***African Journal of Pharmacology and Therapeutics*** Vol. 1 No. 1 Pages 24-29, 2012
Open Access to full text available at http://www.uonbi.ac.ke/journals/kesobap/

Research Article

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

Beatrice N. Irungu [a,*], Murungi J. Mbabu [b], Daniel M. Kiboi [a], Enock Moindi [a], Johnson Kinyua [b], and Mwirichia Romano [b]

[a] *Kenya Medical Research Institute (KEMRI), Centre for Traditional Medicine and Drug Research, Nairobi, Kenya*

[b] *Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya*

_____

*** Corresponding author:** Kenya Medical Research Institute (KEMRI), Centre for Traditional Medicine and Drug Research, P.O Box 54840-00200, Nairobi, Kenya; **Tel**: +254-20-2722541 ext 3349; **Fax**: +254-20-2720030; **Email**: birungu@yahoo.com

**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

**Received**: February, 2012
**Published**: March, 2012

## 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*

malaria in endemic areas. Unfortunately, recent reports indicate a decline in efficacy of artemisinin derivatives along Thai-Cambodia border, a site historically known for the recurrent emergence of drug resistant malaria parasites (Dondorp et al, 2009; Noedl et al, 2008). New classes of antimalarial agents are therefore urgently needed given that drug resistance is likely to eventually compromise the efficacy of currently available antimalarial drugs; identification of lead antimalarial agents from medicinal plants could boost the search.

*Clausena anisata* (Willd.) Benth is a deciduous shrub/tree whose leaves are aromatic. In Kenya, it is known locally as Mjarikali (Swahili) and Mutathi (Kikuyu). Traditionally, soup boiled with the roots of the plant was given to women after birth to cleanse the uterus. This soup is also highly recommended for headache, malaria, influenza and indigestion. Twigs are used as toothbrushes and are believed to cure toothache. Decoctions of the root are also drunk to treat syphilis (Kokwaro, 2009; Beentje, 1994; Gachathi, 1989).

Pharmacological activities associated with extracts from this plant include antifungal and antibacterial activity (Senthilkumar and Venkatesalu, 2009; Hamza et al, 2006; Gundidza et al, 1994), antidiabetic activity (Ojewole, 2002), anticonvulsant activity (Makanju, 1983) angiotensin converting enzyme (ACE) inhibitory activity (Duncan et al, 1999) and antiviral activity (Ayisi and Nyadedzor, 2003). Phytochemical investigations indicate carbazole alkaloids as the major component of this plant (Ito et al, 2000; Ito et al, 2009). Coumarins and limonoids have also been isolated (Ngadjui et al, 1991; Ngadjui et al, 1989; Lakshmia et al, 1984). Pharmacological activities associated with carbazole alkaloids include antifungal, antibacterial and antiviral activities (Ito et al, 2009; Ito et al, 2000; Chakraborty et al, 1995). Coumarins are reported to have anticoagulant properties (Emerole et al, 1981). Steam distillation of fresh leaves yielded sweet smelling, brownish-yellow oil whose major component is the acute toxin estragole (Okunade, 1987).

As part of our continuing efforts to identify antimalarial agents from medicinal plants, *in vivo* antimalarial activity and acute toxicity of *C. anisata* extracts were investigated. The plant was selected as it has been traditionally used to treat malaria (Kokwaro, 2009; Beentje, 1994).

## 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 separately 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 $10^7$ 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 0.01 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

| Type of extract | Dosage (mg/kg/day) | Average parasitaemia | % chemosuppression |
|---|---|---|---|
| **Hexane** | 100 | [b]8.7±1.2 | 31.4 |
| | 250 | [b]7.6±2.5 | 40.1 |
| | 500 | [a]5.6±1.9 | 56.7 |
| **Chloroform** | 100 | [b]9.2±1.5 | 39.8 |
| | 250 | [b]8.0±3.4 | 47.5 |
| | 500 | [a]4.1±1.7 | 73.4 |
| **Untreated group** | - | 15.5±4.1 | - |
| **Chloroquine** | 10 mg/kg/day | [a]0.4±0.2 | 96.4 |

*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

| Type of extract | Dosage (mg/kg/day) | Average parasitaemia | % chemosuppression |
|---|---|---|---|
| **Hexane** | 100 | [b]5.76±0.8 | 11 |
| | 250 | [b]4.8±2.1 | 11.7 |
| | 500 | [b]4.5±0.4 | 30.7 |
| **Chloroform** | 100 | [b]4.23±1.4 | 34.8 |
| | 250 | [b]3.09±0.7 | 52.3 |
| | 500 | [a]2.2±0.5 | 66.1 |
| **Untreated group** | - | 6.49±1.8 | - |
| **Pyrimethamine** | 1.2 mg/kg/day | [a]1.76±0.6 | 72.8 |

*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

| Type of extract | Dosage (mg/kg/day) | Mean survival time in days |
|---|---|---|
| **Hexane** | 100 | [b]7.6±0.5 |
| | 250 | [b]8.2±0.8 |
| | 500 | [b]9.3±1.9 |
| **Chloroform** | 100 | [b]9.2±1.3 |
| | 250 | [b]9.8±0.8 |
| | 500 | [a]12.3±1.7 |
| **Untreated group** | | 7.8±0.5 |
| **Chloroquine** | 10 | [a]21± 6.4 |

*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

| Dose levels in mg/kg | % Mortality in 48 hours | |
|---|---|---|
| | Hexane extract | Chloroform extract |
| 5000 | 0 (0/5) | 60 (3/5) |
| 2811 | 0 (0/5) | 40 (2/5) |
| 1581 | 0 (0/5) | 0 (0/5) |
| 889.2 | 0 (0/5) | 0 (0/5) |
| 500 | 0 (0/5) | 0 (0/5) |

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 $LD_{50}$ 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 21days.

### 3.4 Acute Toxicity Testing and $LD_{50}$ 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 $LD_{50}$ 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 $LD_{50}$ 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.

## Acknowledgements

Funds to carry out this project were provided by International Foundation for Science (IFS), Grant number F/4575-1. The authors gratefully acknowledge the technical assistance received from Simon Mwangi and Margaret Wanjohi.

## References

Ayisi NK and Nyadedzor C (2003). Comparative in vitro effects of AZT and extracts of *Ocimum gratissimum, Ficus polita, Clausena anisata, Alchornea cordifolia,* and *Elaeophorbia drupifera* against HIV-1 and HIV-2 infections. *Antiviral Res.* **58:** 25-33.

Beentje H (1994). Kenya Tree shrubs and lianas National Museum of Kenya, Nairobi, pp 366.

Chakraborty A, Chowdhury BK and Bhattacharyya P (1995). Clausenol and clausenine - two carbazole alkaloids from Clausena . *Phytochemistry* **40:** 295-98.

Dondorp MA, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin MK, Ariey FR, Hanpithakpong W, Lee JS, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, Sam An S, Yeung S, Singhasivan P, Day NPJ, Niklas Lindegardh DM, Socheat D and White NJ (2009). Artemisinin Resistance in *Plasmodium falciparum* malaria. *N. Engl. J. Med*. **361:** 455-67.

Duncan AC, Jager AK and van Staden J (1999). Screening of Zulu medicinal plants for angiotensin converting enzyme (ACE) inhibitors. *J. Ethnopharmacol.* **68:** 63-70.

Emerole G, Thabrew MI, Anosa V and Okorie DA (1981). Structure-activity relationship in the toxicity of some naturally occuring coumarins –chalepin, imperatorin and oxypeucedanine. *J. Toxicol.* **20:** 71-80.

Finney DJ (1964). Probit Analysis: A Statistical Treatment of the Sigmoid Response Curve, 2nd Ed. Cambridge University Press, London.

Gachathi FN (1989). Kikuyu botanical dictionary of plant names and uses. Reprinted 2007. Tropical Botany.

Gundidza M, Chinyanganya F and Chagonda L (1994). Phytoconstituents and antimicrobial activity of the leaf essential oil of *Clausena anisata* (Willd.) J.D. Hook ex. Benth. *Flavour Fragr. J*. **9:** 299-03.

Hamza OJ, van den Bout-van den Beukel CJ, Matee MI, Moshi MJ, Mikx FH, Selemani HO, Mbwambo ZH, Van der Ven AJ and Verweij PE (2006). Antifungal activity of some Tanzanian plants used traditionally for the treatment of fungal infections. *J. Ethnopharmacol.* **108:** 124-32.

Houghton JP and Raman A (1998). Laboratory handbook for fractionation of natural extracts 1st Ed. Chapman and Hall, UK, pp 55-65.

Ito C, Itoigawa M, Aizawa K, Yoshida K, Ruangrungsi N and Furukawa H (2009). Gamma-lactone carbazoles from *Clausena anisata*. *J. Nat. Prod*. **72:** 1202-04.

Ito C, Katsuno S, Itoigawa M, Ruangrungsi N, Mukainaka T, Okuda M, Kitagawa Y, Tokuda H, Nishino N and Furukawa H (2000). New Carbazole Alkaloids from *Clausena anisata* with antitumor promoting activity. *J. Nat. Prod.* **63:** 125–28.

Kokwaro JO (2009). Medicinal Plants of East Africa. 3rd Ed., University of Nairobi Press, Nairobi, pp 254.

Lakshmia V, Prakasha D, Raja K, Kapila RS and Poplia SP (1984). Monoterpenoid furanocoumarin lactones from *Clausena anisata*. *Phytochemistry.* **23:** 2629-31.

Lorke D (1983). A new approach to practical acute toxicity testing. *Arch. Toxicol*. **54:** 275–86.

Makanju OOA (1983). Behavioral and anticonvulsant effects of an aqueous extract from the roots of *Clausena anisata* (Rutaceae). *Pharm. Biol.* **21:** 29-32.

Ngadjui BT, Ayafor JF, Sondengam BL and Connolly JD (1989). Limonoids from *Clausena anisata*. *J. Nat. Prod.* **52:** 832-36.

Ngadjui BT, Mouncherou SM, Ayafor JF, Sondengam BL and Tillequint F (1991). Geranyl coumarins from *Clausena anisata*. *Phytochemistry.* **30:** 2809-11.

Noedl H, Se Y, Smith BL, Schaecher K and Fakuda MM (2008). Evidence of artemisinin-resistant malaria in western Cambodia. *N. Engl. J. Med*. **59:** 2619-20.

Ojewole JA (2002). Hypoglycaemic effect of *Clausena anisata* (Willd) Hook methanolic root extract in rats. *J. Ethnopharmacol*. **81:** 231-37.

Okunade AL (1987). Estragole: An acute toxic principle from the volatile oil of the leaves of *Clausena anisata. J. Nat. Prod*. **50:** 990-91.

Peters W (1965) Drug resistance in *Plasmodium berghei* Vinke and Lips 1984. I. Chloroquine resistance. *Exp. Parasitol.* **17:** 80-7.

Peters W, Portus JH and Robinson BL (1975). The chemotherapy of rodent malaria XXII. The value of drug-resistant strains of *Plasmodium berghei* in screening for schizontocidal activity. *Ann. Trop. Med. Parasitol*. **69:** 155-71.

Ryley JF and Peters W (1970). The antimalarial activity of some quinoline esters. *Am. J. Trop. Med. Parasitol*. **84:** 209-11.

Schlitzer, M (2008). Review; Antimalarial Drugs-what is in use and what is in the pipeline. *Arch. Pharmacol. Chem*. **341:** 149-63.

Senthilkumar A and Venkatesalu V (2009). Phytochemical analysis and antibacterial activity of the essential oil of *Clausena anisata* (Willd) Hook ex .f Benth. *Int. J. Integr. Biol*. **5:** 116-20.

Tona L, Mesia K, Ngimbi NP, Chrimwami B, Okond'ahoka, Cimanga K, de Bruyne T, Apers S, Hermans N, Totte J, Pieters L and Vlietinck AJ (2001). In-vivo antimalarial activity of *Cassia occidentalis*, Morinda morindoides and *Phyllanthus niruri*. *Ann. Trop. Med. Parasitol.* **95:** 47-57.

Uwaifo AO (1984). The mutagenicities of seven coumarin derivatives and a furan derivative (nimbolide) isolated from three medicinal plants. *J. Toxicol. Environ. Health A.* **13:** 521-30.