
P. yeolii^{R} infected + 200 mg/kg b.w of crude extract and 10 mg/kg b.w of CQ	6.67 ± 0.66^{ab}	1.67 ± 0.62^{ab}	0.00 ± 0.00^{ab}	0.00 ± 0.00^{ab}
P. yeolii^{R} infected + 100 mg/kg b.w of C70 : M30 fraction	23.33 ± 3.33	13.33 ± 3.33^{ab}	18.00 ± 3.04^{ab}	23.00 ± 2.05^{ab}
P. yeolii^{R} infected + 200 mg/kg b.w of C70 : M30 fraction	16.67 ± 3.33^{a}	3.33 ± 0.31^{ab}	6.67 ± 0.63^{ab}	10.00 ± 0.50^{ab}
P. yeolii^{R} infected + 100 mg/kg b.w of C70 : M30 fraction and 10 mg/kg b.w of CQ	13.33 ± 3.33^{a}	10.00 ± 0.00^{ab}	6.67 ± 0.13^{ab}	3.33 ± 0.07^{ab}
P. yeolii^{R} infected + 200 mg/kg b.w of C70 : M30 fraction and 10 mg/kg b.w of CQ	6.67 ± 0.34^{ab}	3.33 ± 0.13^{ab}	3.33 ± 0.67 ^{a}	1.67 ± 0.07^{ab}

Table 1: *Mean Parasitemia count of Plasmodium yeolii*^{R} *17x (pr1) infected mice treated with ethanol extract and Bioactive fraction of T. diversifolia Co-administered with CQ.*

n = 4; Data represented in Mean ± SEM. $^{a}P < 0.05$ significant compared with infected mice without treatment.

$^{b}P < 0.05$, significant compared with infected mice treated with 10 mg/kg b. w of chloroquine [CQ: Chloroquine].

Treatment	Percentage Suppression (%)			
	Day 7	Day 14	Day 21	Day 28
Normal Control	-	-	-	-
P. yeolii infected without Treatment	-	-	-	-
P. yeolii infected administered 10 mg/kg b.w of CQ	22.23	19.1	31	15
P. yeolii infected administered 5 mg/kg b.w of Artemeter	88.90	90.5	86.8	83.3
P. yeolii infected administered 100 mg/kg b.w of crude extract	55.57	71.4	60.5	42.5
P. yeolii infected administered 200 mg/kg b.w of crude extract	77.77	80.9	91.2	91.7
P. yeolii infected administered 100 mg/kg b.w of crude extract and 10 mg/kg b.w of CQ	88.90	90.5	82.4	75.0
P. yeolii infected administered 200 mg/kg b.w of crude extract and 10 mg/kg b.w of CQ	77.77	95.2	100	100
P. yeolii infected administered 100 mg/kg b.w of C70 M30 fraction	22.23	61.9	52.6	42.5
P. yeolii infected administered 200 mg/kg b.w of C70 M30 fraction	44.43	90.5	82.4	75.0
P. yeolii infected administered 100 mg/kg b.w of C70 M30 fraction and 10 mg/kg b.w of CQ	55.57	80.9	82.4	91.7
P. yeolii infected administered 200 mg/kg b.w of C70 M30 fraction and 10 mg/kg b.w of CQ	77.77	90.5	91.2	95.8

Table 2: *Mean percentage chemo-suppression of Plasmodium yeolii^R 17x (pr1) infected mice treated with ethanol extract and fraction of T. diversifolia Leaves Co-administered with CQ n = 4; Data represented in Mean ± SEM.*

Therapeutically, plant-drug interaction may be classified into three; additive, synergistic and antagonistic effects [3]. The outcomes of this study are suggestive of synergistic effect. Previous study by Oyewole., *et al.* [18] has revealed that *T. diversifolia* leaves alone possessed chemosuppressive activity on CQ-sensitive *P. berghei* infected mice. The synergistic administration of *T. diversifolia* and CQ reveals significant parasitemia reduction, chemo-suppression and parasite clearance in *P. yoelii^R* infected mice compared with the drugs alone. This result agrees with the findings of Adepiti., *et al.* [3] who evaluated herbal antimalarial MAMA decoction-amodiaquine combination in murine malaria model.

The higher therapeutic efficacy observed in some of the orthodox medicine-plant combination are due to the active phyto-constituents, the anti-plasmodial phyto-constituents reported include; quinoline, coumarins, alkaloids and terpenoids [19]. The last two phyto-constituents have been reported to be present in *Tithonia diversifolia* leaves [20] and may contribute to the observed anti-plasmodial property of the leaf.

CQ at the therapeutic dose in the food vacuole function as a schizonticidal agent via alteration of DNA replication, blockage of haemozoin generation and deprivation of energy due to interactions with parasite lactate dehydrogenase [19]. This CQ action is prevented in resistant strains owing to parasite mediated alkalinisation and efflux of the drug from the food vacuole [25] but are restored by chemo-sensitizer such as chlorpheniramine. Therefore, *T. diversifolia* leaf extract elicit CQ resistant reversal potential when used as an adjuvant with CQ.

Effect of co-administration of ethanolic leaf extract and bioactive fraction of *T. diversifola* with CQ on haemozoin concentration and hematological Indices of *P. yoelii^R* infected mice

The *P. yoelii^R* infected mice administered 5 mg/kg b. w of artemeter, varying concentrations of the extract and varying doses C70:M30 fraction only and in combination with 10 mg/kg b. w of CQ showed non-significant ($P > 0.05$) reduction in haemozoin concentration com-

pared with the parasitized mice without treatment (Figure 1). The infected mice administered 200 mg/kg b. w of the extract and C70:M30 fraction of *T. diversifolia* only and in combination with 10 mg/kg b. w of chloroquine revealed non-significantly (P > 0.05) higher PCV and haemoglobin concentration and significant (P < 0.05) higher RBC count compared with the infected mice without treatment (Table 3).

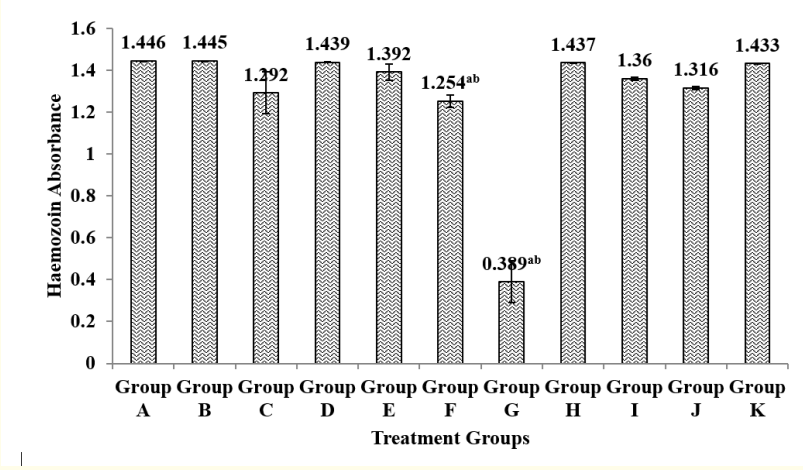

Figure 1: *Effect of co-administration of ethanol leaf extract of T. diversifolia and CQ on haemozoin concentration of Plasmodium yeoliiR (pr1) infected mice. aP < 0.05 significant compared with administered 0.2 ml of distilled water. bP < 0.05, significant compared with infected mice treated with 10 mg/kg b. w of chloroquine.*

	Treatment	PCV (%)	RBC (×10^6 g/dl)	Hb (g/dl)
I	Normal Control	47.3 ± 3.7	456.7 ± 26.8	22.2 ± 5.2
II	*P. yeolii* infected without Treatment	33.7 ± 1.7*	153.3 ± 23.3^{*b}	11.5 ± 0.8*
III	*P. yeolii* infected + 10 mg/kg b.w of Chloroquine	45.7 ± 3.8	223.3 ± 8.3^{*a}	17.0 ± 0.3*
IV	*P. yeolii* infected + 5 mg/kg b.w of Artemeter	49.7 ± 0.3^a	233.3 ± 1.6^{*a}	18.3 ± 0.2
V	*P. yeolii* infected + 100 mg/kg b.w of crude extract	36.7 ± 0.3^{*a}	193.3 ± 16.6^{*ab}	11.9 ± 1.1*
VI	*P. yeolii* infected + 200 mg/kg b.w of crude extract	35.7 ± 0.3*	203.3 ± 6.7*	12.2 ± 1.3*
VII	*P. yeolii* infected + 100 mg/kg b.w of crude extract and CQ	36.3 ± 3.7*	168.3 ± 28.3*	10.9 ± 1.2*
VIII	*P. yeolii* infected + 200 mg/kg b.w of crude extract and CQ	37.3 ± 1.3*	198.3 ± 11.7*	15.2 ± 1.1*
IX	*P. yeolii* infected + 100 mg/kg b.w of C70 M30 fraction	36.3 ± 0.3*	183.3 ± 3.3*	14.9 ± 0.6^{*b}
X	*P. yeolii* infected + 200 mg/kg b.w of C70 M30 fraction	32.0 ± 3.0*	156.7 ± 3.3*	12.7 ± 1.4*
XI	*P. yeolii* infected + 100 mg/kg b.w of C70 M30 fraction and CQ	39.7 ± 1.7*	220.0 ± 5.0*	13.6 ± 0.5*
XII	*P. yeolii* infected + 200 mg/kg b.w of C70 M30 fraction and CQ	39.7 ± 0.7*	200.0 ± 5.0*	13.9 ± 0.3^{*b}

Table 3: *Effect of co-administration of ethanol leaf extract of T. diversifolia and chloroquine on haematological indices of mice infected with chloroquine resistant Plasmodium yeolii (pr1) n =4; Data represented in Mean ± SEM.*P < 0.05 significant compared with normal control; ^{a}P < 0.05 significant compared with infected mice without treatment; ^{b}P < 0.05, significant compared with infected mice treated with 10 mg/kg b. w of chloroquine [CQ: Chloroquine].*

Malarial parasites detoxified haeme by forming crystalline haemozoin. Many clinically used drugs act by inhibition of haemozoin generation in the parasite food vacuole; this inhibits the detoxification of the haeme produced, thereby causing parasite death due to haeme build up [22]. The formation of haemozoin by malaria parasite is an essential process in the survival of the parasite. This assumption pre-supposes that an efficient control strategy against that parasite must necessarily be directed towards inhibition of the process. Many clinically used drugs have been reported to exert inhibitory effect on haemozoin formation by malaria parasites. Therapeutic doses of quinoline drugs such as chloroquine and mefloquine were reported in human erythrocytes and the inhibition caused monomeric haeme to accumulate and kill the parasites [22].

Reduction in packed cell volume of *P. yoelii*^{R} infected mice was observed in this study and this could be attributed to the consumption of red blood cells by the actively feeding merozoites in malaria infected mice during haemozoin formation. It was further indicated that the most conclusive evidence of this reliance on haemoglobin degradation is that, the specific inhibition of haemoglobin is fatal for the parasites. A direct relationship between haemozoin production and haemoglobin depletion or reduction in packed cell volume observed in this study was consistent with the report of Clark [26]. The effect of the plant extract and anti-malarial drugs on haemozoin concentration observed in this study was supported by the evaluation of the inhibitory effect of some medicinal plant extracts on haemozoin concentration via *in vivo* and *in vitro* studies reported by Dibua [22].

Effect of co-administration of ethanol extract and bioactive fraction of *T. diversifolia* with CQ on plasma calcium ion concentration of *P. yoelii*^{R} infected mice

The infected, untreated mice showed significantly ($P < 0.05$) higher calcium ion level compared to normal control mice. The infected mice administered with 10 mg/kg b. w of CQ and 5 mg/kg b. w of arthemeter showed significant ($P < 0.05$) decrease in calcium ion level compared to infected untreated mice. The *P. yoelii*^{R} infected mice treated with 200 mg/kg b. w of C70:M30 fraction singly and in calcium ion concentration compared with infected mice without treatment (Figure 2).

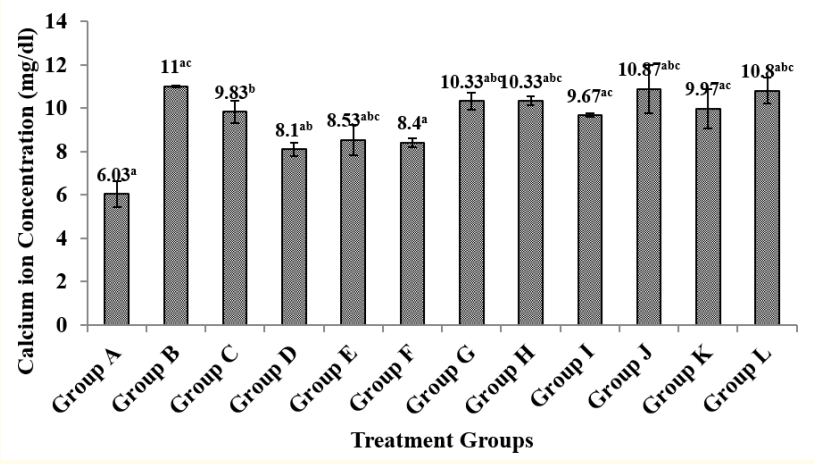

Figure 2: *Effect of ethanol leaf extract of t. diversifolia and chloroquine co-administration on calcium ion (Ca^{2+}) concentration of Plasmodium yeolii*^{R} *(pr1) infected mice. aP < 0.05 significant compared with Normal control mice. bP < 0.05, significant compared with infected mice administered 0.2 ml of distilled water, cP < 0.05, significant compared with infected mice treated with 10 mg/kg b. w*

Many stages in the *Plasmodium* life cycle depend on the controlled release of calcium ion (Ca^{2+}) from intracellular stores to activate stage-specific Ca^{2+}-dependent protein kinases [27]. The result of plasma calcium ion concentration shown in figure 2 revealed significantly ($P < 0.05$) lower Ca^{2+} level in the *P. yoelii*^{R} infected mice administered different doses of the extract in combination with CQ compared with the parasitized mice administered 0.2 ml of distilled water. Elevation of intracellular Ca^{2+} results in decrease membrane deformability, altered monovalent cation transport and modification of membrane protein structure [27]. Reduction in calcium ion concentration is a major mechanism by which membrane stabilization is achieved [24].

The high plasma Ca^{2+} concentration of infected mice treated with 0.2 ml of distilled water might implied that host-cell mechanisms for maintaining low intracellular Ca^{2+} levels have been modified by the parasite, this suggest that the erythrocyte membrane of infected cells may be more permeable to Ca^{2+} or that the Ca^{2+}-Mg^{2+} ATPase that gates efflux from erythrocyte is inoperative. Calcium is required at two intra erythrocytic stages of the asexual cycle i.e. within few seconds during which the erythrocytes are invaded and during normal parasite development [27]. This requirement of calcium for invasiveness and development of the parasite makes relevant the observation of the anti-malarial drug (arthemeter), crude extract and bioactive fraction in combination with CQ treatment of parasitized mice reduced plasma calcium ion concentration and shows, in part, that calcium deprivation could be one of the mechanisms by which the drug and co-administration of crude extract and CQ inhibit both stages i.e. parasite invasion and development in malaria life cycle. This result was consistent with the observation of Nwodo., *et al.* [22] who reported the inhibition of plasma calcium ion concentration by antimalarial drugs (camoquin and mepacrine).

Effect of co-administration of ethanol leaf extract and bioactive fraction of *T. diversifolia* with CQ on plasma free fatty acid concentration of *P. yoelii*^{R} infected mice

The parasitized mice administered 200 mg/kg of the extract singly and 10 mg/kg b. w of CQ showed non-significant ($P > 0.05$) reduction in the free fatty acid (FFA) concentration compared with the infected untreated mice and non-significant ($P > 0.05$) increase compared with parasitized mice treated with 10 mg/kg b. w of CQ. The parasitized mice administered 200 mg/kg of C70:M30 fraction singly and in combination with 10 mg/kg b. w of chloroquine showed significant ($P < 0.05$) decrease in the plasma FFA level compared with the infected untreated treatment.

The malaria parasite has a significant requirement for FFA during the replicative stages that occur in the host. Phospholipase A_2 mobilizes free fatty acid including arachidonic acid from phospholipids, and then arachidonic acid would serve as substrate for cyclooxygenase and lipoxygenase of which their products are mediators of inflammation [26]. The artemether, extract and active fraction in this study therefore act like non-steroidal anti-inflammatory drugs (NSAID) to inhibit substrate mobilization for the two pathways.

Effect of ethanol leaf extract and active fraction of *T. diversifolia* on phospholipase A_2 activity and Percentage inhibition of haemolysis

The crude extract and C70:M30 fraction in habits phospholipase A_2 activity in a concentration-related manner. The crude extract showed higher percentage inhibition of the enzyme activity compared with the C70:M30 fraction. The antimalarial drugs (chloroquine and arthemeter) used in this study showed lower percentage inhibition of phospholipase A_2 activity compared ethanol extract and C70:M30 fraction of the plant (Figure 4). The crude extract and C70:M30 fraction inhibits hypotonicity-induced haemolysis of the erythrocytes in

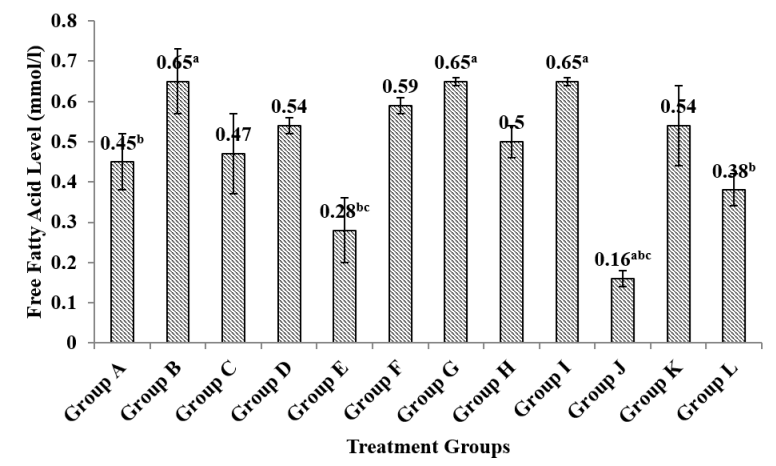

Figure 3: *Effect of ethanol leaf extract of T. diversifolia and CQ Co-administration on free fatty acid (FFA) concentration of Plasmodium yeoliiR (pr1) infected mice. aP < 0.05 significant compared with Normal control mice. bP < 0.05, significant compared with infected mice administered 0.2 ml of distilled water, cP < 0.05, significant compared with parasitized mice treated with 10 mg/kg b. w of chloroquine. of chloroquine.*

concentration-dependent manner. The crude extract showed higher percentage inhibition of haemolysis compared with the C70:M30 fraction. The antimalarial drugs (chloroquine and artemeter) used in this study showed higher percentage inhibition of hypotonicity-induced haemolysis compared to the crude extract and C70:M30 fraction of the plant (Figure 5).

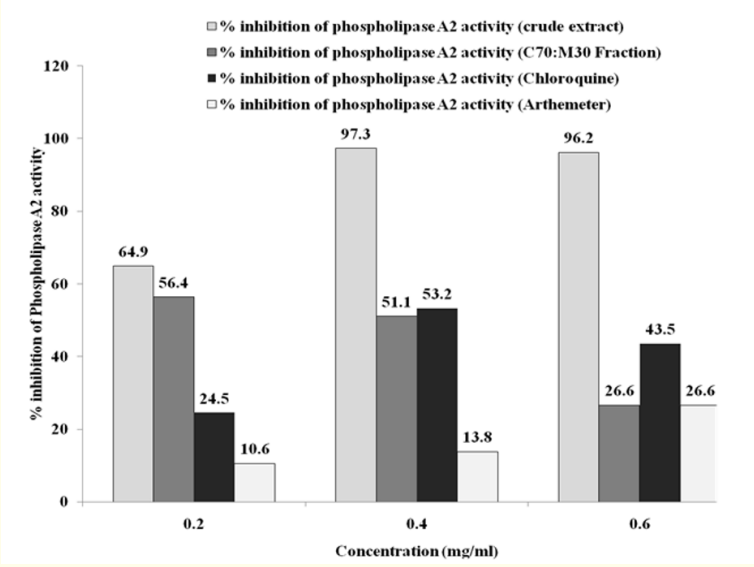

Figure 4: *Percentage inhibition of phospholipase A2 activity against concentration.*

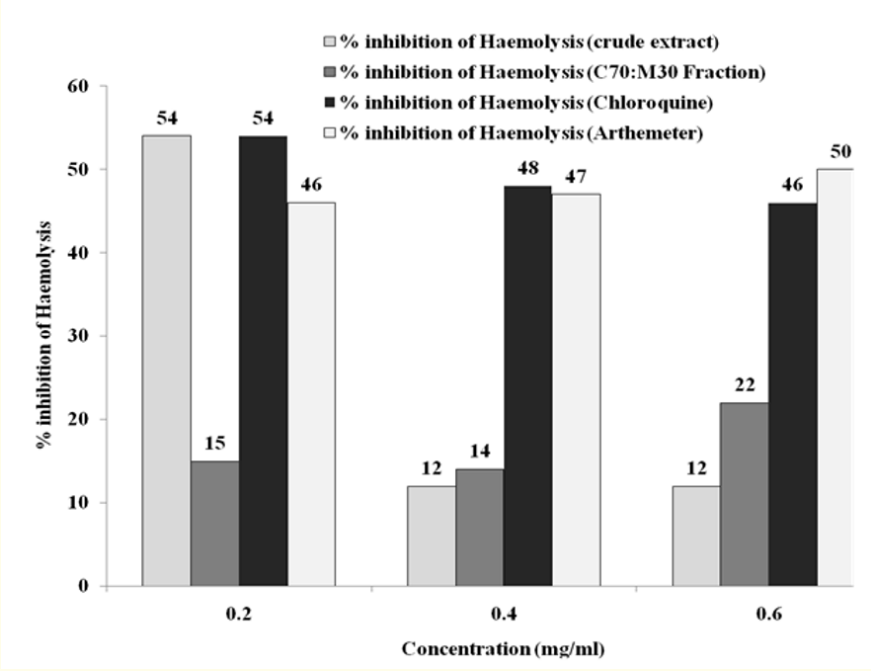

Figure 5: *Percentage inhibition of haemolysis against concentration.*

Calcium is required for the activity of phospholipase A_2 [24], this enzyme liberates FFA from membrane phospholipids leading to increase in the plasma free fatty acid. Thus, the reduction in calcium ion concentration in the parasitized treated mice might contribute to the observed inhibition of plasma free fatty acid concentration. As phospholipase A_2 mobilizes free fatty acids from membrane phospholipids, it creates leakage sites on the membrane. This results in exudation of cellular content (especially haemoglobin in red cells) and cell death. Inhibition of the enzyme activity observed in this study might therefore represent the mechanism by which the extract and fraction reduce plasma free fatty acid concentration of parasitized, treated mice.

Membrane stabilization protects the red cells from haemolysis and the lysosomes from releasing their injurious content. It would further protect mast cells, platelets macrophages and such other cells from releasing their contents including arachidonic metabolites, calcium, microvascular contrictors, acylhydrolases and reactive oxygen species [24], the stabilization of the membrane observed in this study is also significant in the integrity, protection and platelets, other cells and cellular organelles such as lysosomes.

Conclusion

The results obtained from this study have revealed the ability of *Tithonia diversifolia* leaf to enhance chloroquine efficacy when used as synergistically with CQ to manage malaria. The data from this study showed that the administration of ethanol extract of *T. diversifolia* and CQ have a synergistic effect on the rate of parasite clearance of *P. yoelii*^{R} infection in albino mice with a significant ($P < 0.05$) improvement in the haematological indices and decrease the rate of haemozoin formation within seven days of co-administration. This reveals rapid rate of recovery from plasmodial infection administering the drug and plant extract are co-administered.

Development of *T. diversifolia* leaf for this purpose would provide cheap and effective malaria control especially in developing countries where accessibility and affordability of orthodox drugs are the primary challenges of health care.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgment

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