



Phytochemical Analysis and *In vivo* Evaluation of Individual Activity of Ethanolic Leaf Extracts of *Azadirachta indica*, *Senna occidentalis* and *Momordica balsamina* against *Plasmodium berghei* Infected Mice

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AMB and KBD designed the study. Author AMB performed the statistical analysis. Authors AMB, KBD and AIA wrote the protocol and wrote the first draft of the manuscript. Authors AMB, KBD and AIA managed the analyses of study. Author AMB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Malaria is a major cause of morbidity and mortality in tropics and subtropics region with Nigeria accounting for the highest proportions in Africa. This is accompanied with emerging resistance to available drugs, posing it a public health concern. This study is aimed at determining the *invivo* activity of the individual ethanolic leaf extracts of the *Azadirachta indica*, *Senna occidentalis* and *Momordica balsamina*. The leaves of *A. indica*, *S. occidentalis* and *M. balsamina* were subjected to preliminary phytochemical screening. Ethanolic extraction of leaves of plants was carried out and *invivo* evaluation of the individual activity of extracts determined using standard procedures. 55 mice were randomly divided into 11 groups lettered A – K; positive group, negative group and 9

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extract groups. Results showed that *M. balsamina* had the highest yield of 7.6%, followed by *A. indica* with 6.5% and *S. occidentalis* with 5.7%. The preliminary phytochemical screening revealed the presences of alkaloids, flavonoids, saponins, steroids, phenolics and tannin in all plants. The comparison of the individual study groups showed that *Senna occidentalis* is more effective at 600mg/kg dosage and prolonged survival of the mice in its group in the study period. This plant possessed significant (P-value <0.05) antiplasmodial activity, thus lowered parasitaemia in infected mice.

Keywords: *Antiplasmodial; Senna; Momordica; Azadirachta; parasitaemia.*

1. INTRODUCTION

Plasmodium parasites, causative agents of Malaria have been a major health problem in many tropical regions of the world. An undoubtedly life-threatening disease prominent in the less developed and developing countries in tropical and subtropical regions including Africa, Asia and parts of Americas [1]. This disease has a mortality rate of more than one million children annually [2]. Globally, an estimated 216 million cases of the disease occurred in 91 countries making it a global concern with Nigeria accounting for the highest morbidity cases of 23%. In endemic areas of sub-sahara Africa, about 50 million pregnancies occurs every year and pregnancy associated malaria, another public health concern could lead to maternal and fetal morbidity leading to about 100, 000 deaths of infant annually [3]. Notably is the presence of numbers of available drugs to control the disease, but the development of resistant to these drugs has placed it as a major cause of death globally. One of the first effective anti-malaria agent that was used was quinine isolated from the bark of cinchona plant that was later used to synthesize chloroquine and primaquine [4]. The resurgence of herbal remedies in recent times has been attributed to preference of consumers for natural therapies, a greater interest in alternative medicine and dissatisfaction with the result from orthodox pharmaceuticals as a result of ineffectiveness [5], thereby, the need for potential treatment candidates against malaria. *Azadirachta indica* is a member of the Mahogany family with its name derived from 'azadhirakt', a Persian word for 'noble tree' [6]. Its straight trunk measures about 7-15m at maturity producing yellow ellipsoidal drupes called neem fruits. The leaves are alternate, compound with its rachis length 15-25cm long. [7]. *Senna occidentalis* is an annual weed distributed widely in the tropical and subtropical regions of the world [8] with height ranging 60-150cm bearing ovate-lanceolate shaped compound leaves of 15-20 cm long [9],

Momordica balsamina is a trailer with its stem length measuring 4-5 mm. It is an annual/perennial climber plants usually 10-12inch tall and slightly hairy. The tendrils are simple and leaves bright green, palmate (5 – 7 lobed), waxy with lower surface paler more than the upper part. The plant bears red fruits with rows of cream short spines [10], Although, the knowledge of its activity is known, comparing the *in vivo* individual activity of the ethanolic extract of these plants would provide more essential information on their activity against *Plasmodium berghei* ANKA strain.

2. MATERIALS AND METHODS

2.1 Study Area

Adult mice weighing 20–30g of about 6–8 weeks old were purchased from the Veterinary Parasitology Laboratory, Ahmadu Bello University, Zaria and housed in aluminum cages containing wood shavings as beddings in the Biology Laboratory Section, Nigerian Defence Academy, Kaduna. The mice were acclimatized for 2 weeks where they were given pellets in small aluminum plates and drinking water through drinkers.

2.2 Collection, Authentication and Preparation of Plant Materials

Fresh leaves of *A. indica*, *S. occidentalis* and *M. balsamina* were collected from Kurmin Mashi and Zonkwa in Kaduna State. Plants were taxonomically authenticated at the department of Biological Sciences, Nigerian Defence Academy Kaduna. Leaves of plants were spread on a laboratory bench and allowed to air dry under normal room temperature. The dried leaves were sorted out to eliminate plant stems and branches and consequently coarsed into powder using mortar and pestle, followed by an electric mill. The powdered plant leaves were weighed, stored in a polythene bag and kept at room temperature until extraction.

2.3 Extraction of Plant Materials

The leaves of *A. indica*, *S. occidentalis* and *M. balsamina* were extracted using cold extraction. The leaves were soaked in distilled ethanol individually in 3 different glass jar. The solution was shaken at intervals to allow proper mixing in order to derive optimum extraction for 5 days, filtered using a filter paper. The filtrate was concentrated using water bath distillation process and the concentrate left open to air dry. The crude extract of *A. indica* (17g), *S. occidentalis* (16g), *M. balsamina* (20g) were kept in air-tight containers. The percentage yield of plant extracts were calculated using the method described by [11].

$$\text{Percentage yield} = \frac{(W_2 - W_1) \times 100}{W_0}$$

Where W_2 is the weight of extract and container, W_1 is the weight of the container alone and W_0 is the weight of the initial dried leaves

2.4 Phytochemical Analysis

Phytochemical analysis of plants extracts were carried out using the method described by [12]. A small sample of the crude extracts of each of the plant was dissolved in 10 ml distilled ethanol. The solution was divided into various test tubes to test for the presence of the following photochemical:

2.4.1 Test for alkaloids

One cm³ of Hydrochloric acid (HCl) were added to the test fraction in a test tube. The solution was treated with Mayer, Wagner and Dragendorff reagent separately. A creamy-white (Mayer), reddish-brown (Wagner), and orange-brown (Dragendorff) precipitate indicates the presence of alkaloids.

2.4.2 Test for phenolics and tannins

Three drops of ferric chloride were added to the test extract solution in a test tube. A dirty green precipitate indicates a positive test.

2.4.3 Test for saponins

Three drops of olive oil were added to the test extract solution and the mixture was shaken vigorously. A stable emulsion formed indicates the presence of saponins.

2.4.4 Test for steroids

Three drops of H₂SO₄ and acetic anhydride were added to the test extract solution. Color changes from violet to blue or green indicates a positive test.

2.4.5 Test for Terpenoids

Three drops (2 ml) of chloroform were added to the test extract solution and evaporated on the water bath. it was boiled with 3ml of concentrated H₂SO₄. A grey color formed shows the entity of terpenoids.

2.4.6 Test for flavonoids

Three drops (2 ml) of 2.0 % sodium hydroxide solution were added to the test extract. The formation of intense yellow color which becomes colorless on addition of 2 drops of dilute hydrochloric acid indicates the presence of flavonoids.

2.4.7 Test for reducing sugar

To test for the presence of reducing sugar in the extract, 5 - 6 drops each of Fehling solution A and B were added to the solution. The mixture were boiled and a brick red precipitate indicates of a positive test.

2.5 Parasite Strain

Plasmodium berghei ANKA strain was obtained from Veterinary Parasitology Laboratory in Ahmadu Bello University, Zaria where infected mice are being acclimatize. The parasite is subsequently maintained in the laboratory by serial passage of blood from donor infected mice to naive one via intra-peritoneal (IP) route.

2.6 Determination of Body Weight of the Mice

The body weights of each mouse in all groups were measured before and after infections and treatments using sensitive digital weighing balance [13].

2.7 Inoculation of Mice

The parasitaemia of the donor mice were ascertained to be within the range of 20–30%. Blood were collected retro-orbitally and diluted in phosphate buffered saline based on their parasitaemia level such that 1mL contains 5 x 10⁷

parasites. Each experimental mouse was given 0.2mL of diluted infected blood containing 1×10^7 parasitized red blood cells (pRBCs) intraperitoneally.

2.8 Grouping of Experimental Animals

55 Albino mice weighing 20–30g of about 6–8 weeks old purchased were randomly grouped into 11 groups;

Group A – Positive Control (infected and treated with standard drugs)

Group B – Negative Control (infected and not treated)

Group C – 200mg/kg of *Azadirachta indica*

Group D – 400mg/kg of *Azadirachta indica*

Group E – 600mg/kg of *Azadirachta indica*

Group F – 200mg/kg of *Senna occidentalis*

Group G – 400mg/kg of *Senna occidentalis*

Group H – 600mg/kg of *Senna occidentalis*

Group I – 200mg/kg of *Momordica balsamina*.

Group J – 400mg/kg of *Momordica balsamina*.

Group K – 600mg/kg of *Momordica balsamina*.

After inoculation, each mouse was administered 0.2ml each of the drug (Group A) or extract (Group C – K) orally with cannula according to the groupings for 4 days starting from Day 3 (D3) post infection except the negative control which was given distilled water only. Survival was monitored daily till the completion of the experiment at day 29.

2.9 Preparation of the Extracts and Drugs

Stock solution of each plant extracts was prepared by dissolving in distilled water and stored in an air-tight container in the refrigerator at 4°C. 200mg/kg, 400mg/kg and 600mg/kg were consequently prepared from the stock solution by serial dilution and administered to experimental animal. Piperaquine and dihydroartemisin was dissolved to 32mg and 4mg respectively.

2.9.1 Collection of blood samples for determination of parasitaemia and chemosuppression

Thin blood smears were made from blood collected from the tail of each mouse. The cutting was considerable at small inches so as not to cause prolonged damage to each mouse tail. The slide was fixed in methanol, giemsa stained and viewed under the light microscope. The total number of erythrocytes and total number of infected erythrocytes was calculated in random 5

fields from each slides. Percent parasitaemia and chemosuppression were calculated by:

Percentage parasitaemia = $(\text{Number of infected erythrocytes} / \text{Total number of erythrocytes}) \times 100$

Percentage chemosuppression = $\frac{(A - B)}{A} \times 100$

Where

A – parasitaemia of negative group

B – parasitaemia of each of treatment/positive group.

2.10 Statistical Analysis

Parasitaemia were analyzed using One-way Analysis of Variance. The statistical significance was defined as P-value of <0.05 using SPSS version 16.0. The differences between the groups were tested using Duncan Post-hoc test. Graph Pad Prism software was used for preparation of Kaplan-Meier survival curves and parasitemia curves.

3. RESULTS

3.1 Yield of Extract

The ethanol extract of all the plants yielded green solid gummy with a percentage yield of 6.5%, 7.6% and 5.7% accounting for 260g, 210g and 350g was obtained from the dried pounded leaves of *Azadirachta indica*, *Momordica balsamina* and *Senna occidentalis* respectively. Result is presented in Table 1.

3.1.1 Phytochemical components detected in plant extracts

The qualitative phytochemical analysis of the ethanolic plant extracts (Table 2) showed the presence of alkaloids, flavanoids, saponins, steroids, phenolics, tannins in all plants. Reducing sugar was present in *Azadirachta indica* and *Senna occidentalis* while terpenoids was present in *Azadirachta indica* and *Momordica balsamina*.

3.1.2 Results of the antiplasmodial activity of ethanolic extract of individual leaves of *A. indica*, *S. occidentalis* and *M. balsamina* on mice infected with *Plasmodium berghei*

The antiplasmodial activity of the individual plants in the various concentrations showed a

reduced parasitaemia level during the early phase of infection in contrast to the rapid increase observed in negative group. The extracts group produced a parasitaemia count reduction across the day.

On day 6, *A. indica* showed highest mean parasitaemia of 6.2% in 200mg/kg extract group and 400mg/kg and 600mg/kg extract group showed similar mean parasitaemia of 3.3% (Fig. 1), *S. occidentalis* had the highest mean parasitaemia of 9.5% in the 200mg/kg extract group, least mean parasitaemia of 4.2% was observed in the 600mg/kg extract group and 400mg/kg extract group had 4.7 % (Fig. 2), *M. balsamina* had the highest mean parasitaemia of 4.4 % in 200mg/kg extract group, least mean parasitaemia of 3.2 % in the 400mg/kg extract group and mean parasitaemia of 4.3 % in the 600mg/kg extract group (Fig. 3). On the same day, the negative group had a mean parasitaemia of 7.0 % while the positive group showed a mean parasitaemia of 4.2%.

On day 10, the mean parasitaemia of the negative group had increase to 15.50% while the positive group had a mean parasitaemia of 1.30%. The extracts group showed improved parasitaemia levels with *A. indica* highest mean parasitaemia of 7.5% in 200mg/kg extract group, least mean parasitaemia of 6.2% in 400mg/kg

extract group and a mean parasitaemia of 7.2 % in 600mg/kg extract group, *S. occidentalis* had the highest mean parasitaemia of 5.20% in 200mg/kg extract group, least mean parasitaemia of 3.10% in 600mg/kg extract group and a mean parasitaemia of 4.9 % in 400mg/kg extract group, *M. balsamina* had the highest mean parasitaemia of 5.70% in both 200mg/kg and 600mg/kg extracts group and the least mean parasitaemia of 5.40% in 400mg/kg extract group.

On day 10, the percentage chemosuppression of the positive group was 91.6 % while the negative group had 0.0%. *A. indica* had the highest percentage chemosuppression of 60.0 % in the 400mg/kg extract group, least percentage chemosuppression of 51.6 % in the 200mg/kg extract group and percentage chemosuppression of 54.0 in the 600mg/kg extract group. *S. occidentalis* had the highest chemosuppression of 80.0% in the 600mg/kg extract group, least percentage chemosuppression of 66.5% in the 200mg/kg extract group and a percentage chemosuppression of 68.4% in the 400mg/kg extract group. *M. balsamina* had the highest chemosuppression of 65.1% in the 400mg/kg extract group and a percentage chemosuppression of 63.2 % in both 200 mg/kg and 600mg/kg extracts group (Table 3).

Table 1. Yield of plant extracts of *Azadirachta indica*, *Senna occidentalis* and *Momordica balsamina*

Plants	Quantity of plant materials	Yield of extracts	Percentage yield (%)
<i>A. indica</i>	260g	17g	6.5
<i>M. balsamina</i>	210g	16g	7.6
<i>S. occidentalis</i>	350g	20g	5.7

Table 2. Phytochemical components identified from crude extract of *Azadirachta indica*, *Senna occidentalis* and *Momordica balsamina*

Phytochemical components	Plant extracts		
	<i>Azadirachta indica</i>	<i>Senna occidentalis</i>	<i>Momordica balsamina</i>
Alkaloids	+	+	+
Flavanoids	+	+	+
Reducing Sugar	+	+	-
Saponins	+	+	+
Steroids	+	+	+
Phenolics	+	+	+
Tannins	+	+	+
Terpenoids	+	-	+

Keys +: Present -: Absent

The survival proportion of mice treated with individual plant extracts showed a 100% survival rate at day 6. On day 10, the survival proportions of the extracts group had changed with *A. indica* highest survival proportion of 100% in 600mg/kg extract group, least survival proportion of 81.8% in 200mg/kg extract group and a survival proportion of 94.7% in 400mg/kg extract group (Fig 4). *S. occidentalis* had the highest survival proportion of 100.0% in 600mg/kg extract group, least survival proportion of 90.0% in 400mg/kg extract group and a survival proportion of 95.0% in 200mg/kg extract group (Fig 5). Lastly, *M. balsamina* had the highest survival proportion of 100% in 600mg/kg extract group and the least survival proportion of 89.4% in 400mg/kg extract

group. The 200mg/kg extract group had a survival proportion of 95.0% (Fig 6). In comparison, the negative group had the survival proportion of 71.4 % which is significantly lower than the positive group which had survival proportion of 95.0% and considerably lower than the extract groups as discussed above.

3.1.3 Effect of extract on Body weight and rectal temperature

The body weight of the mice in all group were measure on Day zero (0) and Day five (4) before and after treatment respectively. The result of the body weight and rectal temperature are represented in Table 4 and Table 5 respectively.

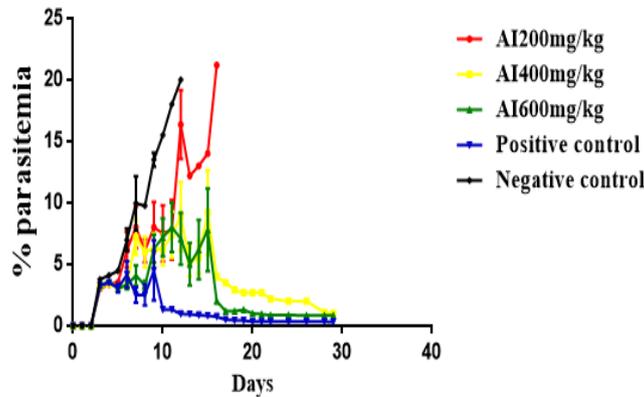


Fig. 1. Effect of the ethanolic leaf extract of *A. indica* on parasitaemia of *P. berghei* infected mice

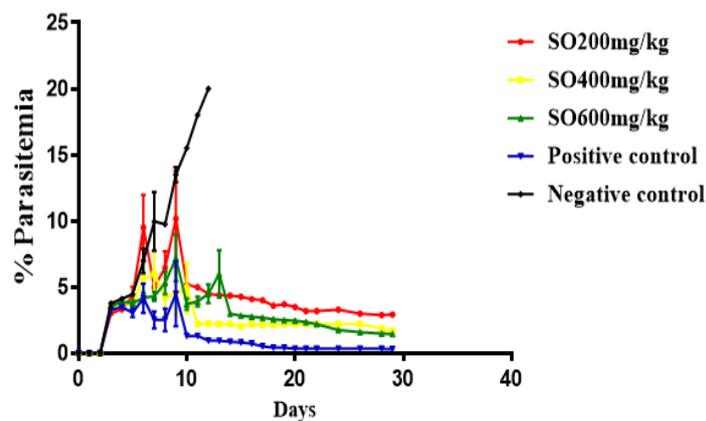


Fig. 2. Effect of ethanolic leaf extract of *S. occidentalis* on parasitaemia of *P. berghei* infected mice

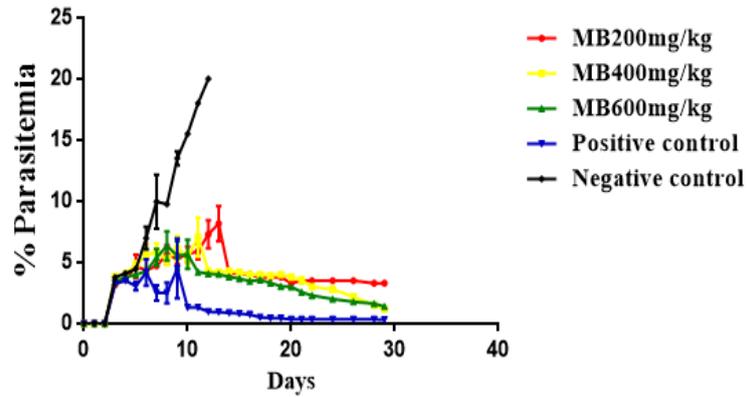


Fig. 3. Effect of ethanolic leaf extract of *M. balsamina* on parasitaemia of *P. berghei* infected mice

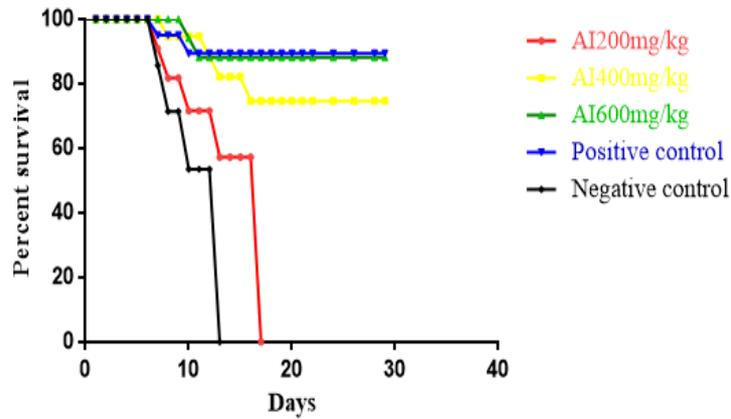


Fig. 4. Survival proportion of *P. berghei* infected mice treated with ethanolic leaf extract of *A. indica*

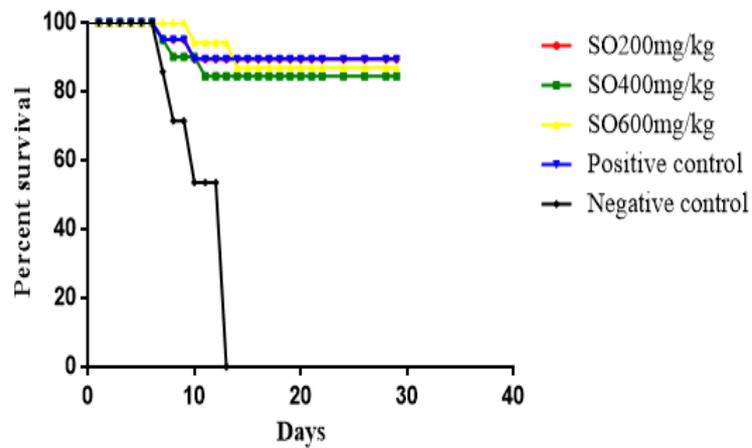


Fig. 5. Survival proportion of *P. berghei* infected mice treated with ethanolic leaf extract of *S. occidentalis*

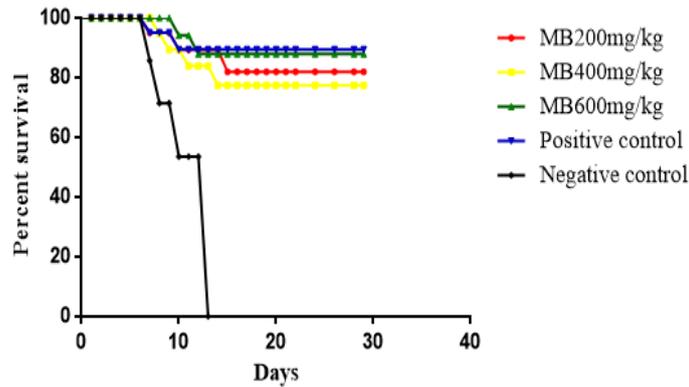


Fig. 6. Survival proportion of *P. berghei* infected mice treated with ethanolic leaf extract of *M. balsamina*

Table 3. Antiplasmodial activity of ethanolic leaf extract of individual plants in mice infected with *P. berghei* on Day 10

Extracts	Dose	% Chemosuppression
Positive	Standard Drugs	91.6
Negative	Distilled water	0.00
<i>A. indica</i> extract	200mg/kg	51.6
	400mg/kg	60.0
	600mg/kg	54.0
<i>S. occidentalis</i> extract	200mg/kg	66.5
	400mg/kg	68.4
	600mg/kg	80.0
<i>M. balsamina</i> extract	200mg/kg	63.2
	400mg/kg	65.1
	600mg/kg	63.2

Table 4. Body weight of mice before and after infection and treatment

Doses	Before	After
Group A – Positive Control (infected and treated with standard drugs)	24.00 ± 1.34	22.70 ± 1.33
Group B – Negative Control (infected and not treated)	24.48 ± 0.71	22.90 ± 0.56
Group C – 200mg/kg of <i>Azadirachta indica</i>	26.08 ± 1.11	24.10 ± 1.26
Group D – 400mg/kg of <i>Azadirachta indica</i>	26.12 ± 1.16	24.54 ± 1.10
Group E – 600mg/kg of <i>Azadirachta indica</i>	25.66 ± 1.03	24.24 ± 1.05
Group F – 200mg/kg of <i>Senna occidentalis</i>	27.04 ± 1.41	25.46 ± 1.38
Group G – 400mg/kg of <i>Senna occidentalis</i>	25.50 ± 1.64	24.22 ± 1.71
Group H – 600mg/kg of <i>Senna occidentalis</i>	25.54 ± 0.77	23.88 ± 0.73
Group I – 200mg/kg of <i>Momordica balsamina</i> .	22.90 ± 0.74	21.78 ± 0.67
Group J – 400mg/kg of <i>Momordica balsamina</i> .	25.42 ± 1.33	23.70 ± 1.24
Group K – 600mg/kg of <i>Momordica balsamina</i> .	25.28 ± 1.62	24.10 ± 1.47

Data is presented as Means ± S.E (n = 5)

4. DISCUSSION

In this study, the yield of *M. balsamina* was high compared to the yield of *S. occidentalis* and *A. indica*. This could be attributed to the pigment-solvent interaction of *M. balsamina* as the plant is known as an important green leafy vegetable

which is a source of important nutrients and supplements according to [14].

This finding is similar to that of [15] whose yield was also high at 11.7% in *Momordica charantia* L. when compared to the extraction of [16] whose *S. occidentalis* yield was low at 7.7%.

Table 5. Rectal temperature of mice before and after infection and treatment

Doses	Before	After
Group A – Positive Control (infected and treated with standard drugs)	35.26 ± 0.29	35.26 ± 0.29
Group B – Negative Control (infected and not treated)	37.34 ± 0.26	33.80 ± 0.24
Group C – 200mg/kg of <i>Azadirachta indica</i>	37.62 ± 0.28	34.26 ± 0.22
Group D – 400mg/kg of <i>Azadirachta indica</i>	36.76 ± 0.17	34.30 ± 0.16
Group E – 600mg/kg of <i>Azadirachta indica</i>	37.28 ± 0.11	36.04 ± 0.34
Group F – 200mg/kg of <i>Senna occidentalis</i>	36.98 ± 0.28	33.42 ± 0.17
Group G – 400mg/kg of <i>Senna occidentalis</i>	37.40 ± 0.31	34.62 ± 0.17
Group H – 600mg/kg of <i>Senna occidentalis</i>	37.76 ± 0.19	36.78 ± 0.13
Group I – 200mg/kg of <i>Momordica balsamina</i> .	37.46 ± 0.14	34.22 ± 0.15
Group J – 400mg/kg of <i>Momordica balsamina</i> .	38.08 ± 0.04	36.36 ± 0.17
Group K – 600mg/kg of <i>Momordica balsamina</i> .	37.12 ± 0.34	35.62 ± 0.42

The quantitative phytochemical screenings of the three plants indicate the presence of alkaloids, flavanoids, saponins, steroids, phenols, and tannin which have been suggested to confer antiplasmodial activity [4]. This is similar to the findings of [17] who confirmed the presence of flavonoids, alkaloids, tannins, saponins, polyphenols and steroids in *A. indica*, also the findings of [18] confirmed the presence of alkaloids, flavonoids, phenols, steroids, saponins, tannins and anthroquinones in *Cassia occidentalis* and the findings of [19] also confirmed the presence of tannins, flavonoids, alkaloids and saponins in *M. balsamina*.

Alkaloids are among the major natural products known to possess antimalarial and various classes of it have been reported to exhibit reliable actions. [20]. The presence of this compound therefore may be responsible for the antiplasmodial activity observed in the plant extract. [21] significantly describe quinine to be a synthetic derivative of the alkaloids. The presence of secondary metabolites like saponins and flavonoids have also been reported to confer antimalarial activity. [22, 23].

The individual study of the plant extract showed that the treatment groups were significant different ($P < 0.05$) from that of the negative control group at day 6 in terms of parasitaemia growth inhibition. Even though the levels of the parasitaemia increase along the days, these were still lower than that observed in the negative group demonstrating the presence of inhibitory properties in them.

The extracts plant being antioxidant may have resulted in free radical scavenging and lipid peroxidation inhibition [24], thus conferring adequate protection enough to counter the effect of the oxidative stress induced in host parasitized

erythrocyte by the malaria parasite [25]. Typically, malaria parasite digest haemoglobin in vacuole to amino acids and heme [26], therefore one of the most important mechanism in its prevention is inhibition of heme polymerization. This inhibition is exhibited by the antioxidant activity before polymerization, and the unpolymerized heme is very toxic for the intraerythrocytic plasmodial[27].

The individual plant extract with the highest effect is the *S. occidentalis* 600mg/kg stating adequacy in its ability to confer antiplasmodial ability. This may be attributed with the presences of phytochemical constituents revealed in the phytochemical studies of the plant [28].

Reduction in body weight, anaemia and rectal temperature decrease are typical features of malaria in mice. [29]. This is closely concerned with the clearance of infected and uninfected RBCs and erythropoies inhibition by malaria parasite. [30].

The *S. occidentalis* although confer 80% chemosuppression while the standard drugs had 91.6% chemosuppression. This substantiates the necessity to determine other details like pharmacokinetic and pharmacodynamics of the plant extracts.

5. CONCLUSION

In conclusion, the extraction solvent yields enough extract of the three plants for the research. The preliminary screening revealed the presence of important phytochemical like alkaloid, flavonoid, phenols, steroids, saponins and tannin in the plants. The individual study of plants extracts had lower percentage parasitaemia and therefore conferred more antiplasmodial activity. This *invivo* study of the

plant extracts therefore demonstrated activity which have individually resulted in prolonged survival of the mice in the groups.

ETHICAL APPROVAL

The experiment management, Animal handling and care were approved by the Research and Ethics Committee of the Department of Biological Sciences, Nigeria Defence Academy, Kaduna, Kaduna State.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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