



Antiplasmodial and antioxidant activities of methanolic leaf extract and fractions of *Alchornea cordifolia*

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ABSTRACT

Introduction: *Alchornea cordifolia* is widely used in Nigeria for the treatment of malaria. This study was aimed at assessing the in vivo antimalarial activities of the extract and fractions (n-hexane, chloroform, ethylacetate, butanol, aqueous) of the *A. cordifolia* in *Plasmodium berghei* infected mice as well as the antioxidant potentials of the crude extract and its fractions. **Methods:** Antioxidant activity was assessed by 1,1-diphenyl-2-picryl-hydrazyl (DPPH), nitric oxide scavenging, reducing power and hydrogen peroxide assays. By orally administering the extract (120 mg/kg, 240 mg/kg and 360 mg/kg) and fractions (240 mg/kg), antimalarial activities were evaluated using suppressive, prophylactic and curative tests. Chloroquine (5 mg/kg), pyrimethamine (1.2 mg/kg) and artesunate (5 mg/kg) were used as positive controls. **Results:** The crude extract showed a significant ($P < 0.05-0.001$) dose-dependent antimalarial activity in the suppressive, prophylactic and curative tests and increased animal survival time. All fractions caused significant reduction in parasitaemia with the ethylacetate fraction showing the highest activity. The extract and the fractions showed a significant ($P < 0.05-0.001$) dose-dependent antioxidant activity. **Conclusion:** *Alchornea cordifolia* exhibited significant antimalarial and antioxidant potentials, which may be useful in the on-going fight against malaria.

Implication for health policy/practice/research/medical education:

Alchornea cordifolia possesses antiplasmodial and antioxidant properties which may be beneficial in combating malaria in the future.

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Introduction

Malaria is a mosquito-borne infectious disease that affects humans and animals. It is caused by protozoans belonging to the genus *Plasmodium* (1). It is one of the most dangerous diseases in the world and is among the most prevalent communicable diseases globally (1). Apart from being a major public health problem with high morbidity and mortality, it has caused major socio-economic problems including global instability and poverty. Malaria is occurred in Africa and Asia and continues to constitute a major threat to mankind (3). *Alchornea cordifolia* is a sprawling, much-branched scandent shrub or tree that grows to about 8 m in height, especially where it is near sea and fresh-water. It is dispersed throughout all countries of the Region and widespread across tropical Africa (4) The

English people call it *christmas bush*, the Igbos (*Ubebe*), the Efiks and Ibibios of southern Nigeria, *mbom*. It is commonly used as a medicinal plant throughout its area of distribution for treatment of malaria. The leaves or leafy stems are used to treat a variety of respiratory conditions such as sore throat, cough and bronchitis, genital-urinary problems including venereal diseases, female sterility, and gastric ulcers, diarrhoea, amoebic dysentery and helminthic infections. It is used as purgative, enema and emetic in high doses when taken orally (4). It is also taken as a blood purifier, as a tonic and to treat anaemia and epilepsy. In Senegal the leaf decoction is taken to treat tachycardia (5).

The phytochemical constituents of the leaf extract include anthraquinones, saponins, flavonoids, tannins,

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steroidal and cardiac glycosides (6). The roots and stem bark have been reported to contain terpenoids, steroid glycosides, flavonoids, tannins, saponins, carbohydrates and the imidazopyrimidine alkaloids such as alchorneine, alchornidine and several guanidine alkaloids (7). The results of tests on anti-HIV activities of the seed extract are inconclusive. Tests done in Africa have shown that HIV-1 strains were sensitive to the seed extract whereas American tests seemed inconclusive. Methanol or ethanol extracts of leaf and root at a concentration of 100 µg/mL did not show cytotoxic activity against 60 different tumor cell lines from 8 organs (8). The ethanol extracts of the leaf and fruit have trypanocidal, anthelmintic and amoebicidal activities. The amoebicidal activity of the root bark was even much higher (9). The ethanol extract of the leaf produced mild in-vitro properties against *Plasmodium falciparum*, whereas chloroform and ether extracts were inactive (10). There is little or no scientific literature on the in vivo antimalarial activities of this plant. This study was therefore embarked upon so as to find out if this plant has in vivo antimalarial potential and hence lend support for its ethnobotanical use.

Materials and Methods

Plant preparation

Leaves of *A. cordifolia* were collected during the month of November, 2015 at Ibiono, Akwa Ibom state, Nigeria. It was identified and authenticated by Prof. Margret Bassey, a taxonomist in the Department of Botany and Ecological Studies, University of Uyo, where a voucher specimen (UUH3501) was deposited. The fresh leaves of *A. cordifolia* were collected and air-dried at room temperature. The dried leaves were then pulverized using a manual blender and 1.8 kg of powder-dried plant was cold-macerated in 12 L methanol at room temperature for 72 hours and thereafter filtered. The filtrate was dried *in vacuo* using rotary evaporator to obtain 215.7 g of dried extract. One hundred grams of the dried extract was partitioned using various solvents: n-hexane, chloroform, ethyl acetate, butanol and water to obtain their fractions (20.5 g, 18.3 g, 15.2 g, 12.4 g and 27 g). The crude extract and its fractions were stored in refrigerator at -4°C until needed.

Phytochemical screening

The extract was screened for phytochemical constituents such as saponins, alkaloids, flavonoids, anthraquinones, phlobotannins and cardiac glycosides using standard procedures (11,12).

Animal stock

Adult albino mice were obtained from the Animal House University of Jos and were maintained in the University of Uyo Animal House and fed with grower pellet feed. Water was given as needed. Ethical approval for the use of animals was obtained from the Animal Ethics Committee

of the Faculty of Pharmacy, University of Uyo.

Micro-organisms

A chloroquine-sensitive strain of *Plasmodium berghei* was obtained from National Institute of Medical Research (NIMER) in Lagos and maintained by sub-passage in mice.

Inoculum preparation

The mice were inoculated intraperitoneally with 0.2 mL of infected blood containing about 1×10^7 *P. berghei* parasitized erythrocytes. This was prepared by determining both the percentage of parasitaemia and the erythrocytes count of the donor mouse, and the blood was diluted with isotonic saline in proportions according to both determinations.

Drug administration

Artesunate, chloroquine and pyrimethamine, extract and fractions used in the antiplasmodial study were orally administered using a stainless metallic feeding cannula.

Acute toxicity test

This was carried out to ascertain the median lethal dose using Lorke's method (13). Adult albino mice were fasted overnight and thereafter administered with different doses of the crude extract and observed for 2 hours and the mortality was recorded for 24 hours. Mortality, body weight changes and spontaneous motor activity were assessed in mice.

Antimalarial activities of the extract

Evaluation of suppressive activity of the crude extract (4-day test)

A 4-day suppressive test was determined as described by Knight and Peters and modified by Ettebong et al (14). On the first day (D_0), 36 mice were inoculated with the parasite and randomly divided into 6 groups of 6 mice in each group. Ten minutes later, the mice in group one were orally administered with 10 mL/kg of distilled water and served as control group. Group 2-4 received 120 mg/kg, 240 mg/kg and 360 mg/kg of crude extract, respectively. Group 5 and 6 received chloroquine and artesunate (standard drugs at 5 mg/kg each) as positive controls. The administration of the extract and drug was continued for 4 consecutive days.

On the fifth day, thin blood film was made from tail blood of each mouse and stained with Leishman stain to reveal parasitized erythrocytes. The percentage of parasitaemia was calculated by counting the number of parasitized red blood cells out of 500 erythrocytes in random fields of microscope using Neubaure counting chamber.

$$\% \text{ Parasitaemia} = \frac{\text{No. of parasitized erythrocyte}}{\text{Total No. of RBC counted}} \times 100$$

Average percentage of chemo-suppression was calculated

as $100 \left(\frac{A-B}{A} \right)$

Where, A is the average percentage of parasitaemia in negative control group and B, average percentage of parasitaemia in the test group.

Evaluation of prophylactic or repository activities of extract

The prophylactic property of the extract was evaluated using the method described by Peters and modified by Ettebong et al (14,15). Mice were randomly divided into 5 groups of 6 mice each. Group one was orally administered with 10 mL/kg of distilled water and served as control. Group 2-4 received 120 mg/kg, 240 mg/kg and 360 mg/kg of crude extract respectively. Group 5 received pyrimethamine (1.2 mg/kg) each as positive control. Administration of the extract/drug was continued for 3 consecutive days (D_0 - D_2). On the fourth day (D_3) the mice were inoculated with *P. berghei* infected red blood cells. The level of parasitaemia was assessed using blood smears 72 hours later. Percentage parasitaemia and average chemosuppression were calculated as stated above.

Evaluation of curative activities of the crude extract (Rane's test)

The antiplasmodial activity of the extract in established infection was done using the method of Ryley and Peters and modified by Ettebong et al (14,16). *P. berghei* infected red blood cells was injected into another 36 mice (i.p) on the first day. Seventy-two hours later, the mice were randomly divided into 6 groups of 6 mice each. Group 1 was orally administered with 10 ml/kg of distilled water as negative control. Group 2-4 received 120 mg/kg, 240 mg/kg and 360 mg/kg of crude extract respectively. Groups 5 and 6 received chloroquine and artesunate (5 mg/kg each) and served as positive controls. The extracts and drugs were administered once daily for 5 days. Tail blood sample from each mouse was collected daily for 5 days. Leishman's stain and thin smears were then prepared from tail blood samples collected daily to monitor parasitaemia level. The mean survival time (MST) of each group was determined over a period of 30 days (D_0 - D_{30}).

$$MST = \frac{\text{No. of days survived}}{\text{Total No. of days (30)}} \times 100$$

Evaluation of suppressive activity of the fractions (4-day test)

The method of Ettebong et al (14) and Knight and Peters (17) was used to evaluate the suppressive effect of the fractions. Forty-two mice were each inoculated on the first day (day 0), intraperitoneally with 0.2 mL of infected blood and randomly divided into 7 groups of 6 mice each. Group 1 was orally administered with 10 mL/kg of distilled water and served as control group. The mice in groups 2, 3, 4 and 5 were administered with n-hexane, chloroform, ethyl acetate, butanol or aqueous fraction of the extract,

respectively while groups 6 and 7 were administered with chloroquine and artesunate (5 mg/kg respectively). On the fifth day, thin blood film was made and stained with Leishman's stain to reveal parasitized erythrocytes. The average percentage suppression of parasitaemia was calculated.

In vitro antioxidant activity

Reducing power assay

The reducing power of n-hexane, ethyl acetate, chloroform, and aqueous extract of *Alchornea cordifolia* was determined using various concentrations of the plant extract and fractions. To 1.0 mL of deionized water was added phosphate buffer (2.5 mL) and 2.5 mL of potassium ferricyanide (30 mM) and incubated at 50°C for 20 minutes. Then 2.5 mL of trichloroacetic acid of the solution was mixed with 2.5 mL of distilled water and 0.5 mL of FeCl_3 and its absorbance was recorded at 700 nm (18).

Nitric oxide scavenging assay

In this model, 3.0 mL of 10 mM sodium nitroprusside in phosphate buffer was added to 2.0 mL of extract/fractions and reference compound at different concentrations (20-100 $\mu\text{g/mL}$). The solution obtained was thereafter incubated at 25°C for 60 minutes. The procedure was repeated with methanol as blank, serving as control. To 5.0 mL of the incubated sample, 5.0 mL of Griess reagent (1% sulphanilamide, 0.1% naphthyethylene diamide dihydrochloride in 2% of H_3PO_3) was added and the absorbance of the chromophore formed was read at 540 nm (19).

Hydrogen peroxide radical scavenging (H_2O_2) assay

The radical scavenging property of the extracts to hydrogen peroxide (H_2O_2) was determined using a solution of hydrogen peroxide (40 mM) prepared in phosphate buffer, pH 7.4. The concentration of hydrogen peroxide was assayed by absorption at 230 nm using a spectrophotometer. The extracts (0.5-2.5 $\mu\text{g/mL}$) in distilled water were added to a hydrogen peroxide solution and the absorbance was read at 230 nm after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as the standard (19).

Determination of DPPH radical scavenging activity

A dose of 0.2 mL of the crude extract and fractions was added to 3.8 mL of ethanol solution of DPPH radical until a final concentration of 0.1 mM was obtained (20). The mixture was agitated vigorously for 1 minute and left to stand at room temperature for 30 minutes. The absorbance of each of the samples (A_s) was read at 517 nm against ethanol as blank. Negative control (A) was taken after adding DPPH solution to 0.2 mL of the respective

extracts. The percentage of DPPH discolouration of the sample was calculated according to the equation.

$$\% \text{ inhibition} = A - \frac{A_s}{A} \times 100$$

Where A is the absorbance for control and A_s is the absorbance for test.

Statistical analysis

Results were expressed as multiple comparisons of mean \pm SEM. Significance was determined using One-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison post-test. A probability level of 5% or less was considered significant.

Results

The median lethal dose (LD_{50}) was calculated to be 1200 mg/kg. The phytochemical screening of the extract showed the presence of alkaloids, flavonoids, saponins, tannins, terpenes, cardiac glycosides and carbohydrates. In the suppressive test (Table 1), there was a dose-dependent decrease in the level of parasitaemia following administration of the extract compared to control group. This decrease was statistically significant ($P < 0.05-0.001$). However, the suppressive effect was less compared with the standard drugs chloroquine and artesunate. The ethyl acetate fraction had the highest chemo-suppressive effect followed by n-hexane, aqueous, butanol and chloroform fractions, respectively as shown in Table 2. In the repository test, the methanol extract of *A. cordifolia* exerted a dose-dependent prophylactic activity at the various doses employed, resulting in significant ($P < 0.05-0.001$) reduction of parasitaemia in extract treated groups when compared to control as shown in Table 3. The extract showed a dose-dependent effect on the parasitaemia. In the curative test, there was a significant dose-dependent antiparasmodial activity of the extract when tested on established infection, which was comparable to chloroquine and artesunate-treated group (Figure 1). The negative control group showed daily increase in parasitaemia. The MSTs of the extract treated groups of mice increased dose-dependently and

Table 1. Suppressive antiparasmodial activity of methanol extract of *Alchornea cordifolia*

Drug/extract	Dose (mg/kg)	Parasitaemia ($\times 10^7$)	% Chemosuppression
Distilled water	10 mL/kg	50.00 \pm 1.00	-
Extract	120	12.00 \pm 0.50 ^a	76.00
	240	4.80 \pm 0.50 ^a	90.40
	360	4.20 \pm 0.08 ^a	91.60
Artesunate	5.0	0.66 \pm 0.05 ^a	98.80
Chloroquine	5.0	0.30 \pm 0.50 ^a	99.40

Values are expressed as mean \pm SEM.

Significance relative to control: ^a $P < 0.001$; $n = 6$.

significantly. The extract increased the MST from 5 to 15 days when compared to control group. However, when compared to standard drug (chloroquine and artesunate), the MSTs were shorter (Table 4).

The antioxidant activity of the extract was demonstrated using Nitric oxide, DPPH, Hydrogen peroxide and reducing power assays. In the nitric oxide scavenging test, the extract and fractions effectively reduced the generation of nitric oxide. When compared with the standard drug, aqueous fraction had more inhibitory effect, followed by crude extract and ethyl acetate fractions (Figure 2).

In the DPPH quenching capacity, the extract and fractions of *A. cordifolia* were capable of neutralizing the DPPH free radicals via hydrogen donating activity at different concentrations. Scavenging activity was increased in a concentration dependent manner. However, ethyl acetate had the highest inhibitory activity when compared with the control, vitamin C (Figure 3).

Table 2. Suppressive antiparasmodial activity of fractions of *Alchornea cordifolia*

Drug/extract	Dose (mg/kg)	Parasitaemia ($\times 10^7$)	% Chemosuppression
Distilled water	10 mL/kg	35.50 \pm 0.40	-
Ethylacetate fraction	240	8.50 \pm 0.66 ^a	76.20
N-hexane fraction	240	14.00 \pm 0.15 ^a	60.50
Aqueous fraction	240	16.30 \pm 0.66 ^a	54.00
Butanol fraction	240	22.70 \pm 0.20 ^a	36.00
Chloroform fraction	240	24.40 \pm 0.54 ^a	31.30
Chloroquine	5.0	0.20 \pm 0.00 ^a	99.40

Values are expressed as mean \pm SEM.

Significance relative to control: ^a $P < 0.001$; $n = 6$.

Table 3. Prophylactic antiparasmodial activity of methanolic extract of *Alchornea cordifolia*

Drug/extract	Dose (mg/kg)	Parasitaemia ($\times 10^7$)	% Chemosuppression
Distilled water	10 mL/kg	35.20 \pm 0.80	-
Extract	120	20.60 \pm 0.50 ^a	27.30
	240	12.10 \pm 0.57 ^a	65.60
	360	6.80 \pm 0.70 ^a	80.00
Pyramethamine	5.0	2.00 \pm 0.15 ^a	94.30

Values are expressed as mean \pm SEM.

Significance relative to control: ^a $P < 0.001$; $n = 6$.

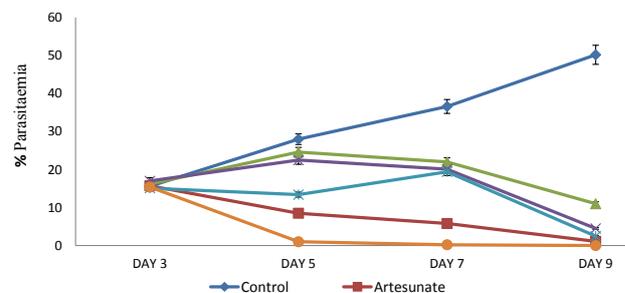


Figure 1. Antiparasmodial activity of methanolic extract *Alchornea cordifolia* during established infection (curative test).

Table 4. Mean survival time of mice receiving various doses of methanol extract of *Alchornea cordifolia*

Drug/extract	Doses (mg/kg)	MST (days)
Distilled water	10 (mL/kg)	12.67 ± 0.66
Extract	120	17.70 ± 0.66 ^a
	240	23.67 ± 0.33 ^a
	360	27.71 ± 0.66 ^a
	5.00	20.67 ± 0.33 ^a
Artesunate	5.00	20.67 ± 0.33 ^a
Chloroquine	5.00	29.33 ± 0.66 ^a

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

On reducing power, the reducing power activity of methanol leaf extracts and fractions of *A. cordifolia* increased consistently with the increase in the volume of extract from 20 µg to 100 µg. The aqueous fraction showed the highest scavenging activity, but was less when compared with the standard drug, vitamin C (Figure 4). In the hydrogen peroxide test, the result showed that hydrogen peroxide scavenging activity of methanol leaf extract and fractions of *A. cordifolia* increased consistently with increase in concentration of extract. It was observed that chloroform and ethyl acetate fractions showed the

highest scavenging activity even more than the standard drug (Figure 5).

Discussion

In this study, the acute toxicity evaluation of the extract revealed that LD₅₀ was 1200 mg/kg, which makes it slightly toxic (21). The phytochemical screening carried out on the leaf extract of *A. cordifolia* revealed the presence of alkaloids, saponins, tannins, glycosides, flavonoids, terpenes and carbohydrates which likely contributed to the observed pharmacological effects. This finding confirmed the phytochemical study of the leaf extract carried out by Osadebe and Okoye (6). The antimalarial activity of the crude extract and fractions of *A. cordifolia* was investigated to establish the antimalarial potentials of the plant. The results obtained show that the leaves of *A. cordifolia* significantly depicted antimalarial activity in a dose-dependent manner which is comparable to that of standard drug used. This antiplasmodial action could be as a result of various phytochemicals present in the leaf of the plant, which may be acting alone or in combination with one another to exert the observed antiplasmodial effect (22).

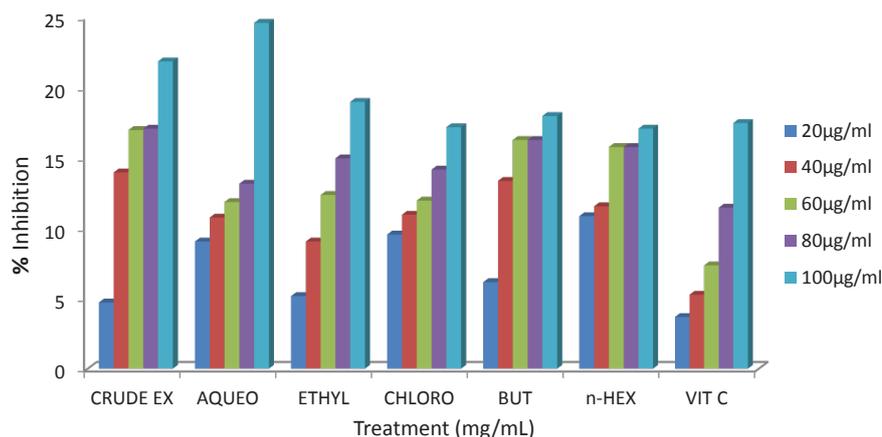


Figure 2. Effect of extract and its fractions on nitrogen oxide assay.

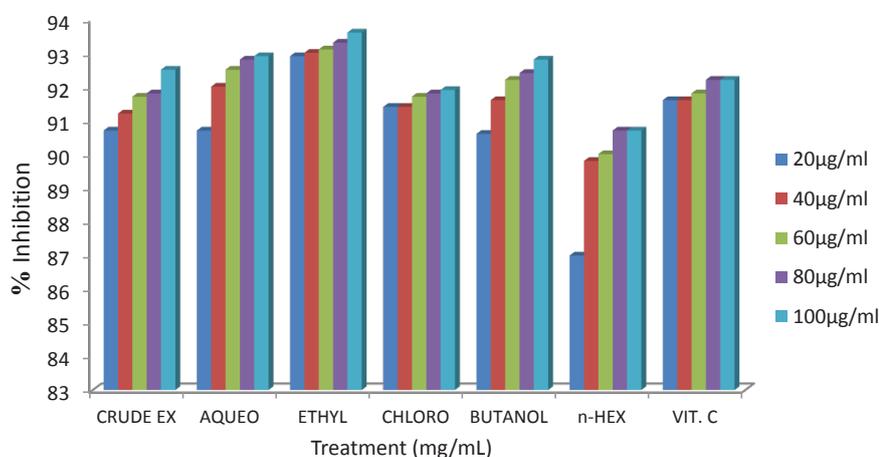


Figure 3. Effect of extract and its fractions on 1,1-diphenyl-2-picrylhydrazyl (DPPH).

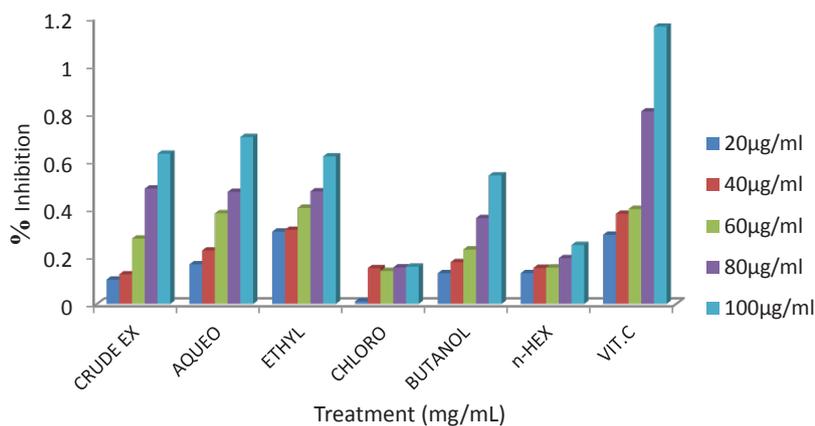


Figure 4. Effect of extract and its fractions on reducing power assay.

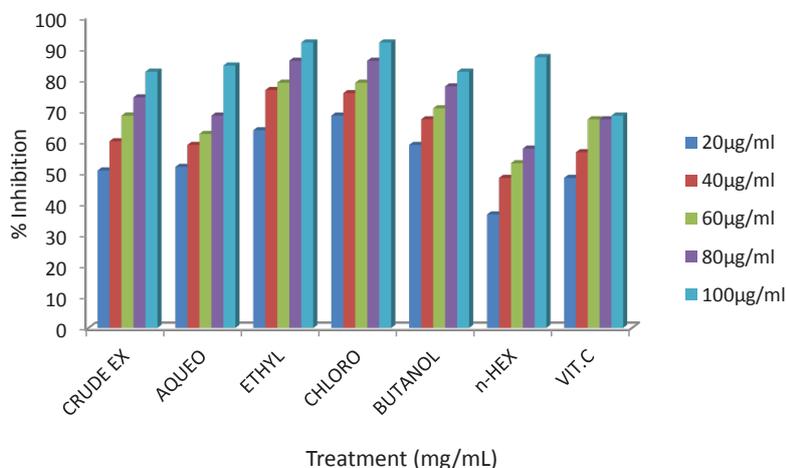


Figure 5. Effect of extract and its fractions on hydrogen peroxide.

Alkaloids have been shown to have in vitro anti-malarial activity by blocking protein synthesis in *P. falciparum* (23). Flavonoids have also been reported to exert their antiplasmodial activity through chelating the nucleic acid base pairs of the malarial parasite (13). The presence of flavonoids in the leaf extract of *A. cordifolia* may contribute to its antiplasmodial activity. Flavonoids may be acting in synergy with alkaloids to exhibit antimalarial effect. Flavonoids are known to inhibit the intra-erythrocytic growth of certain strains of *P. falciparum* (24,25) Tannins are reported to possess antioxidant activity (26). This could be one of the mechanisms of action against malarial parasite observed in the leaf extract of *A. cordifolia*. The fractions were tested for antiplasmodial activity to determine which of the fractions has the highest activity. The fractions exhibited good antiplasmodial activity with ethyl acetate fraction exhibiting the highest antiplasmodial action. This suggests that the bioactive compounds in the plant extract are most probably non-polar compounds. The free radical scavenging potential of leaf extract of *A. cordifolia* and its fractions was studied using different antioxidant models; the in vitro antioxidant activity of methanol leaf extract

of *A. cordifolia* was investigated for the scavenging activity of nitric oxide radical, reducing power assay, hydrogen peroxide (H_2O_2) assay and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay. From the results, a significant correlation existed between concentrations of the extract and percentage inhibition of free radicals, reducing power or inhibition of lipid peroxidation. These results suggest that the polyphenolic compounds of *A. cordifolia* play an important role in reducing ferric ions to ferrous ions and capable of donating electrons that scavenge the free radicals. The antioxidant and free radical scavenging properties of iron chelators are based on their capacities to chelate metal ions, cause reduction of ferric to ferrous ion, scavenge non-biological stable free radical such as DPPH and scavenge active biological oxidants like H_2O_2 and $\bullet OH$ (27,28). This may represent yet another mechanism that contributes to its antiplasmodial activity. The fractions of the extract also showed a dose-dependent scavenging activity with ethyl acetate and aqueous fractions having highest scavenging activity even more than the standard drug. This is an indication that the fractions of *A. cordifolia* are capable of reducing oxidative stress by scavenging ROS which is implicated in the pathogenesis of malaria.

Conclusion

The results of this study show that the extract of *A. cordifolia* has suppressive, repository and curative antiplasmodial properties. The fractions exhibited good antiplasmodial activity with ethyl acetate fraction exhibiting the highest antiplasmodial action. The fractions also showed a dose-dependent scavenging activity with ethyl acetate and aqueous fractions having the highest scavenging activity.

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Author's Contributions

EE designed the study, interpreted the results, and wrote the manuscript. AN and KD did the experiments and assisted in the writing of the manuscript.

Conflict of interests

None.

Ethical considerations

All experimental procedures involving animals were conducted in accordance to Organization for Economic Co-operation and Development guidelines and approved by Animal Ethics Committee, Faculty of Pharmacy, University of Uyo, Nigeria.

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