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The *In Vitro* Antimalarial Activity of Aqueous and Ethanolic Extracts of *Anacardium occidentale* against *Plasmodium falciparum* in Damboa, North-Eastern Nigeria.

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ABSTRACT

Malaria is one of the most important infectious and life-threatening diseases in the tropics and sub-tropics. The search for new antimalarial compounds has largely been necessitated by *P. falciparum* resistance to most antimalarial drugs. In this study, the *in vitro* antimalarial activity of the aqueous and ethanolic crude extracts of *Anacardium occidentale*, was evaluated against fresh isolates of *P. falciparum* from Damboa, North-eastern Nigeria. Acute toxicity test and anti-inflammatory activity of the extracts were also determined. Inhibition of schizont maturation was significant relative to control (P = 0.05). Ethanolic extract exhibited higher antimalarial activity of 75.64.10 %, IC_{50} of 11.7µg/ml and aqueous extract had an activity of 72.38 %, IC_{50} of 16.00µg/ml. Both extracts showed moderate antimalarial activity. The ethanolic extract of *Anacardium occidentale* at 3000mg/kg body weight showed sign of weakness. Other extracts did not show any signs of toxicity in rats and all extracts showed a good measure of anti-inflammatory activity. This result justifies the traditional use of the plant in malaria treatment. As potential source of antimalarial compounds, the plant extracts should be subjected to further research to study their active principles or constituents.

Keywords: *Antimalarial activity, Plasmodium falciparum, Extract concentration, Anacardium occidentale, Malaria.*

1. INTRODUCTION

Malaria is a life-threatening parasitic disease caused by *Plasmodium* species and transmitted by female *Anopheles* mosquitoes. The disease is found throughout tropical and sub-tropical regions of the world and causes more than 300 million acute illnesses and about million deaths annually [1], [2]. Approximately 90% of malaria cases in the world are estimated to be in Africa South of the Sahara, where the disease is endemic [3]. Malaria affects the health as well as the wealth of nations and individuals, and is understood to be both the disease of poverty and the cause of poverty [4].

In Nigeria, malaria transmission occur all-year round in the South, and more seasonal in the North. In addition, the country accounts for a quarter of all malaria cases in the WHO African region [3]. Antimalarial drug resistance is a major health problem, which hinders the control of malaria. The rapid spread of resistance to antimalarial drugs over the few decades [5] has no doubt necessitated the search for more effective antimalarial compounds.

In Africa and other countries where malaria is endemic, traditional medicinal plants are frequently used to treat or cure malaria [6]. It is therefore imperative to investigate the antimalarial activity of medicinal plants in order to determine their potentials as sources of new antimalarial agents [7]. *Anacardium occidentale*, popularly known as cashew is a plant belonging to the family *Anacardiaceae*. It is found throughout the tropics in West Africa. Leaf and bark infusion relieve tooth ache and sore gum [8]. It is used as food and raw materials for confectionary and chocolatery. The back and leaves are traditionally used for malaria cure [9]. In this study, the antimalarial activity of *A. occidentale* was evaluated *in vitro* against *P. falciparum* fresh isolates. Acute toxicity and anti-inflammatory activity of the plant extracts were also determined.

2. MATERIALS AND METHODS

Collection Extraction and Phytochemical Screening of Plant materials

Fresh bark of *A. occidentale* was collected in October, 2008 in Jos, Nigeria. The plant was authenticated by a taxonomist at the Federal College of Forestry, Jos and Herbarium sample specimen with voucher number FCFBM 020 was deposited at the Botanical Museum of the College.Fresh bark of *A. occidentale* was cut into pieces and air dried for three weeks in the laboratory. The dried pieces were then reduced to powder using a laboratory grinder. Eighty grammes of the dried powdered form of the plant materials were extracted with water and ethanol in a soxlet apparatus for 72 hours. All the extracts were concentrated to dryness on a water bath and weighed. The extracts were then stored in well-closed containers and kept in a refrigerator at 4 ºC to protect from light and moisture till used [10]. The phytochemical analysis of the plant extracts was carried out using the thin - layer chromatography (TLC). The standard screening test using standard procedure was utilized for detecting the constituents [11].

Collection of blood samples for *in vitro* sensitivity test

The *in vitro* sensitivity test was carried out at Damboa General Hospital, Borno State, Nigeria. Damboa is the National Malaria Surveillance Sentinel site of the Northeast Nigeria. It was carried out from October to December, 2009, among patients attending the out-patient department of the hospital. The following inclusion criteria was set for this work: children and adults age 6 months and above who had fever in the last 24 hours, auxiliary temperature ≥ 37.5ºC, had not taken any antimalarial in the last 2 weeks, who gave oral or written informed consent after the aim of the study was explained to them (patient/ parent/ or guardian in the case of minors).

Patients who satisfied the inclusion criteria were screened for *P. falciparum* infections. Two drops of blood samples obtained from finger prick, using sterile disposable needles were used to prepare thick and thin smears on clean slides for each patient. Prepared slides were stained for 10 minutes with 10 % Giemsa solution prepared in phosphate buffer of pH 7.3 and examined microscopically for parasites [12]. Patients who had mono infection of *P. falciparum* with parasitaemia of 1,000/µl and not more than 100,000/µl of blood were included in the *in vitro* drug susceptibility test [13].

Fourteen fresh isolates of *P. falciparum* were obtained from symptomatic patients, aged of 5 to 25 years by collecting 3ml to 5ml of blood into EDTA bottles from patients with confirmed *P. falciparum* mono infections. The fresh blood samples were centrifuged at 2000 rpm for 10 minutes, the blood plasma was removed and the blood pellets suspended and washed thrice in RPMI 1640 medium (Gibco USA) before use for parasite cultivation [14]. Patients were then treated with Artemether - Lumefantrine (COARTEM) combination drugs in line with National Malaria Treatment Guidelines.

Preparation of Culture Medium and Extracts Solution

Culture medium was prepared by dissolving 10.43 g RPMI 1640 powder (Gibco), 6 g of N-2 hydroxyethyl piperazine-N-2- ethane sulphonic acid (HEPES), 2 g of NaHCO3 (Sigma Aldrich) in 1 liter of distilled-deionised water. The medium was filtered using 0.22 µm membrane filter and 0.5 ml gentamacin (from 50 mg/ml stock) was added and stored at 4 ºC in aliquots of 45ml. Before cultivation, every aliquot was supplemented with 5 ml of 5 % Albumax II [15]. Water extracts were first dissolved in distilled water and ethanol extracts were dissolved in methanol and diluted in distilled-deionised water, and 2 mg/ml solution of each was prepared. The 2 mg/ml solution was further diluted in the malaria culture medium to give 200 µg/ml stock solution [16]. Extracts were tested in 6 serial two-fold dilution with a final concentration range of 100 – 1.56 µg/ml in 96 wells microtitre plates (Becton Dickinson Lab wares, USA).

In Vitro Cultivation of *P. falciparum* Isolates and Sensitivity test

The assay was performed in duplicate. One hundred µl of distilled water was first distributed into well plates after which 100 µl of culture medium containing extracts at various concentrations was added into well plates. One hundred micro liters of parasite culture were finally added. Titre plates were incubated in CO_2 condition at 37 ºC in candle jar for 24-30 hours [17]. After incubation, contents of the wells were harvested and the red cells transferred to a clean microscopic slide to form a series of thick films. The films were stained for 10 minutes in 10 % Giemsa solution of pH 7.3. Schizont growth inhibition per 200 asexual parasites was counted in 10 microscopic fields. The control parasite culture freed from extracts was considered as 100 % growth. The percentage inhibition per concentration was calculated using the formula: [(% parasitaemia in control wells – % parasitaemia of test wells)/ (% parasitaemia of the control)] x 100 [13], [18]. The IC_{50} values, the concentration required to inhibit schizont growth by 50% were determined by linear interpolation from the schizont growth inhibition curves (Log of concentration versus percent inhibition) generated from each parasite-extract interaction [7].

Experimental Animals

The animals used for acute toxicity test were adult male and female mice (20 – 25 g) and for anti-inflammatory activity were rats (150 – 200 g) obtained from the animal house of the University of Jos, Nigeria. The animals were acclimatized for the period of 7 days to room temperature and humidity before they are used. They were housed in standard cages and maintained on standard animal pellets and water *ad libitum*. Animals were handled according to local rules and regulation of Experimental Animals, University of Jos, Nigeria.

Acute toxicity test

The acute toxicity of plant extracts were tested on rats using 3 doses (500mg/kg, 1000mg/kg and 3000 mg/kg body weight) administered orally. Control rats were kept under the same conditions without any treatments. The animals were routinely inspected for appearances or signs of toxicity such as tremors, weakness and refusal of feeds, falling off of hair, coma or even death for 48 hours.

Anti-inflammatory Test: Topical Oedema of the Mouse Ear

Xylene induced oedema

The effect of water and alcohol extracts of *A. occidentale* on topical acute oedema was assessed using xylene-induced ear oedema in mice [19]. Swiss albino mice (20 – 25 g) received topical application (5 g/ear) of water extract of or ethanol extract of *A.*

occidentale, on the anterior surface of the right ear while xylene (0.08 ml) was instantly applied on the posterior surface of the same ear. Control animals received 0.2 ml of distilled water on the anterior surface and 0.08ml xylene on the posterior surface. The left ear was left untreated.

After 3 hours of xylene application, ear oedema was measured with micrometer screw guage (Moore and Wright, England) [20]. The difference in thickness of ear from right treated and left untreated ears was calculated and used as a measure of oedema [19]. The level of inhibition (%) of oedema was calculated using the relationship:

$$\text{Inhibition (\%)} = 100[1 - (Et/Ec)],$$

Where Et =average oedema of treated ear,
Ec =average oedema of treated control [19].

STATISTICAL ANALYSIS

The Microsoft Excel 2007 was used to calculate mean parasite growth and percentage parasite inhibition. The student t-test was used to statistically analyze the data and values of $P \leq 0.05$ were considered significant.

3. RESULTS

The phytochemical screening of plant material showed the presence of saponins, tannins, flavonoids, carbohydrates, cardiac glycosides, and alkaloids.The crude extracts were tested on trophozoites, mainly ring forms. At each of the seven concentrations of all the extracts (Table 1), there was a significant reduction in the number of parasitized cells relative to control ($P \geq 0.05$). Of the two extracts tested in this study, the ethanol extract of *A. occidentale* showed the highest antimalarial activity of 75.64 %, with IC_{50} of11.7 µg/ml. The water extract had the parasite growth inhibition of 72.38 % and IC_{50} of 16.0 µg/ml (Table 2). Fig. 1 shows schizont growth inhibition curves generated from each parasite-extract combination/interaction. Inhibition Concentration (IC_{50}) values were determined by linear interpolation from each of the inhibition curves. With regard to concentrations administered, dose-dependent antimalarial activity was clearly shown for the two crude extracts. The percentage inhibitions are higher with increasing concentrations**.** Aqueous extract of *A. occidentale* in the doses of 500mg/kg, 1000mg/kg and 3000 mg/kg body weight (administered orally) did not show sign of toxicity or cause any deaths, all parameters were negative. However, ethanolic extract of *A. occidentale* at 3000mg/kg produced weakness in rats. Ethanolic extracts at 500mg/kg and 1000mg/kg were not toxic.The right treated ears exhibited topical oedema induced by xylene in the experimental animals. Ethanol extract of *A. occidentale* exhibited higher anti-inflammatory activity with inhibition of 54.0 % than ethanol extract which had inhibition of 43.0%.

Table 1: **Mean Parasite growth at various concentrations**

	Concentration of Extracts (µg/ml)						
	1.56	3.13	6.25	12.50	25.00	50.00	100.00
Extracts	% Mean Parasite Growth ± S.D.						
Aqueous Extract	88.4±4.2	76.0.9±5.2	64.7±7.5	52.5±6.8	42.3±9.0	35.0±9.8	27.6±9.7
Ethanol Extract	84.8±7.1	71.7±90	60.1±10.8	48.5±10.5	38.7±11.4	31.0±11.1	24.4±9.6

P ≥ 0.05 compared to control; Control is 100 % growth.

Table 2: *In Vitro* Schizont Growth Inhibition of *Plasmodium falciparum* Isolates by Plant Extracts

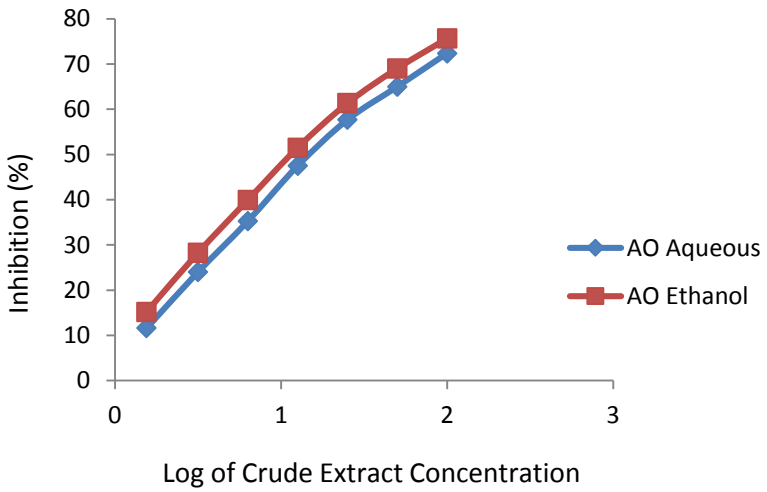

Fig. 1: The Percentage Inhibition of the Extracts showing Dose- dependent Antimalarial Activity

Legend: AO = *Anacardium occidentale*

	Concentration of Extract (µg/ml)						
	1.56	3.13	6.25	12.5	25.0	50.0	100.0
Extract							
Aqueous Extract (% Inhibition)	11.6	24.03	35.26	47.50	57.72	65.0	72.38
Ethanol Extract (% Inhibition)	15.19	28.29	39.94	51.49	61.35	69.03	75.64

4. DISCUSSION

In sub-Saharan Africa and other parts of the world where malaria is endemic, herbal remedies are commonly used to treat the disease. This study analyzed and screened crude extracts *A. occidentale* for antimalarial activity. The ethanol extract gave the highest antimalarial activity of 75.64 %, with IC_{50} value of 11.7 µg/ml compared to aqueous extract which had antimalarial activity of 72.3 8 % with IC_{50} of 16.0 µg/ml. *A. occidentale* (leaf and bark) is used by traditional healers for treatment of asthma, diabetes, urinary disorders, diarrhea, and contains saponins, tannins, flavonoids . These compounds present in the extracts may be responsible for the observed antimalarial activity of the extracts though the active principle is yet to be identified [21]. Flavonoids are compounds with a widespread occurrence in the plant kingdom which have also been detected in *Artemisia* species. They are reported to have exhibited significant *in vitro* antimalarial activity against *P. falciparum* [22]. Their presence in *A. occidentale* extracts justify the antimalarial activities exhibited by the plant extracts.

Both aqueous and ethanolic extracts of *A. occidentale* exhibited antimalarial activity. Traditional healers in Nigeria usually use decoctions of *A. occidentale* and *C. citratus* in combination with other plant parts such as leaves of *Vernonia amygdalina* (bitter leaf) and leaves of *Azadirachta indica* (neem tree) to treat malaria and fever, which results into enhanced antimalarial effect [9], [23].

The thresholds for the *in vitro* antimalarial activity of the plant extracts were based on the classification according to Gessler *et al.* [24]: that is, extract with IC_{50} less than 10µg/ml is considered very good, from 10 to 50µg/ml is moderate and over 50 µg/ml is considered to have low activity. Based on this classification, the water and ethanol extracts of *A. occidentale* with IC_{50} of 16.0µg/ml

and11.7µg/ml respectively, are said to have moderate antimalarial activity.

On acute toxicity, the results obtained imply that aqueous and ethanolic extracts of *A. occidentale* at the dosages tested in this work did not show toxicity in the animals. The extracts can therefore be considered safe since with a 6-fold increase in the therapeutic doses 500mg/kg-3000mg/kg body weight, no deaths or signs of toxicity were observed. This is in agreement with Tedong *et al*. [25] that *A. occidentale* is non-toxic at the dosages tested. The result of this work also agrees with Ofusori *et al*. [26], who reported the non-toxic effect of ethanolic extract of cashew stem bark on the brain and kidney parenchyma of mice.

Both crude extracts exhibited some measure of anti-inflammatory activity. Anti-inflammation properties had been observed in flavonoids, tannins and alkaloids [27]. It is therefore possible that the anti-inflammatory effect observed in water and ethanol extracts of *A. occidentale* may be due to their flavonoids, tannins and alkaloids contents as also observed by Musa *et al.* [28]. In addition, the anti-inflammatory effects exhibited by these extracts to topical model of acute inflammation justify the traditional use of the plants leaves and bark in the management of inflammatory conditions.

5. CONCLUSION

The results of this study have shown that the ethanolic extract of *A. occidentale* exhibited higher antimalarial activity than water extract. Both extracts however, possess a moderate antimalarial activity. This result justifies the traditional use of the plant in the treatment of malaria. As potential source of antimalarial compound, the plant extracts should be subjected to further research to study their active principles or constituents, with a view to making the products available to the people.

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