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Antiplasmodial and analgesic activities of *Clausena anisata*

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ABSTRACT

Objective: Antiplasmodial and analgesic activities of the leaf extract and fractions of *Clausena anisata* (*C. anisata*) were evaluated for antimalarial and analgesic activities. **Methods:** The crude leaf extract (39 – 117 mg/kg) and fractions (chloroform and aqueous; 78 mg/kg) of *C. anisata* were investigated for antiplasmodial activity against chloroquine-sensitive *Plasmodium berghei* (*P. berghei*) infections in mice using suppressive, prophylactic and curative models and analgesic activity against acetic acid, formalin and heat-induced pains. Artesunate, 5 mg/kg and pyrimethamine, 1.2 mg/kg were used as positive controls. Thin films made from tail blood of each mouse were used to assess the level of parasitaemia of the mice. **Results:** The extract and its fractions dose-dependently reduced parasitaemia induced by chloroquine-sensitive *P. berghei* in prophylactic, suppressive and curative models in mice. These reductions were statistically significant ($P < 0.001$). They also improved the mean survival time (MST) from 17 to 21 days relative to control ($P < 0.01 - 0.001$). On chemically and thermally-induced pains, the extract inhibited acetic acid and formalin-induced inflammation as well as hot plate-induced pain in mice. These inhibitions were statistically significant ($P < 0.001$) and in a dose-dependent fashion. **Conclusions:** The antiplasmodial and analgesic effects of this plant may in part be mediated through its chemical constituents and it can be concluded that the *C. anisata* possess significant antimalarial and analgesic properties.

1. Introduction

Clausena anisata (Wild) Hook .F. ex Benth (Rutaceae) (syn. *Clausena abyssinica* (Engl.) Engl., *Clausena inequalis* (DC.) Benth.) (*C. Anisata*) is a tropical shrub or tree up to 10 meters high growing in and on evergreen forests. It is commonly known as 'mbiet ekpene' by the Ibibios of Niger Delta region of Nigeria. The plant is traditionally used as effective remedies for worms infections, respiratory ailments, hypertension, malaria, fever, rheumatism, arthritis and other inflammatory conditions, headaches, pains, toothaches, convulsions and others[1]. The Ibibios use the plant to treat measles, malaria, pains and inflammations[2]. The plant has been reported to contain coumarins, limonoids, carbazole alkaloids, monoterpenoids

furanocoumarin lactones and essential oils[3,4]. Reports of antibacterial[1], antidiabetic[5], antitumor promoting[4], and *in vitro* antimalarial[6] activities have been published. This study was carried out to evaluate the *in vivo* antiplasmodial and analgesic activities of this plant to correlate the reported *in vitro* antiplasmodial activity and confirm its traditional use for malaria and pains.

2. Materials and methods

2.1. Plant materials

The fresh leaves of *Clausena anisata* (Wild) Hook .F. ex Benth (Rutaceae) (*C. anisata*) were collected from the Ukup in Ikono area of Akwa Ibom State and were identified and authenticated by Dr. (Mrs.) Margaret Bassey of the Department of Botany and Ecological studies, University of Uyo and deposited at University of Uyo herbarium (UUh 653).

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2.2. Extraction

The leaves of the plant were air-dried, pulverized using a pestle and mortar and cold-macerated for 72 hours using ethanol. The liquid ethanolic extract that was obtained by filtration was evaporated to dryness in a waterbath at 60 °C and was partitioned with a 50:50 mixture of distilled water and chloroform. The aqueous fraction was evaporated to dryness in a water bath at 60 °C and the chloroform fraction air-dried. The ethanolic extract, the aqueous and chloroform fractions were stored at -4 °C until use.

2.3. Phytochemical screening

Phytochemical screening of the crude leaf extract was carried out employing standard procedures and tests[7], to reveal the presence of chemical constituents such as alkaloids, flavonoids, tannins, terpenes, saponins, anthraquinones, reducing sugars, cardiac glycosides among others.

2.4. Animals

Both male and female animals (Swiss albino mice) used for these experiments were obtained from University of Uyo animal house. The animals were housed in standard cages and were maintained on a standard pelleted Feed (Guinea Feed) and water *ad libitum*. Permission and approval for animal studies were obtained from College of Health Sciences Animal Ethics committee, University of Uyo.

2.5. Microorganism

A chloroquine sensitive strain of *Plasmodium berghei* (*P. berghei*) was obtained from the National Institute of Medical Research (NIMER), Lagos and was maintained by subpassage in mice.

2.6. Determination of median lethal dose (LD₅₀)

The median lethal dose (LD₅₀) of the extract was estimated using albino mice by intraperitoneal (*i.p*) route using the method of Miller and Tainter[8]. This involved intraperitoneal administration of different doses of the extract (100 – 1 000 mg/kg) to groups of three mice each. The animals were observed for manifestation of physical signs of toxicity such as writhing, decreased motor activity, decreased body/limb tone, decreased respiration and death.

2.7. Evaluation of antiplasmodial activity of the extract/fractions

The evaluation of suppressive activity (4-day test) of the extract, fractions and artesunate against early *P. berghei* infection in mice was done with modifications as earlier

described[9,10]. On the first day (D₀), the forty-two mice were infected with the parasite and randomly divided into 7 groups of six mice each. The mice in groups 1– 3 were administered with the 39, 78, and 117 mg/kg of crude extract, groups 4 – 5 were administered with the 78 mg/kg of the chloroform and aqueous fractions respectively, while group 6 was administered with 5 mg/kg of artesunate (positive control), and 10 mL/kg of distilled water to group 7 (negative control) for four consecutive days (D₀ – D₃) between 8 am and 9 am. On the fifth day (D₄), thin blood film was made from tail blood. The film was then stained with leishman stain to reveal parasitized erythrocytes out of 500 in a random field of the microscope. The average percentage suppression of parasitaemia was calculated in comparison with the controls as follows:

$$\frac{\text{Average \% parasitaemia in negative control} - \text{Average \% parasitaemia in positive groups}}{\text{Average \% parasitaemia in negative control}}$$

The evaluation of curative activities of extract and fractions of *C. anisata* in established infection were done with modifications as earlier described[9,10]. *P. berghei* was injected intraperitoneally into another 42 mice on the first day (D₀). Seventy-two hours later (D₃), the mice was divided randomly into seven groups of six mice each and treated as above with extract, fractions and artesunate once daily for 5 days. Leishman's stained thin smears were prepared from tail blood samples collected on each day of treatment to monitor parasitaemia level. The mean survival time (MST) of the mice in each treatment group was determined over a period of 29 days (D₀ – D₂₈).

$$\frac{\text{No of days survived}}{\text{Total No. of days (29)}} \times 100 = \text{MST}$$

Prophylactic or repository activities of extract and fractions were assessed by using the method earlier described[9,11]. The mice were treated as above for three days but 1.2 mg/kg/day of pyrimethamine was given to group 6 (positive control). On the fourth day (D₃) the mice were inoculated with *P. berghei*. The parasitaemia level was assessed by blood smears seventy-two hours later.

2.8. Evaluation of analgesic potential of the extract

Acetic acid induced writhing in mice was carried out according to the procedure earlier described[12–14] The animals were divided into 5 groups of 6 mice per group. Group 1 served as negative control and received 10 mL/kg of normal saline, while groups 2, 3 and 4 were pre-treated with 39, 78 and 117 mg/kg doses of *C. anisata* extract intraperitoneally and group 5 received 100 mg/kg of acetyl salicylic acid. After 30 minutes, 0.2 mL of 2%

acetic acid was administered intraperitoneally (*i.p.*). The number of writhing movements was counted for 30 minutes. Antinociception (analgesia) was expressed as the reduction of the number of abdominal constrictions between control animals and mice pretreated with extracts.

Formalin – induced hind paw licking in mice was carried out according to a modified method earlier described^[14–16]. The animals were treated as above but injected with 20 μ L of 2.5% formalin solution (0.9% formaldehyde) made up in phosphate buffer solution (PBS concentration: NaCl 137 mM, KCl 2.7 mM and phosphate buffer, 10 mM) subcutaneously under the surface of the right hind paw 30 minutes post-treatment. The amount of time spent licking the injected paw was timed and considered as indication of pain. The first phase of the nociceptive response normally peaks 5 minutes after injection and the second phase 15 – 30 minutes after formalin injection, representing the neurogenic and inflammatory pain responses, respectively^[15]. The responses were measured for 5 mins (first phase) and 15 – 30 mins after formalin injection (second phase), respectively.

For hot-plate test, the hot plate was maintained at (45 \pm 1) °C. Each animal was placed into a glass beaker of 50 cm diameter on the heated surface 30 minutes post-treatment with extract/fractions and standard drug as above. The time(s) between placement and shaking or licking of the paws or jumping was recorded as the index of response latency. An automatic 30-second cut-off was used to prevent tissue damage^[12,13,17].

2.9. Statistical analysis

Data obtained from this work were analyzed statistically using Students' *t*-test and ANOVA (One-way) followed by a post test (Tukey-Kramer multiple comparison test). Differences between means will be considered significant at 1% and 5% level of significance i.e $P \leq 0.01$ and 0.05.

3. Results

3.1. Phytochemical screening

The phytochemical screening of the ethanolic extract of the leaves of *C. anisata* revealed the presence of cardiac glycosides, tannins, saponins, terpenes and flavonoids.

3.2. Acute toxicity

The median lethal dose (LD₅₀) was calculated to be (393.70 \pm 25.64) mg/kg. The physical signs of toxicity included excitation, paw licking, increased respiratory rate, decreased motor activity, gasping and coma which was followed by death.

3.3. Effect on suppressive activity of ethanolic leaves extract and fractions of *C. anisata*

The extract and its fractions showed a dose-dependent chemosuppressive effect on the parasitaemia. These effects were statistically significant relative to the control ($P < 0.001$). The chemoinhibitory percentages ranged from 52.51 to 82.02 (Table 1). The aqueous fraction had a higher antiplasmodial potential which was more than that of the standard drug, artesunate.

3.4. Effect on repository activity of ethanolic leaves extract and fractions of *C. anisata*

The ethanolic leaf extract showed a dose-dependent chemosuppressive effect on the parasitaemia. This effect was statistically significant relative to the control ($P < 0.001$). Similarly the aqueous fraction exerted a higher activity that was significant relative to the control but less than that of the standard drug, pyrimethamine (Table 2).

3.5. Antiplasmodial effect of ethanolic leaf extract and fractions of *C. anisata* on established infection

On established infection, it was observed that there was a daily increase in parasitaemia of the control group. However, there was a daily reduction in the parasitaemia levels of the extract/fractions-treated groups as well as that of positive control (artesunate).

The extract and its fractions showed a dose-dependent schizonticidal effect on the parasitaemia. These effects were statistically significant relative to the control ($P < 0.001$) (Figure 1). Though both the extract and its fractions showed a significant dose-dependent mean survival time on established infection ($P < 0.001$), the standard drug, artesunate showed greater protective effect with a much longer mean survival time than the extract and fractions (Figure 2).

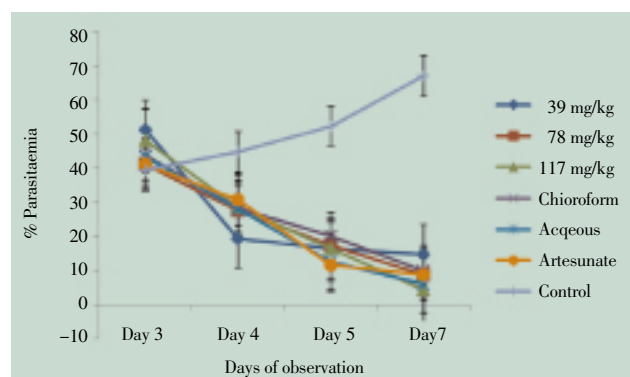


Figure 1. Curative effect of ethanolic leaves extract and fractions of *C. anisata* on *P. berghei* established infection in mice.

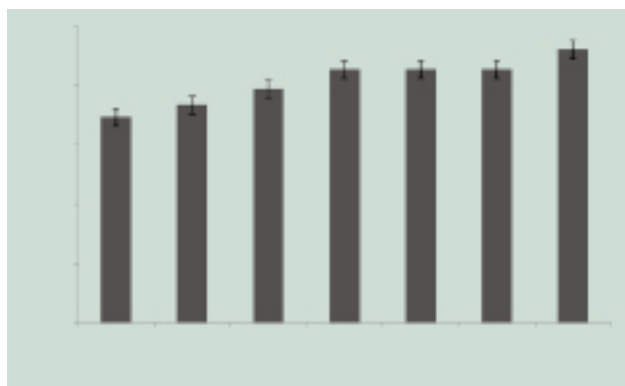


Figure 2. Mean survival time (MST) of mice receiving various doses of ethanolic leaves extract and fractions of *C. anisata* during established infection.

3.6. Effect of ethanolic crude extract of leaves of *C. anisata* on acetic acid–induced writhing in mice

The extract (39–117 mg/kg) demonstrated a dose–dependent reduction in acetic acid–induced writhing in mice. The reductions were statistically significant ($P<0.001$)

relative to control and comparable to that of the standard drug, ASA, at the highest dose, 117 mg/kg. (Table 3).

3.7. Effect of ethanolic leaves extract of *C. anisata* on formalin–induced hind paw licking in mice

The extract exhibited a dose– dependent effect on formalin–induced hind paw licking in mice. This inhibition was significant relative to the control ($P<0.001$) and comparable to that of the standard drug, ASA, at the highest dose, 117 mg/kg. (Table 4).

3.8. Effect of ethanolic crude extract of leaves of *C. anisata* on thermally–induced pain in mice

The extract exhibited a dose – dependent effect on thermally–induced pain in mice. This inhibition was statistically significant relative to the control ($P<0.001$) (Table 5).

Table 1

Suppressive activity of ethanolic leaves extract and fractions of *C. anisata* on *P. berghei* infection in mice(4–day test).

Treatments	Dose(mg/kg)	Parasitaemia	% Chemosuppression
Normal saline	10 mL/kg	46.33 0.33	–
<i>C. anisata</i> Crude extract	39	22.0 4.93 ^a	52.51
	78	15.66 1.85 ^a	66.13
	117	16.66 2.72 ^a	64.04
Acqueous fraction	78	8.33 2.37 ^a	82.02
Chloroform fraction	78	19.02 2.08 ^a	58.94
Artesunate	5.0	9.0 3.00 ^a	80.57

Values are expressed as mean ± SEM. Significance relative to control ^a $P<0.001$, $n = 6$.

Table 2

Repository/Prophylactic activity of ethanolic leaves extract and fractions of *C. anisata* on *P. berghei* infection in mice.

Treatments	Dose	Parasitaemia	% Chemosuppression
Distilled water	10 mL/kg	32.00±5.56	–
<i>C. anisata</i> crude extract	39 mg/kg	19.00±2.08 ^a	40.62
	78 mg/kg	12.00±2.51 ^a	62.50
	117 mg/kg	7.66±1.76 ^a	24.34
Aqueous fraction	78 mg/kg	9.66±3.18 ^a	69.81
Chloroform fraction	78 mg/kg	12.33±3.18 ^a	61.46
Pyrimethamine	1.2 mg/kg	8.00±0.42 ^a	75.00

Values are expressed as mean ± SEM. Significance relative to control ^a $P<0.001$, $n = 6$.

Table 3

Effect of ethanolic leaves extract of *Clausena anisata* on acetic acid induced writhing in mice.

TREATMENT	Dose(mg/kg)	Time interval (MINS)					
		5	10	15	20	25	30
Normal saline	10	5.67±0.87	9.11± 0.67	12.31±0.04	16.32±0.50	15.14±0.11	14.0±0.21
<i>C. anisata</i>	39	1.00±0.11 ^a	6.10±0.26 ^a	8.11±0.21 ^a	7.45±0.30 ^a	5.37±0.65 ^a	4.14±0.66 ^a
	78	0.00±0.00 ^a	5.22±0.32 ^a	7.54±0.42 ^a	5.16±0.38 ^a	4.88±0.42 ^a	3.65±0.35 ^a
	117	0.00±0.00 ^a	3.64±0.18 ^a	4.76±0.20 ^a	3.88±0.72 ^a	3.45±0.14 ^a	3.11±0.25 ^a
ASA		0.43±0.12 ^a	1.34±0.11 ^a	1.40±0.20 ^a	3.95±0.86 ^a	3.67±0.22 ^a	3.48±0.43 ^a

Values are expressed as mean ± SEM. Significance relative to control, ^a $P<0.001$, $n=6$.

Table 4Effect of ethanolic leaves extract of *C. anisata* on formalin induced paw licking in mice.

Treatment	Dose (mg/kg)	0 – 5mins	% Inhibition	15 – 30mins	% Inhibition
Normal saline	10	18.24 ± 0.64	–	25.44 ± 0.35	–
<i>C. anisata</i>	39	9.54 ± 0.36 ^a	47.69	12.45 ± 0.21 ^a	51.06
	78	4.82 ± 0.98 ^a	75.21	7.44 ± 0.28 ^a	70.75
	117	2.14 ± 0.46 ^a	88.26	3.54 ± 0.76 ^a	86.08
	ASA	100	2.28 ± 0.22 ^a	87.50	3.65 ± 0.24 ^a

Values are expressed as mean ± SEM. Significance relative to control. ^a*P* < 0.001, *n* = 6**Table 5**Effect of *Clausena anisata* leaf extract on hot plate test

Group	DoseMg/kg	Reaction time (sec)(mean ± SEM)	% inhibition
Control	–	2.98 ± 0.26	–
<i>C. anisata</i>	39	3.74 ± 0.57	25.50
	78	4.55 ± 0.30 ^a	52.68
	117	7.48 ± 0.15 ^b	151.00
ASA	100	15.32 ± 0.42 ^b	414.09

Data are expressed as mean ± SEM. Significant at ^a*P* < 0.05, ^b*P* < 0.001 when compared to control. *n* = 6.

4. Discussion

C. anisata have been in use traditionally in the treatment of different ailments ranging from malaria, convulsion, inflammation, worm infection to pains[1]. The Ibibios of Niger Delta region of Nigeria have been using it to treat malaria, hemorrhoids, pains and other febrile illnesses. These prompted the need to evaluate the antiplasmodial and analgesic potentials of the crude extract, aqueous and chloroform fractions of the leaves of *C. anisata*.

In this work, median lethal dose (LD₅₀) was determined to be (393.70 ± 25.64) mg/kg and the extract was relatively safe. The antiplasmodial properties of the extract and its fractions were investigated using standard models. The extract and its fractions exerted significant reduction of parasitaemia in prophylactic, suppressive and curative models in a dose – dependent fashion. These activities could be attributed to some secondary metabolites of this plants which have been reported to have antiplasmodial activity with varying mechanisms of action. Among these metabolites are alkaloids, flavonoids and triterpenoids such as limonoids and quassinoids[18]. These compounds (alkaloids, flavonoids, monoterpenes and triterpenoids) present in this plant extract may in part have contributed to the plasmocidal activity of this extract and therefore explained the mechanism of antiplasmodial effect of the extract and its fractions.

The extract significantly reduced acetic acid-induced writhing, formalin-induced hind paw licking as well as delayed the reaction time of animals (mice) to thermally induced pain. Acetic acid causes inflammatory pain by inducing capillary permeability and in part through local peritoneal receptors from peritoneal fluid concentration of PGE₂ and PGF_{2α} [19]. This test alone can not specify the involvement of either central or peripheral activity[20]. Thus, formalin tests as well as hot-plate test is usually carried out in addition to the above to distinguish between

peripheral and central pain. Centrally acting drugs inhibit both abdominal constriction test and hot plate tests[21], while the peripherally acting drugs inhibit only the abdominal constriction[22].

Formalin exhibits neurogenic and inflammatory pains[23] and measures both centrally and peripherally mediated activities that is characteristic of biphasic pain response. The injection of formalin has been reported to cause an immediate and intense increase in the spontaneous activity of C fiber afferent and evokes a distinct quantifiable behavior indicative of pain demonstrated in paw licking by the animals[24–30].

The study also shows that the extract significantly delayed the reaction time of thermally-induced (hot plate) test. This model is selective for centrally acting analgesics and indicates narcotic involvement[31] with opioid receptors.

The antinociceptive activities exerted by this extract may be attributed to the presence of secondary metabolites like saponins, flavonoids, tannins, terpenes. Flavonoids also have anti-inflammatory effects through its inhibition of the cyclo-oxygenase pathway[32]. That the extract inhibited neurogenic and non-neurogenic pains as well as narcotic pains may in part explain the mechanisms of its action and these effects are due to the present of phytochemical components in the extract.

The results of this study support the ethnobotanical use of the plant in the treatment of febrile illnesses, malaria and pains. Further investigation is being advocated especially in elucidating cellular mechanisms and establishing structural components of the active ingredients with a view of standardizing them.

Conflict of interest statement

There is no conflict of interest.

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