In vivo antimalarial activity of yoyo bitters®, a polyherbal formulation against *Plasmodium berghei* in Swiss mice

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ABSTRACT

Background: The emergence of drug resistance necessitates the search for new antimalarial agents. Yoyo Bitters® is a branded polyherbal formulation commonly used in Nigeria.

Objectives: The objective of the study was to investigate the *in vivo* antimalarial activity of Yoyo Bitters® in Swiss mice.

Methods: Yoyo Bitters® was evaluated in a 4-day suppressive test against *P. berghei*. The treatment groups with five animals were inoculated with *P. berghei* on Day 0. Two hours post-infection, the mice in the test groups were treated with 200, 400 and 800mg of Yoyo Bitters® per kg body weight of mice, respectively this was done considering the dosage in humans. Negative control was administered distilled water (5 ml/kg), while positive control received chloroquine phosphate as (5 mg/kg). Blood smears were examined for parasitaemia on Day 4.

Results: The average percentage parasitaemia in the negative control, 200 mg/kg, 400 mg/kg, 800 mg/kg and positive control groups were 24.11 %, 5.06 %, 10.0 %, 3.50 % and 0 %, respectively. Yoyo Bitters® dosage groups: 200, 400 and 800 mg/kg suppressed the parasitaemia by 79.0 %, 58.5 % and 85.5 %, respectively ($p>0.05$). Positive control had 100 % suppression.

Conclusion: The bitters exhibited a dose independent suppressive activity on *P.berghei i*n mice. These results support the use of Yoyo bitters as herbal medicine for the suppression of malaria.

Keywords: malaria, parasitaemia, *Plasmodium berghei*, Yoyo Bitters®

Activité antipaludique *in vivo* de Yoyo Bitters ®, une formulation polyherbale contre *Plasmodium berghei* chez les souris suisses

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RÉSUMÉ

Contexte: L'émergence de la résistance aux médicaments nécessite la recherche de nouveaux agents antipaludiques. Yoyo Bitters® est une formulation polyherbale de marque couramment utilisée au Nigéria.

Objectifs: L'objectif de cette étude était d'étudier l'activité antipaludique in vivo de Yoyo Bitters ® chez des souris suisses.

Méthodes: Yoyo Bitters® a été évalué lors d'un test de suppression de 4 jours contre *P. berghei.* Les groupes de traitement composés de cinq animaux ont été inoculés avec *P. berghei* au jour 0, deux heures après l'infection, les souris des groupes tests ont été traitées avec 200, 400 et 800 mg de Yoyo Bitters® par kg de poids corporel de souris, respectivement, ceci a été fait en tenant compte du dosage chez l'homme. Le témoin négatif a reçu de l'eau distillée (5 ml/kg), tandis que le témoin positif a reçu du phosphate de chloroquine (5 mg/kg). Les frottis sanguins ont été examinés pour la parasitémie au jour 4.

Résultats: Le pourcentage moyen de parasitémie dans les groupes témoin négatif, 200 mg/kg, 400 mg/kg, 800 mg/kg et témoin positif était respectivement de 24,11 %, 5,06 %, 10,0 %, 3,50 % et 0 %. Les groupes de dosage Yoyo Bitters® : 200, 400 et 800 mg/kg ont supprimé la parasitémie de 79,0 %, 58,5 % et 85,5 %, respectivement (p>0,05). Le témoin positif a eu une suppression de 100%.

Conclusion: Les amers ont montré une activité suppressive indépendante de la dose sur *P.berghei* chez la souris. Ces résultats soutiennent l'utilisation des amers Yoyo comme médicaments à base de plantes pour la suppression du paludisme.

Mots clés: paludisme, parasitémie , *Plasmodium berghei* , Yoyo Bitters ®

INTRODUCTION

Malaria is one of the communal diseases of man contributing to stern sociocultural, economic and health influences in humid, middle-income nations, sub-Saharan Africa, Southeast Asia and South America.[1,2] It is caused by *Plasmodium ovale*, *P. malariae, P. falciparum, P. vivax* and *P. knowlesi*.[3] Malarial infections in Africa, the Caribbean, Asia and South America is caused by *P. falciparum*, the most lethal malaria parasite.[4] Other regions such as India, Central America, East Mediterranean, Papua New Guinea and some areas in sub-Saharan Africa experience the causal agent of the disease to be *P. vivax, P. ovale* and *P. malariae* as the case may be.[5]

Malaria epidemic remains enormously high in low socioeconomic empowered regions. As evidence, Africa continues to carry a disproportionately high share of the global burden. More than 94 % of malaria cases and deaths occur in this region with children aged under 5 years being the most affected.[6] According to World Malaria Report 2020, between 2000 and 2015, the global malaria case incidence declined by 27 %, whilst between 2015 and 2019 it declined by less than 2 %, indicating a slowing of the rate of decline since 2015. This trend could be indicative of the widespread drug-resistant malaria and the intricacy of the parasite's life cycle.[7]

Quinoline (QN) derivatives are undoubtedly the commonest form of antimalarial drugs in Africa,[4] but their efficacy has been widely challenged with the emergence of *Plasmodium falciparum* resistance. In recent times, QN derivatives have been an integral component of the current of antimalarial drugs; Artemisinin-based Combination Therapy (ACT)[8] - an effective drug against all malaria parasites which led to regulations against quinine-based drugs in Africa. Recently, despite the predominant achievement of ACT, concerns about its future efficacy have been on the rise due to the build-up of resistance by the parasite.[9] Resistance to artemisinin has been reported in five Asian countries: Cambodia, Laos, Myanmar, Thailand and Vietnam.[2] This event instigates the unrelenting search for antimalarial drugs that are cost-effective, handy, acceptable, and scientifically proven.

Medicinal plants are known to possess several remedial properties and over the years have also been a source of antimalarial drugs i.e. quinine and artemisinin. In Africa, herbal remedies are prepared from them to treat numerous diseases. In the southern parts of Nigeria, these plants are processed into mono or polyherbal formulations and commercialized as patent medicine. Among these bitters is Yoyo Bitters®, one of the most popular and widely marketed in the country to treat several illnesses. The manufacturers claimed it to be effective against malaria, but there is yet to be a published study supporting this claim. Armed with the knowledge of malaria endemicity in the country with persistent concern of drug resistance of the parasites, it has become very important to seek indigenous solution to the problem. This study was therefore initiated to either confirm or debunk the claims of the manufacturers of Yoyo Bitters® as a means of moving forward in the race to eradicate malaria.

Malaria is a major public health challenge and a key problem facing the fight against the disease is the development of resistance to antimalarial drugs and insecticides, hence the need for continuous search for antimalarial agents. Resistance of *Plasmodium falciparum* to previous generations of antimalarial drugs, such as chloroquine and sulphadoxine-pyrimethamine which became widespread in the 1950s and 1960s, including artemisinin derivatives have been documented, undermining malaria control and reversing gains in child survival.[10]

Plants have been major sources of drugs and hence could be a potential antimalarial drug. Yoyo Bitters® is amongst herbal preparations that have gained much publicity through the Nigerian news media and have received wide patronage across the various geo-political zones of the country.[11] Generally, consumers view the product as a safe and effective alternative to conventional therapies. The product, as commonly called in Yoruba language, "gbogbo nishe" meaning *"multipurpose"* or "cure all", claims to be effective in treatment of many ailments including malaria. It is affordable, readily available and registered with National Agency for Food and Drug Administration and Control (NAFDAC). Scientific studies have proven some of its phytoconstituents to have antioxidant,[12] hypoglycemic,[13] and hypolipidemic effects.[14,15] However, none of these studies evaluated its antimalarial activity. Based on this, the present study was carried out to determine the in vivo antimalarial activity of Yoyo Bitters® against *Plasmodium berghei*, a rodent malaria parasite.

Yoyo Bitters® is a Nigerian herbal bitters which was launched into the market by Abllat Company Nigeria Limited in 2003. It is a slightly acidic liquid (pH 5.46) formulated from water, soluble vitamins (e.g., vitamins B1, B2, B3, B6 and B12), minerals (copper, zinc, iron) and

medicinal plants such as *Aloe vera, Acinos arvensis, Citrus aurantifolia, Chenopodium murale, Cinnamomum aromaticum*.[16]

Citrus aurantifolia is one of the constituent plants of Yoyo Bitters®. It is a citric fruit used worldwide in cuisine and belongs to the Rutaceae family.[17] According to Ettebong *et al*. (2019), the preliminary phytochemical screening of the methanol leaf extract of *C. aurantifolia* showed the presence of alkaloids, saponins, flavonoids, cardiac glycosides and tannins.[18] Alkaloids are one of the major classes of compounds possessing antimalarial activity, and one of the oldest and important antimalarial drugs, quinine belongs to these compounds.[19] Saponins, flavonoids and tannins have been suggested to act as primary antioxidant or free radicals scavengers that can counteract the oxidative damage induced by the malaria parasite.[20]

The presence of saponins, alkaloids, flavonoids and tannins in the methanol leaf extract of *C. aurantifolia* justify the anti-plasmodial activity exhibited by the plant extract. In the suppressive test, the methanol leaf extract of *C. aurantifolia* showed a reduction in parasite density/µL/blood in mice treated with low and middle doses of extract. However, parasite density was found to increase in the high dose of extract compared to control group. This depicts that the extract has a partial agonistic activity.[18]

MATERIALS AND METHODS

Study design

This is an exploratory experimental study with a four-day follow-up period.

Drugs: A 0.5 grams salt of chloroquine phosphate (CQ) (Emzor Pharma, Nigeria) was dissolved in 10 ml of distilled water to final doses of 5 mg/kg body weight.

Herbal Bitters: Yoyo Bitters® (Abllat Company, Nigeria) was purchased from a reputable pharmacy in Mushin, Lagos State, Nigeria. The bitters was bought as liquid formulation and stored at room temperature throughout the period of the experiment.

Parasite: A Chloroquine-sensitive strain of *Plasmodium berghei* (NK-65) was obtained from Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria, and was maintained by sub passage in mice.

Experimental animals: Twenty-five (25) pure adult Swiss mice strains weighing between 16-28 g were obtained from the animal house of Nigeria Institute of Medical Research (NIMR), Yaba, Lagos. The animals were observed under 12 hours light/dark cycles in clean cages in the animal house and were fed with mice pellets diet (Ladokun feeds, Ibadan Nigeria) and water for one week so as to acclimatize to room temperature of 29^{o}C. This method was carried out prior to grouping according to body weight into various experimental groups of five animals per group, designated as 200 mg/kg, 400 mg/kg, 800 mg/kg, CQ (chloroquine phosphate) and NC (negative control) groups.

A donor mouse infected with the *Plasmodium berghei* strain of the rodent malaria was used for parasite inoculum preparation. This donor mouse was previously inoculated intra-peritoneally with 0.1 mL of the infected blood containing about 1×10^6 *P. berghei* parasitized red blood cells (RBCs). The mouse was left for 72 hours to allow the parasitaemia build up and a thin blood film was made from the tail of the mouse 72 hours post inoculation. The smear was prepared by spreading the blood on a clean slide, allowed to dry, fixed with methanol, and stained with 10 % Giemsa stain for 10 minutes. The blood film was examined under microscope (Olympus CX, Japan) with the oil immersion objective (x100) to determine the parasitaemia. This is necessary to determine the level of parasitaemia as the mouse can only be used as donor when 30-40 % parasitaemia has been attained. 0.1 ml of blood was drawn through the retroorbital vein and then diluted in 3 mL of phosphate buffered saline to obtain the final inoculum. Each mouse used for this study was injected intra-peritoneally with 0.1 mL (1.0×10^6) of the solution, which is the standard inoculum for the infection of a single mouse.[20]

The body weight of each mouse in all the groups was measured before infection (D0) and on the day before blood collection (D4). It was measured by a sensitive digital weighing balance (SF - 400).

In studying the antimalarial activities of Yoyo Bitters®, the standard 4-day suppressive method was employed.[20] Swiss mice weighing between 16 g and 28 g were inoculated with *P. berghei* and shared into five groups of five mice per cage. The infected mice were grouped into five different groups [three test groups (200 mg/kg, 400 mg/kg and 800 mg/kg) and two control groups (CQ and NC)]. Yoyo Bitters® Solution bought was administered to the three test groups in three different dosage - 200mg/kg, 400mg/kg and 800mg/kg having 0.2 ml, 0.4 ml and 0.8 ml doses respectively. The doses of the three

groups were calculated using the v/v considering the human body weight of 60 kg to 2 table spoons (20 ml). CQ group (positive control) received 5 mg/kg of CQ which is 0.1 ml, while NC group (negative control) received 5mg/kg of distilled water which is 0.1 ml. The bitters and drug were administered as a single dose per day through oral gavage by using oral cannula to ensure safe ingestion. Treatment with Yoyo Bitters® commenced after two hours of infection on D0 and was continued daily for four days (i.e., from D0 to D3) whereas treatment with CQ was for three days. On the fifth day (D4) blood samples were collected from tail snip of each mouse.[22,23]

On the fifth day (D4), thick and thin blood films were made on microscopic slides using blood obtained from the tip of the tail of each mouse in the model. The slides were stained with 3 % Giemsa stain for 45 minutes. Then, each stained slide was examined under the microscope with an oil immersion objective of 100 x magnification power, to evaluate the parasitaemia levels in animals with respect to the control groups.

Parasitaemia determination was done by counting the number of parasitized RBCs against non-parasitized RBCs using a light microscope with an objective lens magnification power of x100. Percentage parasitaemia and percentage suppression were calculated using modified Peters and Robinson formula.[24]

$$\% \text{ Parasitaemia} = \frac{\text{Number of parasitized RBCs}}{\text{Total number of RBCs count}} \times 100$$

$$\% \text{ Suppression} = \frac{\text{Parasitaemia in negative control} - \text{Parasitaemia in study group}}{\text{Parasitaemia in negative control}}$$

Data analysis

All analysis were performed using Microsoft Excel 2013 (Microsoft Office Professional Plus 2013). Result of the study parameters are expressed as mean ± standard error of mean (SEM). The measures of central tendency (mean) and standard error of mean were used to describe the percentage parasitaemia value of the different groups in the results. Significance was determined using Kruskal-Wallis Test Calculator. P value <0.05 was taken to be significant at 95 % confidence limit.

RESULTS

The % parasitaemia was calculated for each group from the data obtained from the study. Then, the mean and standard error of mean (SEM) of the % parasitaemia for each group was calculated. The three test groups (200mg/kg, 400mg/kg, and 800mg/kg) had their mean % parasitaemia and SEM to be 5.1% ± 1.51, 10.0 % ± 0.86 and 3.5 % ± 2.10 respectively. While the CQ group had 0% parasitaemia and the NC group had 24.1 % ± 5.89 SEM. This shows that the three tests' groups suppressed parasitaemia level compared to the NC groups and no significant ($p>0.05$) difference between the outcome of the different doses (Table 1). Thus, their effects were not dose dependent.

The percentage suppression was calculated from the average percentage parasitaemia. From the calculation, the 200 mg/kg group showed 79.0 % suppression, the 400 mg/kg group showed 58.5% suppression, the 800 mg/kg group showed 85.5 % suppression, the CQ group showed 100 % suppression and the NC group showed 0 % suppression of parasitaemia (Figure 1).

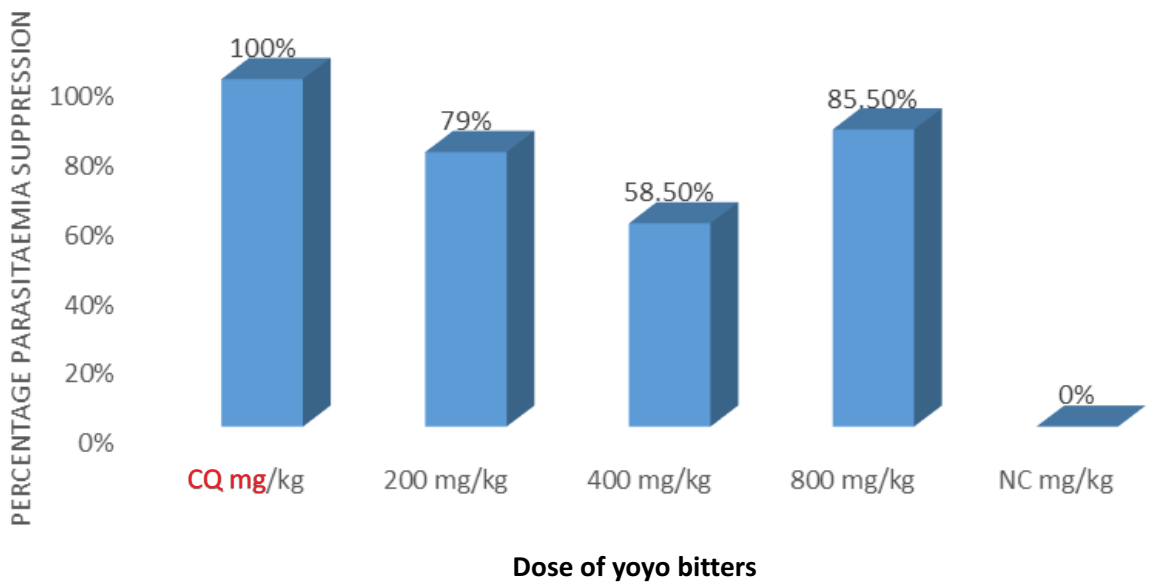

Figure1: Graph Showing Different Doses (Concentration) of Yoyo Bitters® against Percentage Parasitaemia Suppression

Table 1: Average percentage parasitaemia in infected mice

S/N	Groups (Dose)	Mean ± SEM
1.	NC	24.11 ± 5.89
2.	200 mg/kg	5.06 ± 1.51
3.	400 mg/kg	10.00 ± 0.86
4.	800 mg/kg	3.50 ± 2.10
5.	CQ (5mg/kg)	0.00 ± 0.00

Values are expressed as Mean ± SEM; SEM = Standard Error of Mean; NC = negative control; CQ = chloroquine phosphate.

The mean and SD of body weight of mice for each group on the first day (D0) and fifth day (D4) was calculated from the measurements obtained. The percentage Body Weight Change (% BWC) was then determined. From the calculation, the 200 mg/kg group showed 14. 4 % BWC, 400 mg/kg showed 9.6 % BWC, 800 mg/kg showed 16.0 % BWC, CQ group showed 12.9 % and the NC group showed 12.4 % BWC. All showed positive values which signifies weight gain (Table 2).

Table 2: Body Weight Change of Infected Mice Treated with Yoyo Bitters® in the 4-Day Suppressive Test.

S/N	Groups (Dosage)	Body Weight ± SD		
		D0 (Mean ± SD)	D4 (Mean ± SD)	% BWC Change
1.	NC	17.8 ± 0.8	20.0 ± 2.7	12.4
2.	200 mg/kg	22.2 ±0.8	25.4 ± 2.7	14.4
3.	400 mg/kg	23.0 ± 0.7	25.2 ± 1.8	9.6
4.	800 mg/kg	26.2 ± 5.8	30.4 ± 0.9	16.0
5.	CQ (5mg/kg)	18.6 ± 0.9	21.0 ± 1.0	12.9

Values are expressed as Mean ± SD; SD = Standard deviation; BWC = Body Weight Change; D = day

DISCUSSION

Due to the possible prodrug effect and involvement of the immune system in eradication of infection, an in vivo model was adopted in this study.[25] Chloroquine-sensitive *P.berghei* was used for the induction of malaria because of its ability to produce a rodent model malaria similar to human malaria infection.[26] The antimalarial activity of Yoyo Bitters® was evaluated by the 4-day suppressive test, a standard model for the antimalarial screening.[27] The study demonstrated the inherent effects of different doses of Yoyo Bitters® on *P. berghei* in Swiss mice by comparing the parasite suppression of the different doses to CQ, a standard antimalarial agent.

Average % parasitaemia was highest (24.1 %) in NC group. This implies that mice treated with Yoyo Bitters® had lower parasitaemia than the untreated mice (NC group) with average % parasitaemia of the mice treated with the highest dose (800 mg/kg) being 3.5 %. All the mice treated with Yoyo Bitters® at the oral dose range of 200-800 mg/kg daily resulted in greater antimalarial suppressive activity on % parasitaemia compared to the NC group ($p>0.05$). Whereas CQ group which served as positive control group totally suppressed the parasite on third day (D2) under identical conditions. The suppressive effect of the bitters was also found to be dose independent that is, the doses are not the factor for reduction in the parasitemia level. The highest 85.5 % being recorded at 800 mg/kg dose while the lowest is 58.5 % at 400 mg/kg. These results are consistent with previous results from the studies of the antimalarial activity of extracts of the component plants of Yoyo Bitters® (4,18).

Moreover, in vivo antimalarial activity of an agent can be classified as moderate, good or very good if its % suppression of parasitaemia is < 50 % at doses > 100 mg/kg/d[28] and compounds responsible are considered active when suppression in parasitaemia is < 30 %.[27] Based on this, this study clearly showed that Yoyo Bitters® could suppress the level of parasitaemia with % suppressions of 79.0 %, 58.5 % and 85.5 % at doses of 200, 400 and 800 mg/kg respectively and exhibit good *in vivo* suppressive activities comparable to that of CQ, the standard drug tested.

Furthermore, body weight loss is one of the cardinal signs of malaria-infected mice.[29] Therefore, antimalarial agent is expected to mitigate weight loss due to increasing levels of parasitaemia.[30] At the end of the study, a general weight gain was observed with the 800 mg/kg group having the highest % BWC. This shows that Yoyo Bitters® did not have any effect on the body weight.

Lastly, some of the individual bioactive constituents of Yoyo Bitters® - terpenoids, phenols and flavonoids - revealed in the study by James-Okoro *et al*. (2020), proven to possess antimalarial activity[31] either by acting singly or in synergy via various mechanisms, could be ascribed to be responsible for the result of this study's

findings.32

CONCLUSION

Yoyo Bitters® exhibited noticeable antimalarial activity *in vivo* in a dose independent manner. Suppressive activity of Yoyo Bitters® on *P. berghei* in Swiss mice was observed at all the doses administered, but in comparison with the positive control group, which was administered CQ, the polyherbal formulation did not produce a total suppression of 100 %. Also, the bitters was found to not have any effects on the weight of the animals. Therefore, these results support the use of Yoyo bitters as herbal medicine for the suppression of malaria.

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