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The Antimalarial Effect and Mechanism of Action of Methanolic Root Extract of *Boerhaavia diffusa* in Mice

D. I. Adefokun[1*], E. O. Iwalewa[1], N. O. Omisore[1], E. Obuotor[2] and I. J. Idowu[2]

[1]Department of Pharmacology, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.
[2]Department of Biochemistry, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: This study evaluated the antimalarial effect of methanolic root extract of *Boerhaavia diffusa* and its mechanism of action in Mice.

Study Design: One-factor two control groups experimental design.

Place and Duration of Study: Department of Pharmacology, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria, between October 2012 and May 2013.

Methodology: The crude methanolic root extract of the plant was tested for its *in vivo anti-plasmodial activity* against *Plasmodium berghei NK 65* (chloroquine resistant strain) using the three malaria models; suppressive, curative and prophylactic tests. Six different groups of albino mice of both sexes weighing 18 – 20 g (n=5 or 6) were randomly selected for the study. Group 1 was the

**Corresponding author: Email: dolapo.adefokun@gmail.com;*

control (normal saline, 10 ml/kg, p.o.), group 2 was the positive control (chloroquine, 10 mg/kg, p.o.), groups 3, 4 and 5 were treated with methanolic root extract at 125, 250 and 500 mg/kg, p.o., respectively, while group 6 was Nifedipine (15 mg/kg). The method of calcium colorimetry was also adopted for assaying the mechanism through which the extract acted.

Results: *B. diffusa* displayed antimalarial activity at all dose levels in all the three models, though optimum activity of the extract was displayed at the lowest dose (125 mg/kg) in suppressive and prophylactic models and at day 10 in curative model. The dose of 500 mg/kg had the highest activity at day 9 in curative model. The dose of 125 mg/kg again showed the best antipyretic effect in suppressive model at day 3 and this corresponds to its antimalarial activity. At 500 mg/kg, the extract lowered plasma calcium level better than the positive control (1.043 mmol/L compared with 1.35 mmol/L for nifedipine).

Conclusion: The methanolic root extract of *B. diffusa* possessed antimalarial and antipyretic effects which confirm its folkloric use in the treatment of malaria and fever.

Keywords: Boerhaavia diffusa; Plasmodium berghei berghei; malaria; pyrexia; antimalarial activity.

1. INTRODUCTION

Malaria is a vector-borne infectious disease caused by the protozoan plasmodium. The disease is said to be wide spread in tropical and subtropical regions, including parts of the Americas, Asia, and Africa. Each year, there are approximately 515 million cases of malaria, killing between one and three million people, the majority of whom are young children in Sub-Saharan Africa [1]. Compared to a century earlier, the area of malaria risk has reduced from 53% to 27% of the earth's land surface and the number of countries exposed to some level of malaria risk has fallen from 140 to 106.

The most unfortunate thing in the history of malaria chemotherapy is the emergence of drug resistant strains, most importantly *P. falciparum,* to various antimalarials currently in use [2]. A recent systematic analysis has estimated that the global malaria deaths increased from 995 000 in 1980 to a peak of 1,817,000 in 2004, decreasing to 1,238,000 (929,000 - 1,685,000) in 2010 (almost double of the WHO estimate for the same year). This study estimated more deaths in individuals aged 5 years or older than has been estimated in previous studies: 435,000 (307,000 - 658,000) deaths in Africa and 89, 000 (33,000 - 177,000) deaths outside of Africa in 2010 [3].

The increasing resistance of *P. falciparum*, the most virulent of the four human malaria parasite species, to most available antimalarial drugs indicates the need for development of new antimalarial drugs and an understanding of their targets [4].

Boerhaavia diffusa (Spreading Hogweed in English) named in honour of Hermann Boerhaave, a famous Dutch physician of the 18th century [5], is mainly a diffused perennial herbaceous creeping weed of India (known also under its traditional name as *B. diffusa*) and of Brazil (known as Erva tostão) and is up to 1 m or more in length, having spreading branches. It belongs to the family Nyctaginaceae. The leaves are simple, thick, fleshy, and hairy, arranged in unequal pairs, green and glabrous above and usually white underneath. The shape of the leaves varies considerably ovate - oblong, round, or subcordate at the base and smooth above. The upper surface of the leaves is green, smooth and glabrous, whereas it is pinkish white and hairy beneath. The flowers are minute, subcapitate, present together in small bracteolate umbrellas, mainly red or rose, but the white varieties are also known. The achene fruit is detachable, ovate, oblong, pubescent, five-ribbed and glandular, anthocarpous and viscid on the ribs [6]. Due to its sticky nature, the plant gets stuck on the clothes of humans and on the legs of animals, which helps in its dispersal from one place to another. It has a large root system bearing rootlets and is about 30-50 cm deep in soil. Leaves and seeds are cooked but sometimes it can be grounded into a powder and added to cereals when making bread, cakes etc. while roots are rich in carbohydrate and protein used in baked form [7].

In the Ayurvedic herbal medicine, the plant root is mainly used to treat gonorrhea, internal inflammation of all kinds, dyspepsia, oedema, jaundice, menstrual disorders, anaemia, liver, gall bladder and kidney disorders, enlargement of spleen, abdominal pain, abdominal tumours, and cancers [8]. The juice of *B. diffusa* leaves serves as a lotion in ophthalmia. It is also administered orally as a blood purifier and to

relieve muscular pain [9]. In Nigeria, it is used for abscesses, asthma, boils, convulsions, epilepsy, fever, guinea worms, and as an expectorant and laxative. In West Africa, it is used for abortion, guinea worms, menstrual irregularities, and as an aphrodisiac. In tropical Africa, the boiled roots are applied to ulcers, abscesses and to assist in the extraction of Guinea worm.

B. diffusa contains a large number of compounds such as flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and potassium nitrate [10], a glycoprotein [11], boeravinone A-F [12], liriodendrin [13], quercetin and kaempferol [14]. The herb contains 15 amino acids such as argenine (total amino acid in herb 0.47% and in root 0.75%), alanine (total amino acid in herb 0.88% and in root 1.18%), aspartic acid (total amino acid in herb 0.69% and in root 0.95%), methionine (total amino acid in herb 0.41% and in root 0.45%), leucine (total amino acid in herb 0.67% and in root 0.88%), phenylalanine (total amino acid in herb 0.52% and in root 0.71%), proline (total amino acid in herb 0.88% and in root 1.18%), ornithine (total amino acid in herb 0.35% and in root 0.5%), serine (total amino acid in herb 0.73% and in root 0.83%), threonine (total amino acid in herb 0.72% and in root 0.79%), asparagine (total amino acid in herb 0.33% and in root 0.00%), glycine (total amino acid in herb 0.75% and in root 0.00%), valine (total amino acid in herb 0.00% and in root 0.75%), tryptophan (total amino acid in herb 0.53% and in root 0.65%), tyrosine (total amino acid in herb 0.61% and in root 0.72%) [7].

Pharmacological evaluation of extracts of *B. diffusa* has shown that it possessed anti-inflammatory [15], diuretic [16], laxative [17], anti-urethritis [18], anticonvulsant [19], anti-nematodal [20], anti-bacterial [21], anti-hepatotoxic [22].

Despite various ethnomedicinal and pharmacological claims that the plant *B. diffusa* is a good and potent medicinal plant, its antimalarial effect is yet to be elucidated, hence, this study.

2. MATERIALS AND METHODS

2.1 Plant Material and Preparation of *B. diffusa* Extract

Sample of *B. diffusa* was obtained from a dunghill behind Olatunde Memorial Nursery and Primary School Bolorunduro, Ilesa in Ilesa East Local Government, Osun State, Nigeria on 15th August 2012 by 3 pm and transported to the Herbarium Unit of the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria for identification and given IFE Herbarium voucher number, 16900. A specimen of the leaves was kept in the department's herbarium under the same voucher number.

The roots of the plant were dried in the oven at a temperature under 60°C and powdered in the grinding machine while 500 g of the powdered plant was macerated with 2.2 litres of 100% methanol with intermittent shaking twice in a day for 72 hours and then filtered. The filtrate was concentrated to dryness *in vacuo* with a rotatory evaporator (Buchi type TRE121, Switzerland). The residue was collected and stored in the refrigerator (4°C) after a paste level of dryness was attained via the rotary evaporator for onward use.

2.2 Experimental Animals

White albino mice of both sexes, weighing between 18 and 20 g obtained from Animal House, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria were used as experimental animals for this study. This was after they were allowed to acclimatize for two weeks. The animals were kept under conducive laboratory conditions and fed with animal feed (Grower's mash), and water *ad libitum*. The Ethical Committee of the Faculty Postgraduate Committee, Faculty of Pharmacy, Obafemi Awolowo University, approved the research work.

2.3 Drugs and Laboratory Materials

Standard chloroquine (Sulphate salt – Batch No. 442), obtained from May and Baker Nigeria Plc., PMB 21049, 3/5 Sapara Street, Industrial Estate, Ikeja, Lagos was employed as standard reference for the antimalarial screening in this study. Nifedipine (20 mg) tablet, manufactured by Yanzhou Xier Kangtai Pharmaceutical Co. Ltd. Private Economy Garden, Xinyan Town, Yanzhou City, Shandong, China was used as a standard calcium antagonist.

Standard laboratory glasswares were used.

2.4 Acute Toxicity

The acute toxicity (p.o. LD_{50}) of the methanolic root extract of *B. diffusa* was estimated in 13

white albino mice using standard method of Lorke [23]. This test was carried out in two phases; In phase 1 study, three groups of 3 mice each were used. The mice in the three groups were administered with the doses of 10, 100 and 1000 mg/kg body weight of the test extract respectively and monitored for 24 hours, observing for mortality.

In phase 2 study, three groups of 1 mouse in each group were used. The mice in the three groups were administered with the doses of 1600, 2900 and 5000 mg/kg body weight of the extract respectively and similarly monitored for 24 hours, observing for mortality. The phase 2 test was carried out based on the result obtained in phase 1 study.

2.5 Rodent Parasite (*Plasmodium berghei berghei)*

The rodent parasite *Plasmodium berghei berghei* NK 65 was sourced from National Institute for Medical Research (NIMR), Lagos, Nigeria and kept at Animal House, Faculty of Pharmacy Obafemi Awolowo University, Ile-Ife, Nigeria. The parasites were kept alive by continuous intraperitoneal passage in mice [24] every 4 days. The infected mice (donors) were used for the study.

2.6 *In vivo* Antiplasmodial Studies

2.6.1 Suppressive test (4-day test)

The model was carried out using the method of [25]. In this procedure, malaria parasite was obtained by collecting blood samples from mice (donor mice) infected with *P. berghei*. Thirty six (36) mice divided into 6 groups of 6 mice each were used for the study. All the mice were inoculated intraperitoneally with 0.2 ml of infected blood containing about 1 X 10^7 *P. berghei*. All the mice in all the groups were treated orally (starting at 1 hour after parasite inoculation on day 0). Group 1 was treated with normal saline, groups 2 to 4 were treated with 125, 250, and 500 mg/kg body weight of the extract orally, group 5 (positive control) was treated with 10 mg/kg body weight of the standard drug chloroquine, while group 6 was administered 15 mg/kg body weight of Nifedipine. The treatment continued daily for four consecutive days. The temperature of each mouse was taken daily by using a thermaprobe thermometer, which was inserted into the rectum of each mouse and digital reading was taken. On day 5, samples of blood were taken from the caudal vein of each mouse onto a clean slide made into thin film, fixed with methanol and stained with 10% Giemsa stain after which the number of the parasitized cells was determined microscopically and the percentage suppression evaluated by using the following equation:

$$\textbf{Mean chemosuppression} = \left(\frac{\textbf{APC} - \textbf{APT}}{\textbf{APC}}\right) \textbf{X } 100$$

APC= Average Parasitaemia in the Negative Control, **APT=** Average Parasitaemia in the Test group.

2.6.2 Evaluation of Schizontocidal activity of *B. diffusa* (Curative Test)

This was carried out according to [26]. In this procedure, malaria parasite was obtained by collecting blood samples from mice (donor mice) infected with *P. berghei*. Forty two (42) mice divided into 7 groups of 6 mice each were used for the study. All the mice were infected (intraperitoneally) on the first day (Day 0) with the parasites by inoculating them with 0.2 ml of the prepared blood solution. Seventy two (72) hours later, all the mice in all the groups were treated orally. Group 1 was treated with normal saline, groups 2 to 4 were treated with 125, 250, and 500 mg/kg body weight of the extract orally, group 5 (positive control) was treated with 10 mg/kg body weight of the standard drug chloroquine, group 6 was administered 15 mg/kg body weight of Nifedipine, while group 7 was treated with a combination of 15 mg/kg Nifedipine and 500 mg/kg of the extract. The treatment continued daily for five consecutive days and blood smears were collected and examined microscopically to monitor the parasitaemia. The temperature of each mouse was also taken daily by using a thermaprobe thermometer, which was inserted into the rectum of each mouse and digital reading was taken. Blood smears were collected and examined microscopically to monitor the parasitaemia level.

2.6.3 Evaluation of Prophylactic activity of *B. diffusa*

The prophylactic activity of the extract was performed as described by [27]. In this procedure, the adult mice were randomnized into 6 groups of 6 mice each. Group 1 was treated with normal saline, groups 2 to 4 were treated with 125, 250, and 500 mg/kg body weight of the

extract orally, group 5 (positive control) was treated with 10 mg/kg body weight of the standard drug chloroquine, while group 6 was administered 15 mg/kg body weight of Nifedipine. Treatment was initiated on day 0 and continued till day 4 when the mice were all infected (intraperitoneally) with the parasite. The temperature of each mouse was also taken daily by using a thermaprobe thermometer. Blood smears were then made from each mouse 72 hrs after inoculation on day 7 and increase or decrease in parasitaemia was determined.

2.7 Determination of Calcium Blocking Activity

The assay was performed according to the method of [28] using spectrophotometric methods.

2.7.1 Preparation of the samples

Animals were grouped into seven of 3 animals each. All the animals were then infected with the malaria parasite (Day 0). After 72 hours (Day 3), the different doses of the crude extract, nifedipine and nifedipine plus extract (500 mg/kg body weight) were administered to the appropriate groups (making a total of 5 groups). One other group received 10 mg/kg chloroquine (standard) and the remaining one group received no treatment at all (negative control). The test agents were administered for 5 days. Twenty-four hours after the last treatment (Day 7), the animals were sacrificed and the whole blood collected for assessment for calcium quantification in the plasma. The whole blood was centrifuged at 2500 rpm for a period of 15 minutes and the plasma supernatant was collected for the biochemical assay. The plasma samples were stored in the freezer until when biochemical assay was carried out.

2.7.2 Quantification of plasma calcium

The assay was performed according to the method of [28]. The principle of the assay is based on the formation of a violet complex on reaction of O-cresolphtalein complexone with calcium ions in an alkaline medium. Here, the reaction mixture contained 25 µl of the test sample, 0.5 ml of reagent 1 (2-amino-2-methyl-propan-1-ol) and 0.5 ml of reagent 2 (O-cresolphtalein complexone). Control without the sample (blank) was prepared by mixing 25 µl of distilled water with 0.5 ml of reagent 1 and 0.5 ml of reagent 2 and the standard was prepared by mixing 25 µl of calcium standard with 0.5 ml of reagent 1 and 0.5 ml of reagent 2. One drop of reagent 3 (EDTA) was then added to the prepared solutions. After about 5-50 minutes, the absorbance of the sample and the standard were read against that of the reagent blank at a wavelength of 578 nm. Activity of calcium was expressed in mg/dl or mmol/l.

2.8 Statistical Analysis

The One-way analysis of variance (ANOVA), followed by Student-Newman-Keuls' test was used to analyse and compare results. Statistical significance was set at $P < 0.05$. The results were expressed as Mean ± SEM (Standard error of mean). Computer software Graph pad PRISM® version 3.00 was used for the analysis.

3. RESULTS

3.1 Plant Extract

The weight of the brown crude extract obtained from the extraction procedure of the plant was 24.79 g and the percentage yield was 4.95%.

3.2 Acute Toxicity

For the acute toxicity investigation carried out, all the animals were still alive at the end of the experiment and the LD_{50} value was greater than 5000 mg/kg body weight and is of no practical interest. Hence, the extract can be non-toxic [23].

3.3 Suppressive Effect of *B. diffusa* Methanolic Root Extract on *P. berghei*

The % chemosuppression of the extract (125, 250 and 500 mg/kg) were 81.7, 55.9, and 54.8% respectively while that of chloroquine was 18.8% and that of nifedipine was 68.8%. The parasitaemia was observed to be increasing with increase in dose levels of the extract, an indication that the lowest dose of *B. diffusa* root extract exhibited the optimal chemosuppression. These values of the extract were significant compared to control ($p < 0.05$). Similarly, nifedipine gave a decrease % parasitaemia like the extract. Chloroquine exhibited non significant decrease in % parasitaemia compared to control but the % parasitaemia in chloroquine is much higher than in extract and nifedipine (Fig. 1).

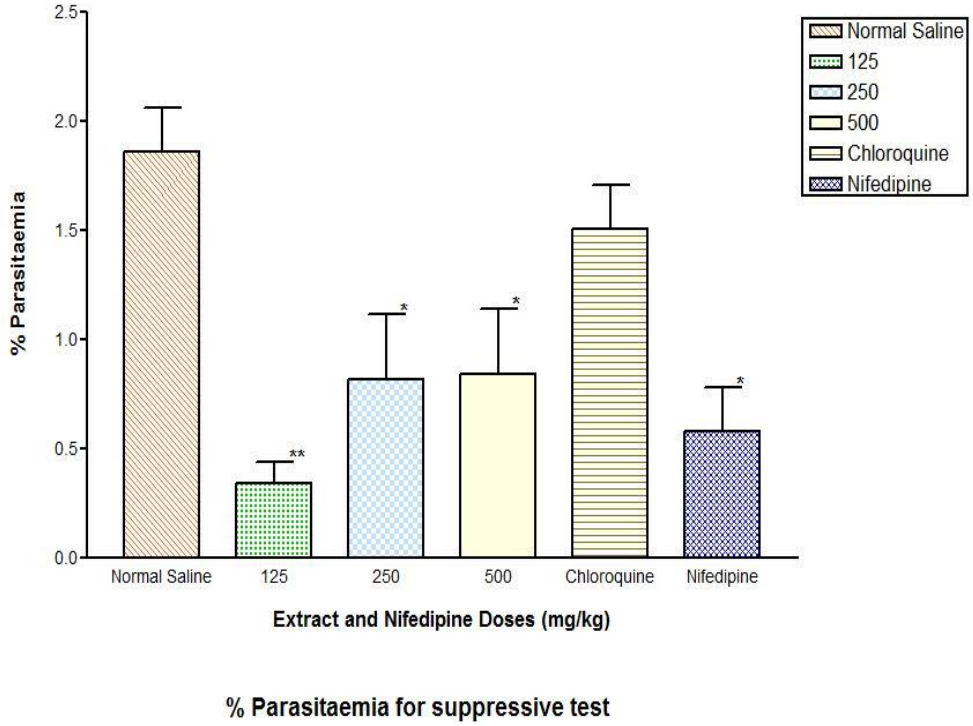

Fig. 1. Suppressive effect of methanolic root extract of *B. diffusa* on chloroquine resistant *P. berghei* in mice

*The bars are as expressed as mean ±SEM, (n = 6), (P < 0.01). * indicates P < 0.05 and ** indicates P < 0.01*

3.4 Curative Effect of *B. diffusa* Methanolic Root Extract on *P. berghei*

From the results shown in Fig. 2, 125 mg/kg displayed activity against the parasite as there was decreasing parasitaemia with increase in days except for day 9, where the parasitaemia increased, and there was also decrease in parasitaemia as compared to the control. Other doses 250 mg/kg and 500 mg/kg showed the same pattern with increase in parasitaemia on days 7 and 10 while decreasing it on days 5 and 9. The two higher dose levels demonstrated activity against the parasite by decreasing the parasitaemia when compared with the control and their activities were lower compared to that of 125 mg/kg which is in line with the activity displayed by the extract in the suppressive and prophylactic models. Chloroquine also decreased % parasitaemia as the days were increasing except for day 10 where it increased and a decrease in parasitaemia was also observed as compared to control (Fig. 2).

3.5 Prophylactic Effect of *B. diffusa* Methanolic Root Extract on *P. berghei*

The % chemosuppression of the extract (125, 250 and 500 mg/kg) were 90.0%, 82.7% and 76.8% respectively while that of chloroquine was 55.7% and that of nifedipine was 83.4%. As it was seen in the suppressive model, the % parasitaemia was observed to be increasing with increase in dose levels of the extract, indicating that optimal chemosuppression was also exhibited at the lowest dose in this model. These values were significant to control ($p < 0.05$). Nifedipine gave a similar significant ($p < 0.05$) reduction in parasitaemia like the extract as compared to control. Unlike in the suppressive model, chloroquine exhibited a significant ($p < 0.05$) decrease in % parasitaemia compared to control. However, the % parasitaemia seen in chloroquine was significantly ($p < 0.05$) higher than in extract and nifedipine (Fig. 3).

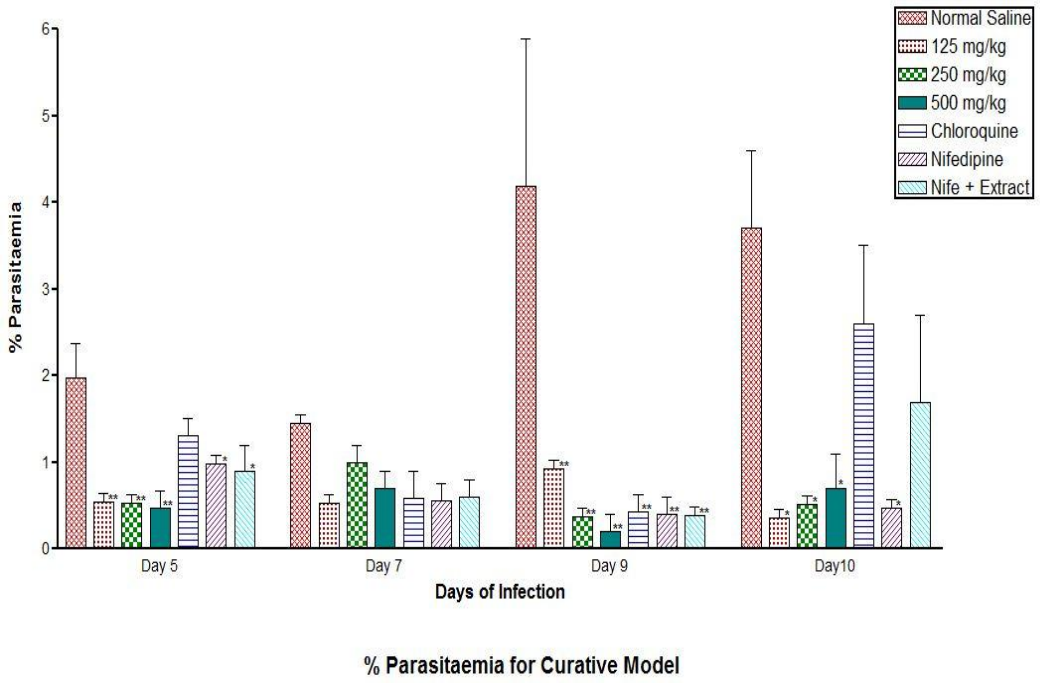

Fig. 2. Curative effect of methanolic root extract of *B. diffusa* on chloroquine resistant *P. berghei* in mice

*The bars are as expressed as mean ±SEM, (n = 6), ** indicates P < 0.01, * indicates P < 0.05*

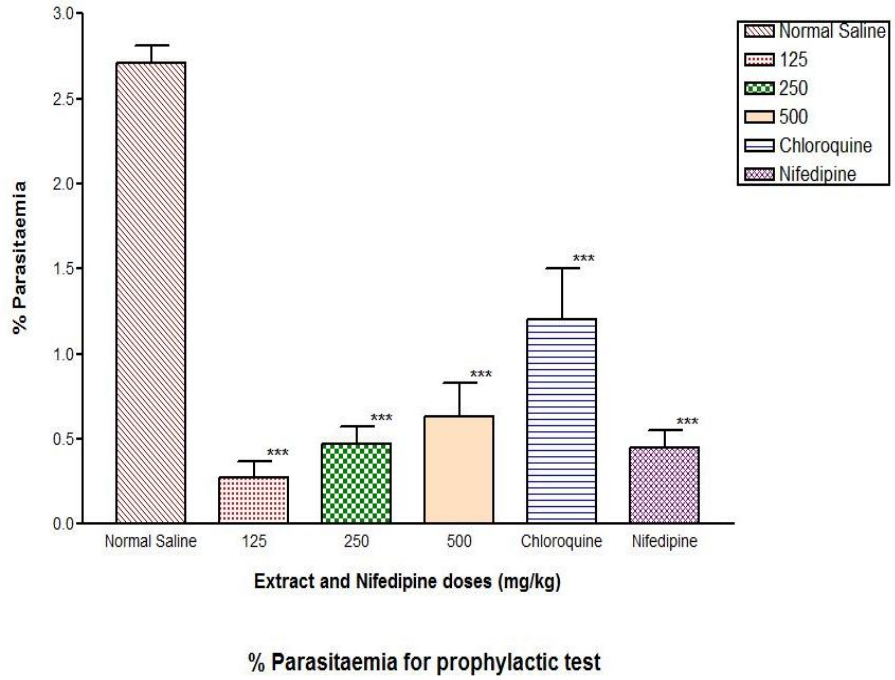

Fig. 3. Prophylactic effect of methanolic root extract of *B. diffusa* on chloroquine resistant *P. berghei* in mice

*The bars are as expressed as mean ±SEM, (n = 6), (P < 0.001). *** indicates P < 0.001*

3.5.1 The Antipyretic activity of methanolic root extract of *B. diffusa* in mice for suppressive model

Fig. 4a summarises the results of the antipyretic investigations of *B. diffusa* root extract in malaria induced mice in suppressive study. The temperature pattern from day 0 to day 5 showed that a reversal of temperature was prominent with extract (125 mg/kg), nifedipine and chloroquine as compared to the control which were all significant at day 3. However, the temperatures of mice that received extract (250 and 500 mg/kg) were lower compared to that of control. The reversal of temperature which was prominent with 125 mg/kg corresponds to an optimal activity displayed by the dose in the suppressive and prophylactic models.

3.5.2 The Antipyretic activity of methanolic root extract of *B. diffusa* in mice for curative model

The results of the antipyretic investigations of *B. diffusa* root extract in malaria induced mice for curative model are summarized in Fig. 4b. Looking at the temperature pattern from day 0 to day 7, it was observed that thebody temperature of the mice increased with the extract (125 mg/kg), nifedipine, chloroquine and nifedipine + extract as compared to the control at day 4 and day 5. However, the temperatures of mice that received extract (250 and 500 mg/kg) were lower than that of 125 mg/kg at day 5.

3.5.3 The Antipyretic activity of methanolic root extract of *B. diffusa* in mice for prophylactic model

In Fig. 4c, the temperature pattern of the extract, nifedipine and chloroquine from day 0 to day 7 as compared with the control are shown. There was an increase in body temperature with the extract (125, 250 and 500 mg/kg), nifedipine and chloroquine as compared with the control, which were all significant at day 3, although 125 mg/kg displayed the highest reversal activity. At day 4, the reversal activity continued with increase in temperature with 125 mg/kg of the extract as compared to the control, whereas the activity decreased with 250 and 500 mg/kg, which is also an evidence of the optimal antimalarial activity of the extract at the lowest dose as seen in the suppressive model.

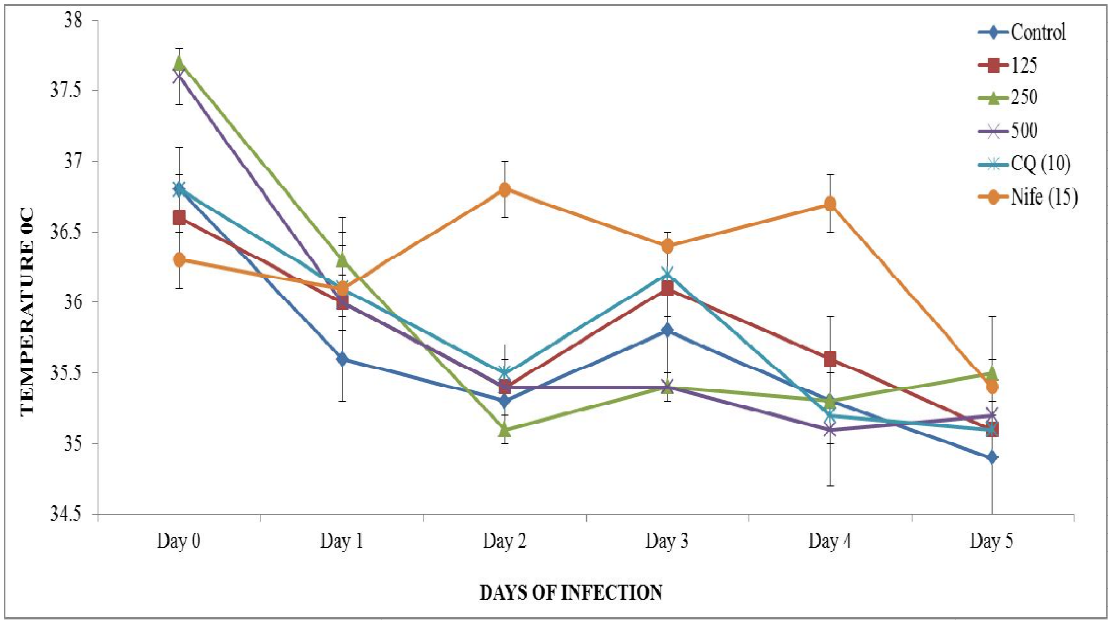

Fig. 4a. Antipyretic activity of methanolic root extract of *B. diffusa* in malaria induced mice for suppressive model

The lines with bars are as expressed as mean ±SEM, (n = 6). The values do not indicate statistical significance to one another

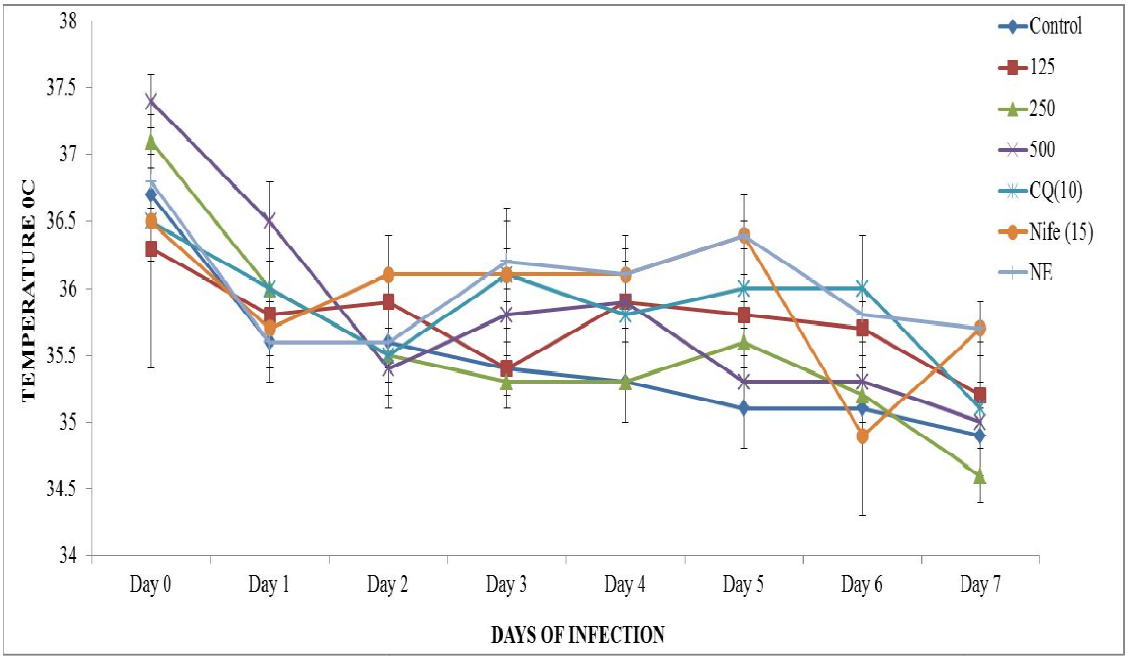

Fig. 4b. Antipyretic activity of methanolic root extract of *B. diffusa* in malaria induced mice for curative model

The lines with bars are as expressed as mean ±SEM, (n = 6). The values do not indicate statistical significance to one another

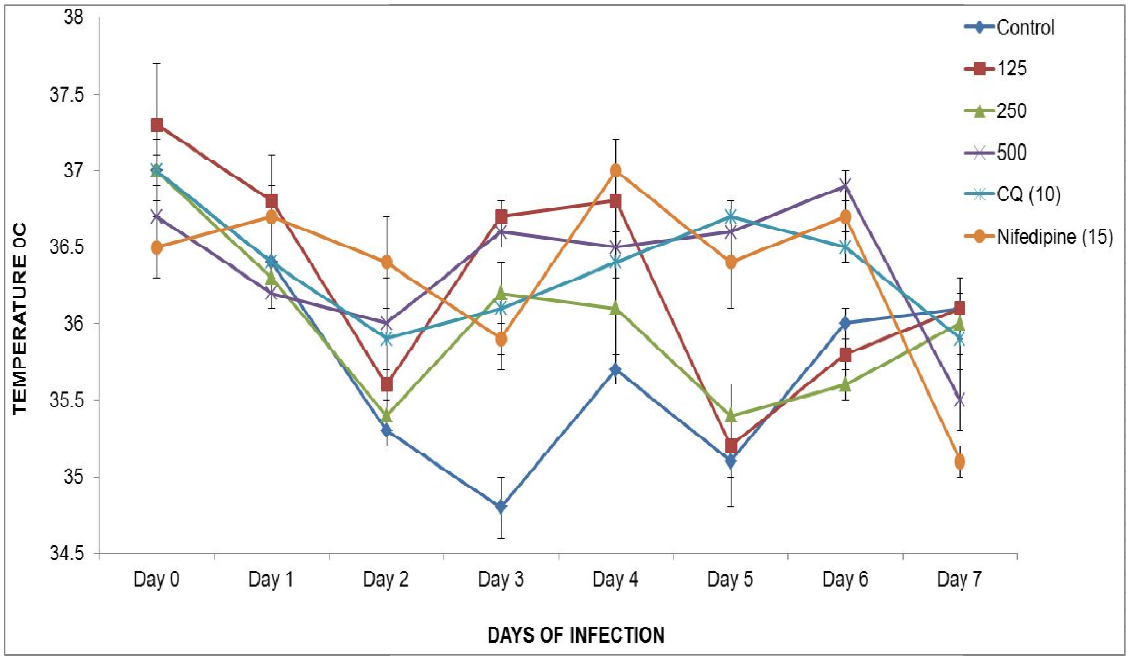

Fig. 4c. Antipyretic activity of methanolic root extract of *B. diffusa* in malaria induced mice for prophylactic model

The lines with bars are as expressed as mean ±SEM, (n = 6). The values do not indicate statistical significance to one another

3.6 Quantification of Plasma Calcium

From the result of the biochemical assay of plasma calcium (Fig 5), it was shown that the three doses of the methanolic root extract demonstrated a dose dependent reduction in the plasma calcium level as compared with the control and the standard drug (chloroquine). Nifedipine, a standard calcium blocker was shown to decrease the plasma calcium level as compared with control but the decrease is higher than that of the extract (500 mg/kg), while it is lower than 125 mg/kg. However, it appears that the level of plasma calcium increased with nifedipine + extract. The decrease in the plasma calcium level observed with all the test agents showed no statistical significance ($p > 0.05$) as compared with the control.

4. DISCUSSION

This study investigated the acute toxicity profile of methanolic root extract of *Boerhaavia diffusa*, its antimalarial activity on chloroquine resistant *P. berghei* in mice and determined its mechanism of action.

According to the method of [23], the oral median lethal dose (LD_{50}) of the crude extract was estimated to be ≥ 5000 mg/kg body weight since there were no mortality at all dose levels used. The absence of death following the oral administration of *B. diffusa* root extract at 5000 mg/kg body weight observed in mice suggests that the extract is practically non-toxic [29]. This is also buttressed by [30] that any chemical that exhibited an LD_{50} more than 5000 mg/kg is practically non-toxic. The toxicological profile of this plant is supported by that of the other specie *B. elegans* which showed no toxicity [31].

P. berghei parasite is used in predicting treatment outcomes of any suspected antimalarial agent due to its high sensitivity to chloroquine making it the appropriate parasite for this study [32]. Chloroquine phosphate was used as the standard antimalarial agent in this study for curative, suppressive and prophylactic antiplasmodial assessment because of its established activity on *P. berghei* [33]. Because the increasing resistance of *P. falciparum* has made malaria a life-threatening parasitic disease worldwide, prompting the need to develop newer antimalarial agents, the resistant strain of *P. berghei* was used in this study to evaluate the antimalarial effect of *B. diffusa.*

According to [34], agents with suppressive activity against *P. berghei* usually possess antimalarial activity, the present study has not only validated the antimalarial activity of *B. diffusa* but has also demonstrated its

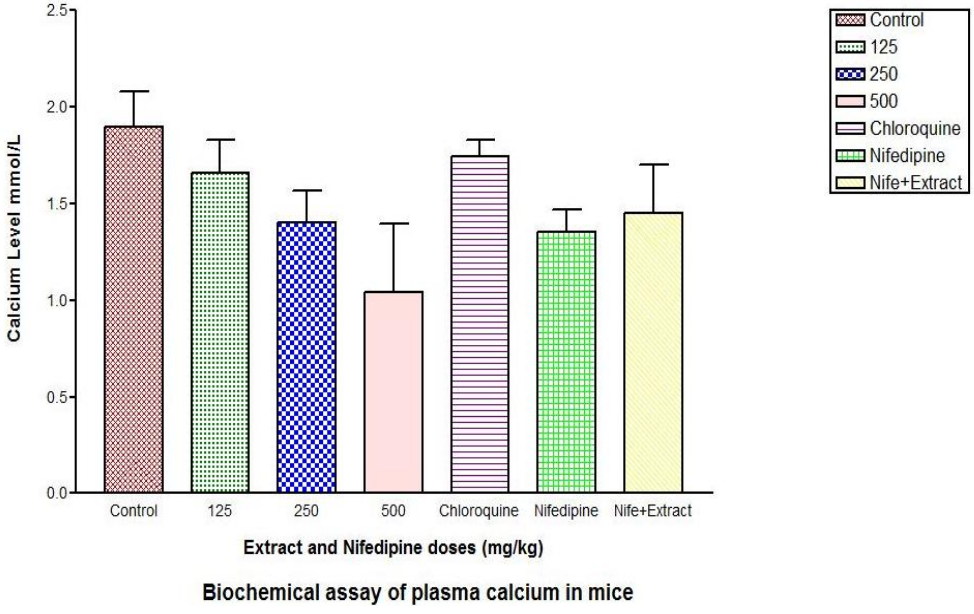

Fig. 5. Quantification of plasma calcium in mice

The bars are as expressed as mean ±SEM, (n = 6). The values do not indicate statistical significance to one another

remarkable activity as a suppressive and prophylactic agent against chloroquine resistant *P. berghei* infection in mice *in vivo*. In the 4-day suppressive and prophylactic assays, the administration of *B. diffusa* methanolic root extract resulted in the suppression of parasitaemia that was greater than the effects observed for chloroquine in the resistant *P. berghei* infected mice, although the activity displayed was optimum at the lowest dose (125 mg/kg) which began to decrease as the dose was increased. The decrease in activity observed in the extract as the dose was increased from 125 – 500 mg/kg in both the suppressive and prophylactic studies explains the fact that the crude extract exerted a pronounced activity against the malaria parasite at an optimal dose of 125 mg/kg which is in conformity to the maximum anxiolytic activity displayed by the hydro-alcoholic leaf extract of the same plant at the optimal dose of 100 mg/kg, followed by a decrease in activity as the dose was increased to 200 mg/kg [35]. This dose-dependent activity displayed by the plant extract in which the low dose gave the highest activity might be attributed to low dose stimulation of response exhibiting more potent effect than the high dose inhibition of response which could tend to produce toxicity as observed in drugs that do not exhibit the classical dose-response relationship [36].

ED_{50} was calculated to buttress the high potency at low dose. The ED_{50} of the extract for the suppressive model was 160 mg/kg and that of prophylactic model was 170 mg/kg. The ED_{50} of *B. diffusa* in the suppressive model is lower than that of *B. erecta* with the ED_{50} of 564 mg/kg [37], an indication that *B. diffusa* is a more potent suppressive agent than *B. erecta.* According to [31], *B. elegans* showed a good antiplasmodial activity against *P. berghei* (ANKA strain) in the 4-day suppressive antimalarial assay in infected mice *in vivo* which complements the antimalarial activity of *B. diffusa.* This study suggests *B. diffusa* as a good antimalarial agent in early malaria infection (in the suppression treatment of malaria) and also in prophylactic mode of treatment.

The antipyretic study of the methanolic root extract was carried out to investigate the traditional claim that the plant is being used ethnomedicinally to treat fever in folkloric medicine [38]. Malaria is known to induce body temperature in the infected host, which in humans raises the body temperature, but in rodent mice, lowers it [39]. From the results of the antipyretic study of the methanolic root extract using the three models of malaria treatment, it was shown that the root extract displayed antipyretic activity at all the models by raising the body temperature in the malaria infected mice. Figs. 4a - 4c showed that the extract displayed a reversal of temperature in all the models. The reversed temperature displayed was optimum at 125 mg/kg when compared to the control and the higher doses. This activity is in correlation with the optimum antimalarial activity by the same dose in malaria suppressive model, validating the claim of the extract in the folkore as an antimalarial agent, seeing that the extract remarkably reversed the body temperature that was lowered by malaria parasites in this study. In ethnomedicine, there are other confirmed works using *Dodonaea angustifolia* seed and *Azadirachta indica* leaves, as antimalarial agents and were able to reverse the temperature induced by malaria parasites [40,41]. *B. diffusa* methanolic crude extract displayed a more remarkable temperature reversal activity in a 4-day suppressive study than *Dodonaea angustifolia* hydroalcoholic and aqueous crude extracts which reduced body temperature in infected mice, although the butanol fraction of the aqueous extract of *D. angustifolia* displayed a strong temperature reversal activity when compared with the control and the standard at days 1 and 3 [41]. This comparison explains the fact that traditional plants are good remedies for malaria infection and fever as claimed in the folklore.

The possible mechanism of the activity displayed by the methanolic root extract of *B. diffusa* against *P. berghei* was also investigated via the blocking of calcium ion; a mechanism needed for the parasite survival and its re-invasion of the erythrocytes. Nagamune et al. [42] has shown that the invasion of red blood cells by plasmodium is known to involve calcium ion (Ca^{2+}) fluxes which might trigger AMA1 (Apical membrane antigen1) phosphorylation. During invasion of malaria parasite into the host cell, the intracellular Ca^{2+} ion in its cytoplasm are released from the stores in ER. Occasionally, parasites replenish its required Ca^{2+} ion from the extracellular into the parasitophorous vacuole [43]. Therefore the blockade of these Ca^{2+} movements could hinder the activity of malaria parasite growth and development in the host cells.

In a normal mouse, the ionized calcium range is generally 1.1 – 1.4 mmol/L [44]. However, from the results of this study, it is evident that calcium level in control malaria mice is very high, an indication that the calcium level is increased in malaria infection.

The methanolic root extracts decreased plasma calcium as compared to control and the standard calcium blocker (Nifedipine). The calcium blocking activity of the methanolic root extract was shown to be dose dependent which may be an indication that the extract displays antimalarial activity by blocking calcium channels in the erythrocytes [45] when compared with the standard calcium blocker. Evidences have shown the involvement of Ca^{2+} antagonist (verapamil) reversing chloroquine resistance *P. falciparum* in vivo and in-vitro [46,47]. The reports of these authors buttressed the involvement of calcium ions in malaria activity and also support the results obtained in this study. To further indicate that the activity of *B. diffusa* involves calcium ions, the earlier study of Mandeep and Rajesh [48] pointed to the fact that the anticonvulsant effect of *B. diffusa* was due to its calcium ion channel antagonistic action as this activity was retained only in the liriodendrin-rich fraction. Hence, the antimalarial activity of the crude extract could be as a result of its calcium blocking activity.

5. CONCLUSIONS

The remarkable activity of *B. diffusa* root extract on malaria parasites were clearly demonstrated in this study. Also, its action in reducing pyrexia induced by malaria, which corroborates its use in treating fever as claimed in the folklore shown in this experiment, affirms its usefulness in the treatment of malarial infection. The outcome of the investigation of the involvement of plasma calcium throws light on the plausible mechanism by which the plant exhibits its antimalarial action through blocking the calcium channel in the erythrocytes. It can therefore be concluded that the extract of *B. diffusa* is safe and possesses a potent antimalarial and antipyretic activities.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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