Phyto Chemical Screening and Antiplasmodial Potential of Morinda Lucida (Brimstone Leave) In Infected Mice

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Abstract. Medicinal plants with proven chemical properties have been used for centuries as remedies for human diseases most especially those caused by bacterial, fungi, and parasite. Traditional medicine is estimated to be used by 80% of the population of developing countries. This study attempted to investigate phytochemical components and anti-plasmodial activity (in vivo) of *Morinda Lucida*. The crude extract of the plant's leaves was carried out using aqueous water, this was followed with the phytochemical screening and fractionating process using different solvents. The experimental mice used in this study were subjected to an acute toxicity test with different concentrations of the fractionated leave extract. Albino-Mice behavior, weight loss, and different histological structures of the liver and the kidney served as major indicators for the toxicity. The result revealed no obvious acute toxicities on mice up to the highest (5000mg/kg) dose given. The experimental animal used for the suppressive test were divided into test and control groups, the test groups with an average weight of 18-24g were administered with different concentrations of the extracted leaves at a lower dose(100mg/kg) and a higher dose (200mg/kg) for four days, the parasite density was determined at day5 and day8 respectively, PCV was also carried out before and after the administration of the extract to determine the blood level. The present result shows the significant reduction of PCV for low and high doses at day five . ($P<0.05$). The reverse was the case for the methanol, ethyl-acetate, and water fractionated extract where no significant reduction in the PCV and the parasite density was observed ($P>0.05$). However, none of the extract concentration was able to clear the parasite completely in the experimental animals. The *Morinda Lucida* fractionated extract shows the promising effect as an anti-malarial agent, most especially the methanol, ethyl acetate, and the water fractionated extracts at both low and high dosages.

To cite this article
[Adebayo, N. S., Abubakar, A. A., Emmanuel, A. S., Oluwabunmi, S. B., Ifeoluwa, J. D. & Blessing, O. (2020). Phyto Chemical Screening and Antiplasmodial Potential of Morinda Lucida (Brimstone Leave) In Infected Mice. *The Journal of Middle East and North Africa Sciences*, 6(06), 24-31]. (P-ISSN 2412- 9763) - (e-ISSN 2412-8937). www.jomenas.org**. 3**

Keywords: Phytochemical screening; Antiplasmodial potential; *Morinda Lucida; Plasmodium beigheie*

1. Introduction:

Medicinal plants are planted with proven chemical properties that have been used for centuries as remedies for human diseases especially those caused by bacterial, fungal, viruses, protozoans, and associated diseases because they contain chemical components of therapeutic values (Tapsell *et al* 2006). While synthetic therapeutic agents continue to dominate research, attention has increasingly been directed to natural products. Similarly, studies have shown that the drug-resistant strain of *P. falciparum* has compromised malaria therapy and has led to the search for a new, safe, affordable, and effective antimalarial drug (Tapsell *et al* 2006; Ogbunugafor *et al* 2008; Bickiia et al 2007). Phytochemical screening of plants used in traditional medicine becomes necessary most especially in Africa where medicinal plants have been part of Africa culture. (Bickiia *et al* 2007; Murray *et al* 2013). In African setup, the study has shown that generally the use of medicinal n plants is far gaining recognition compare to synthetic products (Tolu *et al* 2017; Rynkowski *et al* 2008; Chakraborty *et al* 2009).

The phytochemicals analysis of some of these local plant has shown the presence of some secondary metabolite like tannins, saponins, steroids, flavonoids, protein, reducing sugar (Chaturvedi, 1974; Davis *et al* 1994; Ram *et al* 2015) and the presence of these secondary metabolites in this extract proved to be responsible for their antiplasmodial activity (Waako *et al* 2005). The screening of plants known to cure malaria in

ethnomedicine is, therefore, an important strategy in the treatment and control of malaria. This study therefore aimed at evaluating the in-vivo antiplasmodial activity of *Morinda Lucida* leaves and its toxicity level.

2. Material and Methods:

2.1. Collection of plant material:

The fresh plant was collected from their natural habitat of Dada Estate, Osogbo, Osun state Nigeria. The selection of plants was done based on the frequency of its usage of traditional treatment and management of malaria by local health practitioners in these communities. The plant was identified and authenticated by a Botanist, at Obafemi Awolowo University (OAU) Ife, Osun state Nigeria with herbarium number (FPI 2222).

2.2. Extraction procedure:

The fresh leaves were thoroughly washed with water and dried under normal temperature. They grounded into powder (homogenous sample) using a hammer mill and stored at room temperature inside a clean container (Leighton & Foster, 1993; Lorke, 1983). Aqueous extraction was obtained by infusing 300g of the powdered form in boiling de-ionized water (100 C). The filtrate was evaporated to dryness using a rotary evaporator under vacuum, the crude extract was freeze-dried and weighed for further assessments (Mengiste et al., 2012).

2.3. Phytochemical screening:

A preliminary phytochemical screening of the powdered materials of the leaves was carried out employing the standard procedures by Sofowora, 1978 for detecting the presence of alkaloids, flavonoids, tannins, saponins, glycosides, and volatile oil

2.4. Fractionationprocess:

Through Vacuum Layer Chromatography (VLC) method, five different solvents (N- hexane, Chloroform, Ethyl Acetate, Methanol, and Water) with different polarities were used to partition the crude extract into five fractions and each fraction was therefore available and ready for use in pure form (Debela *et al* 2016; Mohammed *et al* 2014; Newman *et al* 2015)

2.5. Experimental animals:

The albino mice (18-24kg) were obtained from the Animal House, Institute of Nigeria Medical Research and Advanced Technology(INMRAT), University College Hospital, Ibadan, Oyo State, and the mice were infected with NK65 strain of *Plasmodium berghei* which is sensitive to Chloroquine used for the study was obtained from the Institute of Nigeria Medical Research and Advanced Technology (IMRAT). Ibadan, Oyo state. The inoculum consisted of 5 X 107 Plasmodium berghei berghei parasitized erythrocytes per ml. This was prepared by determining both the percentage parasitemia and the erythrocyte count of the donor mouse and diluting the blood with phosphate buffer saline in proportions indicated by both determinations. Each mouse was inoculated on day zero (0) intra-peritoneally with 0.2 ml of infected blood containing about 1 X 107 Plasmodium berghei berghei parasitized red blood cells.

2.6. Blood sample collection:

Blood was obtained from the tail of the mouse to determine the parasitemia level while the blood used for determining hematological parameters was obtained from the eyes skillfully without puncturing the eyes. Before the commencement of the experiment, the animals were screened for the presence of blood parasites. Parasite (Obih *et al* 1985; Ogbunugafor et al 2008)

2.7. Invivo acute toxicity study of fractionated plant extract:

The fractionated extracts were assessed for their toxicity in noninfected albino mice aged 5-7 weeks and weighing 18-24grams (Okokon *et al* 2005). A total of 64 mice were selected, for the test of each fractionated plant extract and randomly divided into 16 groups of four mice per cage: one control group and fifteen test groups. All mice were fasted overnight for at least 4 hours before and 2 hours after the administration of the extracts. 0.2ml of n-hexane fractionated extract of the selected medicinal plants was given orally in an increasing dose, 2000mg/kg, 3000mg/kg and 5000mg/kg for the mice in group one, two, and three, respectively, 0.2ml of methanol fractionated extract given orally in an increasing dose, 2000mg/kg, 3000mg/kg, and 5000mg/kg for the mice in group four, five and six, respectively, likewise Chloroform, Ethyl Acetate and Water for 24hours. The mice in the control group received 0.2 ml of normal saline. They were observed continuously for 24 hours for gross behavioral changes such as feeding, lacrimation, mortality, and other signs of acute toxicity manifestations according to the standard toxicity study guideline (Onawunmi, et al., 1984).

2.8. Invivo antiplasmodial investigation:

The parasites were maintained by serial passage of blood from infected mice to non-infected ones. To infect the mice, the blood sample was collected through cardiac puncture of a donor mouse with a rising parasitemia of 24%, the blood sample was diluted with normal saline. Each mouse was passaged with 0.2 ml of the infected blood containing 1*10 7 *Plasmodium berghei* parasitized red blood cell via the intraperitoneal route (IP).

The forty-eight (48) mice with different weight of 18g to 24g were housed in plastic cages in the animal

house at IMRAT, Ibadan. They were kept under standard 12 hours light and 12hours dark schedule where room temperature, humidity, and ventilation were controlled during the acclimatization period of seven days. The mice were randomly distributed into twelve groups of four per group as shown in table1.

Protocol Table 1: *Showing how the mice were grouped and the amount of dose received by the mice*.

Group	Numbers of Animals	Administered Dose
Group 1	4	*P.berghei* + 100mg/kg of chloroform extract (Low dose) of 0.2ml
Group 2	4	*P.berghei* + 200mg/kg of chloroform extract (High dose) of 0.2ml
Group 3	4	*P.berghei* + 100mg/kg of water extract (Low dose) of 0.2ml
Group 4	4	*P.berghei* + 200mg/kg of water extract (High dose) of 0.2ml
Group 5	4	*P.berghei* + 100mg/kg of N-Hexane extract (Low dose) of 0.2ml
Group 6	4	*P.berghei* + 200mg/kg of N-Hexane extract (High dose) of 0.2ml
Group 7	4	*P.berghei* + 100mg/kg of Ethyl acetate extract (Low dose) of 0.2ml
Group 8	4	*P.berghei* + 200mg/kg of Ethyl acetate extract (High dose) of 0.2ml
Group 9	4	*P.berghei* + 100mg/kg of Methanol extract (Low dose) of 0.2ml
Group 10	4	*P.berghei* + 200mg/kg of Methanol extract (High dose) of 0.2ml
Group 11 (Positive)	4	*P.berghei* + 25mg/kg of Chloroquine (Standard drug)
Group 12 (Negative)	4	Mice infected with *P.berghei* received no treatment.

2.9. *In vivo Suppressive test of Plasmodium berghei*

The Suppressive test with Chloroform, Water, N-Hexane, Ethyl acetate and Methanol fraction of *Morinda lucida* was carried out according to the method described by Ryley and Peter, 1970 (Olson *et al* 2000; Omosa *et al* 2016). On the first day (D 1), standard inoculums of 1* 10 7 *P. berghei* (NK65) infected red blood cells were injected intraperitoneally into the mice.

Three hours later, the infected mice were randomly re-grouped (Table 1) and were administered with the extracts. The fractionated extract was administered orally to ten groups. Chloroquine (10mg/kg/day) was given to the positive control group and 0.2ml of normal saline was given to the negative control groups. The extract was given once daily for 4 days using an oral cannula. On days 5 and 8 of the test, thin blood smears were made from the tail blood of each mouse. The blood films were fixed with methanol, stained with 10% Giemsa of pH 7.0 for 10 minutes, and then assessed using a microscope. The parasite density was determined based on infected erythrocytes calculated per 1000 erythrocytes and the percentage of suppression was also calculated (Omosa *et al* 2016).

Packed Cell Volume (PCV); The packed cell volume (PCV) of each mouse was measured before infection and on day 5 after infection. For this purpose, blood was collected from the tail of each mouse in the heparinized microhematocrit capillary tube up to 3/4 of their length. The tubes were sealed by centrifuged at 12,000 rpm for 5 minutes. PCV was measured to predict the effectiveness of the test extracts in preventing hemolysis resulting from increasing parasitemia associated with malaria, using the Wintrobe method (Murray *et al* 2014).

$$\text{PCV} = \frac{\textit{Volume of erythrocytes in a given volume of blood}}{\text{Total blood volume}} \times 100$$

Body Weight; The body weight of each mouse in all the groups was measured before infection and on day 4 in case of treatment; it was measured before and after the different doses were given by a sensitive digital weighing balance.

3. Results:

The result of the general phytochemical screening of the crude extract of (*Morinda Lucida)* showed the presence of five phytochemicals based on the reagents used. Phytoconstituents detected include; Tannins, Saponins, Phenols, Resins, and Alkaloids as shown in table 2.

The acute oral toxicity test was determined using ranges of low dose (2000mg/kg) to a high dose (5000mg/kg) of the fractionated extract. The results showed no significant difference between various concentrations of the solvents used compared with the weight of the mice as showed in table 3. ($p>0.05$) at a low dose and high dose respectively. There was neither mortality, convulsion nor physical changes when the experimental.

Table 2: *Results of the phytochemical screening of Morinda Lucida*.

S/N	PHYTOCHEMICALS	OBSERVATION
1.	Tannins	+++
2.	Glycosides	-
3.	Resins	+
4.	Saponins	++
5.	Phlobatannins	-
6.	Flavonoids	-
7.	Sterols	-
8.	Phenols	+
9.	Carbohydrate	-
10.	Alkaloids	+++
11.	Terpenoids	-

(-): Negative test (absence of turbidity, flocculation, and precipitation).
(+): Weak positive test (if the reagent has a slight opacity).
(++): Positive test (if the reactive product and not turbidity flocculation).
(+++): Test strongly positive (if the reagent produces a precipitate or flocculation.

difference, but there was no significant difference in the weight of experimental mice between Day 1 and Day 4 respectively ($p>0.05$).

The suppressive test study as shown in Tables 4 and 5 indicated that all infected experimental mice with *Plasmodium berghei* treated with the fractionated extract of *Morinda Lucida* resulted in reduced parasitemia (parasite density) as compared to the negative control group. The fractionated extracts did not clear the parasite completely, whereas a positive control group treated with CQ used as a standard anti-plasmodial drug, at a daily dose of 25mg/kg body weight cleared the parasite on day four under identical condition. Generally, there was a significant difference in parasite density when the infected experimental mice were treated with the fractionated extracts except for n-hexane fractionated extract between Day5 and Day8 respectively ($P<0.05$).

4. Statistical Analysis:

The result of this study was expressed as a mean ± standard deviation (M ± SD). Comparison of parasitemia between tests and control groups and their

Table 3: *Showing the acute toxicity effect of the fractionated extract of Morinda lucida on the weight of experimental mice.*

		BEFORE			AFTER			
S/N	Fractionated Extract	2000 mg/kg	3000 mg/kg	5000 mg/kg	2000 mg/kg	3000 mg/kg	5000 mg/kg	P-value
1.	Chloroform	21.00 ± 1.41	20.00 ± 0.82	20.50 ± 1.29	21.00 ± 1.41	20.90 ± 0.72	20.71 ± 1.87	0.889
2.	Methanol	20.75 ± 1.50	20.00 ± 0.82	19.75 ± 1.26	20.76 ± 1.55	20.38 ± 1.25	19.50 ± 1.35	0.848
3.	Water	20.50 ± 1.63	21.00 ± 1.63	20.00 ± 0.82	21.00 ± 1.65	21.13 ± 1.65	20.25 ± 0.50	0.311
4.	Ethyl acetate	21.00 ± 1.63	19.75 ± 1.26	21.00 ± 0.82	20.75 ± 3.20	19.70 ± 1.45	21.25 ± 0.58	0.937
5.	N-Hexane	20.00 ± 0.82	20.50 ± 2.08	19.50 ± 1.00	19.75 ± 1.71	20.75 ± 2.06	19.55 ± 0.58	0.577
6.	NC	20.50 ± 1.26	20.75 ± 2.22	20.75 ± 0.96	20.75 ± 0.96	21.50 ± 1.73	21.75 ± 0.96	0.080

NC: Negative control

Table 4: *Showing the effect of the fractionated extracts on the PCV and Weight of the mice.*

S/N	Fractionated ligands	PCV Day 0	PCV Day4	Weight Day0	Weight Day 4
1.	Chloroform L	23.75±6.78	20.00±6.78	19.25±0.50	19.13±1.31
	H	23.00±6.27	20.50±5.87	22.50±1.73	16.63±5.64
2.	Methanol L	24.75±4.11	23.75±3.77	20.00±0.82	21.13±0.63
	H	24.50±4.73	24.50±4.51	20.00±1.83	22.00±1.41
3.	Aqeous L	24.50±7.77	23.50±8.06	18.00±0.82	19.75±0.96
	H	21.50±3.69	20.50±3.51	18.25±0.96	20.38±0.49
4.	Ethyl acetate L	28.50±5.80	26.70±5.35	19.50±3.00	20.25±3.20
	H	22.50±2.38	21.75±1.71	21.00±2.45	22.00±2.45
5.	N-Hexane L	25.75±5.12	19.00±4.69	21.25±2.22	20.01±2.36
	H	25.75±3.77	17.75±6.14	19.75±3.59	15.50±4.84
6.	Positive control CQ	34.00±7.05	34.00±7.07	23.00±071	24.20±1.64
7.	Negative control NS	24.20±5.39	19.00±5.39	21.45±3.13	17.40±2.88

Legend: H= High dose , L= Low dose, CQ= Chloroquine, NS= Normal saline.

Comparing the effect of the fractionated extracts on the hematological parameter (PCV) and the difference in weight loss. The result shows little significant difference in the PCV except n-hexane fractionated extract group that shows some significant

statistical were determined by one-way analysis of variance and Dunnett's Multiple Comparison Test.

All data were analyzed at a 95% confidence interval ($P=0.05$). Differences between means at 5% level ($P<0.05$) were considered significant.

Table 5: *Effect of Table each fractionated extract on % parasitemia of the Plasmodium berghei infected mice in the 4-day and 8-day suppressive test. (MEAN ±SD)*

S/N	Fractionated Extract	Day5%Parasitaemia	t value	P value	Day8(%) Parasitaemia	t value	**p-value**
1.	Chloroform						
	L	5.61±0.40	9.929	<0.0001	5.12±0.47	**5.988**	**0.001**
	H	4.99±0.43			4.58±0.48		
2.	Methanol						
	L	3..39±0.72	5.071	0.002	2.52±0.61	**7.472**	**0.003**
	H	2.99±0.35			2.04±0.09		
3.	Water						
	L	2.89±1.09	3,174	0.039	2.05±0.35	**5.616**	**0.004**
	H	2.24±0.34			1.99±0.17		
4.	Ethyl acetate						
	L	2.98±0.25	5.881	0.004	2.39±0.41	**5.322**	**0.002**
	H	2.70±0.30			2.11±0.24		
5.	N-Hexane						
	L	6.24±0.68	12.222	<0.0001	9.96±0.34	**7.681**	**0.003**
	H	6.01±0.28			9.88±0.64		
6.	PC	1.31±1.03	NA	NA	0.00	**NA**	**NA**
7.	NC	7.53±0.47	NA	NA	11.99±1.03	**NA**	**NA**

L- Low dose, H- High dose, NA- Not Applicable, PC- Positive control, NC-Negative control.

Table 6: *Showing the suppressive activities of the Morinda lucida fractionated extracts.*

S/N	Fractionated Ligands		Day 5	Day 8
1.	Chloroform	L	25.50±2.11	57.30±3.12
		H	33.72±1.32	61.80±2.22
2.	Methanol	L	54.98±1.40	78.98±0.35
		H	60.29±2.07	83.00±1.76
3.	Water	L	61.62±1.80	82.90±1.22
		H	70.25±3.10	83.40±3.00
4.	Ethyl acetate	L	60.42±0.99	80.07±2.12
		H	64.14±1.53	82.40±0.76
5.	N-Hexane	L	17.13±2.31	16.93±1.32
		H	20.19±1.04	17.59±1.43
6.	CQ		82.60±0.95	100
7.	NS		0.00	0.00

L- low dose, H- high dose, CQ- chloroquine, NS- normal saline.

5. Discussion:

The phytochemical analysis of *Morinda Lucida* was found to contain Tannins, saponins, resins, phenols, and alkaloids. However, tannins and alkaloids were of high concentration than others as shown in table 2 whereas, glycosides, phlorotannins, flavonoids, sterols, carbohydrate, and terpenoids were generally absent as compared with the previous study of some of the medicinal plants where such were predominant. Very few studies show the phytochemical analysis of Morinda Lucida (Pedroni *et al* 2006; Peter & Anatoli, 1998; Gul *et al* 2017). The acute oral toxicity test was determined using ranges of low dose (2000mg/kg) to a high dose (5000mg/kg) of the fractionated extract. The results showed no significant difference between various concentrations of the solvents used compared with the weight of the mice as showed in table 3. ($p>0.05$) at a low dose and high dose respectively. There was neither mortality, convulsion nor physical changes when the experimental.

Comparing the effect of the extract on the weight, physical changes, mortality, and anticonvulsant potential, the present study shows a low level of significant effect on the above indicators. This is in line with the study carried out by Gahlot *et al* 2013. Significant body weight loss in the untreated group was observed compared with a group treated with the high dosage of chloroform fractionated extract and high dosage of N-Hexane fractionated extracts. This can be

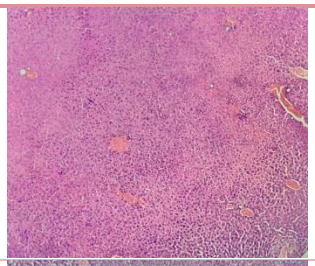
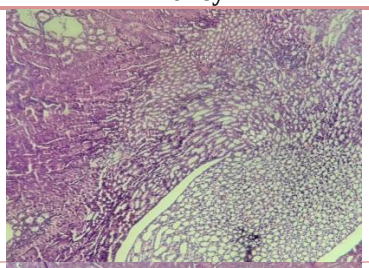
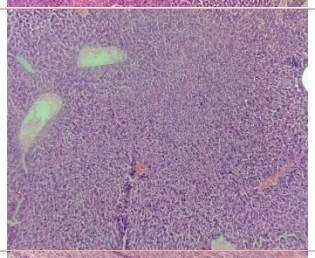
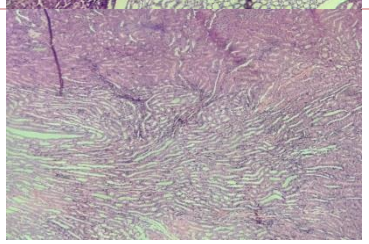
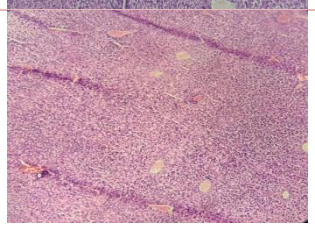
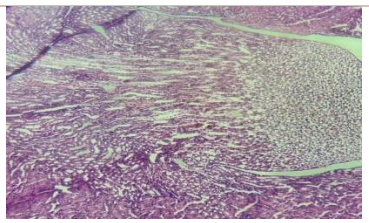
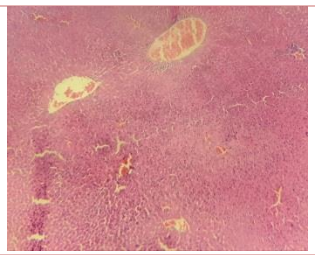
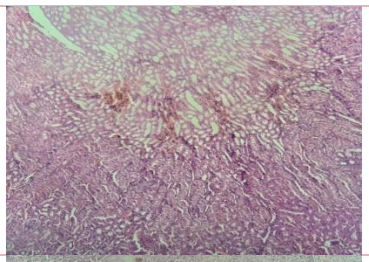
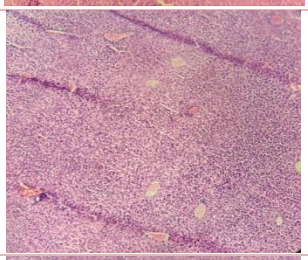
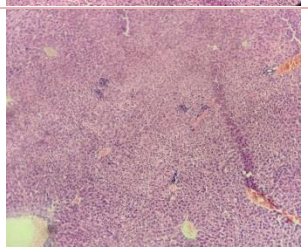
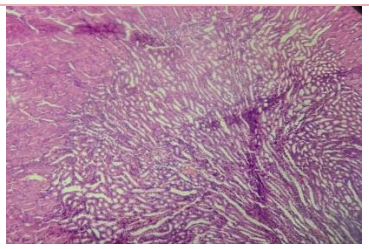
Fractionated Extracts	Liver	Kidney
Chloroform		
Methanol		
Water		
Ethyl acetate		
N-Hexane		
Control		

Figure 1. Showing the morphological appearance of the organs of the experimental mice after 24 hours of oral acute toxicity investigation.

due to the depressant action on the appetite of the mice and the disturbed metabolic function of the malaria parasite (Pedroni *et al* 2006).

The result shows little significant difference in the PCV except n-hexane fractionated extract group that shows a more significant difference, but there was no

significant difference in the weight of experimental mice between Day 0 and Day 4 respectively ($p>0.05$). The influence of malaria on PCV reduction is considered a hallmark of both human and rodent malaria infections (Gul *et al* 2017). A significant reduction of PCV and hemolysis was observed in the untreated group, then Chloroform fractionated group and N-Hexane fractionated groups. However, the absence of a significant reduction of PCV among methanol, water fractionated extract, and ethyl acetate at both low and high doses suggests for further research. In this study, the fractionated extract of *Morinda Lucida* showed considerable Antiplasmodial properties except the chloroform and N-Hexane fractionated extracts. The suppression activities of the fractionated extract were increased with an increase in the concentration. The suppressive effect of *Morinda Lucida* fractionated extracts could be due to the presence of a high concentration of tannins, saponins, and alkaloids that are the characteristic constituents of the genus Morinda. Moreover, the antimalarial activities exhibited by extracts may also be due to the presence of other active compounds such as Resins and phenols which are metabolites that have been proved to possess potential Immune-modulatory effects in other plants which as a consequence might have some impact on the host-parasite inter-relationship (Gul *et al* 2017). The *Morinda Lucida* fractionated extract shows the promising effect as an antimalarial agent, most especially the methanol, ethyl acetate, and the water fractionated extracts at both low and high dosages, though no case of complete malarial parasites clearance in the experimental mice was recorded throughout the study. Going by the findings from this study, consumption of *Morinda Lucida* extract by mice to assess kidney and liver cells morphology produced the least toxic effect (Figure 1) indicated that the treatment with the fractionated extracts could still be safe for humans at these concentrations. The extract shows the promising effect as the antimalaria agent most especially the methanol, ethyl acetate, and water fractionated extracts at both low and high doses. Further studies are suggested by using more solvents for fractionation so that the possibility of assessing pure ligand for antimalaria agents from the promising leave could be achieved.

Acknowledgment:

We are particularly grateful for the supports of Professor Ademowo, The Dean, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan and the entire staff of the Institute of Nigeria Medical Research and Advanced Technology (INMRAT), College of Medicine, University College Hospital, Ibadan, Oyo State, Nigeria for their support during the period of the study.

Conflict of Interest

The authors have no conflict of interest to declare.

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Received April 14, 2020; reviewed April 24, 2020; accepted May 16, 2020; published online June 01, 2020