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IN VITRO ANTIMALARIAL ACTIVITY OF THE EXTRACTS OF *Vernonia amygdalina* COMMONLY USED IN TRADITIONAL MEDICINE IN NIGERIA.

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ABSTRACT

Malaria is one of the most important of infectious diseases in the tropics and sub-tropics. The search for antimalarial compounds has been necessitated by *P. falciparum* resistance to almost all antimalarial drugs. In this study, the *in vitro* antimalarial activities of the aqueous and ethanolic crude extracts of *Vernonia amygdalina*, a plant used by traditional healers to treat malaria and other diseases, was evaluated against 14 fresh isolates of *P. falciparum* from Damboa, Borno State, Nigeria. Acute toxicity test and anti-inflammatory activity of the extracts were also determined. There was a significant inhibition in schizont maturation relative to control (P = 0.05). Ethanolic extract exhibited higher antimalarial activity of 78.10 %, IC_{50} of 11.2 µg/ml and aqueous extract had an activity of 74.02 %, IC_{50} of 13.6 µg/ml. Both extracts showed moderate antimalarial activity. The extracts exhibited negligible toxicity in rats and showed a good measure of anti-inflammatory activity. This result justifies the traditional use of the plant in malaria treatment. Further work is suggested to isolate, identify and characterize the active principles from the plant.

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

INTRODUCTION.

Malaria is one of the most important tropical diseases and the greatest cause of hospitalization and death among children age 6 months to 5 years (Molta *et al*., 2006; Quattara *et al*., 2006). The World Health Organization reported that there were an estimated 246 million malaria cases distributed among 3.3 billion people at risk in 2006, causing at least a million deaths. These were mostly children under five years. One hundred and nine countries were endemic in 2008 and 45 within the WHO African region (WHO, 2008). Approximately 80% of malaria cases in the world are estimated to be in Africa where the disease is endemic (WHO, 2008). The disease is a major cause of the continents high infant mortality, killing 1 in every 20 children below 5 years of age (Goose, 1993). In Nigeria, malaria transmission occurs all-year round in the South, and is more seasonal in the North. The country accounts for a quarter of all malaria cases in the WHO African region (WHO, 2008).

The alarming rate at which *Plasmodium falciparum* has developed resistance to chloroquine and other synthetic antimalarial drugs makes it necessary to search for more effective antimalarial compounds (Bhat & Surolia, 2001). In Africa and other countries where malaria is endemic, traditional medicinal plants are frequently used to treat or cure malaria (Gessler *et al*., 1994; Jenett-Siems *et al*., 1999). It is a fact that conventional antimalarials such as chloroquine, quinine and artemisinin derivatives originated from plants (Taylor *et al*., 1993). It is therefore important to investigate the antimalarial activity of medicinal plants in order to determine their potentials as sources of new antimalarial agents (Mustofa *et al*., 2007).

Vernonia amygdalina, popularly known as bitter leaf, is an under shrub of variable height with petiolate green leaves of about 6mm diameter. The leaves are usually bitter and are very popular soup vegetable in Nigeria (Ojiako & Nwanjo, 2006). The plant is found in savannah regions and through central and south tropical Africa. All parts of the plant (leaves, stem and roots) are said to have medicinal uses. These include promotion of dieresis, cure of tonsillitis, fever, malaria, diabetis, pneumonia, jaundice, anaemia, stomach problem and ascaris (NNMDA, 2006; Odugbemi *et al.,* 2007).

Both aqueous and ethanolic extracts of the stem bark and leaves are reported to have been used as purgative, antimalarial and in the treatment of eczema (Ojiako & Nwanjo, 2006). In this study, the antimalarial activity of *Vernonia amygdalina* was evaluated *in vitro*. Acute toxicity and anti-inflammatory activity of the plant were also determined.

MATERIALS AND METHODS.

Collection and Extraction of Plant materials: Fresh leaves of *V. amygdalina* were 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 008 was deposited at the Botanical Museum of the College.

Fresh leaves of *V. amygdalina* was cut into pieces and air dried 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 in a soxlet apparatus for 72 hours. Another 80 g powder of the plant was extracted with ethanol in soxlet extractor 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 (Sutharson *et al.,* 2007).

Phytochemical Screening of extracts: The preliminary 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 active constituents (Harborne, 1984). This was done at the Pharmacognosy Department, Faculty of Pharmacy, University of Jos, Nigeria.

Collection of blood samples for *in vitro* sensitivity test: The study was conducted at Damboa General Hospital, Borno State, Nigeria. Damboa is the North-east zone National Malaria Surveillance Sentinel site. The study was carried out between October - 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 ≤ 37.5 ºC, 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. Few 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 (Molta *et al.,* 1992). Patients who had mono infection of *P. falciparum* with parasitaemia of 2000/µl and not more than 80,000/µl of blood were included in the *in vitro* drug susceptibility test (WHO, 2001).

Fourteen fresh isolates of *P. falciparum* were obtained from symptomatic patients, aged of 5 to 25 years by collecting 2.5 to 3 ml 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 (Flyg *et al.*,1997). Patients were then treated with Artemether - Lumefantrine (COARTEM) or Amodiaquine - Artesunate combination drugs.

Preparation of Culture medium and Extracts solution: Culture medium was prepared by dissolving 10.43 g RPMI 1640 powder (Gibco), 6 g of 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 (Cranmer *et al*., 1997).

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 (Clarkson *et al.*, 2004). Extracts were then tested in 6 serial two-fold dilution with a final concentration range of 1.56 – 100 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 (Trager & Jensen, 1976). 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 (WHO, 2001; Ngemenya *et al*., 2006).

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 (Mustofa *et al*., 2007).

Experimental Animals: The animals used for acute toxicity test and anti-inflammatory activity were adult male and female mice (20 – 25 g) and 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*. All experiments involving animals were conducted in the animal house of the University of Jos. 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 (500, 1000 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. The Lethal Dose LD_{50} was estimated from the graph of percentage mortality converted to probit against log-dose of the extract, probit 5 being 50 %.

Anti-inflammatory Test: Topical Oedema of the Mouse Ear: The effect of water and alcohol extracts of *Vernonia amygdalina* on topical acute oedema was assessed using xylene-induced ear oedema in mice (Okoli *et al*., 2005). Swiss albino mice (20 – 25 g) received topical application (5 g/ear) of water extract of *V. amygdalina* or ethanol extract of *V. amygdalina*, 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) (Inoue *et al.*, 1989). The difference in thickness of ear from right treated and left untreated ears was calculated and used as a measure of oedema (Okoli *et al.*, 2005). 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.

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

RESULTS.

The phytochemical screening of plant material showed the presence of saponins, tannins, flavonoids, carbohydrates, cardiac glycosides, alkaloids, steroids and anthaquinones.

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 ($P \geq 0.05$). The basic measurement of antimalarial activity used in this study was the reduction in number of parasitized cells in the test cultures compared to control at 24 - 30 hours of incubation. Of the two extracts tested, the ethanol extract of *V. amygdalina* showed the highest antimalarial activity of 78.1 %, with IC_{50} of 11.2 µg/ml. The water extract had the parasite growth inhibition of 74.0 % and IC_{50} of 13.6 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**.**

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**
	% Mean Parasite Growth ± S.D.						
Water Extract	88.7±4.6	77.9±6.1	67.9±6.4	54.1±8.7	43.3±8.5	35.2±9.4	26.0±9.6
Ethanol Extract	87.5±5.0	74.6±6.8	61.7±9.5	47.6±9.3	37.6±9.0	30.3±8.0	21.9±9.6

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

TABLE 2. *IN VITRO* SCHIZONT GROWTH INHIBITION OF *P. falciparum* ISOLATES BY PLANT EXTRACTS.

	Concentration of Extracts (µg/ml)						
	1.56	**3.13**	**6.25**	**12.50**	**25.00**	**50.00**	**100.00**
Water Extract (% inhibition)	11.35	22.14	32.10	45.9	56.74	62.81	74.02
Ethanol Extract (% inhibition)	12.54	25.38	38.33	52.44	62.44	69.74	78.10

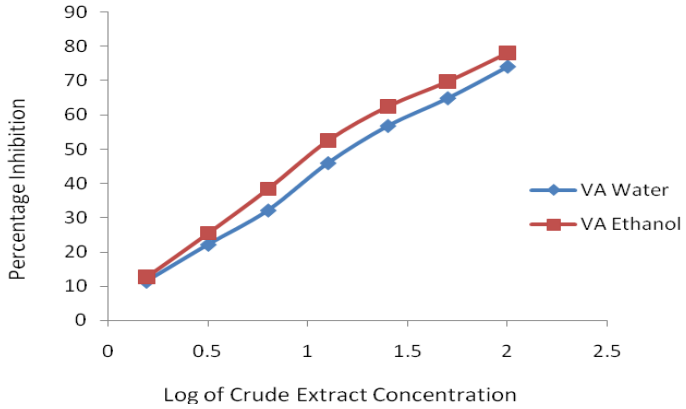

FIG. 1. THE PERCENTAGE INHIBITION OF THE EXTRACTS OF *V. amygdalina* SHOWING DOSE- DEPENDENT ANTIMALARIAL ACTIVITY.

Water extract of *V. amygdalina* in the doses of 500, 1000 and 3000 mg/kg body weight (orally) did not cause any deaths. In addition, only 3000 mg/kg body weight showed signs of weakness, all other parameters were negative. The ethanol extract of *V. amygdalina* in the doses of 500 and 1000 mg/kg body weight also did not cause any deaths. However, deaths were recorded at 3000 mg/kg body weight of ethanol extract of *V. amygdalina*. Lethal dose (LD_{50}) using probit was estimated at LD_{50} of 1950 mg/kg body weight.

The right treated ears exhibited topical oedema induced by xylene in the experimental animals. Water extract of *V. amygdalina* exhibited higher anti-inflammatory activity with inhibition of 46.4 % than ethanol extract which had inhibition of 44.5 %.

DISCUSSION.

In this study, the *in vitro* antimalarial activity of water and ethanol extracts of *V. amygdalina*, a plant used in traditional medicine in Nigeria is reported. The ethanol extract gave the highest antimalarial activity of 78.1 %, with IC_{50} value of 11.2 µg/ml compared to water extract which had antimalarial activity of 74.3 % with IC_{50} of 13.6 µg/ml.

The antimalarial activity of *V. amygdalina* was earlier reported by Abosi & Raseroka (2003) in an *in vivo* antimalarial activity test of the plant against resistant *P. berghei*. In their findings, leaf extract produced 67 % suppression of parasitaemia in a four day test. The result of this work is in agreement with their result, though experimental methods differ. This work also agrees with Iwalokun (2008), who reported the *in vivo* antimalarial activity of *V. amygdalina* . In his work, the aqueous extract of the plant was used to enhance the antimalarial effects chloroquine in mice infected with chloroquine resistant and sensitive *P. berghei* strains. He revealed the potential of *Vernonia* to reverse chloroquine resistance when used as an adjuvant with chloroquine. This study validates the traditional use of *V. amygdalina* in the treatment of malaria in Nigeria.

The thresholds for the *in vitro* antimalarial activity of the plant extracts were based on the classification according to Gessler *et al.,* (1994) where: extract with IC_{50} less than 10 µg/ml is considered very good; 10 to 50 µg/ml considered moderate and over 50 µg/ml considered to have low activity. Based on this classification, result from this study of the water and ethanol extracts of *V. amygdalina* with IC_{50} of 13.6 µg/ml and11.2 µg/ml respectively, are said to have moderate antimalarial activity.

The acute toxicity test in this work shows that the water extract of *V. amygdalina* is non-toxic. This seems to agree with Idowu *et al*., (2010) who reported that the plant had a negligible toxicity. The ethanol extract which showed sign of toxicity at the dose of 3000 mg/kg body weight also had an LD_{50} of 1950 mg/kg body weight. The LD_{50} of this study seems to disagree with Ojiako & Nwanjo (2006) who recorded an LD_{50} of 500 mg/kg body weight in rats administered intraperitoneally. The wide difference in LD_{50} could

be due to mode of administration of the extracts or any determinants. However, LD_{50} should not be regarded as biological constant since different results are obtained on repetition or when the determinations are carried out in different laboratories (Lorke (1983).

Both crude extracts showed a good measure of anti-inflammation. Anti-inflammation properties have been observed in flavonoids, tannins and alkaloids (Iwalewa *et al.,* 2007). It is therefore possible that the anti-inflammatory effect observed in water and ethanol extracts of *V. amygdalina* may be attributable to their flavonoids, tannins and alkaloids contents as also observed by Musa *et al.,* (2008). 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 painful inflammatory conditions.

CONCLUSION.

The results of this study have shown that the ethanolic extract of *V. amygdalina* exhibited higher antimalarial activity than water extract. Both extracts possess a moderate antimalarial activity. This result justifies the traditional use of the plants in the treatment of malaria. Further work is suggested to isolate, identify and characterize the active principles from these plants.

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