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Evaluation of the Antiplasmodial Activity of the Aqueous and n-hexane fractions of *Citrullus lanatus* leaf in *Plasmodium berghei beghei* infected Mice

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There is still high morbidity and mortality from malaria in Sub-Saharan Africa and Nigeria. Reports of the emergence of resistance to artemisinin combination therapy, will aggravate the situation. This study evaluated the *in vivo* antiplasmodial activities of aqueous and n-hexane fractions of *Citrullus lanatus* leaf extracts in mice infected with *Plasmodium berghei berghei*. Median lethal-dose (LD_{50}) of the extracts were determined using Lorke's method. The antiplasmodial activity was investigated using three standard methods: a 4-day suppressive, curative and prophylactic tests. Tests groups were administered three graded doses (50, 100 and 200 mg/kg) of the extracts. Chloroquine (5 mg/kg) and pyrimethamine (1.2 mg/kg) were used as positive controls while normal saline (10 mL/kg) was the negative control. Preliminary phytochemical screening revealed the presence of alkaloids, saponins, flavonoids, tannins, phytosterols, reducing sugars and phenolic compounds. The antimalarial activity of both fractions showed significant ($p < 0.05$) dose-dependent reductions in parasitaemia compared to the negative control. For suppressive test, the aqueous and n-hexane fractions of *Citrullus lanatus* at their highest doses (200 mg/kg), gave maximum of 90.95% & 74.07% chemo-suppression respectively comparable to 94.65% for chloroquine. For curative test, the aqueous fractions showed significant ($p < 0.002$) schizonticidal activity at all doses. The prophylactic effect of the n-hexane fraction showed significant reductions in parasitaemia ($p < 0.05$) at all doses. This result demonstrates that the leaf of *Citrullus lanatus* possesses significant antiplasmodial activity and is safe for the ethnomedicinal management of malaria.

Keywords: Antiplasmodial, *Citrullus lanatus,* Leaf, *Plasmodium berghei berghei,* Fractions.

1. Introduction

Malaria is a parasitic disease of high morbidity and mortality. It accounts for 7.8% of the fraction of deaths in children less than 5 years of age globally, has been and is still a major health concern especially in the tropics and subtropics where malaria is more prevalent affecting more than 3 billion people annually [1, 2]. The fight to eliminate malaria from endemic countries is still in progress, although meaningful results have been achieved over the last 10 years, however the battle is not completely over [3]. Recent reports have shown a stable rate of infection, with the 69, 000 increase in deaths and 47, 000 new cases reported for the year 2020, which was attributed to disruptions in services and interventions for malaria due to the COVID-19 pandemic outbreak, as well as new methods adopted for computing burden of malaria [4].

In year 2020, 95% of morbidity and 96% mortality of the global burden of malaria was recorded in Africa, of which, 80% were children under 5 years of age [4]. Nigeria currently has the highest burden and infection rates for malaria globally, and is primarily due to *Plasmodium falciparum* [4]. Malaria interventions in Nigeria, both preventive and chemotherapeutic are still being challenged by parasite resistance [4], although the recently approved malaria vaccine is been deployed in some endemic countries and have shown to reduce the severity of the disease in young children, however in some countries, the vaccine is yet to be deployed [5]. Recent reports of the emergence of partial resistance to the artemisinin combination therapies (ACT),

currently the most effective and reliable treatment against malaria is worrisome [4, 6]. The ACTs are used as first- and second-line treatments for malaria in countries endemic for the disease [4]. Hence, there is an urgent need to search and develop new lead compounds that are potent and safe alternatives. *Plasmodium falciparum* the most predominant causative parasite in the sub-Sahara Africa and the most virulent *species* capable of progressing into severe malaria and has developed resistance to all currently available antimalarials [4]. To achieve the African Union Assembly set goals of malaria elimination and significant reductions in both morbidity and mortality by the year 2030, will require commitment, coordinated efforts of all stakeholders and unwavering focus on reaching out to the vast population of the endemic areas of the developing world [3].

Traditional medicines have track records of accessibility, cost effectiveness, safety and valuable phytochemicals of medicinal importance [7]. The recent focus on sourcing medications from natural origin (such as plants) is to a large extent a realistic approach to containing the problem of malaria in the short and long run, as there exist so much to be discovered from plants. The search for a long-lasting solution to the menace of malaria is a top priority, as claims need to be validated for better understanding of safety, efficacies and maximum utilization of ethnomedicines.

Citrullus lanatus, English name water melon, is a prostrate annual herbaceous plant belonging to the family *Cucurbitaceae.* The leaves are 60-200mm long and 40-150mm broad. it produces fruit which is usually globose to oblong or ellipsoidal, sometimes ovoid containing about 92% of water by weight, 6% of sugar [8,9,10]. The plant is widely distributed in temperate regions of Africa, central Asia and Mediterranean. Different parts of *Citrullus lanatus* have been used in the treatment of various ailments, the leaves have been traditionally used to treat malaria, pains, infections and other diseases [11, 12,13, 14]. This study therefore seeks to evaluate the antiplasmodial activity of the aqueous and n-hexane fractions of *Citrullus lanatus* leaf in *Plasmodium berghei berghei* infected mice in order to achieve the best result in the utilization of this plant.

2. Materials and Methods

2.1 Collection and identification of plant material

The fresh leaves of *Citrullus lanatus* were collected from Isa local government area of Sokoto State, Nigeria, in June 2021. It was identified and authenticated by a botanist in the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto, Nigeria where a voucher specimen (PCG/UDUS/CURC/004) was deposited in the herbarium for reference.

2.2 Preparation of the Extract

The leaves of *Citrullus lanatus* were shade dried for two weeks and the dried leaves were reduced to powder using pestle and mortar; 500 g of the powdered leaves was macerated in 2 L of methanol for 48 hours at ambient temperature. The mixture was then filtered using Whatman filter paper size No.42 with a pore size 125 mm and the filtrate was dried at 40^0C. The percentage yield of the crude extract was calculated and the crude extract obtained was further suspended in distilled water separately, filtered and partitioned successively with solvents of increasing polarity. The fractions were concentrated using rotary evaporator at 40^0C, the extracts were labelled and stored away in airtight containers for subsequent use.

2.3 Experimental animals

Both sexes of Swiss *albino* mice locally bred, aged between 6 and 8 weeks and weighing 25 - 32 g were used for this study. The mice were purchased from the animal facility unit of Faculty of Pharmaceutical Sciences Ahmadu Bello University Zaria, Kaduna State, Nigeria and transported to the animal house of the Department of Pharmacology and Toxicology, Usmanu Danfodiyo University Sokoto where they were housed. The animals were fed with commercial feeds and water *ad libitum* and maintained under standard conditions (12 hours light and 12 hours dark cycle) in propylene cages at 25^0C room temperature. All experimental procedures were performed with the guidelines of the Animal Right Ethics Community of Usmanu Danfodiyo University, Sokoto. Ethical animal approval number PTAC/CI/(He)/OT/59-23 was issued.

2.4 Malaria parasite (*Plasmodium berghei* parasite)

Chloroquine sensitive strain of *Plasmodium berghei* (*NK65*) was purchased from the Faculty

of Pharmaceutical Sciences, Ahmadu Bello University (A.B.U), Zaria, Nigeria. The parasite was retained in the Department of Pharmacology and Toxicology Laboratory, Usmanu Danfodiyo University, Sokoto by continuous intraperitoneal passage of infected blood into healthy mice after every 3-5 days of blood from infected mice into healthy mice.

2.5 Parasite inoculation

A standard inoculum was prepared by diluting blood containing infected red cells (blood from infected donor mice) with normal saline to obtain $1x10^7$ in 0.2 mL. The mice were then inoculated with 0.2 mL of the infected blood intraperitoneally using hypodermic needle fitted to 1 mL syringe.

2.6 Preliminary Phytochemical Screening of Extract

Qualitative phytochemical screening for the presence of different secondary metabolites such as carbohydrates, alkaloids, flavonoids, tannins, phytosterol, and reducing sugars was carried out using standard procedures [15, 16].

2.7 Determination of Acute toxicity

The LD_{50} was evaluated by the procedure described by Lorke in 1983 [17], the route of administration of extract was oral. In the first Phase, 3 groups of animals (n=3) received the extract at doses of 10, 100, and 1000mg/kg body weight, respectively and observed for signs of toxicity such as behavioural, neurological and death in the first 24 hours. In the second phase, 3 mice were given different doses of the extract, 16000, 2900 and 5000 mg/kg body weight and were observed for any sign of toxicity and death within 24 hours. The median lethal dose (LD_{50}) was calculated using Equation 1.

$$LD_{50} = \sqrt{\text{minimal lethal dose} \times \text{maximum survival dose}}$$

Equation 1

2.8 Determination of Antiplasmodial activity

Evaluation of suppressive activity of extracts

The *Plasmodium* suppressive activity carried out using the Peter's 4 days suppressive test [18] on both the aqueous and n-hexane fractions of *Citrullus lanatus*. Twenty-five (25) *Albino* mice were inoculated with 0.2 mL of the standard inoculum of $1x10^7$ parasitized erythrocytes. After 3 hours of infection, the animals were randomly allotted into 5 groups of 5 mice each. Group 1 which served as the negative control received 10 mL/kg normal saline. Groups 2, 3 and 4 received 50, 100 and 200 mg/kg doses of the aqueous fraction of *Citrullus lanatus*, respectively while group 5 served as the positive control and received 5 mg/kg dose of chloroquine. All drugs were administered per oral. All animals were treated once daily for four consecutive days. The above procedure was repeated in another set of 25 animals but the aqueous fraction of *Citrullus lanatus* was replaced with n-hexane fraction of the plant. On the fifth day blood was taken from the tail vein of each mouse and a thick blood film was prepared. The slide was fixed with methanol and then diluted Giemsa stain was applied with buffer at the ratio of 1:9. The slides were first examined at x40 magnification to check the staining distribution of cells. The best fields of viewed were used to count the parasitaemia level using the x100 objective lens. The parasite count (Equation 2) was recorded and the suppression of parasitaemia was expressed as percentage of each dose by comparing the parasitaemia in the control group with the treated group (Equation 3). Parasites were counted using the formula:

$$\text{Number of parasites per } \mu L \text{ of blood} = \frac{\text{No. of parasite counted}}{\text{No. of WBC counted}} \times 8000$$

…. *Equation* 2.

$$\%\ \text{Parasite suppression} = \frac{PC - PTG}{PC} \times 100$$

…. *Equation* 3

Where:

PC = average parasite count in the negative control group

Q = average parasite count in the extract treated group.

2.9 Evaluation on established infection (Curative or Rane Test)

Evaluation of the curative antiplasmodial activity of extract on established infection was carried out by the method described by Ryley *et al.* [20]. Twenty-five mice were inoculated intraperitoneally with 0.2 mL of blood containing the standard inoculum ($1x10^7$) on Day 1. Three days (72 hours) later, parasitemia count was carried out in all the animals, mice with established infection were randomly distributed into five (5) groups of 5 mice each and treated for another 4 consecutive days (days 4, 5, 6 and 7). The negative and the positive controls were also administered 10 mL/kg normal saline and 5mg/kg chloroquine respectively. Groups 2 - 4 were treated orally with the aqueous fraction of *Citrullus lanatus* at daily doses of 50, 100 and 200 mg/kg respectively. On the final day of the treatment (Day 8), blood samples were collected

from the tail vein of each mouse and both thick and thin blood smears were prepared and stained with Giemsa and buffered at 7.0, the slides were left for 10 minutes, cleaned with cotton wool and allowed to dry before it was viewed at x100 magnification. The parasitaemia level determined using equation 1 above, while percentage parasitaemia suppression was determined using Equation 4.

$$\% \text{ Suppression} = \frac{PC - PTG}{PC} \times 100$$

…….. *Equation* 4

Where:
PC = average parasite count in the negative control group
PTG = average parasite count in the extract treated group.

2.10 Evaluation of the prophylactic activity of extracts

Ryley and Peter's method of evaluating the prophylactic antimalarial activity was used [19]. Twenty-five (25) mice were randomly divided into five (5) groups of 5 animals each. Group 1 (negative control) received normal saline (10 mL/kg). Groups 2-4 received oral doses of 50, 100 and 200 mg/kg doses of the n-hexane fraction *Citrullus lanatus* respectively. Group 5 received the standard drug Pyrimethamine (positive control) at a dose of 1.2 mg/kg. All treatments were given daily for 4 consecutive days and on the fifth day all the mice were then infected with the parasite, with 0.2 mL inoculum. Blood smear was made by taking blood from the tail vein of each mouse after 72 hours of infection. Parasitaemia levels were then determined using the method describe above, while average percentage prophylaxis was calculated using equation 5.

$$\% \text{ Prophylaxis} = \frac{PC - PTG}{PC} \times 100$$

…….. *Equation* 4

Where:
PC = average parasite count in the negative control group
PTG = average parasite count in the extract treated group.

2.11 Statistical analysis of data

All data were analyzed using the Statistical Package for Social Sciences (SPSS) version 25.0, results were expressed as mean ± standard error of mean. The differences between means were compared using One-way Analysis of Variance (ANOVA) followed by Dunnett's test, the probability level of $p \leq 0.05$ was considered to be significant compared to the control

3. Results

3.1 Percentage yield of *Citrullus lanatus* leaf extract

The percentage yield of the crude methanol extract was calculated to be 4.83%. following fractionation of the crude extract, the percentage yield of the n-hexane fraction was calculated to be 2.29% while that of the aqueous fraction was 9.66%.

3.2 Qualitative Phytochemical Screening

The methanolic leaf extract of *Citrullus lanatus* was found to contain: carbohydrates, alkaloids, saponins, flavonoids, tannins, phytosterols, reducing sugars and phenolic compounds and the result is presented in Table 1.

Table 1: Phytochemical constituents of the leaf extract of *Citrullus lanatus*

S/N	Constituents	Test	Inference
1.	Carbohydrates	Molisch's/Fehling's	+
2.	Alkaloids	Mayer's/Dragendorff's	+
3.	Saponins	Frothing	+
4.	Anthraquinone glycosides	Bontrager's	-
5.	Flavonoids	NaOH/Ferric chloride	+
6.	Phytosterols	Salkowski's	+
7.	Tannins	Ferric chloride	+
8.	Reducing sugar	Benedict's	+
9.	Lipids	Sudan III	-
10.	Phenolics	Ferric chloride	+

Key: + = Present; - = Not detected

3.3 Acute toxicity

The oral median lethal dose (LD_{50}) of the leaf of *Citrullus lanatus* was estimated to be 5000 mg/kg, no mortality was recorded in both phases at doses up to 5000 mg/kg, however, the aqueous and n-hexane fractions showed signs of toxicity such as restlessness, salivation and head tilting at 2900 and 5000 mg/kg respectively.

3.4 Suppressive antiplasmodial activity

Peter's 4-day suppressive test of the aqueous and n-hexane fractions of *Citrullus lanatus* showed a significant ($p < 0.05$) percentage suppression with increasing doses when compared to the control group (normal saline: 48.6 ± 3.71). Percentage Chemo-suppression was found to be at the highest dose of 200 mg/kg body weight of the aqueous fraction of extract (90.95%) which was comparable to the standard chloroquine (94.65%). The n-Hexane fraction, although showed a dose dependent activity, the percentage suppression (74.07%) was much lower than the standard chloroquine (94.65%) as shown in Tables 2 and 3 respectively.

Table 2: Suppressive and Curative antimalarial effect of the aqueous fraction of *Citrullus lanatus* leaf in mice infected with *Plasmodium berghei berghei*

Activity	Treatment	Dose (mg/kg)	Average parasitemia ± SEM	Percentage suppression (%)
	NS	10 (mL/Kg)	48.6±3.71	-
	AF	50	13.2+1.30*	72.84
Suppressive	AF	100	9.6±1.04*	80.25
	AF	200	4.4±1.04*	90.65
	Chloroquine	5	2.6±0.28**	94.65
	NS	10 (mL/Kg)	114.94±6.67	-
	AF	50	34.70±4.60*	68.80
Curative	AF	100	29.01±4.18*	74.76
	AF	200	23.80±4.28*	90.25
	Chloroquine	5	11.20±1.53*	97.20

Key: NS = Normal saline; AF= Aqueous fraction *Citrullus lanatus*; values are expressed as Mean± SEM (n=5). Values of the group with * are significant at p<0.02 from normal saline (curative) and * are statistically significant at p<0.05 (Suppression) from normal saline.

Table 3: Suppressive and Prophylactic effect of n-hexane fraction of *Citrullus lanatus* leaf extract against *Plasmodium berghei berhgei* infected mice

Activity	Treatment	Dose (mg/kg)	Average parasitemia ± SEM	Percentage suppression (%)
	NS	10 (mL/Kg)	48.6±3.71	-
	HF	50	25.8+2.3*	46.91
Suppression	HF	100	17.8±2.8*	63.37
	HF	200	12.6±1.53*	74.07
	Chloroquine	5	2.6±0.82**	94.65
	NS	10 (mL/Kg)	6.11±0.32	
	HF	50	3.29±0.60*	46.15
Prophylaxis	HF	100	3.21±0.61*	47.46
	HF	200	2.58±0.53*	57.77
	Pyrimethamine	1.2	1.82±0.48	70.21

Key: NS = Normal saline; HF= n-hexane fraction of *Citrullus lanatus*; *P.O* = per oral; values are expressed as Mean± SEM (n=5). Values of the group with * are significant at p<0.001 from normal saline for prophylaxis and * are statistically significant at p<0.05, for suppression.

3.5 Curative antiplasmodial activity

The aqueous fraction of the methanol extract of *Citrullus lanatus* also showed a significant and dose-dependent schizonticidal effect on *Plasmodium berghei berghei* infected mice. At doses of 50, 100 and 200 mg/kg body weight, the percentage suppressions were 68.80%, 74.76% and 90.25% respectively while chloroquine at a dose of 5 mg/kg body weight gave level of inhibition with mean parasitemia level on day 7 as 11.20 ± 1.53 and percentage suppression of 97.20% significant at $p < 0.02$ compared to the extract (Table 2).

3.6 Prophylactic antiplasmodial activity

The n-hexane fraction of *Citrullus lanatus* leaf extract showed an increasing anti plasmodial prophylactic activity at doses of 50, 100 and 200 mg/kg body weight when compared to the negative control. The dose-dependent activity of this extract revealed that the highest percentage suppression was at the dose of 200 mg/kg body weight (57.77%). However, the highest level of chemo-suppression achieved with the prophylactic treatment was much lower than what was achieved with the positive control (Pyrimethamine 1.2 mg/kg). The Kruskal-Wallis test showed that there is a statistically significant difference in the average parasitemia among the different doses of treatment ($H = 18.549$, $df = 4$, $p < 0.001$), indicating that the different doses have effect on parasitemia.

4. Discussion

Secondary metabolites have been reported to be responsible for the various pharmacological activities of plants; [21]. Phytochemicals such as saponins, alkaloids, tannins and triterpenoids detected in the methanolic leaf extract of *Citrullus lanatus* have been shown to be responsible for various therapeutic activities including antimalarial activities [22 -27]. The n-hexane

fractions have been shown to also contain saponins, carbohydrate, steroids and terpene [28], however, further studies would be needed to single out the exact compound responsible and the possible mechanism through which it exerts its action.

The median lethal dose (LD_{50}) was estimated to be greater than 5000 mg/kg body weight observed in this study suggests the relative safety of the leaf extract of *Citrullus lanatus*; this is similar to the findings reported by Ettebong *et a*l. (2021), where the LD_{50} of the methanol mesocarp extract of *Citrullus lanatus* was found to be greater than 5000 mg/kg [14].

Both the aqueous and n-hexane fractions of the leaf extracts gave dose-dependent antimalarial activities, with increase in doses resulting in decrease in parasite counts. The aqueous fraction demonstrated a good suppressive and curative activity. Chemo-suppression of 90.95% exhibited by the aqueous extract was comparable to the standard drug chloroquine (94.65%) which was found to be statistically significant ($p < 0.05$) at the highest dose (200 mg/kg body weight). The high parasite clearance observed in both the suppressive and schizonticidal effect by the aqueous fraction of the extracts suggests that the secondary metabolites present may be responsible for its antimalarial activity and also justifies the use of the aqueous decoction of *Citrullus lanatus* leaf in ethnomedicine. The schizonticidal parasite clearance of the aqueous fraction which increased with increased doses was statistically significant at ($p < 0.02$), although not as much as chloroquine which shows that the curative effect may not be as good as chloroquine.

The suppressive and prophylactic effect of the n-hexane fraction also followed a similar dose-dependent activity, the percentage suppression of both the suppressive and prophylactic antiplasmodial activity were 74.07% and 57.77% and were statistically significant ($p < 0.05$ and $p < 0.001$) respectively when compared to the standard drugs used as positive controls.
Once again, this indicates that the leaf of *Citrullus lanatus* could potentially be utilized for preventive purposes or prophylaxis, and it is possible that the n-hexane fraction may not have contained the constituents responsible for the level of antimalarial effect observed with the aqueous fraction. Although the presence of certain constituents such as alkaloids has been reported to demonstrate antimalarial activity by inhibiting protein synthesis in *plasmodium* parasites, tannins and saponin are known as free radical scavengers that counteract the oxidative damage caused by malaria parasites. Additionally, several flavonoids have been shown to exert antimalarial activity (Ahmed *et al*., 2011). Nevertheless, the specific compound responsible for the antiplasmodial activity in this study is unclear.

5. Conclusion

The LD_{50} for oral administration of both the aqueous and n-hexane fractions of *Citrullus lanatus* stem bark has been demonstrated to exceed 5000 mg/kg, indicating their relative safety. Both fractions contain phytochemicals that may contribute to their antiplasmodial effects, and they have shown significant antiplasmodial activity. However, the aqueous fraction exhibited superior suppressive and curative activity, supporting its traditional use in the management of malaria in folk medicine.

Conflict of interest

The authors declare no conflict of interest.

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