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Antiplasmodial and Biochemical Effects of Combination of Ethanolic Leave-extracts of *Azadirachta indica* and *Ocimum gratissimum* on *Plasmodium berghei*-infected Mice

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ABSTRACT

Malaria is an infectious disease that continues to be linked with considerable morbidity and mortality. The present study was to compare antimalarial activities of *Azadirachta indica* and *Ocimum gratissimum* as well as to evaluate the combined effect of the combination on *Plasmodium berghei*-infected mice. Curative effects against established infection were studied in chloroquine-sensitive *P. berghei* NK65-infected mice using the method described by Ryley and Petes in 1970. Hematological studies were carried out using the methods described by Cheesbrough, while biochemical parameters were determined by the method of Reitman and Frankel. Infected mice orally treated with ethanolic extract of *A. indica* leaves and *O. gratissimum* as well as the combination produced significant ($P<0.05$), dose-dependent activity against the parasite throughout the treatment period. Higher reduction was observed at days 4 and 7, and maximum reduction (95.07%) was attained with 24 mg/kg of the combination of each of the extracts on the seventh day. This demonstrates that the combination of *O. gratissimum* and *A. indica* had better antimalarial activity than when used individually. The extracts also improved PCV and Hb, in addition to offering a protective effect to the liver. The findings from this work provide justifiable evidence and need to combine plant extracts in malaria treatment in order to improve their efficacy and slow the development of resistance as well.

Keywords:
Malaria, *Azadirachta indica, Ocimum gratissimum, Plasmodium, berghei*

Introduction:

Malaria is a devastating global health problem and it remains the greatest cause of hospitalization and deaths among the parasitic infections that afflict man (1). According to the World Health Organization (WHO), approximately 214 million cases and 0.44 million deaths were reported due to malaria worldwide in 2015 (2). Nigeria accounts for more cases and deaths than any other country in the world and there are an estimated 100 million malaria cases with over 300,000 deaths per year in Nigeria (3).

Plasmodium falciparum, the deadliest form of the malaria parasite, is responsible for the vast majority of the mortality and morbidity associated with malarial infection. Drug resistance is a factor in the economic constraints of malaria control (4). *P. falciparum* has developed resistance to almost all antimalarial drugs (5) and combination drug regimens have become the practice of choice because of their increased therapeutic efficacy over monotherapy and the other benefits including decreased cytotoxicity, delay or prevention of the development of drug resistance (6). Despite the emergence and spread of resistance to artemisinin-based compounds, artemisinin combination therapy (ACT) is still effective globally and it remains the first-line treatment for uncomplicated *P. falciparum* malaria and has contributed to the reduction in malaria-related mortality and morbidity.

Affordability and accessibility limit the use of ACT (7,8) and these can lead to poor treatment practices, production of substandard forms of the drug, inadequate patient adherence to prescribed antimalarial regimens, which may in turn lead to treatment failures. In view of the problems associated with the implementation of ACT, majority of the populations depend on traditional medical remedies (9), mainly from plants. Traditional herbal medicines are often more available, affordable, and sometimes perceived as being more effective than conventional anti-malarial drugs including artemisinin combination therapy (ACT) (10). Antimalarial plants used in combination might promote the effectiveness of each plant, with efficacy being achieved using a lower dose of each extract. Pharmacological benefits would accrue, with one extract clearing infection from one body system while the other clears it from a different site (11). In addition, synergism with antimalarial agents could be utilized in an attempt to prevent or delay the emergence *in vivo* of resistant populations of the parasite (12).

Azadirachta indica plants from the Meliaceae family are extensively used as traditional remedies against malaria in the tropics (13, 14 and 15). Several studies demonstrated that *A. indica* leaf, seed and stem bark extracts possess *in vitro* inhibitory activity on *P. falciparum* asexual stages (16-18). Antimalarial activity of *Ocimum* species have also been demonstrated (19). Although these plants are used in the traditional treatment of malaria, there is the need to assess the combination for increased antimalarial activity and delay the development of resistance

Therefore, the aim of the present study was to compare antimalarial activities of *A. indica* and *O. gratissimum* as well as to evaluate the synergistic effect of the combination in *P. berghei* infected mice.

Plant material

The fresh leaves of *A. indica* and *O. gratissimum* were collected from Amikwo village, Awka, Anambra State, Nigeria and

were identified at the Herbarium unit of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka-Nigeria. The specimen was documented and assigned voucher number 1320 and 1321 for *A. indica* and *O. Gratissimum* respectively

Extraction Procedure

The fresh leaves of *A. indica* and *O. gratissimum* plant were air-dried separately in the laboratory at room temperature for three weeks, after which the leaves were pulverized into coarse form with a crestor high speed milling machine. The coarse form (300g) was then macerated in absolute ethanol. This was left to stand for 48 hours after which each extract was filtered through muslin cloth on a plug of glass, wool in a glass column. The resulting ethanol extracts were concentrated and evaporated to dryness using rotary evaporator (Buchi Rotavapor, Switzerland) at an optimum temperature between 40 and 45 °C to avoid denaturation of the active ingredients. The concentrated extracts were stored in the refrigerator.

Animals

Forty-five male Swiss albino mice, weighing between 20 to 22 g were purchased from College of Veterinary Medicine, University of Nigeria, Nsukka. All animals were fed with formulated feeds (Vital feeds, Enugu, Nigeria) and water was administered *ad libitum*. The caring and experimental use of the mice was in accordance with the National Institutes of Health Guidelines for Care of Laboratory Animals. The animals were acclimatized for 7 days prior to their randomization into the various experimental groups.

Parasite and standard inoculation

P. berghei, donated by National Institute of Medical Research (NIMR) was obtained from College of Veterinary Medicine, University of Nigeria, Nsukka. Parasites were maintained through serial blood passage in mice wherein the mice previously infected with *P. berghei* and with high parasitemia level served as the donor. Blood samples was taken from the donor and diluted with phosphate-buffered saline such that 0.2 mL injected intraperitoneally into the experimental animals contained 1×10^7 infected erythrocytes.

Curative study

The method described by Ryley and Peters (20) was used in this study. The animals were infected with *P. berghei* NK 65 and then divided into nine groups of five mice per group. Parasitemia was established 72 hours after infection and was taken as day 0. Group I was the control. Group II received quinine (Q) 100 mg/kg only. Group III and IV received 250 mg/kg and 500 mg/kg doses of ethanolic leaf extract of *A. indica* respectively while groups V and VI received 250 mg/kg and 500 mg/kg doses of ethanol leaf extract *O. gratissimum* respectively. Group VII received combination of 12 mg/kg of ethanolic leaves extract each of *A. indica* and *O. gratissimum* while group VIII was treated with combination of 24 mg/kg of ethanolic extract each of *A. indica* and *O. gratissimum*. Group IX was the infected and untreated. Administration was done orally once daily for four days for the standard drugs or the extract/vehicle respectively.

Parasitemia Determination

Blood samples were collected by bleeding via the tail vein of *P. berghei*-infected mice and thin blood smears were made on microscope slides, fixed in methanol and stained with 10% Giemsa solution (Merck, Tokyo, Japan) and observed under the binocular microscope (Olympus, Japan). The percentage parasitemia was determined by counting the percentage pRBC for at least ten different fields.

Determination of Mean Survival Time

Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse. The mean survival time (MST) was calculated as follows:

$$MST = \frac{\text{Sum of survival time of all mice in a group}}{\text{Total number of mice in that group}}$$

Hematological Parameters

Using the methods described by Cheesbrough (21), percentage Packed Cell Volume (% PCV), haemoglobin and erythrocyte counts were determined. These parameters were determined for each mouse before infection, before treatment and after treatment. Blood samples were collected from the ocular orbit of each mouse with a heparinized capillary tube with one end sealed with plasticine. For PCV determination, the sealed capillary tubes were centrifuged at 10,000 revolutions per minute for five minutes using a Seward Haematocrit centrifuge (model 800D, England). The PCV was read on the haematocrit reader and recorded. The % PCV of each mouse was then calculated using the formula:

$$PCV = \frac{Volume\ of\ \ erythrocytes\ in\ a\ given\ volume\ of\ \ blood}{Total\ \ blood\ \ volume\ examined} x100$$

To determine the haemoglobin concentration, 20 µL of blood was mixed with 4 ml of Drabkin solution in a test tube. The mixture was allowed to stand for 5 min, and then read with a colorimeter (Jenway model 255, England) at 540 nm wavelength. The optical density was recorded and haemoglobin concentration read from the cyanmethaemoglobin standard curve.

2.8. Determination of Alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

Blood from cardiac puncture was dispensed into plain bottles and allowed to clot after which it was centrifuged at 1372 g-force for 10 min and the clear sera aspirated off. Then, sera were thawed and assayed for the levels of ALT and AST respectively using the method of Reitman and Frankel (22).

Determination of Alkaline Phospahatase (ALP) Activity

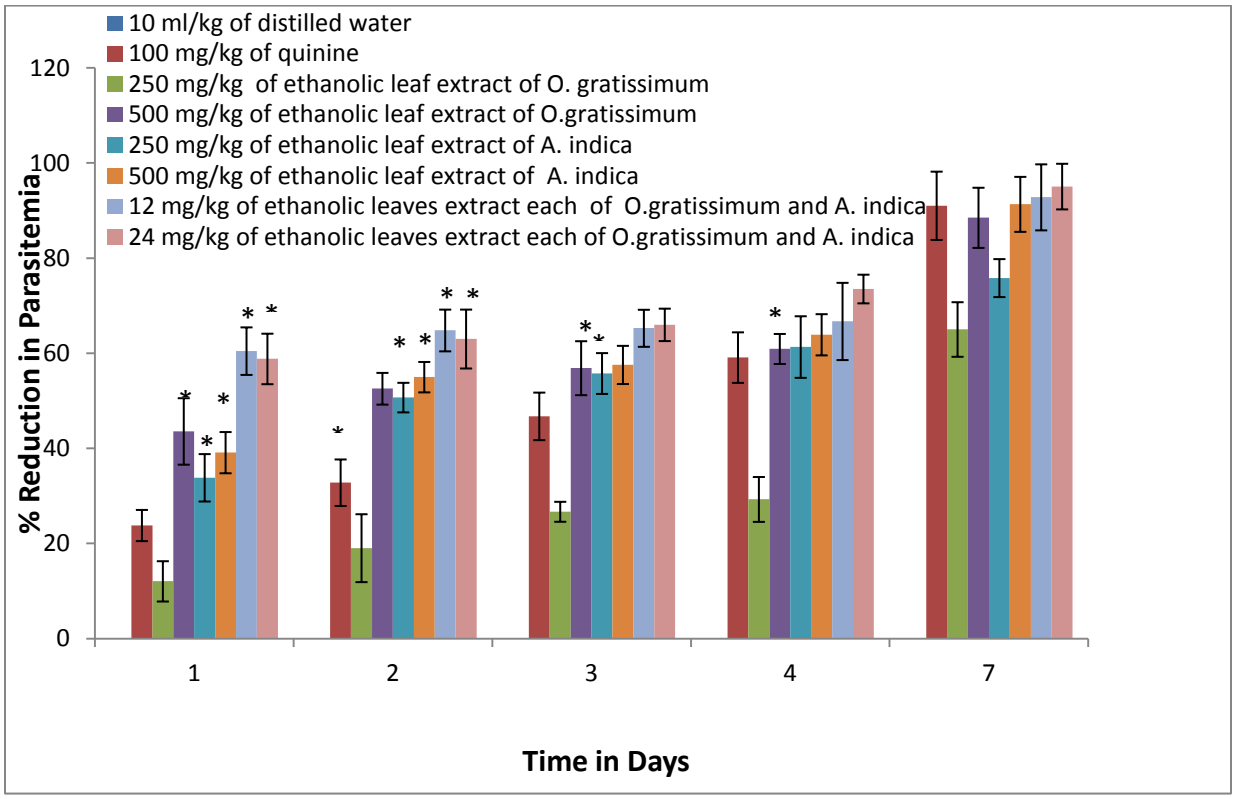
The activity of alkaline phosphatase (ALP) was assayed by the method of Klein et al (23) as outlined in Randox kits, UK.

Statistical Analysis

The results were expressed in terms of mean ± standard deviation (SD). Parameters in the groups were compared by one-way (ANOVA) using the computer software Statistical Package for Social sciences (SPSS) Version 16.0. All data was analyzed at a 95% confidence interval and values were considered statistically significant at $p<0.05$.

RESULTS:

Figure 1: **Percent Reduction in Parasitemia**



* Significant at $p<0.05$ when compared to the infected group administered 10 mg/ kg of distilled water

Percentage reduction in parasitemia of mice placed on different treatments

Results of *in vivo* antiplasmodial assay of ethanolic leaves extracts of *A. indica* and *O. gratissimum* against *P. berghei* (chloroquine sensitive NK65 strain) are given in *Figure 1*. *Figure 1* shows that the parasitemia in the infected untreated group increased with days of infection while the parasite-infected treated groups had significant decrease ($p<0.05$) in parasitemia level from day one to seven. Three days after infection and before commencement of treatment, all groups were not similar in parasitemia levels ($P>0.05$). From day 1 to 7 all groups were significantly different compared to untreated control group ($p<0.05$). Infected mice treated with ethanolic extract of *A. indica* and infected mice treated *O. gratissimum* extract, as well as the combination showed a dose dependent reduction in parasitemia throughout the treatment period. Higher reduction was observed at days 4 and 7.

Extract of *O. gratissimum* at 250 mg/kg exhibited parasitemia reduction of 29.29 % and 65.03 % on days 4 and 7, respectively, while the group treated with 500 mg/kg of *O. gratissimum* recorded 60.93 % parasitemia reduction on day 4 and 88.5 % on day 7. Values observed with 250 mg/kg of *A. indica* were 61.33 and 75.83 % on days 4 and 7 respectively while 500 mg/kg of *A.*

indica showed 63.92 % and 91.33 % reduction on days 4 and 7 respectively *(Figure 1*). Combinations with 12 mg/kg or 24 mg/kg of each extract allowed a significantly higher reduction in parasitemia compared to the other treated groups. Maximum reduction (95.07%) was attained for combination with 24 mg/kg of each extract on the seventh day. By day 28, the parasitemia had gone below detectable levels in all treated groups.

Table 1:

Mean percent change in AST and ALT after infection and after oral administration of ethanolic leaf extracts of *A. indica* and *O. gratissimum* in *P. berghei* infected mice

	AST		ALT	
GROUPS	**AFTER INFECTION**	**AFTER TRERATMENT**	**AFTER INFECTION**	**AFTER TRERATMENT**
10 ml/kg distilled water	101.2 ± 2.9	102.2±4.6*	113.4±3.5	101.5±3.3*
100mg/kg of quinine	119.5±3.4	101.3±7.8*	111.3±8.8	104.4±7.9*
250mg/kg of *O. gratissimum*	118.2±1.9	99.4±3.3*	111.2±99.8	100 ± 6.3*
500 mg/kg *O. gratissimum*	120.9±7.5	99.0±1.7*	117±7.1	100±1.8*
250 mg of *A. indica*	119.1±1.9	110.2±1.8*	118.5±3.5	112±2.9*
500 mg/kg of A. *indica*	118±5.6	103±1.1*	111.8±5.4	99.3±8.7*
12 mg/kg each of O. *gratissimum and A. indica*	121.7±3.4	107.9±4.1*	118.3±1.5	103.2±8.8*
24 mg/kg each of *O. gratissimum and A. indica*	118.4±1.3	103.4±2.2*	116.4±1.2	106.6±4.1*
Infected and untreated	118.2±2.9	129.7±3.1	114±0.8	127±2.1

**Significant at P<0.05 compared to the infected and untreated group*

The levels of AST and ALT are presented in *Table 1*. The values of AST and ALT at day 7 was significantly (*p<0.05) lower than that obtained 3 days after infection. The results indicate that the infected and untreated mice (Group 9) expressed a significantly (P<0.05) higher serum AST and ALT compared with all other groups of the experimental animals. After treatment, there was no significant difference (P>0.05) between AST and ALT activity of the group of healthy animals compared to the treated groups. Also, after treatment the group treated with 500 mg/kg of *O. gratissimum* extract had the lowest AST activity while the group treated with 500 mg of *A. indica* had the lowest ALT activity.

Table 2:

Mean percent change in ALP after infection and after oral administration of ethanolic leave extracts of *A. indica* and *O. gratissimum* in *P. berghei* infected mice

	ALP	
GROUPS	**AFTER INFECTION**	**AFTER TREATMENT**
10 ml/kg distilled water	107.2±2.9	106.3±6.9*
100mg/kg of quinine	106.8±4.2	107.8±4.7*
250mg/kg of *O. gratissimum*	100.4±4.3	102.2±2.4*
500 mg/kg *O. gratissimum*	102.0±6.4	95.2±4.8*
250 mg of *A. indica*	120.7 ±2.5	99.6±2.8*
500 mg/kg of A. indica	101.1±4.6	101.3±1.07*
12 mg/kg each of O. *gratissimum and A. indica*	112.8±2.2	107.1±4.7*
24 mg/kg each of *O. gratissimum and A. indica*	97.6±1.9	98.5±3.1*
Infected and untreated	100.2±6.3	124±3.8*

**Significant at P<0.05 compared to the infected and untreated group*

Table 2 shows that there was a significant (P<0.05) increase in ALP in the infected untreated group when compared to that of other groups. It was also noted that there was no significant (P>0.05) difference between the treated groups and the uninfected and untreated. 500 mg/kg of *O. gratissimum* recorded the least percentage change in ALP (95.2±4.8) after treatment.

Table 3:

Mean percent change in PCV and Hb after infection and after oral administration of ethanolic leave extract of *A. indica* and *O. gratissimum* in *P. berghei* infected mice

	PCV		HB	
GROUPS	**AFTER INFECTION**	**AFTER TRERATMENT**	**AFTER INFECTION**	**AFTER TRERATMENT**
10 ml/kg distilled water	99.4±3.9	98.8±4.2*	55.6±6.1	78.3±10.2*
100 mg/kg of quinine	51.1±2.3	88.5±0.9*	51.2±2.2	76.2±8.5*
250 mg/kg of *O. gratissimum*	51.6±3.8	85.4±3.1*	51.±3.6	74±4.1*
500 mg/kg *O. gratissimum*	50.1±6.7	91.4±0.3*	50±6.5	77.2±8.5*
250 mg of *A. indica*	53.4±3.1	91.3±1.3*	53.6±3.0	81.8±10.6*
500 mg/kg of A. *indica*	48.4±8.2	92.6±2.0*	47.5±8.5	68.9±8.5*
12 mg/kg each of O. *gratissimum* *and A. indica*	47.6±7.8	96.9±3.6*	49.42±6.3	75.4±3.3*
24 mg/kg each of *O. gratissimum* *and A. indica*	53.1±9.8	98.8± 3.6*	50.8±3.9	77.5±5.5*
Infected and untreated	52.0±2.0	38.6±1.5	48.5±1.3	32.1±3.0

**Significant at P<0.05 compared to the infected and untreated group*

Table 3 indicates that at day 7 the PCV and Hb increased significantly in all the treated groups when compared with the untreated group. The group treated with 24 mg/kg combination of each of *O. gratissimum* and *A. indica* had the highest value of PCV but this was not significant ($p<0.05$) when compared with other treated groups. *Table 3* shows that the uninfected untreated group had no significant difference in PCV and Hb when compared to other treated group.

DISCUSSION:

The *in vivo* model was employed for this study because it takes into account the possible pro-drug effect and possible involvement of the immune system in eradication of infection (24). The *in vivo* antimalarial activity of the extracts was carried out in *P. berghei* infected mice. *P. berghei* parasite is used in predicting treatment outcomes of any suspected antimalaria agent due to its high sensitivity to antimalarial, making it the appropriate parasite for this study (25, 26). The significant increase in the level of parasitemia in the infected untreated group recorded from day 1 to 7 is in line with the view that parasitemia increases progressively after infection until the point of death in the absence of suitable treatment (27). It was clearly observed among the treated groups that there were significant increases in parasitemia reduction with increase in doses of the extracts. The dose effect observed in this study correlates with the findings of Awe *et al*., (28); Tona *et al*., (29); Iwalokun (30), who separately reported greater reduction in parasitemia at higher doses.

From the result (*Figure 1*), it was noted the that combination with 12 or 24 mg/kg each of the extracts showed higher efficacy than those treated with single plant extracts. This could be as a result of synergistic effect of the compounds or their metabolites. Phytochemicals such as flavonoids, phenolics, saponins and alkaloids which have been implicated as the antimalarial agents in many plants (31-34) have been reported to be present in *A. indica* (35) and *O. gratissimum* (36, 37).

Phenol elevates red blood cell oxidation and inhibits the parasite's protein synthesis (38, 39). Saponin, flavonoids and tannins have been suggested to act as primary antioxidant or free radical scavengers that can counteract the oxidative damage induced by the malaria parasite (22, 40). Furthermore, studies have shown that effect of natural products of plant origin may be mediated via inhibition of protein synthesis (26). Furthermore, these metabolites may be acting individually or in synergy with one another to produce the observed anti-malarial activity observed with the combination.

Liver and acute kidney injuries could occur as a result of dreaded complications of severe malaria (41, 42). Diagnostic enzymes are enzymes that are used in diagnosing or differentiating between certain or specific diseases (43). The two transaminases in use in diagnostic enzymology are AST and ALT. The reduction in the activity of AST and ALT observed from groups 3 – 8 after treatment may portend its protective effect against the onset of injury to the hepatic parenchyma cells, since the *P. berghei* infected untreated group did not show the same trend.

The liver enzymes amelioration was more pronounced at a higher dose of 500 mg/kg *O. gratissimum* and 500 mg/kg of *A. indica* and the activities of the enzymes in the groups treated with the combination were comparable with that of the single extracts. This means that the combination did not cause hepatic injury. It has also been reported that *A. indica* and *O. gratissimum* offered protective effect on the liver (44, 45).

The effect of the extract in this study was dose dependent and this is consistent with Effraim *et al.*, (46). Measurement of the enzymatic activities or marker enzymes in tissues plays a significant role in the diagnosis and assessment of toxicity of plant extracts (47). The increase in enzyme activities of AST and ALT experienced by the infected and untreated groups may be as a result of the *Plasmodium* infection which caused degenerative changes in the hepatocytes and consequent release of the enzyme in the blood stream.

The elevation of these enzyme levels recorded in this study are consistent with earlier reports from natural and experimental infected animals (48-50). Alanine aminotransferase is cytosolic and is present in large concentrations in the liver and, in smaller amounts in the kidney, heart and skeletal muscle (51) and a better assay of liver damage. Ethanol leaf extract of *A. indica* and *O. gratissimum* as well as the combination showed a significant decrease ($p<0.05$) in ALP compared to the infected and untreated group. Alkaline phosphatase is present in all tissues throughout the body, but is particularly concentrated in the liver, bile duct, kidney, bone and the placenta. It is therefore not a specific liver marker (52).

Hematological indices were considered in this study because the most pronounced changes related to malaria involve the blood and the blood-forming system (53). Anemia is usually assessed by evaluating packed cell volume, haemoglobin (54). Expectedly, the reduction in PCV of the infected untreated group is due to the progressive

increase in parasitemia. The PCV in the uninfected and untreated group was not significantly different from the treated group at day 7. The treated group had improved PCV and this was comparable to the group of normal healthy rats. This was more pronounced in the group treated with 24 mg/kg of the combination extract, which showed 100% improvement. The ability of *A. indica* and *O. gratissimum* to reduce the severity of anemia in *P. berghei* infected animals have all been reported (55, 56).

From *Table 3*, it is clear that the untreated *P. berghei* infected mice had the lowest value of Hb at day 7. This may result from a reduction in erythrocyte number or a reduction in the concentration of haemoglobin in each erythrocyte (55). The treated group had no significant difference in Hb when compared to the healthy group suggesting that the extract of this plant has anti-anaemic properties.

The reference values of Hb and PCV for mice are 10.2 – 16 6 g/dl, 39 – 49 % respectively. From the result obtained, it is evident that the PCV for group II treated with 100 mg/kg of quinine, Group III and IV treated with 250 mg/kg and 500 mg/kg doses of ethanolic extract of *A. indica* respectively; groups V and VI treated with 250 mg/kg and 500 mg/kg doses of ethanol extract *O. gratissimum* respectively as well as groups VII and VIII treated with 12 mg/kg and 24 mg/kg each of the combination were all within the approved reference range but group IX, infected and untreated had PCV and Hb that falls below the normal range.

Conclusion:

This study thus showed that the combination of *O. gratissimum* and *A. indica* had better antimalarial activity than when used individually. There was also improved haematological and biochemical parameters. This potential can be explored for development of antimalarial phytomedicine in this era of increasing resistance.

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