**In vivo** antimalarial and acute toxicity properties of hexane and chloroform extracts from *Clausena anisata* (Willd.) Benth.

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**Background:** Drugs are an important tool for control of malaria. However, drug resistance is likely to compromise available antimalarial drugs with time. As a result, efforts are being directed towards discovery and development of novel and affordable malaria drug molecules including those from medicinal plants.

**Objectives:** To investigate suppressive, curative, prophylactic and acute toxicity properties of hexane and chloroform extracts of *Clausena anisata* against murine malaria.

**Method:** Activity of the extracts was tested against *Plasmodium berghei* ANKA strain. Extracts were administered orally to mice (n=5) at 500, 250 and 100 mg/kg/day. Median lethal dose was evaluated after oral administration of the extracts in doses ranging from 500 to 5000 mg/kg.

**Results:** 500 mg/kg/day of the chloroform extract exhibited 66.1% and 73.4% parasite reduction in the prophylactic and suppressive tests, respectively, while the same dose of the hexane extract comparatively lower suppressive and prophylactic properties (56.7% and 30.7 %, respectively). In the curative test, 500 mg/kg/day of the chloroform and the hexane extracts resulted in mean survival times (MST) of 12.3 days and 9.3 days, respectively. No mortality was observed in mice that received the hexane extract, whereas the LD$_{50}$ of mice that received the chloroform extract was 4166.7 mg/kg. These results suggest that the chloroform extract at 500 mg/kg/day had notable in vivo antimalarial activity and partly explain therapeutic efficacy claimed for this plant in traditional medicine.

**Key words:** *Clausena anisata*, *Plasmodium berghei*, antimalarial activity, acute toxicity

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1. Introduction

Malaria is a disease caused by *Plasmodium* parasites and, though preventable and curable, is still one of the greatest global public health problems especially in sub-Saharan Africa. This can be partly attributed to the development of resistance by malaria parasites to most of the established antimalarial drugs such as chloroquine, sulphadoxine/pyrimethamine and amodiaquine (Schlitzer, 2008). Currently, fixed dose artemisinin-based combination therapies are being used as first-line treatment of uncomplicated *falciparum*...
2. Material and Methods

2.1 Plant materials

Stem bark of *C. anisata* was collected from Ngong’ forest, Kajiado County in Kenya. Authentication was carried out at the University of Nairobi Herbarium, School of Biological Sciences, University of Nairobi, where a voucher specimen was deposited (voucher specimen number BN/2008/1).

2.2 Extraction

Plant material was chopped, air-dried for a period of two weeks and ground using a mill. 200 g was extracted by repeated soaking (2 x 24 hr) successively with hexane and chloroform. Filtrates were separated dried *in vacuo* using a rotary evaporator yielding 1.26 g hexane extract (yellowish gum) and 10.03 g chloroform extract (dark brown gum). The solvent free extracts were stored at 4 °C until needed for bioassay.

2.3 Animals

Male Swiss mice, 8 weeks old, weighing 20±2 g were obtained from KEMRI animal house. They were housed in clearly labelled, standard Macrolon type II cages at 22 °C and 60–70% relative humidity. They were fed on commercial rodent feed and water *ad libitum*.

2.4 Drug (extracts) preparation

These were freshly prepared on the day of treatment. Extracts were suspended in a solvent mixture consisting of 70% Tween-80 (d = 1.08g/ml) and 30% ethanol (d= 0.81g/ml), then diluted 10-fold in water. Three different stock solutions were prepared, i.e. 10 mg/ml, 25 mg/ml and 50 mg/ml for 100, 250 and 500 mg/kg/day dose levels, respectively.

2.5 4-day Suppressive test

The four day test described by Peters et al (1975) was adapted. The animals were randomly infected intraperitoneally with blood containing 2 x 10<sup>7</sup> parasitized (*Plasmodium berghei* ANKA) red blood cells contained in 0.2 ml inoculum on Day zero (D0). They were divided into 3 groups of 5 mice each (test, untreated, and positive control). 4 hours post infection, the test animals were orally treated with three dose levels, (500, 250 and 100 mg/kg/day) at a volume of 0.81 ml/gram mouse. Positive control received chloroquine at 10 mg/kg/day while negative controls received 7% Tween-80 and 3 % ethanol in water. Drug administration was repeated 24, 48, and 72 hours post infection. On Day 4 (96 hours post infection), blood smears were taken by making a thin film from a tail snip of each mouse, fixed in methanol for about 2 minutes and stained with 10% Giemsa for 15 minutes. Blood smears were used to estimate the level of parasitaemia. Percentage chemosuppression (parasite reduction) was calculated as described by Tona et al (2001).

2.6 Prophylactic Test

Prophylactic properties were tested as described by Peters (1965). Briefly, the test groups were treated orally with three dose levels 500, 250 and 100 mg/kg/day for three consecutive days (Days 0 - 2). The positive control group received pyrimethamine 1.2 mg/kg/day while the negative control group received 7% Tween-80 and 3 % ethanol in water. On Day 3, the animals were infected intraperitoneally and left for a further four days after which blood smears were made and used to estimate the level of parasitaemia (%) (as described in Section 2.5 above).
Table 1: % Chemosuppression by *C. anisata* extracts in the suppressive test against *P. berghei* ANKA

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>Dosage (mg/kg/day)</th>
<th>Average parasitaemia</th>
<th>% chemosuppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>100</td>
<td>b$^b$7.2±1.2</td>
<td>31.4</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>b$^b$7.6±2.5</td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>a$^a$5.6±1.9</td>
<td>56.7</td>
</tr>
<tr>
<td>Chloroform</td>
<td>100</td>
<td>b$^b$9.2±1.5</td>
<td>39.8</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>b$^b$8.0±3.4</td>
<td>47.5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>a$^a$4.1±1.7</td>
<td>73.4</td>
</tr>
<tr>
<td>Untreated group</td>
<td>-</td>
<td>15.5±4.1</td>
<td>-</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>10 mg/kg/day</td>
<td>a$^a$0.4±0.2</td>
<td>96.4</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM  
$^a$ Significantly different from the untreated group (p<0.05)  
$^b$ Not significantly different from the untreated group (p<0.05)  
n=5 mice per group  
Score definition >70% active: 50-69 % moderate: < 50% low

Table 2: % Chemosuppression by *C. anisata* extracts in the prophylactic test against *P. berghei* ANKA

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>Dosage (mg/kg/day)</th>
<th>Average parasitaemia</th>
<th>% chemosuppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>100</td>
<td>b$^b$5.76±0.8</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>b$^b$4.8±2.1</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>b$^b$4.5±0.4</td>
<td>30.7</td>
</tr>
<tr>
<td>Chloroform</td>
<td>100</td>
<td>b$^b$4.2±1.4</td>
<td>34.8</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>b$^b$3.09±0.7</td>
<td>52.3</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>a$^a$2.2±0.5</td>
<td>66.1</td>
</tr>
<tr>
<td>Untreated group</td>
<td>-</td>
<td>6.49±1.8</td>
<td>-</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>1.2 mg/kg/day</td>
<td>a$^a$1.76±0.6</td>
<td>72.8</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM  
$^a$ Significantly different from the untreated group (p<0.05)  
$^b$ Not significantly different from the untreated group (p<0.05)  
n=5 mice per group  
Score definition >70% active: 50-69 % moderate: < 50% low

2.7 Curative test

This test was carried out as was described by Ryley and Peters (1970). All animals were infected intraperitoneally on Day 0 and left for 72 hours before commencement of treatment. Thereafter, test animals were orally given the extracts at three dose levels of 500, 250 and 100 mg/kg/day for 4 consecutive days. Positive controls received chloroquine at 10 mg/kg/day while the untreated group received a solution consisting of 7% Tween-80 and 3% ethanol. Animals were observed and any death that occurred noted to determine the mean survival time.

2.8 Acute Toxicity Testing and LD$_{50}$ Determination

Median lethal dose was estimated following the method described by Lorke, (1983). Briefly, on Day 1 mice were starved for 24 hours prior to drug administration. They were divided into two groups (test and negative control) of five mice each. For each extract, a single dose of between 500-5000 mg/kg/day was administered orally on Day 2. Five different dosing levels were used. Negative controls received a solution consisting of 7% Tween-80 and 3% ethanol. The animals were given food and water four hours post drug administration. On Day 2 and 3 they were observed for signs of toxicity such as writhing, decreased motor activity, decreased body/limb tone, decreased respiration and death. The number of deaths that occurred within 48 hours was recorded.
Table 3: Mean Survival Time (MST) of mice treated with hexane and chloroform in the curative test

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>Dosage (mg/kg/day)</th>
<th>Mean survival time in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>100</td>
<td>b7.6±0.5</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>b8.2±0.8</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>b9.3±1.9</td>
</tr>
<tr>
<td>Chloroform</td>
<td>100</td>
<td>b9.2±1.3</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>b9.8±0.8</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>a12.3±1.7</td>
</tr>
<tr>
<td>Untreated group</td>
<td></td>
<td>7.8±0.5</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>10</td>
<td>a21±6.4</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM

a Significantly different from the untreated group (p<0.05)
b Not significantly different from the untreated group (p<0.05)
n=5 mice per group

Table 4: Effects of C. anisata extracts on uninfected mice

<table>
<thead>
<tr>
<th>Dose levels in mg/kg</th>
<th>% Mortality in 48 hours</th>
<th>Hexane extract</th>
<th>Chloroform extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000</td>
<td>0 (0/5)</td>
<td>60 (3/5)</td>
<td></td>
</tr>
<tr>
<td>2811</td>
<td>0 (0/5)</td>
<td>40 (2/5)</td>
<td></td>
</tr>
<tr>
<td>1581</td>
<td>0 (0/5)</td>
<td>0 (0/5)</td>
<td></td>
</tr>
<tr>
<td>889.2</td>
<td>0 (0/5)</td>
<td>0 (0/5)</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>0 (0/5)</td>
<td>0 (0/5)</td>
<td></td>
</tr>
</tbody>
</table>

Values in brackets indicate the number of mice that were dead at the end of 48 hours

2.9 Statistical Analysis

StatView Version 5.0.1 (SAS institute Inc.) was used for statistical analysis. In suppressive and prophylactic tests Student’s t-test (Minitab Inc. software, State College, PA, USA) was used to evaluate the differences between average parasitaemia of untreated and treated groups. In curative test, Student’s t-test was used to evaluate the differences between mean survival time of untreated and treated group. P-values of 0.05 or less were considered significant. For LD50 determination, probit-log analysis was used (Finney, 1964).

2.10 Ethical considerations

Permission to carry out this study was approved by KEMRI Animal Care and Use Committee.

3.0 Results

Suppressive and prophylactic activities were evaluated by comparing the % reduction of parasitaemia (chemosuppression) between the treated and untreated groups while the curative test compared the mean survival time (MST) of the treated group in comparison to the untreated group.

3.1 Suppressive test

Results are as shown in Table 1. The chloroform extract at 500 mg/kg/day showed 73.4% chemosuppression. Average parasitaemia at this dosage was significantly lower (p< 0.05) than that of the untreated group. The 100 and 250 mg/kg doses showed lower suppressive activity, 39.8% and 47.5% respectively. The hexane extract at all the three dose levels had moderate to low chemosuppression (56.6%, 40.1% and 31.4% at 500, 250, and 100 mg/kg/day respectively).

However, none of the observed activities of the extracts was comparable to that of chloroquine (10 mg/kg/day) which exhibited a 96.4% chemosuppression.

3.2 Prophylactic test

As shown in Table 2 prophylactic activities were slightly lower than the suppressive activities for both extracts. At 500 mg/kg/day, the chloroform extract exhibited high (66.1%) parasite reduction; at 250 mg/kg/day, the chloroform extract exhibited moderate activity at 52.3% parasite reduction. The hexane extract at all three dose levels showed parasite reduction of less than 50%. Pyrimethamine (1.2 mg/kg/day) was used as a reference drug and showed a parasite reduction of 72.8%.

3.3 Curative test

There was no curative property observed in mice treated with different doses of the hexane extract as shown in Table 3. The life span of treated mice was also not significantly different (p>0.05) from that of untreated group which had a mean survival time (MST) of 7.8 days.

The chloroform extract at 100 and 250 mg/kg/day also exhibited no curative properties. However, the group of mice treated with 500 mg/kg/day of the chloroform extract had an MST of 12.3 days. This was significantly different (P<0.05) from that of the untreated group (7.8 days) but still incomparable to the group treated with chloroquine which had a MST of 21 days.

3.4 Acute Toxicity Testing and LD50 Determination

There was no mortality observed within 48 hours for mice that received hexane extract as shown in Table 4. However, physical signs such as decreased motor activity, respiration and feeding, as well as closure of eyes were observed.

The chloroform extract at 5000 and 2811 mg/kg produced 60% and 40% mortality, respectively, within 48 hours. Oral LD50 of the extract was calculated to be 4166.7 mg/kg.
4 Discussion and Conclusion

Two extracts were tested at 3 different dose levels for their in vivo antimalarial properties and also for their acute toxicity in mice.

The chloroform extract at 500 mg/kg/day exhibited promising antimalarial activity in both suppressive and prophylactic tests. In the curative test, the same dose level was associated with an MST that was significantly longer than that of the untreated group though incomparable to that of chloroquine. The hexane extract at 500mg/kg/day had moderate to low suppressive and prophylactic properties. In the curative test life span of mice was also not significantly different from that of untreated group. This lower activity may probably be due to the fact that hexane extract mainly consists of fats and oils that are often not biologically active (Houghton and Raman, 1998).

The chloroform extract displayed some mild oral acute toxicity as shown in Table 4. Coumarins like chalepin isolated from C. anisata are reported to be toxic with an intraperitoneal LD₅₀ of 100 mg/kg in rats (Emerole et al, 1981). Coumarins are also reported to be mutagenic (Uwaifo, 1984) while carbazole alkaloids, the major constituents of C. anisata, are reported to be cytotoxic (Ito et al, 2009; Ito et al, 2000). These compounds may have contributed to the observed oral mild acute toxicity.

The results of this study indicate that the chloroform extract of the C. anisata stem bark possesses antimalarial activity. Further studies are required to determine if the antimalarial activity of C. anisata is attributable to the alkaloid and coumarin constituents. Meanwhile, the results partly corroborate claims made in traditional medicine of the antimalarial efficacy of this plant.

Conflict of Interest declaration

The authors declare no conflict of interest.

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References


