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Investigating the *In-vivo* Antiplasmodial Properties of Aqueous Extract of *Moringa oleifera* Lam (Moringaceae) Leaves

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Authors' contributions

This work was carried out as a collaboration among all authors. Authors PA and MFO designed the in-vivo study, wrote the protocols and supervised it. The study was then performed by authors EO and RKA designed the phytochemical analysis. Author RKA also supervised the phytochemical analysis. Author EO managed the literature search, performed the statistical analysis and wrote the first draft of the manuscript. Authors PA, MFO and RKA reviewed the manuscript and approved the final copy.

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Original Research Article

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ABSTRACT

Aim: The study investigated *Moringa oleifera* Lam (Moringaceae), for its *in-vivo* antiplasmodial properties, using a murine model involving *Plasmodium berghei*.
Study Design: Experimental.
Places of Study and Duration: Department of Pharmaceutical Chemistry, KNUST, Department of Immunology, NMIMR and Department of Animal Experimentation, NMIMR, May – November,

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2013.
Methodology: Phytochemical investigation was conducted on powdered plant material and aqueous leaf extract (ML), to determine the presence of secondary metabolites using standard methods. Using 4-day suppressive and 7-day curative tests respectively, ML at 250-1000 mg/kg, was evaluated for *in-vivo* antiplasmodial activity in *P. berghei* infected male ICR mice (25-30 g), using Artemether-Lumefantrine (A/L) at 4 mg/kg as reference drug. The effect of ML on the body weights and the survival of the mice, as well as the effect on improvement in clinical signs was also monitored.
Results: ML extract was found to contain alkaloids, saponins, flavonoids and tannins. The extracts (250-1000 mg/kg) produced significant reduction in parasitemia in the four-day suppressive test ($F_{6.49}$ = 4.309; p =.0014). However, 250 mg/kg (69.31%; p< .001) and 500 mg/kg (77.26%; p< .001) extracts exhibited relatively higher activities compared to 750 mg/kg (25.28%; p< .001) and 1000 mg/kg (07.12%; p> .05). In the curative test, similar results were obtained with significant parasitemia reduction for 250 mg/kg (AUC = 52.52±6.732; p< .01) and 500 mg/kg (AUC = 49.62 ± 3.804; p< .01) compared to the positive control group (AUC = 101.3±14.32). Piloerection, lethargy and decreased locomotion were observed to be progressive in all infected experimental groups except A/L. Survival data showed that, although 750 mg/kg and 1000 mg/kg groups recorded relatively higher mortalities, statistical analysis didn't indicate significant difference.
Conclusion: ML extract demonstrated *in-vivo* antiplasmodial activity; probably due to the alkaloids, saponins, flavonoids and tannins in its composition.

Keywords: Moringa oleifera; antiplasmodial; phytochemicals; suppressive; piloerection; lethargy; survival and mortality.

1. INTRODUCTION

Malaria is a potentially deadly parasitic disease of global public health relevance. The infection is known to cause death and illness in children and adults, especially in tropical countries. In Ghana, malaria is termed to be endemic and perennial in all parts, with seasonal variations more pronounced in the Northern part [1]. According to the 2010 national census, 24.2 million Ghanaians are at risk of malaria infection. Children under five years and pregnant women however stand a higher risk of severe illness due to declined immunity [1]. The control of malaria requires an integrated approach, including prevention, which deals primarily with vector control and prompt treatment with effective antimalarials [2]. Management of malaria has seen a lot of changes, mainly as a result of resistance development of *P. falciparum* against antimalarials in use. For instance, Chloroquine, which used to be one of the most effective drugs has now been proven to be ineffective in malaria treatment [3]. Currently, WHO recommends a combination therapy involving any of the artemisinins and other classes of antimalarials for the treatment of uncomplicated malaria. Some of the recommended combinations include, Artesunate - Amodiaquine, Artemether - Lumefantrine,Atovaquone - Proguanil, Chloroquine - Proguanil, and Mefloquine – Sulphadoxine - Pyrimethamine [2].

A school of thought holds that, the solution to plasmodial resistance development rests in the use of traditional medicinal plants [4]. Several authors have documented medicinal plants that are used in the treatment of malaria in Ghana and other African countries [4-8]. The story behind the discovery of the artemisinins, as an example, seeks to provide aheadway in the discovery of bioactive constituents from medicinal plants for combating malaria [9]. Armed with information from successful traditional treatments of malaria, it is possible to discover novel compounds from plants that could be developed into potent antimalarials. This study was thus carried out to determine the fortunes of *Moringa oleifera* Lam (Moringaceae), as a candidate plant for traditional management of malaria infection and potential source for future antimalarial agents.

2. MATERIALS AND METHODS

2.1 Sample Preparation

2.1.1 Collection of plant sample

Fresh leaves were collected from the environs of Appiadu – Kokoben, a suburb in the Kumasi Metropolis, Ghana, within the hours of 6 am – 8 am. The collected sample was identified and authenticated in the Department of Herbal

Medicine, KNUST before use and specimen placed in the Department Herbarium. The voucher number was KNUST/HM1/2014/L044. It was then dried at room temperature in the laboratory for 21 days and pulverized upon drying.

2.1.2 Preparation of extract

A decoction of 1.000 kg of dried powdered leaves was prepared with distilled water for 1 hour. The extract was then filtered with sterile Whatman®Grade 1 filter paper and the marc obtained, also dried and re-extracted for similar period for two more times. The filtrates were combined and concentrated on a Büchi Rotavapor R-215® (Büchi Labortechnik AG Switzerland)at 40ºC. The concentrate was then freeze dried to obtain dried lyophilized extract, which was kept at -18ºC for future use.

2.2 Phytochemical Investigation

Phytochemical tests were carried out on dried powdered sample and aqueous extract of the leaves of the plant to detect the presence of alkaloids, sterols, coumarins, flavonoids, tannins, reducing sugars, saponins, cyanogenetic and anthracene glycosides. The standard methods employed for the tests are as described elsewhere [10-12].

2.3 *In-vivo* Antiplasmodial Test

2.3.1 Materials and equipment

Equipment employed in the study included a Leica DM 2500 Mlight microscope, centrifuge, water bath (37°C), liquid Nitrogen freezer and Neubeaur counting chamber [13]. The materials and reagents included syringes and needles for intraperitoneal (i.p) injections, 25×75-mm microscope slides, cryovials (Nunc), Giemsa stain (Gurr's improved Giemsa, BDH Laboratory Supplies), heparinized vacutainer tubes, phosphate buffer (Na_2HPO_4 - 1g/L; KH_2PO_4- 0.7 g/L = pH 7.0) and absolute methanol. Other materials essential for the study are discussed below.

2.3.1.1 Animal and diet

The study was approved by the Noguchi Memorial Institute for Medical Research Institutional Animal Care and Use Committee (NIACUC). The animal procedures were carried out in accordance with the suggested ethical guidelines for care of laboratory animals by NIACUC and Principles of laboratory animal care [14] and given the code number, 2013-01-1Z.The test animals employed in the study were specific pathogen free male ICR mice [15]. The mice (25-30 g) were obtained from the Department of Animal Experimentation, NMIMR, of the University of Ghana and were between 8-10 weeks old. They were maintained in an Animal Biosafety Level 2 facility (ABSL2) with controlled temperature (23±2ºC) and illumination (12h; 6:00 am to 6:00 pm). Animals were randomly assigned into seven groups, consisting of 5 animals each. Each group was housed in asterile standard stainless steel cage (34 × 47 × 18 cm^3) with sterile soft wood shavings as bedding. Mice were fed with sterile rodent pellet diet (AGRICARE, Kumasi) and administered water, *ad libitum* respectively. The animals were transferred from the Barrier Breeding Facility to the Infectious Animal Experimentation Unit, for which reason they were allowed to acclimatize for 3 days prior to their randomization into the various experimental groups for experimentation.

2.3.1.2 Parasite strain

The parasites were cryopreserved *Plasmodium berghei* (NK 65) strain, preserved in liquid nitrogen at -196ºC. They were donated by the Department of Immunology, NMIMR, of the University of Ghana, Legon, Accra. Parasite stock was sustained by serial passage of blood from infected mice to uninfected mice. Parasitemia was monitored regularly. At parasitemia between 25-30%, the mice were euthanized and bled [16,17]. Blood samples were collected into heparinized tubes and injected intraperitoneally into uninfected mouse to keep parasites alive.

2.3.2 Methods

2.3.2.1 Thawing, Inoculum preparation and infection of mice

The cryopreserved parasites were removed from liquid nitrogen and immediately thawed in a water bath set at 37ºC, followed by spinning at 1500 rpm in a refrigerated centrifuge for 5 minutes and supernatants discarded [13]. Disinfection with 70% ethanol was always carried out before placing in the Biological Safety Cabinet (BSC). After discarding off the freezing mixture (supernatant), the pellets (infected red blood cells) were thoroughly mixed with a thawing mixture (3.5% NaCl in distilled water) [13]. The mixture was then centrifuged at 1500 rpm for 7 minutes and the supernatants

immediately discarded. The cells were then washed using 500 µL of Complete Parasite Medium (CPM) by spinning at 1500 rpm for 7 minutes. The washing step was repeated one more time and the supernatant discarded. Finally, the cells were suspended in 1.0 ml CPM. Using 200 µL of the final parasites suspension, each donor mouse was intraperitoneally injected and thin blood smears prepared daily to monitor parasite load, to establish parasitemia and confirm infection after 3-4 days [13,18].

In infecting experimental mice, heparinized blood was taken from an infected donor mouse with approximately 30% parasitemia, through cardiac puncture after euthanasia [18], (AVMA Guidelines on Euthanasia, 2013). The total number of erythrocytes/ml of the whole blood collected was estimated with the aid of a Neubeaur counting chamber and Trypan Blue stain [19]. The blood was then diluted with phosphate buffered saline (PBS) to 10^8 parasitized erythrocytes/ml. An aliquot of 0.2 ml or 200 µL (= $2x10^7$ parasitized erythrocytes/ml) of this suspension was injected intraperitoneally (i.p.) into the experimental mice [18]. The total number of parasitized red blood cells was then estimated using the relation, Total number of parasitized rbcs/ml = total rbcs/ml × % parasitemia.

2.3.2.2 Estimation of parasitemia

A thin blood film from tail blood was prepared and fixed with methanol for 2 min [13]. The smear was then stained with 10% Giemsa in phosphate buffer and allowed to stand for 10-15 minutes before washing off gently with distilled water [13,18]. Counting was done with the aid of a Leica DM 2500 M light microscope at a magnification of X100 under oil of immersion [18] and five fields counted each of averaging 300-500 red blood cells. The estimation for parasitemia was done as follow:

Percentage parasitemia =

$$\frac{\text{number of infected red blood cells/field}}{\text{total number of red blood cells/field}} \times 100\%$$

2.3.2.3 Four - day suppressive test

The Peters' 4-day test [20] with minor modifications [21] was employed. Four consecutive days' treatment with four doses of ML extract (250, 500, 750 and 1000 mg/kg) and standard drug, A/L (Artemether/Lumefantrine – 4 mg/kg) were initiated after three days of infection. This was done to ensure parasitemia establishment in the experimental animals before treatment. Blood samples were taken to prepare thin smears for examination and estimation of parasitemia after the four days of treatment. Percentage suppressions were then calculated from the relation below, and analysed using Two-Way ANOVA from GraphPad Prism v 5 at 95% confidence interval.

Percentage suppression or activity =

$$\frac{\Delta\left(\begin{matrix}\text{mean parasitemia}\\ \text{of positive control}\end{matrix}\right)-\Delta\left(\begin{matrix}\text{mean parasitemia}\\ \text{of treated group}\end{matrix}\right)}{(\Delta\text{mean parasitemia of positive control})} \times 100\%$$

Where positive control represents the untreated infected group.

2.3.2.4 Curative test

In the 7-day Curative test, treatment was initiated on day-3 post-infection and continued to day-9 post-infection with daily blood sampling [18]. Daily parasitemia was estimated from three thin blood smears prepared from each mouse and five fields counted for each smear. The results obtained from the estimation were then statistically analysed using both One- and Two-way ANOVA followed by Newman-Keuls and Bonferroni post-tests respectively from GraphPad Prism v 5 at 95% confidence level.

2.4 Monitoring of Other Parameters

In addition to the daily parasitemia estimation, parameters such as body weight [18] as well as visual inspection of clinical changes [22] and mouse survival time [18] were also monitored in the seven day- curative test.

2.4.1 Body weight distribution

The body weights of the mice were taken daily in the morning before feeding and drug administration. The results obtained were then analysed using One-Way ANOVA followed by Newman-Keuls post-tests from GraphPad Prism v 5.

2.4.2 Visual inspection of clinical signs

The changes in clinical conditions of the experimental animals throughout the duration of the investigation were also visually inspected. Parameters such as, feed and water consumption (lethargy), nature of faeces

(indication of presence or absence of diarrhoea), nature of fur (smooth or piloerection) and locomotor activity were monitored. An arbitrary scale grading the degree of clinical deterioration was adopted and these are: Absent (-); Mild (+); Moderate (++) and Severe (+++).

2.4.3 Survival record

The survival or mortality of the animals as infection worsened and treatment progressed in the experimental groups was also monitored. Mice were monitored on a daily basis and any death per day, recorded. A survival curve was plotted. Results were analysed using Mantel-Cox test and Log-rank test for trend from GraphPad Prism v 5.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Investigation

Phytochemical investigation of the powdered plant material as well as the ML extract revealed the presence of secondary metabolites, some of which have been shown in other studies to elicit antiplasmodial activity [7]. The powdered plant material was shown to contain alkaloids, phytosterols, saponins, flavonoids and tannins. ML extract was also shown to contain similar constituents with the exception of phytosterols, which were absent (Table 1). Both powdered material and extract did not contain coumarins and anthracene glycosides. The results from this investigation showed minimal variation in the constitution of the plant material when compared to ML extract. It could be inferred thus, that, the activity of the plant material may be comparable to that of ML extract, especially when traditional mode of preparation of the extract for treatment was simulated in preparation of the ML extract.

From literature, *Moringa oleifera* Lam (Moringaceae) contain alkaloids [23], phytosterols (sterols and terpenes), tannins, flavonoids [24], glycosides, volatile oils and carbohydrates [23]. The results obtained from the current study confirm that in literature, which also report of the absence of coumarins [24]. On the contrary, anthraquinones, reported to be present in the same study [24], was shown to be absent in the current study. Bioactivity of most plants have been attributed to the presence of phytochemicals. Thus, the confirmation of the presence of some classes of compounds in ML extract, could be inferred to contribute to its bioactivity. For example, alkaloids have been shown to possess antimicrobial effects [23-25], antihypertensive effects [25], anti-inflammatory effects [26], anticancer effects [27], antimalarial effects [28-30] among others. Flavonoids, which are mostly found in the aerial parts of plants, are chiefly responsible for most antioxidant and antiradical effects of natural products [31-35]. They possess anticancer effects [36], anti-allergic effects [37], wound healing effects [38] and antimicrobial effects [39]. It is therefore proposed, from the phytochemical investigation conducted that, the presence of alkaloids, phytosterols, saponins, flavonoids, tannins and glycosides in the plant samples and the extracts could be responsible for their antiplasmodial activity.

Table 1. Phytochemical investigation of the powdered *Moringa oleifera* Lam (Moringaceae) and aqueous extract

Test	Powdered plant material	Aqueous extract (ML)
Alkaloids		
1. Dragendorff's	+	+
test	+	+
2. Mayer's test		
Phytosterols		
1. Salkowski's test (triterpenes)	+	-
2.Libermann's test (sterols)	+	-
Coumarins	-	-
Glycosides		
1. Fehling's test	+	+
2. Saponins	+	+
3. Anthracene	-	-
4. Cyanogenetic	-	-
Flavonoids	+	+
Tannins		
1. Gelatin test	+	+
2. Lead acetate test	+	+
3. Ferric chloride test	+	+

KEY: Present (+), Absent (-)

3.2 *In vivo* Antiplasmodial Activity Test

The initial investigation into the bioactivity of ML extract was a four-day suppressive test. The percentage suppressions were determined in relation to their parasitemia on initiation of therapy. The results showed that suppression was not entirely dose dependent (Table 2), as the two lower doses, that is, 250 mg/kg

(69.31%) and 500 mg/kg (77.26%), exhibited better suppression of parasite multiplication when compared to the two higher doses, that is, 750 mg/kg (25.28%) and 1000 mg/kg (7.12%) by the end of the investigation. In addition to that, statistical analysis showed that the suppression from 1000 mg/kg was insignificant (t = 2.453, p> .05). Thus, the suppression increased with the administration of 250 mg/kg (69.31%) and 500 mg/kg (77.26%) and decreased with 750 mg/kg (25.28%) and 1000 mg/kg (7.12%). The treatment control exhibited the highest suppression (105.52%), (t = 38.00, p< .001).

In the curative test, ML extracts exhibited similar trend of parasite inhibition (Figs. 1a & b) as observed in the four-day suppression test. A One-Way ANOVA showed significant reduction in the parasitemia of the treated groups ($F_{6,98}$= 14.89, N = 15, p< .0001; Fig. 1b). The total parasitemia (calculated as area under curve, AUC) for the first two doses were significantly lower than that of the positive control; 52.32±6.732 (N = 15, q = 4.694, p< .01; Fig. 1b) was the estimate for 250 mg/kg, and 49.62 ± 3.804 (N = 15, q = 4.974, p< .01; Fig. 1b) for 500 mg/kg. That of 750 mg/kg (87.77±13.36, N = 15, p> .05; Fig. 1b) and 1000 mg/kg (81.16±17.63, N = 15, q = 1.936, p> .05; Fig. 1b) were shown not to be significantly different from the positive control (101.3±14.32). The treatment control also showed significant reduction in parasitemia (3.201±0.1569, N = 15, q = 9.446, p< .001).

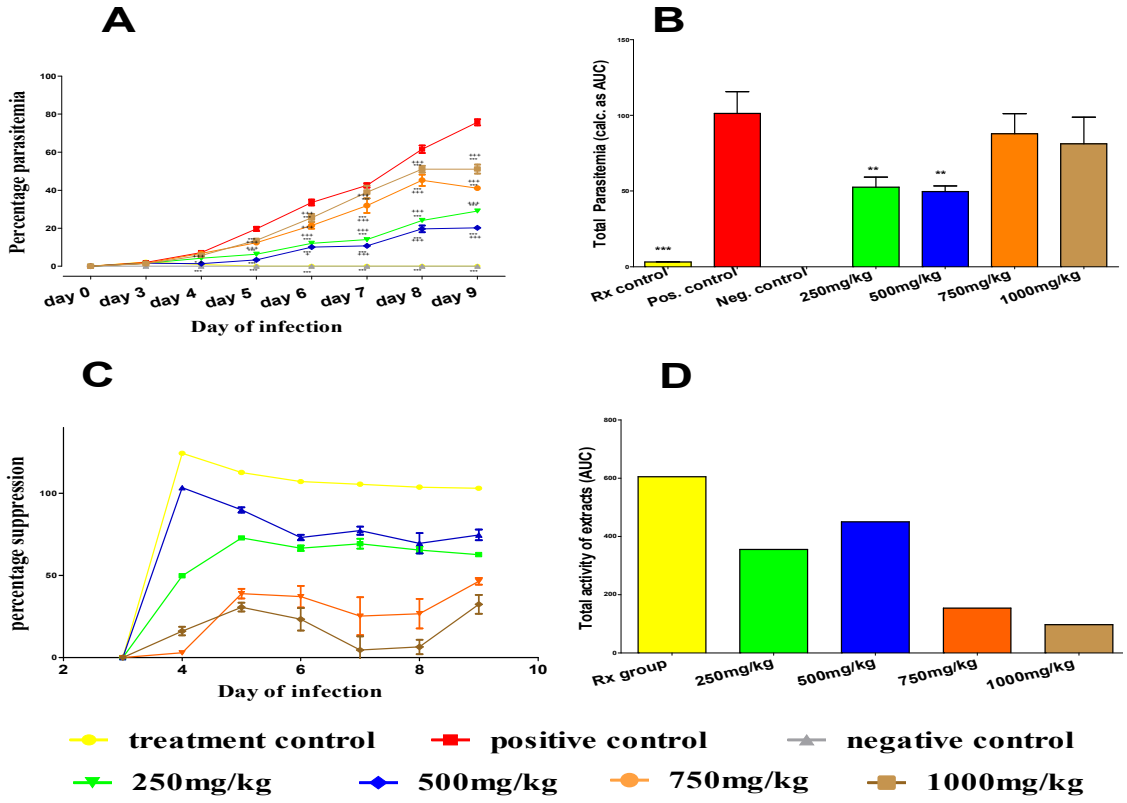

Fig. 1. Dose dependent evaluation of antiplasmodial effects of ML extracts using Curative test model

*[A] - Daily estimation of parasitemia from thin film smears. ML at doses 250, 500, 750 and 1000 mg/kg administered twice daily orally. Each point represented Mean ± SEM; [B] - Estimation of total parasitemia calculated as Area under curve for each experimental group. Each bar represented Mean ± SEM of AUC; [C] - Daily estimation of the percentage suppression of extracts. Each point represented Mean ± SEM; [D] - Estimation of the total activity or suppression of the extracts as Area under Curve. Each bar represented Mean AUC. N = 5. Results analysed with One- and Two-way ANOVA followed by Newman-Keuls and Bonferroni post-tests at 95% Confidence level respectively from GraphPad Prism v5. (*p< .05, **p < .01 and ***p< .001 denoted significance levels in comparison to positive control. +p < .05, ++p < .01 and +++p< .001 denoted significance levels in comparison to treatment control)*

The ML extracts exhibited different patterns in daily suppression of parasite growth (Fig. 1c). For the treatment control group (A/L), the percentage suppression on the day after initiating the therapy was 124.32%±0.246; however it declined in the subsequent days, finally to 103.04%±0.0 on day-9. The next higher activity was exhibited by the 500 mg/kg group, which started with 103.43%±0.639 and finally declined to 74.65%±3.235 on day-9 post infection. The 250 mg/kg group recorded 49.75%±0.756 initially on day-4 but the activity increased to 72.83%±1.218 on day-5 and then declined to 66.63%±1.676 on day-6, finally ending with 62.68±1.148 on day-9. For the 750 mg/kg group, the initial suppression recorded was low, that is, 2.97%±0.730 on day-4 but increased gradually and finally to 46.51%±2.031 on day-9. In the case of the 1000mg/kg group, initial suppression was 16.18%±2.654 on day-4, but increased to 23.34%±6.823 on day-6. It however declined to 4.66%±8.264 on day-7 and then increased again, finally to 32.44%±5.792 on day-9. It was also shown from the average area under curve (AUC) calculations (Fig. 1d) that, activity was greatest for the treatment control (604.9) group, followed by 500 mg/kg (456.0), 250 mg/kg (355.4), 750 mg/kg (154.2) and 1000 mg/kg (97.61) in that order. Similar observation was reported by Harini *et al*., during an investigation of biochanin A, a soy isoflavone for its antihyperglycaemic effects on streptozotocin diabetic rats [40]. In that study, administration of 10 mg/kg of the compound induced a 21.16% reduction in plasma glucose after 2 hours as compared to 13.07% from 5 mg/kg and 10.81% from 20 mg/kg. After 15 days, the reduction were as follows; 30.94% (10 mg/kg), 25.41% (5 mg/kg) and 20.53% (20 mg/kg) [40]. On the contrary, results observed in another study involving the seeds of *Moringa oleifera* showed the *in-vivo* antiplasmodial suppression to be dose dependent for the ethanolic and n-hexane extracts [41]. However, the difference in inhibitory patterns could be as a result of differential phytochemical composition of the seeds and the leaves [42].

The significantly higher activity for the two lower doses, 250 and 500 mg/kg as compared to 750 and 1000 mg/kg of ML extract, in both the four-day suppressive and the curative models, indicated a reversal of activity beyond a certain concentration of extract in the animal. The results obtained showed that, as the dose increased for the 750 and 1000 mg/kg, there was a decrease in anti-plasmodial activity (Figs. 1b; 1c & d).This may probably be due to functional antagonism, where increasing dose lead to increasing the concentration of constituents, which act independently of each other but exhibit effects opposite to each other [43]. At lower doses, the inhibitory effect exerted would be attributed to the dominant compound (s) in the extract but as the dose of individual compounds increase in the extracts, the opposite effects contributed by other compounds would become paramount, thereby reducing the activity. It could also be competitive antagonism, where increasing the concentration of the compounds, allow for competition in occupation for the receptor sites on the parasite [14,43]. Thus, a relatively higher concentration of an antagonistically acting constituent would either prevent inhibition or rather enhance parasite growth. This could be the likely case for the 1000 mg/kg ML extract (Figs. 1a &b).

3.3 Monitoring of Other Clinical Parameters

3.3.1 Body weight distribution

The weights of the animals treated with the ML extracts were also monitored. From Fig. 2e, the negative control group experienced a gradual increase in their body weights throughout the duration of the investigation. This was expected since they were uninfected. For the treatment control group, there was a small decline in their weights on the 4th day (-1.52%) and 5th day (-2.27%) of infection. The body weights then increased gradually afterwards, with a slight decline again on day 7 (-0.34%). In the case of the 250 mg/kg group, the body weights declined on day 3 post-infection (-4.07%) and then increased to a peak of (+6.40%) on day 6 post-infection.

The 500 mg/kg, 750 mg/kg and 1000 mg/kg groups also experienced varying degrees of decline in their body weights, with the 750 mg/kg group presenting the least decrease. The decrease in body weights of the animals were confirmed by the Two-Way ANOVA analysis (p< 0.0001). The positive control group, experienced the greatest of percentage reductions in body weight from day 6 (-13.82%) to day 9 (-38.18%). This decline was significant when compared to the uninfected (negative control) group. On the contrary, these percentage changes in body weights were notsignificantly different when analysed using One-Way ANOVA and Newman-Keuls post- test ($F_{6,26}$ = 0.5253, N = 5, p = 0.7839; Fig. 2f).

3.3.2 Visual inspection of clinical signs

From Table 3, it is shown that the clinical conditions of the infected experimental groups deteriorated progressively, with the treatment control group presenting only mild piloerection. The negative control group did not show any deterioration clinically. In the positive control group, there was moderate piloerection and a mild decline in locomotion on the third day, which became pronounced by the fifth day and with attendant deaths in subsequent days. For the ML treated groups, one key observation made was the one-day delay in the appearance of symptoms like piloerection and lethargy when compared to the positive control group. This notwithstanding, the clinical conditions of the treated groups also deteriorated but were less severe in the 250 mg/kg and 500 mg/kg groups compared to the 750 mg/kg and 1000 mg/kg groups. In view of the severe changes noted in the untreated (positive control) group, it could be inferred that ML mitigated the progression of experimental malaria in the 250 mg/kg and 500 mg/kg treated groups.

3.3.3 Survival analysis

From the survival records, it was observed that as days of infection progressed, none of the animals in the treatment control group was lost, thus, recording a 100% survival by the end of the investigation (Fig. 2g). In the ML treated groups, one animal was lost from the 250 mg/kg group on day 6 post-infection (that is, 80% survival); however, on day 9 post-infection, survival reduced to 40% and then maintained. For the 500 mg/kg group, survival reduced to 80% on day 8 post-infection and was then maintained. For the 750 mg/kg, survival reduced drastically on day 8 post-infection to 40% (that is, loss of three animals) and then on day 10 post-infection, none of the rest survived (0% survival).

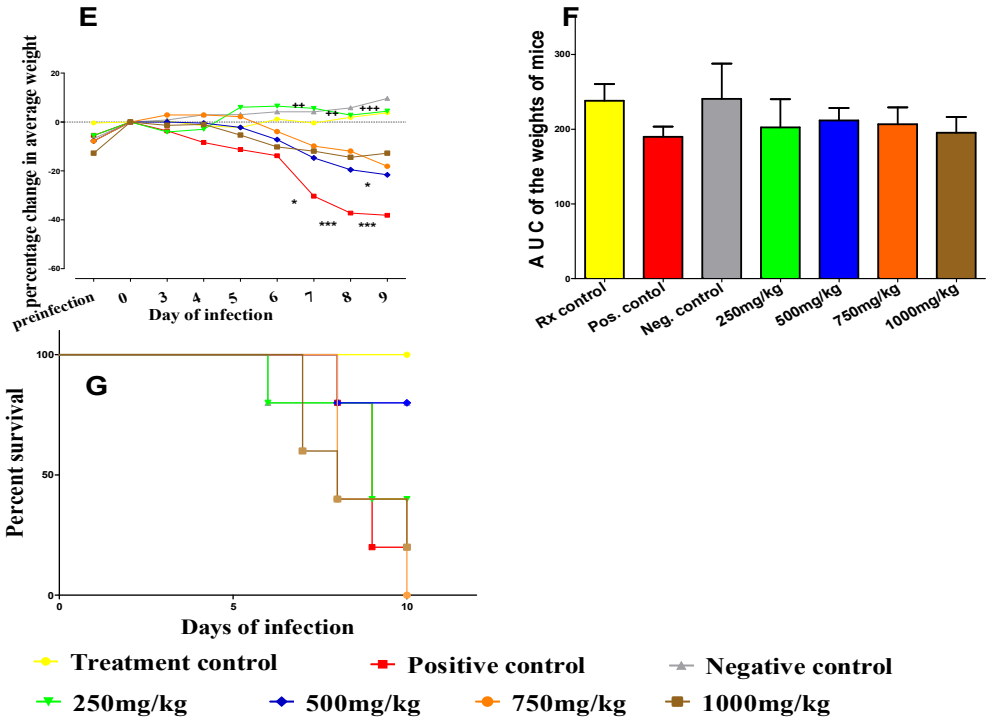

Fig. 2. Evaluating the effects of ML extract on the body weight and survival of *P. berghei* infected ICR mice in the curative test

*[E]- Estimation of the percentage change in weights of the infected mice treated with ML extracts. Each point represented Mean ± SEM. [F] - Total estimation of the weights of each experimental group by the end of the investigation. Each bar represented Mean ± SEM. N=5. Statistical analysis using One - and Two-way ANOVA followed by Newman - Keuls and Bonferroni post-tests respectively. *p < .05, **p < .01 and ***p< .001 denoted significance levels when compared with negative control. [G] - Percentage survival of the experimental groups employed in the curative test. Each point represented percentage of survived animals on a given day of infection. N = 5. Results were analysed using Mantel-Cox test and Log-rank test for trend from GraphPad Prism v5*

Table 2. Percentage suppression in parasitemia in *P. berghei* infected ICR mice with aqueous *Moringa oleifera* Lam (Moringaceae) (ML) extracts (250 - 1000 mg/kg) in the 4-day suppressive test

	Rx control	250 mg/kg	500 mg/kg	750 mg/kg	1000 mg/kg	Pos. control
Parasitemia on initiation (%)	2.239±0.584	1.571 ± 0.313	1.569 ± 0.318	1.637 ± 0.545	1.343±0.438	2.000±0.458
Parasitemia at end of therapy (%)	0 ***	14.010±3.004***	10.787±2.452***	31.926±11.567***	38.992±8.264	42.337±4.099
Percentage suppression (%)	105.52	69.31	77.26	25.28	07.12	

*N= 5. Data expressed as mean ± SEM (average of 15 readings from each group, that is, 3 readings from each mouse) and analysed using Two-Way ANOVA at 95% Confidence level from GraphPad Prism v5. *p < .05, **p < .01 and ***p< .001 denote significance level in comparison with positive control*

Table 3. Physical signs of illness associated with the experimental malaria infection and *Moringa oleifera* Lam (Moringaceae) therapy

Day of infection	Treatment control				Positive control				Negative control				250 mg/kg				500 mg/kg				750 mg/kg				1000 mg/kg			
	D	L	P	M	D	L	P	M	D	L	P	M	D	L	P	M	D	L	P	M	D	L	P	M	D	L	P	M
1	-	-	+	-	-	-	+	-	-	-	-	-	-	-/+	+	-/+	-	-	+	-	-	-	+	-	-	-	+	-
2	-	-/+	+	-	-	-/+	+	-	-	-`	-	-	-	-	+/++	-/+	-	-	+	-	-	-	+	-	-	-	+	-
3	-	-	+	-	-	-	++	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-
4	-	-	+	-	-	-	++	++	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-
5	-	+	+	+	-	+++	+++	+++	-	-	-	-	-	+	++	+	-	++	++	+	-	++	++	++	-	++	++	+++
6	-	-/+	+	-/+	++	+++	+++	+++	-	-	-	-	-	++/+++	++	++	-	++	++	++	-	+++	+++	+++	-	+++	+++	+++
7	-	-	+	-	++	+++	+++	+++	-	-	-	-	-	+++	++	+++	-	++	+++	++	-	+++	+++	+++	-	+++	+++	+++
8	-	-	+	-	++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	+++	+++	++	-	+++	+++	+++	-	+++	+++	+++
9	-	-	+	-	++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	+++	+++	+++	-	+++	+++	+++	-	+++	+++	+++

Physical signs of the experimental ICR mice were monitored to determine the effects of the infection on the animals and how these effects could be reversed with the administration of ML extracts. N = 5. KEY: (-) absent; (+) mild; (++) moderate and (+++) severe, signifying severity of illness; D –Diarrhoea; L – Lethargy; P – Piloerection and M – decreased Locomotion. (-), (+), (++) and (+++) assigned when 4-5 mice in the group exhibited similar signs; (-/+) and (+/++) assigned when at least 2 mice in the group exhibited one similar sign while the others also exhibited another similar sign

In the case of 1000 mg/kg group, survival reduced to 60% on day 7 post-infection and then to 40% on day 8 post-infection and finally to 20% on day 10 post-infection. The infected but untreated (positive control) group maintained 100% survival up to the 8th day of infection, when it reduced to 80%, with the loss of one animal. The next day recorded loss of three more animals in the same group, reducing survival to 20% and this was maintained to the end of the study. In the case of the uninfected (negative control) group, survival reduced to 80% on day 6 post-infection, with loss of one animal for unexplained reasons; and then maintained throughout the rest of the days of the study. Not with standing these observations, comparison of the survival curves from the different experimental groups showed that they were not significantly different from each other, using the Mantel-Cox test analysis from GraphPad Prism (χ^2 = 12.48, *df* = 6, *p* = .0522). On the other hand, it was shown that there was a significant linearity between the doses and the median survival using the Log-rank test for trend (χ^2 = 4.910, *df* = 1, *p* = 0.0267). This meant that, the survival of the animals to a greater extent depended on the dose administered. It could therefore be inferred that, the higher the dose administered, the more toxic it was to the animals; with the optimum dose offering a balance between the activity or efficacy, survival and toxicity. Therefore in this study, the 500 mg/kg body weight dose emerged as the optimum dose.

4. CONCLUSION

Moringa oleifera aqueous extract contains alkaloids, saponins, flavonoids, tannins and reducing sugars. These phytochemicals may be responsible for the *in-vivo* antiplasmodial activity exhibited by the extracts. *Moringa oleifera* has been shown to be effective within the range 250–500 mg/kg body weight; above this range, activity declines. In addition, *Moringa oleifera* Lam (Moringaceae) administration has been shown not to significantly affect the weights or the survival of treated *P. berghei* infected ICR mice. Thus, in traditional medicinal practice, *Moringa oleifera* Lam (Moringaceae) could be considered in malaria treatment, in view of results of this study and other studies done on the same plant.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The study was approved by the Noguchi Memorial Institute for Medical Research Institutional Animal Care and Use Committee (NIACUC). The animal procedures were carried out in accordance with the suggested ethical guidelines for care of laboratory animals by NIACUC and Principles of laboratory animal care [17].

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COMPETING INTERESTS

Authors have declared that no competing interest exist.

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