

Antimalarial Potentials of Leaf and Stem Bark Methanol Extracts of *Diospyros mespiliformis* on *Plasmodium Berghei* (Nk65) Infected Mice

Abstract

Purpose: The aim of this work is to determine the Antimalarial Potential of Leaf and Stem bark Methanol Extracts of *Diospyros mespiliformis* on *Plasmodium berghei* (NK-65) Infected Mice.

Methodology: The mice were randomly divided into thirteen (13) groups of five (5) mice per group. The leaf extract (100, 200 and 400 mg/kg), stem bark extract (50, 100 and 200 mg/kg) of ethylacetate, n-butanol and aqueous fractions, chloroquine (5mg/kg) and Artemisinin combination therapy (10mg/kg) were administered orally for four days. Qualitative, quantitative, parasitemia, packed cell volume and relative body weight analysis of the mice were monitored.

Results: The phytochemical studies revealed the presence of carbohydrates, free anthraquinone, cardiac glycosides, glycosides, saponins, tannins, alkaloids and flavonoids in the crude leaf extract and absence of cardiac glycosides, flavonoids, free anthraquinone and alkaloids in the stem bark extract. The quantitative phytochemical analysis of the fractions of leaf and stem bark extract of *Diospyros mespiliformis* showed that, saponins (0.57 ± 0.06 mg/g), (0.36 ± 0.21 mg/g), alkaloids (0.12 ± 0.04 mg/g), (0.67 ± 0.01 mg/g), tannins (0.73 ± 0.36 mg/g), (0.51 ± 0.22 mg/g), and glycoside had the highest concentration of (1.15 ± 0.10 mg/g), (0.97 ± 0.33 mg/g) respectively.. At the end of the four (4) days suppressive test, PCV and Haemoglobin concentration showed significant decrease ($P < 0.05$) in the vehicle control group and significance increase in the infected but treated with chloroquine. Relative organ weight showed significant increase ($P < 0.05$) in liver and kidney weight of n-butanol and aqueous fractions of vehicle control group.

Conclusion: The present work establishes for the first time antiplasmodial activity of the methanol extract of *Diospyros mespiliformis* which can be a potential source of new chemotherapeutic agent.

Key words: Antimalarial, *Diospyros mespiliformis*, *Plasmodium berghei*, Mice

1. Introduction

Human malaria is caused by four different species of the protozoan parasite Plasmodium: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*, transmitted by the female anopheles mosquito^[1]. Although all the four species of malaria parasite can infect and cause illness. Only the malaria caused by *Plasmodium falciparum* is known to be potentially life threatening in humans. Infection with *P.falciparum* is therefore a medical emergency^[2]. The severity of *P.falciparum* infections (48hrs) is due to high percentage of red blood cells (RBCs) that are infected by this particular Parasite. In extreme infections, up to 80% of RBCs can be parasitized and destroyed.

It has been estimated that approximately 250 million clinical cases of malaria occur every year^[3]. Malaria is estimated to kill nearly one million people annually, with most of the deaths occurring in children under 5 years of age^[4]. Drugs such as quinine related drugs, antifolate combination drugs and artemisinin and its derivatives are antimalarial drugs used for effective treatment^[5]. The discovery of new and effective antimalarial drugs based on new mechanisms of action or with new structures, is urgently needed to overcome the problem of rapid emergence of drug resistance and achieve long-term clinical efficacy^[6].

Despite the substantial progress made in the treatment of parasitic diseases, malaria remains a significant therapeutic challenge especially because of the wide spread resistance of malaria parasites to currently available anti-malarial agents. These have stimulated the search for new pharmacologically active agents. The plant kingdom remains a major target in the search of lead compounds and new drugs to treat this debilitating parasitic disease. *Diospyros mespiliformis*, also known as African Ebony is a large deciduous tree found mostly in the savannas of Africa. The ethnopharmacological uses of different parts of *Diospyros mespiliformis* have been reported e.g. leaf decoctions are used against fever, whooping cough and wounds^[7]. It also possesses valuable medicinal properties such as anti-diabetic, antihelminthic, analgesic, antioxidant and anti-inflammatory activities^[8]. From the foregoing, it is necessary to subject these plants to detailed scientific studies, to determine its antimalarial activities and its efficacy in the treatment of malaria.

2. MATERIAL AND METHODS

2.1 Material

2.1.1 Plant material

Fresh leaf and stem bark of *Diospyros mespiliformis* were collected from Zango village, in Sabo-Gari Local Government Area of Kaduna State, in the month of June 2013 and authenticated in the herbarium unit, Department

of Biological sciences, Ahmadu Bello University Zaria where a voucher specimen with voucher number 901431 was deposited.

2.1.2 Experimental animals

A total of a hundred and thirty (130) mice of both sex weighing between 18-28g were purchased from the animal house, Department of Pharmacology, Ahmadu Bello University, Zaria. The animals were housed in well-ventilated cage and allowed to acclimatize under standard laboratory condition for a period of two weeks before commencement of the experiment. The mice were randomly divided into thirteen (13) groups, with five animals per group: Non infected and not treated (Normal control), Infected and administered 0.2ml normal saline (Negative control), Infected and treated with standard chloroquine (5mg/kg Positive control), Infected with parasite and treated with (ACT) Artemisinin Combination Therapy (10mg/kg Positive control), Infected and treated with low, medium and high dose (100, 200 and 400mg/kg leaf extract) and (50, 100, and 200mg/kg stem bark extract) of individual fractions of ethylacetate, n-butanol and aqueous.

2.2 Methods

2.2.1 Preparation and fractionation of plant extract

Fresh leaf and stem bark of *Diospyros mespiliformis* were rinsed in clean water and air dried at room temperature for two weeks. The dried leaf and stem bark were pulverized into powder using Thomas-Wiley laboratory mill (Model 4) U.S.A. A portion 500g of the pulverized plant leaves and stem bark were suspended each in 2.5 L of methanol and the solution was left standing for 48 hours in large amber bottles with intermittent shaking. At the end of the extraction, the crude methanol extract was filtered using Whatman No. 1 filter paper (1mm mesh size) and then concentrated in a water bath maintained at 45° C until greenish black residues were obtained. The crude methanol extract of *Diospyros mespiliformis* was subjected to liquid-liquid partition to separate the extract into different fractions. The reconstituted extract (250ml) was placed in a separatory funnel and 250ml each of ethylacetate and n-butanol solvents were added sequentially as a 1:1 (v/v) solution and rocked vigorously ^[9]. The sample was left standing for 30 minutes for each solvent on the separating funnel until a fine separation line appear clearly indicating the supernatant from the sediment before it was eluted. The process was repeated thrice in order to get adequate quantity for each fraction. The ethylacetate, n-butanol as well as the aqueous residue fractions were

concentrated over a water bath maintained at 45°C. The concentrated fractions were kept in sealed containers and refrigerated at 2-4°C until required for analysis.

2.2.2 Parasites

The choloquine sensitive *Plasmodium berghei* used in this study was obtained from, Nigerian Institute for Medical Research (NIMR) Yaba, Lagos, Nigeria. The parasite was maintained by sub-passaging into healthy mice on a weekly basis throughout the duration of this study using the method described by ^[10] and ^[11]. *Plasmodium berghei* infected red blood cells were intraperitoneally injected into the mice from the blood diluted with Phosphate Buffered Saline (PBS) so that each 0.2ml administered per kg body weight contains approximately 10⁶-10⁷ infected red cells.

2.2.3 Qualitative Preliminary Phytochemical Screening

Qualitative Phytochemical screening of the leaf and stem bark methanol extract of *Diospyros mespiliformis* were carried out according to standard methods ^[12, 13 and 14].

2.2.4 Antiplasmodial Activity (4-Days Suppressive Test)

In-vivo study of antimalarial property of the plant was evaluated by determining its suppressive antiplasmodial properties by using the method ^[15]. In this procedure, malaria parasite inoculum was prepared and collection of blood samples from the mice (donor mice) infected with *Plasmodium berghei* was diluted with normal saline such that 0.2ml will contain 10⁶-10⁷ of the parasite then 130 mice divided into 13 groups of 5 mice each was used for this study; all the mice were infected with the parasites by inoculating them with 0.2ml of the prepared blood solution.

$$\% \text{ Suppression} = \frac{\text{APC} - \text{APT}}{\text{APC}} \times \frac{100}{1}$$

APC = Average Parasitaemia in the Negative Control.

APT = Average Parasitaemia in the Test group.

2.2.5 Haematological assays

Packed cell volume (PCV) and haemoglobin concentration (Hb) were determined using the cyanomethaemoglobin and PCV by microhaematocrit methods ^[16].

2.2.6 Estimation of parasitemia

Parasitemia was monitored in all the groups for 14 days starting from day 1 using thin smears of blood films made from tail vein puncture of mice ^[11]. The smears were stained with 10% Giemsa stain at pH 7.2 for 15 min and examined under the microscope. The Percentage parasitemia was calculated by Giemsa-stained thin blood films using the blood collected from the tail of each mouse in all the groups. The mean % parasitemia were recorded for each mice and for each group as described ^[17].

$$\text{Percentage parasitemia} = \frac{\text{No of parasites in treated}}{\text{No of parasites in control}} \times 100$$

2.3 DATA ANALYSIS

The results obtained from the present study were analyzed by the analysis of variance (ANOVA) and expressed as mean $\pm$ Standard Deviation (SD) except where otherwise stated. P value less than 0.05 were regarded as significant ($P < 0.05$).

3.0 RESULTS

TABLE 1: QUALITATIVE PHYTOCHEMICAL ANALYSIS (mg/kg) OF DIOSPYROS MESPILIFORMIS

SAMPLE

Phytoconstituents	Leaf	Stem bark
Carbohydrate	+	+
Glycoside	+	+
Saponin	+	+
Cardiac glycoside	+	-
Flavonoids	+	+
Tannin	+	+

Anthraquinone	+	-
Alkaloids	+	-

(+ve)=present; (-ve)=Absent

TABLE 2: QUANTITATIVE PHYTOCHEMICAL ANALYSIS OF LEAF METHANOL EXTRACT OF *DIOSPYROS MESPILIFORMIS*

PHYTOCHEMICALS (mg/g)	AQUEOUS FRACTION	N-BUTANOL FRACTION	ETHYLACETATE FRACTION
Saponins	0.22±0.03 ^{ab}	0.17±0.03 ^a	0.24±0.04 ^b
Glycosides	0.45±0.03 ^b	0.34±0.03 ^a	0.35±0.03 ^a
Alkaloids	0.04±0.01 ^a	0.06±0.01 ^a	0.06±0.02 ^a
Tanins	0.14±0.04 ^a	0.37±0.05 ^c	0.25±0.04 ^b

Values are mean ± Standard Deviation (n=3) .Values with different superscript in the row differ significantly (P<0.05).

TABLE 3: QUANTITATIVE PHYTOCHEMICAL ANALYSIS OF STEM BARK METHANOL EXTRACT OF *DIOSPYROS MESPILIFORMIS*

PHYTOCHEMICALS (mg/g)	AQUEOUS FRACTION	N-BUTANOL FRACTION	ETHYLACETATE FRACTION
Saponins	0.24±0.03 ^b	0.08±0.04 ^a	0.06±0.02 ^a
Glycosides	0.46±0.03 ^c	0.38±0.03 ^b	0.23±0.04 ^a
Alkaloids	0.03±0.02 ^a	0.04±0.02 ^a	0.05±0.03 ^a

Tanins	0.16±0.03^a	0.14±0.03^a	0.25±0.04^b
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Values are mean ± Standard Deviation (n=3) .Values with different superscript in the row differ significantly (P<0.05).

TABLE 4: IN-VITRO PARASITEAMIA LEVEL AND PERCENTAGE SUPPRESSION OF *PLASMODIUM BERGHEI* BY DIFFERENT FRACTIONS OF THE LEAF METHANOL EXTRACT OF *DIOSPYROS MESPILIFORMIS*

TREATMENT/DOSE	AQUEOUS FRACTION		N-BUTANOL FRACTION		ETHYLACETATE FRACTION	
(n=5)	PARASITEAMIA	%	PARASITEAMIA	%	PARASITEAMIA	%
	LEVEL	SUPPRESSION	LEVEL	SUPPRESSION	LEVEL	SUPPRESSION
NC	0.00±0.00 ^a	0	0.00±0.00 ^a	0	0.00±0.00 ^a	0
IM+ normal saline	1.94±0.08 ^d	0	1.94±0.08 ^e	0	1.94±0.08 ^e	0
IM+ CQ(5mg/kg)	0.22±0.21 ^a	88.66	0.22±0.21 ^{ab}	88.66	0.22±0.21 ^b	88.66
IM+ ACT(10mg/kg)	0.37±0.22 ^{ab}	80.93	0.37±0.22 ^{bc}	80.93	0.37±0.22 ^{bc}	80.93
IM + 100mg/kg	0.84±0.47 ^c	56.70	0.62±0.31 ^{cd}	68.04	0.42±0.81 ^c	78.35
IM+ 200mg/kg	0.77±0.27 ^c	60.30	0.48±0.26 ^{bc}	75.25	0.85±0.21 ^d	56.19
IM+ 400mg/kg	0.73±0.52 ^{bc}	62.37	0.81±0.21 ^d	58.24	0.54±0.92 ^c	72.16

Values are Means ± SD (n=5). Values with different superscript down the columns are significantly different (P<0.05).

NC=Normal Control, IM=Infected Mice CQ=Chloroquine, ACT=Arthemisinin combination therapy.

TABLE 5: IN-VITRO PARASITEAMIA LEVEL AND PERCENTAGE SUPPRESSION OF *PLASMODIUM BERGHEI* BY DIFFERENT FRACTIONS OF THE STEM BARK METHANOL EXTRACT OF *DIOSPYROS MESPILIFORMIS*

TREATMENT/DOSE	AQUEOUS FRACTION		N-BUTANOL FRACTION		ETHYLACETATE FRACTION	
(n=5)	PARASITEAMIA	%	PARASITEAMIA	%	PARASITEAMIA	%
	LEVEL	SUPPRESSION	LEVEL	SUPPRESSION	LEVEL	SUPPRESSION
NC	0.00±0.00 ^a	0	0.00±0.00 ^a	0	0.00±0.00 ^a	0

IM+ normal saline	1.94±0.08 ^d	0	1.94±0.08 ^c	0	1.94±0.08 ^c	0
IM+ CQ(5mg/kg)	0.22±0.21 ^a	88.66	0.22±0.21 ^{ab}	88.66	0.22±0.21 ^b	88.66
IM+ ACT (10mg/kg)	0.37±0.22 ^{ab}	80.93	0.37±0.22 ^{bc}	80.93	0.37±0.22 ^{bc}	80.93
IM + 50mg/kg	0.46±0.22 ^b	76.29	0.69±0.22 ^d	64.43	0.29±0.28 ^b	85.05
IM+ 100mg/kg	0.11±0.09 ^a	87.5	0.62±0.13 ^d	68.04	0.53±0.30 ^c	72.68
IM+ 200mg/kg	0.50±0.20 ^c	94.33	0.56±0.19 ^{cd}	71.13	0.22±0.07 ^b	88.66

Values are Means ± SD (n=5). Values with different superscript down the columns are significantly different (P<0.05).

NC=Normal Control, IM= Infected Mice , CQ=Chloroquine, ACT=Artemisinin Combination Therapy.

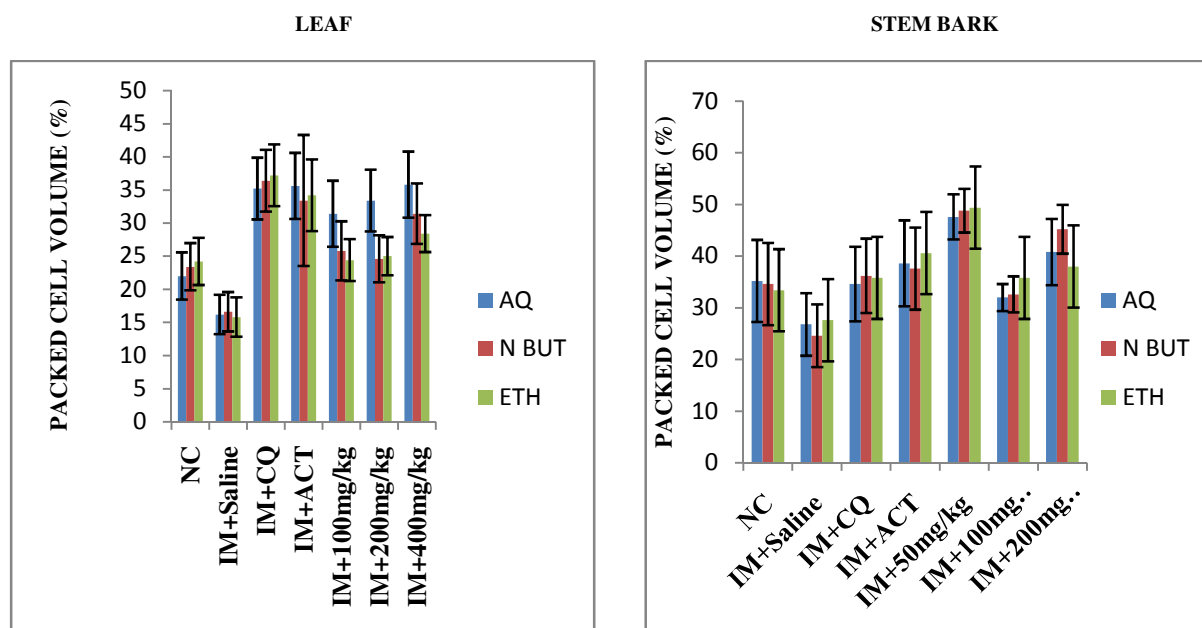


FIGURE 1: THE EFFECT OF DIFFERENT FRACTIONS OF LEAF AND STEM BARK METHANOL EXTRACTS OF *DIOSPYROS MESPILIFORMIS* ON PACKED CELL VOLUME (PCV) OF *PLASMODIUM* INFECTED MICE.

Values are Means ± SD. (n=5). Values with different superscript down the columns are significantly different (P<0.05).

NC=Normal Control, IM=Infected Mice, CQ=Chloroquine, ACT=Artemisinin Combination Therapy.

LEAF

STEM BARK

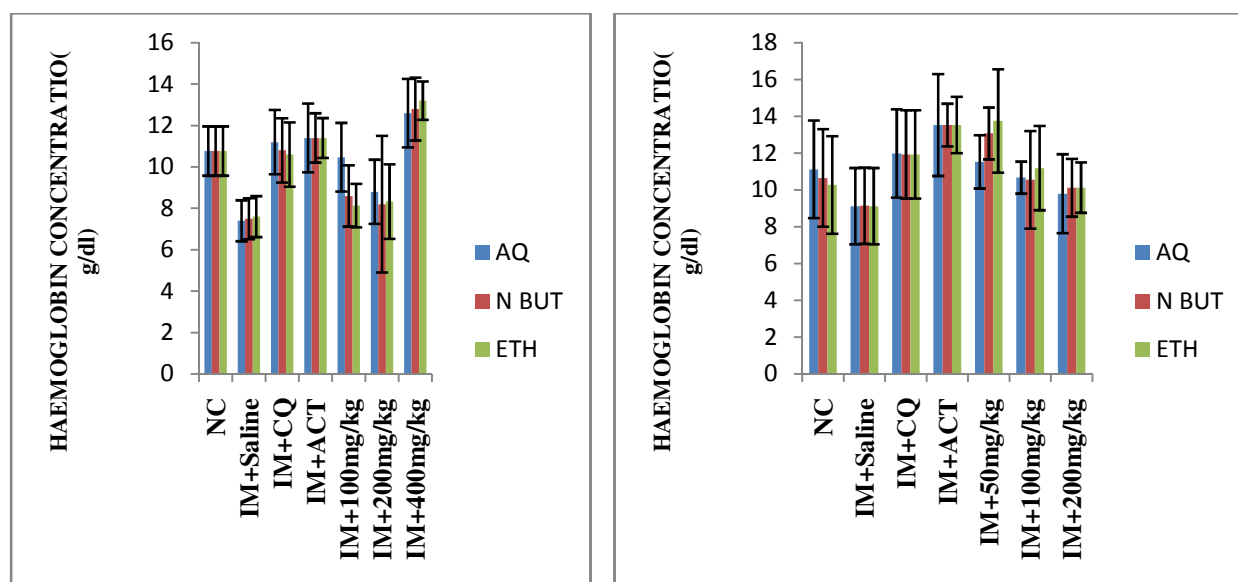


FIGURE 2: THE EFFECT OF DIFFERENT FRACTIONS OF LEAF METHANOL EXTRACTS OF *DIOSPYROS MESPILIFORMIS* ON HAEMOGLOBIN LEVEL (HB) OF *PLASMODIUM* INFECTED MICE.

Values are Means $\pm$ SD. (n=5). Values with different superscript down the columns are significantly different ($P < 0.05$).

NC=Normal Control, IM=Infected Mice, CQ=Chloroquine, ACT=Artemisinin Combination Therapy.

TABLE 6: EFFECT OF DIFFERENT FRACTIONS OF THE LEAF METHANOL EXTRACT OF *DIOSPYROS MESPILIFORMIS* ON RELATIVE LIVER AND KIDNEY ORGAN WEIGHT OF *PLASMODIUM BERGHEI* INFECTED MICE

TREATMENT/DOSE	AQUEOUS FRACTION		N-BUTANOL FRACTION		ETHYLACETATE FRACTION	
(n=5)	LIVER(g)	KIDNEY(g)	LIVER(g)	KIDNEY(g)	LIVER(g)	KIDNEY(g)
NC	1.33 $\pm$ 0.22 ^{ab}	0.96 $\pm$ 0.06 ^a	1.33 $\pm$ 0.22 ^a	0.96 $\pm$ 0.06 ^a	1.33 $\pm$ 0.22 ^a	0.96 $\pm$ 0.06 ^a
IM+ normal saline	4.65 $\pm$ 1.17 ^{de}	2.12 $\pm$ 0.97 ^c	4.65 $\pm$ 1.17 ^c	2.12 $\pm$ 0.97 ^c	4.65 $\pm$ 1.17 ^c	2.12 $\pm$ 0.97 ^b
IM+ CQ (5mg/kg)	2.29 $\pm$ 0.53 ^{abc}	1.82 $\pm$ 0.58 ^{ab}	2.29 $\pm$ 0.53 ^b	1.82 $\pm$ 0.58 ^{ab}	2.29 $\pm$ 0.53 ^b	1.82 $\pm$ 0.58 ^{ab}
IM+ ACT (10mg/kg)	1.55 $\pm$ 0.54 ^{ab}	1.54 $\pm$ 0.76 ^{ab}	1.55 $\pm$ 0.54 ^{ab}	1.54 $\pm$ 0.76 ^{ab}	1.55 $\pm$ 0.54 ^{ab}	1.54 $\pm$ 0.76 ^{ab}
IM + 100mg/kg	1.84 $\pm$ 0.65 ^{ab}	1.77 $\pm$ 0.85 ^{ab}	1.86 $\pm$ 0.30 ^{ab}	1.39 $\pm$ 0.21 ^{ab}	3.74 $\pm$ 3.03 ^{bcd}	2.00 $\pm$ 0.40 ^b
IM+ 200mg/kg	1.72 $\pm$ 0.31 ^{ab}	1.31 $\pm$ 0.26 ^{ab}	1.01 $\pm$ 0.27 ^a	1.20 $\pm$ 0.30 ^a	3.80 $\pm$ 1.45 ^{bcd}	1.97 $\pm$ 0.95 ^b
IM+ 400mg/kg	0.92 $\pm$ 0.16 ^a	1.74 $\pm$ 0.18 ^{ab}	1.85 $\pm$ 0.78 ^{ab}	1.53 $\pm$ 0.37 ^{ab}	4.00 $\pm$ 0.39 ^{cd}	2.43 $\pm$ 0.74 ^b

Values are Means $\pm$ SD. (n=5). Values with different superscript down the columns are significantly different ($P < 0.05$).

NC=Normal Control, IM=Infected Mice, CQ=Chloroquine, ACT=Artemisinin Combination Therapy.

TABLE 7: EFFECT OF DIFFERENT FRACTIONS OF THE STEM BARK METHANOL EXTRACT OF *DIOSPYROS MESPILIFORMIS* ON RELATIVE ORGAN WEIGHT OF *PLASMODIUM BERGHEI* INFECTED MICE

TREATMENT/DOSE	AQUEOUS FRACTION		N-BUTANOL FRACTION		ETHYLACETATE FRACTION	
(n=5)	LIVER(g)	KIDNEY(g)	LIVER(g)	KIDNEY (g)	LIVER(g)	KIDNEY(g)
NC	1.33±0.22 ^a	0.96±0.06 ^a	1.33±0.22 ^a	0.96±0.06 ^a	1.33±0.22 ^a	0.96±0.06 ^a
IM+ normal saline	4.65±1.17 ^{bc}	2.12±0.97 ^a	4.65±1.17 ^{cd}	2.12±0.97 ^b	4.65±1.17 ^c	2.12±0.97 ^{abc}
IM+ CQ (5mg/kg)	2.29±0.53 ^{ab}	1.82±0.58 ^a	2.29±0.53 ^{ab}	1.82±0.58 ^{ab}	2.29±0.53 ^{ab}	1.82±0.58 ^{ab}
IM+ ACT (10mg/kg)	1.55±0.54 ^a	1.54±0.76 ^a	1.55±0.54 ^a	1.54±0.76 ^{ab}	1.55±0.54 ^a	1.54±0.76 ^{abc}
IM + 50mg/kg	1.93±0.34 ^a	2.15±1.35 ^a	6.21±2.10 ^d	1.04±0.29 ^a	2.26±1.45 ^{ab}	2.11±0.61 ^{abc}
IM+ 100mg/kg	8.43±4.10 ^d	2.13±0.96 ^a	3.94±1.00 ^{cd}	1.45±0.46 ^{ab}	4.20±1.42 ^{bc}	2.00±1.44 ^{abc}
IM+ 200mg/kg	6.69±3.00 ^{cd}	1.48±0.52 ^a	6.30±2.23 ^d	1.71±0.40 ^{ab}	5.42±2.91 ^c	2.68±0.88 ^{bc}

Values are Means ± SD (n=5). Values with different superscript down the columns are significantly different ($P<0.05$).

NC=Normal Control, IM=Infected Mice, CQ=Chloroquine, ACT=Arthemisinin Combination Therapy.

4. DISCUSSION

Qualitative phytochemical analysis of leaf methanol extract of *Diospyros mespiliformis* revealed the presence of saponin, alkaloids, tannin, cardiac glycosides, anthraquinone, glycosides, flavonoids and absence of cardiac glycosides, flavonoids, free anthraquinone and alkaloids in the stem bark extract (Table 1). Phytochemicals constitute an integral part of medicinal plants and are responsible for their numerous bioactivities^[18]. Table 2 and 3 showed significantly higher level of glycosides, saponin and tannins content compared to the stem bark extract. At the end of the four (4) days suppressive test, there were significant increase ($P<0.05$) in parasiteamia level in the vehicle control group and infected but treated with chloroquine group compared to the normal control and treated groups which is shown in Table 4 and 5. The chlooroquine group almost cleared the parasite at dose of 5mg/kg body weight which exhibited higher suppressive antiplasmodial activities by the extent of inhibition of parasitemia^[19]. The effect of oral administration of leaf and stem bark fractions of *Diospyros mespiliformis* on packed cell volume and heamoglobin level is shown in Figure 1 and 2. The result showed that, the packed cell volume (PCV) and haemoglobin (Hb) level in the vehicle control group showed significant decrease ($P<0.05$) and significant increase ($P<0.05$) in the infected but treated with chloroquine^[20]. Relative organ weight of different fractions of the leaf and

stem bark methanol extracts of *Diospyros mespiliformis* is shown in Table 6 and 7. The results showed that there were no significant difference ($p < 0.05$) in relative change of the liver and kidney weight.

5. CONCLUSION

The results of this study have scientifically validated for the first time, the traditional use of *Diospyros mespiliformis* in the management and treatment of malaria. The results indicate that leaf and stem bark methanol extracts of *Diospyros mespiliformis* are relatively safe at doses $\leq 400\text{mg/kg}$ and $\leq 200\text{mg/kg}$ body weight which possess antiplasmodial effects. It is therefore concluded that, the fractions of leaf and stem bark methanol extracts of *Diospyros mespiliformis* possess active principle with antimalarial potential and has opened up a fresh line of research into discoveries of new drugs.

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