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

Ette Ettebong1*, Peace Ubulom2, Aniekeme Etuk3

1Department of Clinical Pharmacology and Therapeutics, Faculty of Clinical Sciences, University of Uyo, Nigeria
2Department of Animal and Environmental Biology, Faculty of Science, University of Uyo, Nigeria.
3Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, University of Uyo, Nigeria

*Corresponding author: Ette Ettebong, Email: ettebong@yahoo.com

Implication for health policy/practice/research/medical education: The methanolic leaf extract of Ricinus communis is a low toxic plant with antiplasmodial activity which can be exploited as a potential source of new antimalarial drug to combat malaria.


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 ± 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.
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, diarrhea 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, gonorrhea 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 antymycobacterial (13, 14), as well as antifungal and antiaflatoxigenic 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_ 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 ×10⁷), 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 (LD₅₀) 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 LD₅₀ value. The standard error (SE) of the LD₅₀ value was calculated using the formula:

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

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 $1 \times 10^7$ *P. berghei* parasitized erythrocytes. The animals were then randomly divided into five groups of 6 animals each. On day one ($D_1$) 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 ($D_0$–$D_4$), between 8.00 AM and 9.00 AM. On the fifth day ($D_5$), 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 Ettebong 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 $1 \times 10^7$ *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 Ettebong et al, (28) and Adu-Gyasi et al, (26) was used. A quantity of 0.2 ml of blood containing *P. 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 ($D_0$–$D_{30}$).

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

**Data analysis**

Results were expressed as multiple comparisons of mean ± 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
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<sub>50</sub>) of the extract in mice was calculated to be 3280 mg/kg ± 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 1. The suppressive antiplasmodial activity of methanol leaf extract of *Citrus aurantifolia* in mice (4 days test).

<table>
<thead>
<tr>
<th>Drug/Extract</th>
<th>Dose (mg/kg)</th>
<th>Parasite (µL/blood)</th>
<th>Density/% Chemosuppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>10 mL</td>
<td>269.792.00 ± 4.67</td>
<td>99.83*</td>
</tr>
<tr>
<td>Extract</td>
<td>320</td>
<td>65.680.00 ± 1.49*</td>
<td>97.75*</td>
</tr>
<tr>
<td></td>
<td>640</td>
<td>180.256.67 ± 2.70*</td>
<td>33.19*</td>
</tr>
<tr>
<td></td>
<td>960</td>
<td>371.575.67 ± 4.17*</td>
<td>17.71*</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>5.0</td>
<td>894.33 ± 1.30*</td>
<td>99.67*</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Drug/Extract</th>
<th>Dose (mg/kg)</th>
<th>Parasite (µL/blood)</th>
<th>Density/% Chemosuppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>10 mL</td>
<td>52.961.54 ± 4.55</td>
<td>99.83</td>
</tr>
<tr>
<td>Extract</td>
<td>320</td>
<td>91.82 ± 0.00*</td>
<td>97.75</td>
</tr>
<tr>
<td></td>
<td>640</td>
<td>118.941 ± 2.00*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>960</td>
<td>369.187.35 ± 5.30*</td>
<td>-</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>5.0</td>
<td>0.00 ± 0.00*</td>
<td>100</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Drug/Extract</th>
<th>Dose (mg/kg)</th>
<th>Parasite Densities/µL of blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 mL</td>
<td>148.883.67 ± 4.40</td>
</tr>
<tr>
<td>Extract</td>
<td>320</td>
<td>149.109.00 ± 4.06*</td>
</tr>
<tr>
<td></td>
<td>640</td>
<td>150.849.33 ± 4.45*</td>
</tr>
<tr>
<td></td>
<td>960</td>
<td>148.345.45 ± 3.70*</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>5.0</td>
<td>149.210.35 ± 4.40*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± 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

<table>
<thead>
<tr>
<th>Drug/Extract</th>
<th>Dose (mg/kg)</th>
<th>MST (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>10 mL</td>
<td>9.01 ± 0.25</td>
</tr>
<tr>
<td>Extract</td>
<td>320</td>
<td>27.33 ± 0.55*</td>
</tr>
<tr>
<td></td>
<td>640</td>
<td>23.33 ± 0.20*</td>
</tr>
<tr>
<td></td>
<td>960</td>
<td>16.35 ± 0.66*</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>5</td>
<td>27.15 ± 0.53*</td>
</tr>
</tbody>
</table>

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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