Original Article

TIPS Trends in Pharmaceutical Sciences 2020: 6(4): 271-278.

Antiplasmodial activity of *Anchomanes difformis* aqueous leaf extract on *Plasmodium* berghei infected mice

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Abstract

Challenges including treatment failure, parasite resistance, and adverse effects associated with antimalarial drugs have increased the use of medical plants as alternative treatment. *Anchomanes difformis* (*A. difformis*) is used traditionally for the treatment of a variety of ailments including malaria, but with a paucity of scientific evidence. This study assessed the antiplasmodial activity of *A. difformis* aqueous leaf extract (AEA) on *Plasmodium berghei* (*P. berghei*) infected mice. AEA (100, 200 and 400 mg/kg) was orally administered to *P. berghei* infected mice in the curative, suppressive and prophylactic groups. The untreated parasitized control (UP) and the positive control (PC) were treated orally with normal saline (0.2 mL) and chloroquine (CQ) (10 mg/kg), respectively. After treatment, blood samples were analyzed for percentage parasitemia levels, hematological parameters and liver samples were evaluated for histology. In the curative, suppressive and prophylactic groups treatment with AEA decreased percentage parasitemia levels and prolonged mean survival time in a dose-dependent fashion with significance observed at 100 mg/kg ($p<0.05$), 200 mg/kg ($p<0.01$) and 400 mg/kg ($p<0.001$) when compared to UP. The antianemic effect of AEA was characterised by increased red blood cells, haemoglobin, packed cell volume and decreased white blood cells in a dose-dependent fashion with significance obsevred at 100 mg/kg ($p<0.05$), 200 mg/kg ($p<0.01$), 400 mg/kg ($p<0.001$) when compared UP. Reductions in liver hemozoins, parasitized red blood cells and central vein congestion occurred in a dose-dependent fashion in AEA-treated mice. The findings in this study support the traditional use of the leaves of *A. difformis* for the treatment of malaria.

Keywords: *Anchomanes difformis*, Antimalaria, Hematology, Liver, Mice

1. Introduction

Malaria is caused by *Plasmodium* species. It is one of the greatest and oldest health predicaments affecting 40-50 % of the world's population (1). It affects 350-500 million people and causes mortality in 1.5-2.7 million people annually (2). Malaria is a major impediment to the economic growth of developing nations especially Africa countries with heavy poverty burden (3). The current malaria treatment, which is artemisinin based combination therapy, offers several advantages. However, it can be characterised by some drawbacks such as treatment failure, cost, parasite resistance, and adverse effects, which are quite challenging in some parts of the world (4). This has necessitated the quest for alternative treatment sources including the use of medical plants in most parts of the world (5). The use of medicinal plants to treat ailments has been in existence since the onset of time. In Africa, about 80% of people use medicinal plants for health related problems (6). Over the years, medicinal plants have become

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Recieved: 01/10/2020; Accepted: 01/12/2020

remarkable sources of newer, effective and potent therapeutic agents and have become a central research point in the world (6).

Anchomanes difformis (*A. difformis*) is a herbaceous plant, which has stout prickly stem. It grows up to 2 m high bearing a huge much-divided leaf spathe (20-25 cm long). Both stem and spathe arise from a horizontal tuber of 80 cm long and 20 cm wide. The latex is mostly milky or watery, but rarely colored (7). It widely grows in terrestrial lands and swamp lands of West tropical Africa which include Ghana, Côte d'Ivoire, Sierra Leone, Nigeria, Senegal and Togo (8). In herbal medicine, literature showed that people of different cultural background use *A. difformis* for the treatment of different health conditions. In Nigeria, decoctions of its root are used as remedies for diabetes, dysentery, cough and throat related problems (9). Vesicants and rubefacients produced from the rhizomes are employed for external use against smallpox and measles (10). Rhizome aqueous extract is used as treatment for inflammation, pain, and fever (11). Ethnomedicinal information showed it is also used for the treatment of edema, kidney disease, jaundice, and urethral discharge (11). In some African countries, its roots, leaves and stems are employed for the treatment of malaria. Available literature showed lack of scientific information on the antimalarial effect of its aqueous leaf extract. Hence, this study evaluated the antiplasmodial activity of *A. difformis* aqueous leaf extract (AEA) on *P. berghei* infected mice.

2. Materials and methods

2.1. Plant collection

Fresh leaves of *A. difformis* were collected from Obi/Akpor Local Government Area of Rivers State, Nigeria. The leaves were identified and authenticated by Mr. Ozioko. O a taxonomist at the International Centre for Ethnomedicine and Drug Development in Nsukka, Enugu State, Nigeria.

2.2. Preparation of plant extract

A. difformis leaves were washed properly with clean water, spread and allowed to dry at room temperature for 3 weeks. The leaves were minced into a uniform powder using a milling machine. The powder was soaked in distilled water and allowed to stay for 48 hours at room temperature. The extract was filtered through Whatman filter paper. The filtrate was evaporated using a water bath at 50 °C leaving the extract in a paste form. Chloroquine (CQ) (Evans Medicals PLC) was used as a standard drug for this study.

2.3. Qualitative phytochemical analysis

Tests for flavonoids, tannins, carbohydrates, glycosides, saponins, resins, terpenoids and alkaloids were carried out using standard methods (12, 13).

2.4. Animals

Swiss albino mice (25-30 g) of both sexes were used. The mice were obtained from the animal facility of the University of Nigeria, Nsukka, Enugu State, Nigeria. The mice were housed in wooden cages (6/group) under standard conditions (ambient temperature, 28.0+2.0 °C, and humidity 46%, with a 12 hour light/dark cycle). The mice were fed with growers mash and allowed access to water *ad libitum*.

2.5. Acute toxicity study

A. difformis aqueous leaf extract (AEA) was evaluated for toxicity in mice using modified Lorke's (1983) method (14). The test was carried out in two phases. In phase 1, 9 mice randomized into 3 groups of n=3 were orally administered with AEA (10, 100 and 1000 mg/kg) respectively. The mice were observed for behavioural changes and mortality in the first four hours and subsequently daily for ten days. Due to lack of evident signs of toxicity and mortality, phase 2 study was performed.

In phase 2, fresh set of 3 mice randomized into 3 groups of n=1 were orally administered with AEA (1500, 2500 and 5000 mg/kg). The mice were observed for signs of toxicity and mortality for the first four hours and thereafter daily for ten days.

2.6. Plasmodium parasite

A chloroquine sensitive strain of *Plasmodium berghei* (*P. berghei*) (NK 65) was sourced from the Department of Pharmacology, Faculty of Basic Clinical Sciences, University of Port Har-

court, Rivers State, Nigeria. The *P. berghei* was subsequently maintained in the laboratory by serial blood passage from mouse to mouse every 5-7 days. Blood samples were collected from donor infected mice with a parasitemia of 20-30% into heparinized tubes. Blood samples were diluted with 0.9% normal saline and mice were infected with 0.2 mL of the diluted blood containing $1x10^7$ parasitized erythrocytes intraperitoneally (i.p). Parasitemia was monitored daily by microscopic examination of Giemsa stained thin blood smears, and percentage parasitemia was calculated using the formula below.

$$\%\ parasitemia = \frac{Number\ of\ parasitized\ erythrocytes \times 100}{Total\ number\ of\ erythrocytes} \quad \text{Eq.1}$$

$$\%\ Inhibition = \frac{(\%\ parasitemia\ of\ untreated\ control - \%\ parasitemia\ of\ treated\ group) \times 100}{\%\ parasitemia\ of\ untreated\ control} \quad \text{Eq.2}$$

2.7. Curative antiplasmodial study

The curative effect of AEA was evaluated using the method described by Ryley and Peter (1970) (16). Thirty mice randomized into 6 groups (I-V1) of n=5 were used. Groups II-VI were inoculated with 1 X 10^7 *P. berghei* parasitized erythrocytes i.p. Seventy two hours later (day 3), the mice were treated as follows: Group1 (Normal control) (NC), and group 11 (untreated parasitized control) (UP) were orally treated with normal saline (NS) 0.2 mL once daily for 4 days. Group III (Positive control) was orally treated with CQ (10 mg/kg) once daily for 4 days. Groups IV- V1 were orally treated with AEA (100, 200 and 400 mg/kg) once daily for 4 days. On day 5, thin films were made from the tail blood of each mouse. The blood films were fixed with methanol, stained with 10% Giemsa at pH 7.2 for 10 min and parasitemia examined microscopically. Parasitemia levels were determined by counting the number of parasitized erythrocytes out of 200 erythrocytes in random fields of the microscope. Average percentage inhibitions were calculated as shown above.

2.8. Suppressive antiplasmodial study

The suppressive antiplasmodial effect of AEA on *P. berghei* infected mice was evaluated using the method described by Knight and Peters (1980) (15). Twenty five mice randomized into 5 groups (I-V) of n=5 were inoculated with 1 X 10^7 *P. berghei* parasitized erythrocytes i.p. Two hours later, groups III-V were orally treated with AEA (100, 200 and 400 mg/kg/day) once daily for 4 days. Group I (UP) was orally treated with normal saline (0.2 mL) whereas group II (PC) was treated with CQ (10 mg/kg) once daily for 4 days. On day 5, thin films were formed from tail blood samples and stained with 10% Giemsa at pH 7.2 for 10 min. Percentage parasitemia levels were determined and percentage inhibitions calculated as described above.

2.9. Prophylactic antiplasmodial study

The method reported by Peters (1967) (17) was use for the evaluation of the prophylactic antiplasmodial effect of AEA. Twenty five mice were randomize into 5 groups (I-V) of n=5. Groups III-V were pretreated with AEA (100, 200 and 400 mg/kg) once daily for 4 days. Group 1 (UP) was treated with normal saline (0.2 mL) whereas group II (PC) was treated with CQ (10 mg/kg). Thereafter, the mice were inoculated with $1x10^7$ *P. berghei* infected erythrocytes i.p and treatment continues. On day 8, parasitemia levels were determined using microscopic examination of Giemsa stained thin blood smears and percentage inhibitions were calculated as described above.

2.10. Determination of mean survival time

The mice were monitored for mortality daily and the number of days from the onset of infection to death for each mouse in the control and experimental groups. Mean survival time (MST) in days was calculated using the formula below.

$$MST = \frac{Sum\ of\ survival\ time\ of\ all\ mice\ in\ a\ group}{Total\ number\ of\ mice\ in\ that\ group} \quad \text{Eq. 3}$$

2.11. Determination of Hematological
2.11.1. Parameters

Hemoglobin (Hb), packed cell volume (PCV), red blood cells (RBCs) and white blood cells (WBCs) were evaluated using an auto analyzer.

2.11.2. Histology of the liver

Liver samples were collected from the mice in the curative test group and fixed in 10%

Table 1. Phase 1 of acute toxicity test.

Dose (mg/kg)	No. of mice per group	No. of death
10	3	0/3
100	3	0/3
1000	3	0/3

formalin for 24 hours. The liver samples were dehydrated in graded solutions of isopropyl alcohol. After dehydration, the liver samples were fixed in paraffin wax and 5µm sections were produced using a microtome. Sections were stained with hamatoxylin and eosin and examined for histological changes using a light microscope.

2.11.3. Statistical analysis

Statistical analysis of data was performed using GraphPad Prism (GraphPad Prism software, Inc., US).The results are expressed as mean±standard error of mean (SEM). The means were compared using *student-t-test* followed by *Tukey*'s post hoc test. P values <0.05;<0.01;<0.001 were regarded as statistically significant.

3. Results

3.1. Phytochemical analyses and acute toxicity study

Phytochemical analyses of AEA showed the presence of tannins, flavanoids, flavanol, carbohydrates, proteins, alkaloids, terpeniods and glycosides. Acute toxicity assessment of AEA showed no signs of toxicity and mortality (Tables 1 and 2).

3.2. Currative antiplasmodial effect of Anchomanes difformis

Curative antipasmodial evaluation of AEA showed dose- dependent decreases in percentage parasitemia with significance observed at 100 mg/kg ($p<0.05$), 200 mg/kg ($p<0.01$) and 400mg ($p<0.001$) when compared to UP. CQ significantly decreased percentage parasitemia at $p<0.0001$ when compare to UP (Table 3). Treatment with AEA prolonged MST in a dose-related fashion, with significance observed at 100 mg/kg ($p<0.05$), 200 mg/kg ($p<0.01$) and 400 mg ($p<0.001$) when compared to UP.

3.3. Suppressive antiplasmodial effect of Anchomanes difformis

In the suppressive study, reductions in percentage parasitemia in dose-dependent fashion were observed in AEA treated mice. Significance occurred at 100 mg/kg ($p<0.05$), 200 mg/kg ($p<0.01$) and 400mg ($p<0.001$) of AEA when compared to UP. CQ produced reduction in percentage parasitemia at $p<0.0001$ when compared to UP (Table 4). AEA produced dose-related prolongation of MST with observed significance at 100 mg/kg ($p<0.05$), 200 mg/kg ($p<0.01$) and 400mg ($p<0.001$) when compared to UP.

3.4. Prophylactic antiplasmodial effect of Anchomanes difformis

Prophylactic antiplasmodial activity of AEA showed reductions in percentage parasitemia in a dose-related fashion. The reductions were significant at AEA 100 mg/kg ($p<0.05$), 200 mg/kg ($p<0.01$) and 400mg ($p<0.001$) when compared to UP. On the other hand, significant reduction in percentage parasitemia in CQ treated mice occurred at $p<0.0001$ when compared to UP (Table 5). Treatment with AEA prolonged MST in a dose-dependent fashion. Significance was observed at 100 mg/kg ($p<0.05$), 200 mg/kg ($p<0.01$) and 400mg ($p<0.001$) of AEA when compared to UP.

Table 2. Phase 2 of acute toxicity test.

Dose (mg/kg)	No of mice per group	No of death
1500	1	0/1
2500	1	0/1
5000	1	0/1

Table 3. Curative antiplasmodial effect of *Anchomanes difformis* on *Plasmodium berghei*-infected mice

Treatment	% Parasitaemia±SEM	% Inhibition	MST
UP 0.2 mL (NS)	45.2±4.11	0.00	9.10
AEA (100 mg/kg)	29.2±3.71*	35.3	20.4*
AEA (200 mg/kg)	21.5±2.01**	52.4	27.2**
AEA (400 mg/kg)	11.7±0.74***	74.2	30.5***
PC CQ (10 mg/kg)	4.47±0.24 π	90.7	35.7***

UP; Untreated parasitized control, NS: Normal saline, AEA: Aqueous extract of *A. difformis*, PC; Positive control; CQ: Chloroquine, MST: Mean survival time. * p<0.05 difference when compared to UP, **p<0.01 difference when compared to UP, ***p<0.001 difference when compared to UP; π p<0.0001 difference when compared to UP

3.5. Effect Anchomanes difformis on hematological parameters of parasitized mice

Parasitized mice showed significant (p<0.001) decreases in RBCs, PCV, and Hb levels with significant (p<0.001) increases in WBCs when compared to NP (Table 6). However, treatment with AEA produced significant and dose-dependent increases in RBCs, PCV, and Hb lev-

Table 4. Suppressive antiplasmodial activity of *Anchomanes difformis* on *Plasmodium berghei*-infected mice.

Treatment	% Parasitaemia±SEM	% Inhibition	M ST
UP 0.2 mL (NS)	30.2±1.22	0.00	9.30
AEA (100 mg/kg)	18.6±1.01*	38.4	23.2*
AEA (200 mg/kg)	11.9±1.06**	60.7	30.1**
AEA (400 mg/kg)	6.61±0.80***	78.1	35.4***
PC CQ (10 mg/kg)	1.99±0.05 π	93.4	40.4***

Data as mean ± SEM, n=5, UP: Untreated parasitized control, NS: Normal saline, AEA: Aqueous extract of *A. difformis*, PC; Positive control; CQ: Chloroquine, MST: Mean survival time. * p<0.05 difference when compared to UP, **p<0.01 difference when compared to UP, ***p<0.001 difference when compared to UP; π p<0.0001 difference when compared to UP, SEM; Standard error of mean

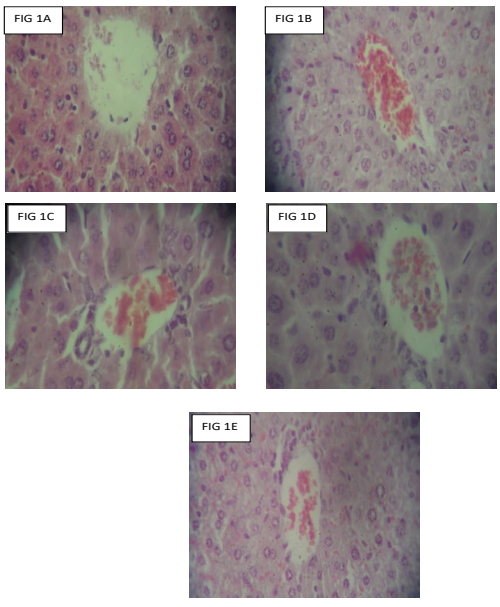

Figure 1 A: Liver of the control mice showed normal histology. Figure 1 B: Liver of *P berghei* infected mice showed large amount of hemozoins with kupfer cells inside the sinusoids. It also showed central vein congestion, large number of parasitized red blood cells, swollen hepatocytes and distended liver sinusoids. Figures 1 C and D: Liver of AEA (100 and 200 mg/kg) treated mice showed central vein congestion, parasitized red blood cells and sinusoids containing hemozoins and kupfer cells. Figures 1 E: Liver of EEA (400mg/kg) treated mice showed decreased hemozoin, decreased central vein congestion and parasitized red blood cells.

Table 5. Prophylactic antiplasmodial activity of *A. differmis* on *Plasmodium berghei*-infected mice.

Treatment	% Parasitaemia±SEM	% Inhibition	M ST
UP 0.2 mL(NS)	25.2±3.95	0.00	9.60
AEA (100 mg/kg)	14.8±1.92*	41.1	25.3*
AEA (200 mg/kg)	8.29±0.22**	67.1	33.2**
AEA (400 mg/kg)	5.24±0.09***	79.2	37.5***
PC CQ (10 mg/kg)	1.23±1.24 π	95.1	43.7***

Data as mean ± SEM, n=5, UP: Untreated parasitized control, NS: Normal saline, AEA: Aqueous extract of A. differmis, PC; Positive control; CQ: Chloroquine, MST: Mean survival time. * p<0.05 difference when compared to UP, **p<0.01 difference when compared to UP, ***p<0.001 difference when compared to UP; π p<0.0001 difference when compared to UP, SEM; Standard error of mean

els with decreases in WBCs levels at 100 mg/kg (p<0.05), 200 mg/kg (p<0.01) and 400mg (p<0.001) when compared to UP. CQ produced significant effect on hematological parameters at p<0.001 when compared to UP (Table 6).

3.6. Effect Anchomanes differmis on liver histology of parasitized mice

The liver of control mice showed normal histology (Figure 1 A). On the other hand, the liver of UP showed large amount of hemozoins with kupfer cells inside the sinusoids. Also, it showed central vein congestion, large number of parasitized red blood cells, swollen hepatocytes and distended liver sinusoids (Figure 1 B). The liver of mice treated with AEA (100 and 200 mg/kg) showed central vein congestion, parasitized red blood cells and sinusoids containing hemozoins and kupfer cells (Figures 1 C and D). However, the liver of mice treated with EEA (400 mg/kg) showed decreased hemozoin, decreased central vein congestion and parasitized red blood cells (Figure 1 E).

4. Discusion

Historically, plants have been used to supply and meet the basic needs of man such as shelter food, clothing and medicines. Plants have been playing central and essential functions in traditional systems of medicine for the treatment and prevention of diseases in the world especially in developing nations. It is highly imperative to know that before the advent of orthodox medicine, the traditional systems of medicine used plant based medicines to meet the basic health need of humans (18). *A. differmis* is a multipurpose plant broadly used traditionally for the treatment of a variety of ailments including malaria (19). *A. differmis* extracts have shown potential anti-inflammatory, antioxidant, antimicrobial, antidiabetic, antiasthmatic and analgesic activities (20). Despite its traditional use for the treatment of malaria there is a paucity of scientific study on the antiplasmodial potential of its aqueous leaf extract. This study evaluated the suppressive, curative and prophylactic antiplasmodial effects of the aqueous leaf extract of *A. differmis* (AEA) on *P. berghei* infected mice. Impacts on MST, hematological parameters

Table 6. Effects of *A. differmis* on hematological parameters of *Plasmodium berghei*-infected mice.

Treatment	RBCs($\times 10^6$/µl)	WBCs($\times 10^3$/µl	PCV (%)	Hb(g/dl)
NP 0.2 mL (NS)	5.46	6.00	55.9	17.2
UP 0.2 mL (NS)	2.73	16.0	20.4	6.00
AEA (100 mg/kg)	3.04*	12.6*	27.1*	9.01*
AEA (200 mg/kg)	3.81**	9.43**	35.7**	12.1**
AEA (400 mg/kg)	4.91***	7.00***	42.5***	16.2***
PC CQ (10 mg/kg)	5.30***	6.36 ***	53.5***	16.8 ***

Data as mean ± SEM, n=5, NP: Non-parasitized control, UP: Untreated parasitized control, NS: Normal saline, AEA: Aqueous extract of A. differmis, PC; Positive control; CQ: Chloroquine, MST: Mean survival time. * p<0.05 difference when compared to UP, **p<0.01 difference when compared to UP, ***p<0.001 difference when compared to UP, Standard error of mean.

and liver histology of *P. berghei* infected mice were also studied. This study showed the presence of tannins, flavanoids, flavanol, carbohydrates, proteins, alkaloids, terpenoids and glycosides as essential phytochemicals in AEA. The presence of these phytochemical showed the potential medicinal value of AEA. In this study, the acute toxicity study of AEA did not show any sign of toxicity and mortality. This observation showed it may be safe. The curative, suppressive and phrophylactic evaluations of AEA showed reductions in percentage parasitemia in a dose-dependent fashion. These findings showed that AEA may have antiplasmodial activity. However, this observation is at variance with studies that showed lack of antiplasmodial activity in the methanolic extract and fraction of its root tubers (21). One of the goals of malaria treatment is to improve survival rate or prevent malaria associated mortality. In the curative, suppressive and phrophylactic studies, AEA prolonged MST in a dose-dependent fashion. This finding lends credence to the antiplasmodial potential of EEA. Severe Malaria infection causes anemia, which has been associated with mortality in children and pregnant women (21), The erythrocytic stage of malaria parasite affects hemoglobin by converting blood meal to harmless hemozoin, thus decreasing the oxygen carrying capacity of the RBCs. The etiology of anemia caused by severe malaria can be characterized by overlapping and distinct features, such as lyses of parasitized RBCs, sequestrating of RBCs in the spleen, and the transmission of chronic malaria in holoendemic regions (21). Therefore an antimalarial drug candidate should have the ability to curtail malaria induced anemia. The current study observed that treatment AEA inhibited *P. berghei*-induced anemia, which was characterized by increased RBCs, Hb, PCV levels and decreased WBCs levels. This finding showed that AEA could prevent malaria induced anemia. Interestingly, in a dose-dependent fashion, AEA reduced the number of hemozoin, parasitized RBCs and decreased central vein congestion in the liver. The mechanisms behind the antiplasmodial activity of AEA are not well understood, but it contains alkaloids which can prevent the polymerization of heme in hemozoin in digestive vacuoles (22). The presence of terpenoids can generate free radicals inside the parasite, thus alkylating macromolecules and disrupting intracellular transport process (23). Its phenolic contents can act as antioxidant that can prevent or reduce oxidative stress induced by malaria parasites (24).

5. Conclusion

AEA reduced blood and liver parasitemia levels in a dose-dependent fashion. This supports its traditional use for the treatment of malaria.

Conflict of Interest

None declared.

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