



Antiplasmodial activity of methanol leaf extract of *Citrus aurantifolia* (Christm) Swingle

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ABSTRACT

Introduction: *Citrus aurantifolia* (Christm) is a plant used for the treatment of various ailments including malaria. This study aimed to evaluate the *in vivo* antiplasmodial efficacy of methanol leaf extract of *C. aurantifolia* in Swiss albino mice.

Methods: The median lethal dose (LD₅₀) was determined by intraperitoneal administration of different doses of the extract (100–4000 mg/kg) to 6 groups of 3 mice each and the animals were observed for 24 hours for physical signs of toxicity. To evaluate the antiplasmodial activity of the extract, three models were used: suppressive, curative and repository. Doses of the extract used were 320, 640 and 960 mg/kg/d in mice, with Chloroquine (5 mg/kg/d) as standard drug. Pyrimethamine (1.2 mg/kg/d) was used as the standard drug for the repository test and distilled water (10 mL/kg/d) as control in all models.

Results: In all models, the low dose (320 mg/kg) of the extract produced the highest chemosuppressive effects in all models ($P < 0.001$). Mice treated with extract lived longer than those in the control group ($P < 0.001$). Phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, tannins and cardiac glycosides and the LD₅₀ of 3280 mg/kg \pm 0.01 shows that the extract has low toxicity.

Conclusion: The result of this study shows that *C. aurantifolia* has antiplasmodial properties which support its use in ethnomedicine in the treatment of malaria.

Implication for health policy/practice/research/medical education:

The methanolic leaf extract of *Citrus aurantifolia* is a low toxic plant with antiplasmodial activity which can be exploited as a potential source of new antimalarial drug to combat malaria.

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Introduction

Malaria is a disease of the blood that is caused by the protozoan microparasite of the genus *Plasmodium*, which affects red blood cells (1). About 156 species of *Plasmodium* that infect many types of animals have been identified, however, only *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi* are known to infect humans (1).

The disease is as old as humanity itself (2). Numerically, the most important of the life-threatening protozoan diseases is malaria, which is responsible for at least 750 000 deaths a year (3). There were 212 million new cases of malaria worldwide in 2015 (range 148–304 million). In 2015, there were an estimated 429 000 malaria deaths (range 235 000–639 000) worldwide. Most of these

deaths occurred in the African region (92%), followed by the South-East Asia Region (6%) and the Eastern Mediterranean Region (2%). Between 2010 and 2015, malaria incidence rates (new malaria cases) fell by 21% globally and in the African region. During this same period, malaria mortality rates fell by an estimate of 29% globally and by 31% in the African Region (4).

Treatment of malaria requires the use of antimalarial drugs. Recently, there was a major breakthrough by Chinese researchers in the discovery of antimalaria agent Artemisinin (Quinghao), an endoperoxide sesquiterpene lactone as the active component of *Artemisia annua*, an herbal remedy used in Chinese folk medicine for over 2000 years (5). This antimalarial drug was developed due to the problem of resistance to older forms of antimalarial

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drugs like chloroquine. Although clinically relevant Artemisinin resistance has not been demonstrated, it is likely to occur since artemisinin resistance has been obtained in laboratory models (5).

Majority of populations in many tropical countries depend on traditional medical remedies using herbs (6). There is increased dependence on herbal remedy as a result of high cost of artemisinin-based combination therapy (ACT) to populations in third world countries because of the high number of peasants and low-income earners who are at most affected by the disease (7).

Citrus aurantifolia in its natural state is widely used in West Africa, particularly in Nigeria, where it is employed in herbal medicine to treat several illnesses. Crushed leaves are applied to forehead to treat headache and it is squeezed near the nostril for irritant inhalation to treat nausea and resuscitate fainting individuals (8). A decoction of the flower is believed to help induce sleep for those with insomnia (8). The fruit juice has been found useful to treat irritation, diarrhoea and swelling due to mosquito bites. It is sometimes mixed with oil and used as vermifuge and also incorporated into weight management diet (9). Decoction of roots is used to treat dysentery, diarrhea, colic, gonorrhoea and fever (10). The extracts of the fruit peels, leaves and the essential oil of the leaves of *C. aurantifolia* have been shown to possess anticholinesterase and butyrylcholinesterase activity (11). Similarly, the hydroalcoholic extract of *C. aurantifolia* in the form of tincture has been found to have anti-platelet aggregation activity (12). Extracts of the root have been found to have antibacterial and antimycobacterial (13, 14), as well as antifungal and antiaflatoxic activities (15). *C. aurantifolia* has been shown to inhibit colon cancer (16) and has antioxidant (17), immune-modulatory (18), antiobesity (19), antifertility activities (20). In southwest Nigeria, the roots, bark, stem twigs, leaves and fruits are used in the treatment of malaria (21).

This study was aimed at investigating the *in vivo* antiplasmodial activity of the leaf extract of *C. aurantifolia*.

Materials and Methods

Collection and identification of plant material

Fresh leaves of *C. aurantifolia* were collected from the Faculty of Pharmacy Medicinal Plants Farm, University of Uyo in October 2017.

Plant extraction

The leaves of *C. aurantifolia* were washed; air-dried and pulverized using a mortar and pestle. The powdered plant material was weighed (26.97 g) and cold macerated for 72 hours at room temperature using methanol. The methanol extract that was obtained by filtration was concentrated *in vacuo* at 40°C using a rotary evaporator. The dried extract obtained was weighed and stored in a refrigerator prior to further studies.

Phytochemical screening

The qualitative phytochemical screening was carried out on the methanol extract using standard methods (22).

Animals

Both male and female animals (Swiss albino mice) weighing 15–27 kg used for these experiments were obtained from the Animal House of the Faculty of Pharmacy, University of Uyo. The animals were kept in well ventilated standard cages, fed with growers pellet feed and water *ad libitum*.

Parasite and preparation of inoculum

A chloroquine-sensitive strain of *Plasmodium berghei berghei* (NK-65) was obtained from Nigeria Institute for Medical Research (NIMR), Lagos and was maintained by sub passage in mice. The parasitized blood donor with high parasitaemia was obtained by first anaesthetizing the mouse with chloroform, and through cardiac puncture blood was collected using sterile syringe. The percentage parasitaemia was determined by counting the number of parasitized red blood cells against the total number of red blood cells. The inoculum was prepared by diluting 2 mL of the parasitized blood with 10 mL of sterile normal saline, to obtain the final inoculum of 0.2 mL (1.0×10^7), which is the standard inoculum for the infection of a single mouse (23).

Acute toxicological study

Acute toxicological study was carried out to determine the median lethal dose (LD_{50}) and the safety profile of the methanol leaf extract of *C. aurantifolia* using the modified method of Miller and Tainter as described by Nwafor et al (24) and Jigam et al (25). This involved intraperitoneal administration of different doses of extract (100–4000 mg/kg) to 6 groups of 3 mice each. The animals were observed for the first 2 hours and then at the 6th and 24 hours period for manifestation of physical signs of toxicity such as writhing, decreased motor activity, decreased body/limb tone, decreased respiration and death. The number of deceased mice was counted in each group and percentage mortality calculated. The calculated percentage mortalities were transformed into probit values and were plotted against log-doses and then the dose corresponding to probit 5, i.e., 50% was recorded as the LD_{50} value. The standard error (SE) of the LD_{50} value was calculated using the formula:

$$SE \text{ of } LD_{50} = \frac{(\log \text{probit}6 - \log \text{probit}4)}{\sqrt{2N}}$$

Where, N= number of animals in each group.

Determination of antiplasmodial activities

Suppressive activity

Suppressive effect of the extract on early infection (4 -

day test) was determined. The mice were each inoculated on the first day (day 0), intraperitoneally with 0.2 ml of infected blood containing about 1×10^7 *P. berghei* parasitized erythrocytes. The animals were then randomly divided into five groups of 6 animals each. On day one (D_0) 0.2 ml of infected blood containing *P. berghei* was administered intraperitoneally to all mice. Groups 1-3 received 320, 640, and 960 mg/kg/d of the leaf extract respectively. Group 4 received 5 mg/kg/d of chloroquine (standard drug) which served as the positive control, while Group 5 served as the negative control group and received 10 mL/kg of distilled water. The administration of extract and drug was continued daily for 4 days (D_0 – D_3), between 8.00 AM and 9.00 AM. On the fifth day (D_4), 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 Leishman's stain and examined under the 100x oil immersion microscope to determine the effect of the extract (26). The World Health Organization (WHO) method of counting parasites was adopted. This was to determine the presence of malaria parasites. Here, two tally counters were used to count parasites and white blood corpuscles (WBCs) with one counter used for the WBCs and the other for the parasites. Counting was commenced at the top most part of the film by looking for a field with a good number of WBCs. After all the parasites and WBCs in one field were counted, it was moved to the next field until 200 WBCs were counted then the counting stopped. Care was taken not to overlap fields. The parasite density was calculated using the formula (27):

$$\text{Parasite density} = \frac{\text{Parasite count}}{\text{WBC count}} \times 8000$$

Repository/prophylactic activity

The method of Etebong et al (28) was also employed to evaluate the prophylactic activity of the extract. Groups 1-3 were administered with 320, 640 and 960 mg/kg of the extract orally. Group 4 animals served as positive control and were administered with 1.2 mg/kg/d of pyrimethamine. Group 5 animals served as negative control and received 10 mL/kg of distilled water. All the groups were treated for three consecutive days (D_0 - D_2) and on day 4 (D_3), the mice were intraperitoneally injected with 0.2 mL of infected blood that contained 1×10^7 *P. berghei* parasitized red blood cells (RBCs). The parasite density was assessed using thin films obtained from tail blood of each mouse, 72 hours after parasite inoculation. The earlier method of counting parasites described for suppressive model was also adopted for this model.

Curative activity

The earlier method described by Etebong et al, (28) and Adu-Gyasi et al, (26) was used. A quantity of 0.2 ml of blood containing *P. berghei berghei* parasitized blood

was administered intraperitoneally to the mice on the first day (D_0) and left for 72 hours for parasitaemia to be established. The mice were randomized into 5 groups of 6 animals each. Groups 1-3 received 320, 640 and 960 mg/kg/d of the leaf extract respectively. Group 4 received 5 mg/kg/d of the chloroquine (Standard drug) and served as the positive control. Group 5 received 10 mL/kg of distilled water and served as the negative control. All the drugs were administered to the animals once daily for five days. Tail blood sample from each mouse was collected daily for 5 days and stained with Leishman's stain. Thereafter, thin films were prepared and used to determine the parasite density. The earlier method of counting parasites described for suppressive model was also adopted for this model.

The mean survival time (MST) of each group was determined over a period of 30 days (D_0 - D_{29}).

$$\text{MST} = \frac{\text{Number of days survived}}{\text{Total of Number of days}} \times 100$$

Data analysis

Results were expressed as multiple comparisons of mean \pm standard error of the mean (SEM). Significance was determined using one-way analysis of variance (ANOVA). A probability level of 5% or less was taken as significant.

Results

Phytochemical constituents of methanol leaf extract of *Citrus aurantifolia*

The phytochemical screening of methanol leaf extract of *C. aurantifolia* revealed the presence of saponins, alkaloids, tannins, flavonoids and cardiac glycosides.

Acute toxicity study

The mean lethal dose (LD_{50}) was determined to be 3280 ± 0.01 mg/kg.

Antiplasmodial activities

Suppressive test

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 and these reductions were statistically significant ($P < 0.001$) compared to control but less than that of standard drug. The low dose gave 75.66% chemosuppression. However, parasite density was found to increase in the high dose of extract compared to control. This depicts that the extract has a partial agonistic effect (Table 1).

Repository test

In the repository test, there were reductions in parasite densities in animals treated with low and middle doses of extract. These reductions were statistically significant ($P < 0.001$) but more pronounced in animals treated with low dose of the extract. The percentage chemosuppression

Table 1. The suppressive antiplasmodial activity of methanol leaf extract of *Citrus aurantifolia* in mice (4 days test).

Drug/Extract	Dose (mg/kg)	Parasite ($\mu\text{L}/\text{blood}$)	Density/% Chemosuppression
Distilled water	10 mL	269 792.00 \pm 4.67	
Extract	320	65 680.00 \pm 1.49*	75.65*
	640	180.256.67 \pm 2.70*	33.19*
	960	317 575.67 \pm 4.71*	17.71*
	Chloroquine	5.0	894.33 \pm 1.30*

Values are expressed as mean \pm SEM; * P < 0.001 compared to control, n=6.

for low and middle doses were 99.83% and 97.75% respectively and these were comparable to that of the standard drug, Pyrimethamine (Table 2).

Curative test

In the curative test it was observed that there was a reduction in parasite density in mice treated with the extract from the 4th to the 7th day. Low doses of the extract had more curative effects than the middle and high doses. These reductions were statistically significant (P < 0.001) compared to control. The low dose of the extract exhibited more curative effect than the standard drug chloroquine (Table 3).

Mean survival time

The result showed that mice treated with the extract lived longer (16.35–27.33 days) than those in the control group (9.01 days) (P < 0.001). Survival times of mice treated with low doses of extract compared favorably with those of standard drug, chloroquine (Table 4).

Discussion

The preliminary phytochemical screening of the methanol leaf extract of *C. aurantifolia* showed the presence of alkaloids, saponins, flavonoids, cardiac glycosides and tannins. 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 (29). 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 (29). However, the lack of oxidizing action in some plant does not rule out antiplasmodial activity since they may be active through other biochemical mechanisms (30). The presence of alkaloids in *C. aurantifolia* extract might have contributed to antiplasmodial activity exhibited by the plant extract. Also the presence of saponins, flavonoids and tannins in the methanol leaf extract of *C. aurantifolia* justify the antiplasmodial activity exhibited by the plant extract.

The median lethal dose (LD_{50}) of the extract in mice was calculated to be 3280 mg/kg \pm 0.01, which is slightly toxic according to the toxicologic testing methods by Loomis and Hayes (31). This indicated that the extract is not very toxic and somewhat safe for oral use.

Standard antimalarial drugs like chloroquine, halofantrine, mefloquine and artemisinin have been tested using animal models. The *in vivo* antiplasmodial activity of the methanol leaf extract of *C. aurantifolia* was investigated by evaluating the chemosuppression during the early infection (suppressive test), established infection

Table 2. The repository antiplasmodial activity of methanol leaf extract of *Citrus aurantifolia* in mice

Drug/Extract	Dose (mg/kg)	Parasite ($\mu\text{L}/\text{blood}$)	Density/% Chemosuppression
Distilled water	10 mL	52 961.54 \pm 4.55	
Extract	320	91.82 \pm 0.00*	99.83
	640	1189.41 \pm 2.00*	97.75
	960	369 187.35 \pm 5.30*	- 597.08
	Pyrimethamine	5.0	0.00 \pm 0.00*

Values are expressed as mean \pm SEM; * P < 0.001 compared to control, n=6.

Table 3. The curative antiplasmodial activity of methanol leaf extract of *Citrus aurantifolia* in mice

Drug/Extract	Dose (mg/kg)	Parasite Densities/ μL of blood				
		Day 3	Day 4	Day 5	Day 6	Day 7
Distilled water	10ml	149 883.67 \pm 4.40	138 680.15 \pm 2.10	101 869.82 \pm 1.67	79 950.18 \pm 1.45	80 630.32 \pm 1.70
Extract	320	149 109.00 \pm 4.06*	92 906.66 \pm 1.21*	27 862.70 \pm 1.33*	851.67 \pm 0.95*	287.33 \pm 0.63*
	640	150 849.33 \pm 4.45*	130 037.40 \pm 1.40*	51 198.80 \pm 0.90*	23 671.60 \pm 0.65*	4235.50 \pm 1.35*
	960	148 345.45 \pm 3.70*	132 540.20 \pm 1.35*	57 280.06 \pm 0.85*	42 495.03 \pm 1.95*	8506.00 \pm 1.22*
	Chloroquine	5.0	149 210.35 \pm 4.40*	20 899.33 \pm 1.00*	16 520.50 \pm 0.66*	4528.65 \pm 0.97*

Values are expressed as mean \pm SEM; * P < 0.001 compared to control, n=6.

Table 4. Mean survival time (MST) of mice receiving various doses of methanol leaf extract of *Citrus aurantifolia*

Drug/Extract	Dose (mg/kg)	MST (days)
Distilled water	10 mL	9.01 ± 0.25
Extract	320	27.33 ± 0.55*
	640	23.33 ± 0.20*
	960	16.35 ± 0.66*
	Chloroquine	5

Values are expressed as mean ± SEM; * $P < 0.001$ compared to control, n=6.

(curative test), the repository test and the mean survival time.

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. Similar findings were also reported for the aqueous root extract of *Berberis aristata* (32).

In the curative test it was observed that there was a reduction in parasite density in mice treated with the extract from the 4th to the 7th day. Low doses (320 mg/kg) of the extract had more curative effects than the middle and high doses of the extract. *C. aurantifolia* has antioxidant properties (18) and it is known that such properties may become prominent in low doses than in high doses. For instance, vitamin C is a naturally occurring organic compound and a potent antioxidant preventing oxidative damage to lipids and other macromolecules. It can also exhibit bimodal activity as a pro-oxidant at a higher concentration (33). It is certainly well established that vitamin C can serve as a pro-oxidant through formation of ascorbyl radical (34). This result may therefore be associated with the pro-oxidant property of *C. aurantifolia*. The low dose (320 mg/kg) of the extract exhibited more curative effect than the standard drug chloroquine. The methanol leaf extract of *C. aurantifolia* displayed chemosuppression in a dose-independent manner. As expected, 320 mg/kg body weight of the plant extract prolonged the mean survival time of the study mice indicating that it suppressed *P. berghei* and reduced the overall pathologic effect of the parasite on the mice. Similar findings were also reported for extracts of *Lecaniodiscus cupanioides* and *Echinops hoehnelii* (35, 36).

In the repository test, there were reductions in parasite densities in animals treated with low and middle doses of extract. The percentage chemosuppression for low and middle doses were comparable to that of the standard drug, pyrimethamine.

In the mean survival time, the result showed that mice treated with the extract lived longer than those in the control group. Survival times of mice treated with low doses of extract compared favorably with those of the

standard drug, chloroquine.

Chloroquine has shown a greater chemosuppressive activity than the extract of *C. aurantifolia* except for the curative test where the low dose (320 mg/kg) of the extract showed a greater chemosuppressive activity than chloroquine. This implies that the low dose of methanol leaf extract of *C. aurantifolia* has an advantage over chloroquine since it is effective and with no known resistance as compared to chloroquine.

Conclusion

The results of this study show that the leaf extract of *C. aurantifolia* possesses significant antiplasmodial activity *in vivo*, which is dose independent. Preliminary phytochemical screening of the extract showed the presence of alkaloids, saponins, flavonoids, cardiac glycosides and tannins. The antiplasmodial activities of this plant may be ascribed to these constituents. This study has established a rationale for the ethnomedicinal use of the plants in the treatment of malaria in Nigeria.

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Authors' contributions

EE and UP designed the study and interpreted the results. EE did statistical analysis of the results. EE, UE and AL did the experiments and assisted in the writing of the manuscript. All authors read and confirmed the last edition of the manuscript and confirmed it for publication.

Conflict of interests

Authors declare that no competing interest exists.

Ethical considerations

Approval for the use of animals in the study was obtained from the Animal Ethics Committee of the Faculty of Pharmacy, University of Uyo (UUP41).

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