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Antiplasmodial activities of hydro-ethanol extracts of *Ancylobotrys amoena Hua* leaf and twig on Swiss mice

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Abstract

Aim: The study evaluated the antiplasmodial activities potential of the leaf, twig and combination of leaf and twig hydroethanol extracts of *Ancylobotrys amoena* in Swiss mice.

Methodology: The plant samples were collected, separated, dried, powdered and extracted in 70 % ethanol. The extracts were concentrated in vacuo and freeze-dried. The LD_{50} of the extracts was assessed using the Lorke's method. Each extract was subjected to Peters four-day chemosuppressive and the residual infection test respectively to evaluate the suppressive and the prophylactic activities. In the chemosuppressive test, adult Swiss mice (18-20 g) infected with chloroquine-sensitive *Plasmodium berghei* NK65 parasite, were divided into five groups (n=5 per group), for three doses (50, 100, and 200 mg/kg) of the extracts, 0.2 ml of normal saline (negative control) and 10 mg/kg chloroquine (positive control) for 4 days and the average percentage parasitaemia taken on the fifth day. In the residual infection test, the same doses above except 1.2 mg/kg pyrimethamine used in place of chloroquine, were administered to another five groups of 5 mice for 3 days prior to inoculation with the parasites. The average percentage parasitaemia was assessed after 72 hours. Statistical analysis was done using Graph Pad Prism 5.0 (Graph Pad Software, San Diego, CA).

Results: The leaf, twig and combination of leaf and twig extracts of *Ancylobotrys amoena* at 50, 100 and 200 mg/kg showed significant ($P < 0.05$) antiplasmodial activities which was dose dependent compared with standard drug (Chloroqiune). The results also demonstrated that the percentage chemo-suppression at doses of 50, 100 and 200 mg/kg twig extract (80.55%, 89.67% and 91.03% respectively) were better than the leaf extract (60.33 %, 63.83 % and 79.18 % respectively), and combination of leaf and twig extract (69.6%, 70.36% and 74.62% respectively) when compared to chloroquine (77.05 %) in the suppressive test. For the prophylactic test, the percentage chemo-suppression at doses of 50, 100 and 200 mg/kg; the leaf extract (68.75 %, 77.72 % and 77.72 % respectively), twig extract (64.95 %, 67.66 % and 75.82 % respectively) and combined extract (39.94 %, 61.96 % and 80.16 %), revealed that only at 200 mg/kg were they comparable to pyrimethamine (73.37 %).

Conclusion: The study concluded that *Ancylobotrys amoena* extracts had potential antimalarial activities and is safe for oral administration.

Keywords: Antiplasmodium; *Ancylobotrys amoena*; Antimalaria

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1. Introduction

Ancylobotrys amoena is a genus of plant under the family Apocynaceae found in tropical and southern Africa. *Ancylobotrys amoena* is found in the wild in Tropical Africa [1]. In Nigeria, it is common in parts of South-Western and North-Eastern of the country and has been used recently in Nigeria traditional medicine. In Nigeria, it is called "Ehu in Hausa" or "Ubo in Yoruba". *Ancylobotrys amoena* plant are used as fruit seed food, general fruit medicines, generally healing latex medicines, eye treatments products, withies and twigs products and exudations-gums [2]. The plant is a naïve plant with no know reported scientific activities than its folkloric medicinal uses. Although recently being used traditionally to treat fever and body pains, there has been no scientific study to verify the antimalarial efficacy and safety of its preparations, thus the reason for this study to establish or otherwise the antiplasmodial activities of the plant.

2. Materials and methods

2.1. Plant Materials

The leaf and twig of *Ancylobotrys amoena* was collected from the Zoological Gardens, Obafemi Awolowo University, Ile-Ife, Nigeria the samples of which were authenticated by Mr. I. I. Ogunlowo of the Faculty of Pharmacy Herbarium, Department of Pharmacognosy, Obafemi Awolowo University Ile-Ife where voucher specimen FPI 2150 was deposited.

Extraction of the plant materials: The leaves and twigs of *Ancylobotrys amoena* were air-dried and powdered separately. A 300 g each of the powdered leaf and twig was macerated in 3L of 70 % ethanol for 72 h and then filtered. The filtrates were concentrated in vacuo with the rotary evaporator. The product which was a gel-like material was allowed to dry in the desiccator weighing and finally yielded 14.02 g (4.67%) and 11.47 g (3.82%) respectively.

2.2. Experimental Animal

Swiss mice were raised to adults up to weight range 18 - 20 g. The mice were allowed free access to food and water *ad libitum* throughout experimental period. Good hygiene was maintained by constant cleaning and removal of feces from cages daily. The mice were maintained and cared for according to the international guidelines for the use and maintenance of experimental animals [3].

2.3. Acute toxicity test

A 0.8 mg, 8 mg and 80 mg of the leaf extract was dissolved in 0.8mls of normal saline to give a concentration of 1, 10, 100 mg/ml respectively which was used for the first phase of acute toxicity experiment. Similarly, 128 mg, 232 mg and 400 mg of the leaf extract was dissolved in 0.8mls of normal saline to give concentration of 160, 290 and 500 mg/ml respectively for the second phase of the acute toxicity experiment. The twig extract and the combination of the leaf and the twig were similarly prepared. Combination of the leaf and the twig was prepared by mixing equal ratio of the concentrated and freeze-dried extracts.

2.4. Antiplasmodial experiment

A 25.0 mg, 50.0 mg and 100.0 mg of the leaf extract was dissolved in 5.0 mls of normal saline to give concentration of 5, 10, 20 mg/mL respectively which was used for the chemosuppressive and the prophylactic experiments.

2.4.1. Determination of the LD_{50}

The LD_{50} which is a measure of the acute toxicity of the extracts were determined in two phases using the Lorke's method (1983). Briefly, three doses (10, 100 and 1000 mg/kg) of the leaf extract were administered orally to three different groups of mice (n = 3). The animals were observed for mortality for 24 h. If no mortality therefore the experiment proceeded to the second phase with the administration of doses 1600, 2900 and 5000 mg/kg of the extract orally to another three groups respectively of three mice each. The animals were observed again for mortality for 24 h. The same was repeated separately for the twig and combination of twig and leaf in other to determine the LD_{50} respectively. There was no mortality recorded at the second phase which confirmed the extract as being non-toxic according to Lorke (1983). The LD_{50} was calculated by the formula: $LD_{50} = \sqrt{(D_0 \times D_{100})}$ where D_0 = Highest dose that gave mortality, D_{100} = Lowest dose that produced mortality.

Parasite: Chloroquine-sensitive *Plasmodium berghei* NK65 inoculated into mice was obtained from Professor O. G. Ademowo of the Institute of Advanced Medical Research and Training, University College Hospital, Ibadan. It was

maintained by serial passaging in mice and close monitoring of parasitaemia level until the selected donor mice attained a parasitaemia of about 30 %.

2.5. Antiplasmodial activities of the extracts

The antiplasmodial activities of the extracts were determined using both chemosupprssive and the prophylactic models.

2.5.1. Suppressive assay (4-day schizonticidal test)

The chemosuppresive activities was determined using the Peters four-day test (Peters, 1980). Briefly, 25 Swiss mice weighing 18-20 g each were divided into 5 groups (I – V) of 5 mice each and inoculated each with 0.2mls of inoculum of the parasite prepared above (2.2.2.1). Two hours later, Group I was given normal saline (Negative control), Group II - IV administered with doses 50, 100 and 200 mg/kg while Group V received 10 mg/kg chloroquine positive control. Same was administered similarly for three consecutive days. The parasitemia was assessed on the fifth day after preparing a smear of the blood obtained from the tail of each animal.

2.5.2. Assessment of prophylactic activity (Residual infection test)

In the prophylactic assay, the mice were divided into groups of five and treated with the various test agents for 3 days. They were then inoculated intraperitoneally with 0.2 ml of parasitized blood containing 1 x 10^7 *Plasmodium beghei* (NK65) parasites on the 4th day and the tail smears were taken 72h after the inoculation (Peters, 1965). The positive control was 1.2 mg/kg of pyrimethamine.

2.5.3. Assessment of the percentage parasitaemia

Thin smear of each mouse blood was done by taken a drop of blood from a tiny cut to the tail of the animal onto a slide and spreading it thinly with the aid of another slide placed on the blood spot at an angle of 45º. The smear was allowed to air-dry, then fixation done with methanol. The fixed smear was then copiously stained with the prepared Giemsa stain (3 %) which was diluted with distilled water in ratio 1:9. The stain was poured sufficiently on each slide and allowed to stand for 15 minutes, decanted and rinsed with slowly running water, then air-dried. The slides were individually placed on the light microscope and viewed under the ×100 objective with the aid of immersion oil. The numbers of parasitized red blood cells were counted against the total number of red blood cells in ten fields. This gives the percentage (%) parasitaemia in each field. The average values were calculated and further the percentage (%) chemosuppression.

Percentage parasitaemia in each field was calculated as:

$$\text{Percentage (\%) parasitaemia} = \frac{\text{Total number of PRBC}}{\text{Total number of RBC}} \text{ x } 100\%$$

PRBC = Parasitized red blood cells, RBC= red blood cells.

Calculation of average percentage chemosuppression i.e reduction in parasitaemia:

$$\text{Mean \% chemosuppression of test group} = \frac{\text{Mean \% parasitaemia in control} - \text{Mean \% parasitaemiain test group}}{\text{Mean \% parasitaemia in control}} \text{ x } 100\%$$

2.6. Statistical Analysis

Statistical analysis was carried out using Graph Pad Prism 5.0 (Graph Pad Software, San Diego, CA). The data was analysed by one-way analysis of variance (ANOVA) followed by post hoc test using Students Newman Keull's test, the significance set at $P < 0.05$. All values were expressed as mean ± standard error of mean (SEM).

3. Results

3.1. Acute oral toxicity test

No mortality was observed at any of the test doses in both phases of the procedure for the leaf, twig and combination of the two after 24 h observation in both phases.

3.2. Effect of the ethanol leaf extract of *Ancylobotrys amoena* on *Plasmodium berghei* in 4-day schizonticidal (suppressive) test

In this test, the percentage chemosuppression of the hydro-ethanol leaf extract of *Ancylobotrys amoena* obtained was 60.33%, 63.83% and 79.18% at 50 mg/kg, 100 mg/kg and 200 mg/kg respectively (Table 1). The activity of the leaf extracts increased with increase in dose although the difference was not statistically significant. Highest dose 200 mg/kg compared favourably with standard drug chloroquine which exhibited 77.05%.

3.3. Effect of the ethanol twig extract of *Ancylobotrys amoena* on *Plasmodium berghei* in 4-day schizonticidal (suppressive) test.

In this test, the percentage chemosuppression of the hydro-ethanol twig extract of *Ancylobotrys amoena* obtained was 80.55%, 89.67% and 91.03% at 50 mg/kg, 100 mg/kg and 200 mg/kg, respectively (Table 1). The activity of the twig extracts also increased with increase in dose although the difference was not statistically significant. All the three doses compared favourably with standard drug chloroquine.

3.4. Effect of the ethanol leaf + twig extract of *Ancylobotrys amoena* on *Plasmodium berghei* in 4-day schizonticidal (suppressive) test.

In this test, the percentage chemosuppression of the hydro-ethanol leaf + twig extract of *Ancylobotrys amoena* obtainedwas 69.60%, 70.36% and 74.62% at 50 mg/kg, 100 mg/kg and 200mg/kg respectively (Table 1). The activity of the leaf and twig combination extracts increased slightly with increase in dose although the difference was not statistically significant. All the three doses also compared favourably with standard drug chloroquine.

The leaf, twig and combination of the twig and leaf at the three doses of 50 mg/kg, 100 mg/kg and 200 mg/kg showed comparable antiplasmodial activity with positive control (CQ). There activities increased with increase dose. However, the percentage (%) Chemosuppression was highest with the twig, the combination of the twig and leaf and least with the leaf. The activities of all doses of the extracts were statistically significant ($P < 0.001$) when compared with the negative control. However, the differences of antiplasmodial activity produced by all doses of the extracts were statistically not significant ($P < 0.05$) when compared to the positive control (CQ). The results are shown in Table 1.

3.5. Effect of the ethanol leaf extract of *Ancylobotrys amoena* on *Plasmodium berghei* in prophylactic antiplasmodial test

It was observed that the leaf extract of *Ancylobotrys amoena* exhibited percentage chemosuppresion of 68.75 %, 77.72 % and 77.72% at 50 mg/kg, 100 mg/kg and 200 mg/kg respectively (Table 2). The prophylactic activities increased with dose. The mean percentage (%) parasitaemia values for extract doses of 50, 100 and 200 mg/kg are significantly lower than that of the negative control. The standard drug (pyrimethamine 1.2 mg/kg) exhibited 73.37% chemosuppression. At doses of 50 mg/kg, 100 mg/kg and 200 mg/kg the difference was statistically significant compared to negative control.

3.6. Effect of the ethanol twig extract of *Ancylobotrys amoena* on *Plasmodium berghei* in prophylactic antiplasmodial test.

It was observed that the twig extract of *Ancylobotrys amoena* exhibited percentage chemosuppresion of 64.95 %, 67.66 % and 75.82 % at 50 mg/kg, 100 mg/kg and 200 mg/kg respectively (Table 2). The prophylactic activity of the twig extract was increasing with increase in dose.

3.7. Effect of the ethanol leaf and twig extract of *Ancylobotrys amoena* on *Plasmodium berghei* in prophylactic antiplasmodial test.

The leaf and twig extract of *Ancylobotrys amoena* exhibited percentage chemosuppresion of 39.94 %, 61.96 % and 80.16% at 50 mg/kg, 100 mg/kg and 200 mg/kg respectively (Table 2). The prophylactic activity the combination of leaf and twig extract also increased with increased doses.

Table 1 Effects of the ethanol leaf, twig and combination extract of *Ancylobotrys amoena* on *Plasmodium berghei* in 4-day schizonticidal (suppressive) test.

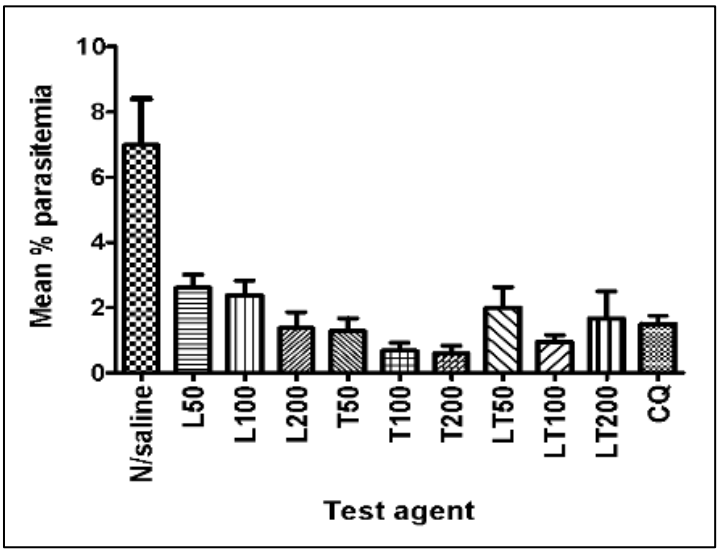
Treatment (mg/kg)	Mean % parasitemia (Leaf)	% Chemo suppression (Leaf)	Mean % parasitemia (Twig)	% Chemo suppression (Twig)	Mean % parasitemia (Leaf+twig)	%Chemo suppression (Leaf+twig)
N/saline (0.9%)	6.58 ± 1.55	0	6.58 ± 1.55	0	6.58 ± 1.55	0
50	2.61 ± 0.40***	60.33	1.28±0.40***	80.55	2.00±0.62***	69.60
100	2.38± 0.46***	63.83	0.68±0.25***	89.67	0.95±0.22***	70.36
200	1.37± 0.49***	79.18	0.59±0.24***	91.03	1.67±0.83***	74.62
CQ (10mg/kg)	1.51±0.25***	77.05	1.51±0.25***	77.05	1.51±0.25***	77.05

Data are expressed as mean ± SEM; n = 5; CQ = chloroquine (positive control), N/saline = Normal saline (Negative control).; *** p < 0.001 when compared with Negative control (ANOVA, Students Newman Keull's test). The values are significant with the negative control.

Table 2 Effects of the ethanol leaf, twig and combination extract of*Ancylobotrys amoena*on *Plasmodium berghei* in 4-day schizonticidal (prophylactic) test.

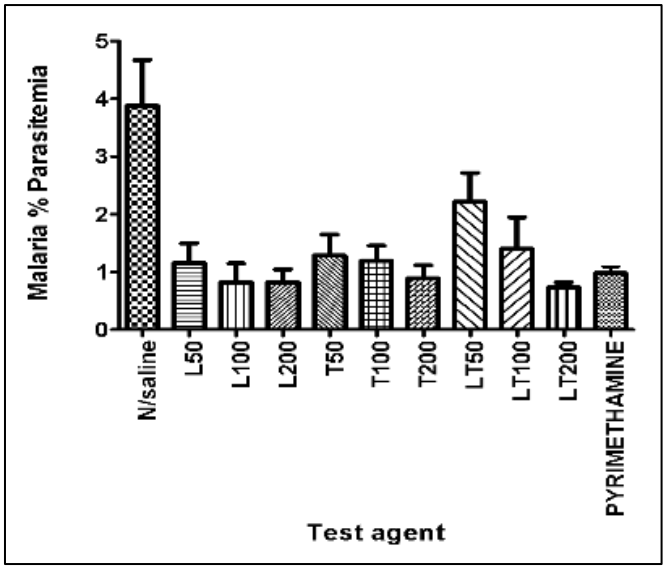
Treatment (mg/kg)	Mean % parasitemia (Leaf)	% Chemo suppression (Leaf)	Mean % parasitemia (Twig)	% Chemo suppression (Twig)	Mean % parasitemia (Leaf+twig)	% Chemo suppression (Leaf+twig)
N/saline (0.9%)	3.68±0.91	0	3.68±0.91	0	3.68±0.91	0
50	1.15±0.35**	68.75	1.29±0.36**	64.95	2.21±0.51*	39.94
100	0.82±0.33***	77.72	1.19±0.26**	67.66	1.40±0.55**	61.96
200	0.82±0.22***	77.72	0.89±0.23***	75.82	0.73±0.09***	80.16
PYM (1.2 mg/kg)	0.98±0.12***	73.37	0.98±0.12***	73.37	0.98±0.12***	73.37

Data are expressed as mean ± SEM; n = 5; PYM = Pyrimethamine (positive control) ; The statistical significance is indicated as: *p < 0.05, **p < 0.01 and *** p < 0.001 when compared with the negative control



L =Leaf, T = Twig, LT = Combination of Leaf and Twig. Test agent (mg/kg); CQ = Chloroquine, N/Saline = Normal saline

Figure 1 Mean percentage parasitemia against test agents (L, T and LT) (Suppressive model)



N/Saline = Normal saline, L =Leaf, T = Twig, LT = Combination of Leaf and Twig

Figure 2 Mean percentage parasitemia of the extracts in the Residual infection (Prophylaxis) model

4. Discussion

From the literature, *Ancylobotrys amoena* has been reported to be used as fruit seed food, general fruit medicines, generally healing latex medicines, eye treatments products, withies and twigs products, exudations-gums, resins and worms expellant. Different parts of this plant are used in the various functions. However, the leaves and twigs are increasingly used as antimalarial purposes folklorically without scientifically documentation (Persoon, *et.al.*, 1992). Most of the ailments quoted in these ethnomedical reports (viz diarrhoea, dysentery, worms, and eye treatments) are borne out of infection. The ethnomedicinal claims paved way for curiosity on the plausible activity of the plant on malaria infection. *Ancylobotrys amoena*is a naïve plant with no previous scientific work to verify its ethnomedicinal claims or screened for constituent.

The result of the acute toxicity assay (LD_{50}) in this work showed that *Ancylobotrys amoena* leaf and twig extract could be declared safe for use; having no incidence of mortality of the test animals up to the dose of 5000 mg/kg (on acute exposure). There was no significant toxicity reported as no signs of toxicity such as inactiveness, dizziness or mortality was observed even at the highest dose of 5000 mg/kg body weight. According to Locke (1983) [4], an LD_{50} greater than 5000 mg/kg is of no practical interest.

Although some research investigation on antimalarial plants have observed some degree of toxicity in the animals used, many have noticed no signs of toxicity up to 48 hours post drug administration. The antiplasmodial assays of *Ancylobotrys amoena* leaf and twig extract showed the plant's potentials as an alternative antimalarial. The suppressive assay, leaf extract percentage chemosuppression effect ranges from 60.33 % - 79.18 %, the highest dose 200 mg/kg having a higher value than the reference drug chloroquine which gave a value of 77.05%. These chemosuppressive effect reported is similar to previous active antimalaria plant extracts [5,6,7]. The twig extract also exhibited percentage chemosuppression range of 80.55 % - 91.03 % and increases with increased dose, with all doses having a higher value than the reference drug chloroquine. In the suppressive assay, the twig is the most effective. However, the combination of the leaf and twig extract exhibited percentage chemosuppression in between the leaf and twig activity. The prophylactic assay also demonstrated significant antimalarial and chemosuppressive activities where value ranges from 68.75 % - 77.72 %, 64.95 % - 75.82 %, 39.94 % - 80.16 % was obtained for the leaf, twig and combination of leaf and twig respectively. In the prophylactic assay, at highest dose, the twig demonstrated greatest chemosuppression followed by the leaf then the combination of the leaf and twig. Okokon *et al*., (2012) [8] noticed 52.12 to 82.02 % chemosuppressive effect of the leaves extract and fractions of *C. anisata* on the parasitaemia in the suppressive model similar to the result of the leaf extract suppressive model in this study where chemosuppressive effect was 60.33 % to 79.18 %. Also, in the findings reported by Malebo *et al*., (2015) [9], *M. senegalensis* ethanol root extract exhibited comparable chemosuppression of malaria parasites to that of the standard antimalarial drug, sulphadoxine/pyrimethamine similar to this present study where the prophylactic assay of the leaf, twig and combination exhibit slightly higher chemosuppression compare with standard antimalarial drug, pyrimethamine. The suppressive and prophylactic assay of the plant *A. amoena* demonstrated similar activities. Giving that no toxicity of the

plant extracts was demonstrated, *A. amoena* could be suitable antimalaria source. The 4-day suppressive test is a standard test commonly used for antimalarial screening [10]. It is the most widely used preliminary test, in which the efficacy of a compound is assessed by comparison of blood parasitemia and mouse survival time in treated and untreated mice [11], and the determination of percent suppression of parasitemia is the most reliable parameter [10,12].

5. Conclusion

It can be concluded from the results of this work that *Ancylobotrys amoena* is a potential target as an alternative treatment of malaria.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors have declared that no conflict of interest exist. The authors alone are responsible for the content and writing of this article.

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