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Toxicity of *Anchomanes difformis,* An Antimalarial Herb in Murine Models

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Authors' contributions

This work was carried out in collaboration between all authors. Author JOO designed the experiment, did microscopy, wrote the manuscript, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author COB treated the animals, prepared slides, managed the literature searches and did histology. Author OOO approved the study, provided reagents, read and corrected the manuscripts. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: *Anchomanes difformis* (*A. difformis*) is commonly used in folkloric medicine for the treatment of malaria. However, there had been no scientific evidence to substantiate this folkloric claim in murine models; hence the study.
Study Design: We employed murine models for this *in vivo* experiment and Vacuum Liquid Chromatography as our separation techniques for the plant extracts.
Place and Duration of Study: Laboratories for Biomembrane Research and Biotechnology, Department of Biochemistry, Faculty of Basic Medical Sciences and Institute of Advanced Medical Research and Training, College of Medicine, University of Ibadan, Nigeria between June 2014 and August, 2015.
Methodology: Methanol Extract (ME) and methanol Fraction (MF) obtained from *A. difformis* were used to treat mice for curative and prophylactic experiments. Therapeutic doses (methanol extract therapy [MET] and methanol fraction therapy [MFT] 100, 200 and 400 mg/kg body weight [bw])

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were administered daily for seven days after confirming parasitemia. Prophylactic groups (methanol extract prophylaxis [MEP] and methanol fraction prophylaxis [MFP]) were pretreated for seven days before experimental infection.

Results: Observed slides showed that there was no significant suppression, reduction in parasitemia or increase in clearance compared with the positive control. There was a significant reduction in Packed Cell Volume (PCV) in the curative experiment compared with the unparasitized control (UTA). The PCV did not change significantly across the groups in the prophylactic experiment. White Blood Cell (WBC) values decreased significantly ($p<0.0001$) among the treated groups for MET and MFT compared with Artesunate (ART). The ART's WBC value increased significantly ($p<0.0001$) when compared with parasitized control (MCT). In the prophylactic group, WBC values decreased significantly with both MEP and MFP compared with the Sulfadoxine-Pyrimethamine (SP) group. In both curative and prophylactic groups, survival rate decreased significantly as the dose increased. While ME-treated group survived better than MF-treated group, no animal survived under the MFP 400 mg/ kg bw. Histopathology of the liver revealed toxic effects of all drugs used.

Conclusion: The results revealed that doses used did not have significant antiplasmodial activity compared with the control drug used in this research. Extra caution must be taken while taking antimalarial drugs because of their possible toxicity.

Keywords: Antimalaria; prophylaxis; toxicity; survival; phytomedicine; mice.

1. INTRODUCTION

There is a continuous search for antimalarial drugs which will be readily available, cost effective and overcome the recurrent multidrug resistance of *P. falciparum* which is common in Sub Saharan Africa and in Asian continent. There is an urgent need to verify the potency of some of the herbs used for the treatment of malaria in order to substantiate their indigenous claims and to improve on their extraction procedures with a view to maximize their medicinal use. The vast majority of people still rely on their traditional *materia medica* for health care needs. It is also a fact that one quarter of all medical prescriptions is formulation based on substances derived from plants or plant-derived synthetic analogs. A large proportion of the population of developing countries uses traditional medicines, either as a result of the high cost of Western pharmaceuticals and health care, or because traditional medicines are more acceptable from a cultural and spiritual perspective [1]. In tropical regions where malaria is endemic, alternative therapies based on traditionally used antimalarial plants are used [2]. Many, but not all, drugs introduced into the therapeutic arsenal are mostly derived from natural products [3]. Plants provide secondary metabolites that have therapeutic effects and some of these metabolites serve as reaction intermediates in the synthesis of orthodox drugs which are useful for the treatment of protozoan diseases such as malaria [4], leishmaniasis, and African and American trypanosomiasis [5]. Traditionally-used antimalarial plants are the origin of the alkaloid quinine (isolated from *Cinchona* spp) and the sesquiterpene lactone artemisinin (isolated from *Artemisia annua* L.) that gave rise last century to the synthetic quinoline antimalarials (chloroquine) and semi-synthetic artemisinin derivatives (sodium artesunate). Plants also help to combat malaria by providing mosquito repellents and insecticidal oils (*citronella*, neem, etc.), solvent extracts and isolated chemicals (chrysanthamic acid, nicotine, etc.) that have given rise to the pyrethroid, neonicotinoid and other insecticides and repellents [4].

Anchomanes difformis (Blume) Engl. Pallidus commonly known as forest anchomanes is a plant of the family Araceae. *Anchomanes difformis* (*A. difformis*) is a native plant of the African continent and grows widely in wetlands and terrestrial areas of west tropical Africa including Nigeria, Ghana, Côte d'Ivoire, Sierra Leone, Senegal and Togo. *A. difformis* is a large herbaceous perennial plant with stout prickly stem (leaf petioles) of about 0.8 to 2 m high. The plant is erect on an enormous horizontal tuber, often reaching 50 to 80 cm long and 10 to 20 cm in diameter. It usually contains milky or watery latex, which is rarely colored.

In Nigeria, *A. difformis* is locally called Olikhoror by the Bini tribe of Edo state and Ogirisako by the Yoruba tribe of the South-Western part of Nigeria. Despite the toxic effects of Araceae, species of several genera are also cultivated as

food plants, mainly as subsistence crops in the tropical areas. The major edible Araceae are *Colocasia esculenta* and several species of Xanthosoma, grown primarily for their corms and sometimes for their leaves. Most of the North American species of Araceae were historically used by the Native Americans as both food and medicine [6]. Furthermore, claims from cellular reports is that its leaves, stem and roots (Rhizome) serve as food and are believed to have medicinal properties [7]. Traditionally, *A. difformis* has been used as diuretic, antidiabetic, anti-tuberculosis, antimalarial as well as for the treatment of oral and anal lesions [8].

Previous work by Bero et al. [8] showed the *in vitro* antiplasmodial activities of some medicinal plants used in Benin Republic for the treatment of malaria (*in vitro*) including *A. difformis*. Inspite of this, there is paucity of information to justify its antimalarial action. We present therefore, in this study, the curative, prophylactic, survival rates and toxicity studies of the various solvent extracts of the root tubers of this plant against chloroquine-sensitive strains of *Plasmodium berghei* in mice.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The root tubers of *A. difformis* were collected from uncultivated farmlands in Ado-Ekiti area of Ekiti State, Nigeria. The plant was identified by Mr. Omotayo, F.O. and a Voucher specimen was deposited at the Herbarium Unit of Plant Science Department, Ekiti State University, Nigeria. The root tubers were washed, sliced and air-dried for two weeks in the laboratory. The dried root tubers were powdered using electric blender and weighed.

2.2 Extraction

The powdered root tubers of *A. difformis* were extracted with methanol in a glass jar at room temperature for 72 hours. The crude methanol extract concentrate was heated in a water bath at 50°C to obtain a solvent free extract.

2.3 Partitioning of the Crude Methanol Extract

A known concentration of the methanol extract (ME) was adsorbed on silica gel and loaded on a column of Vacuum Liquid Chromatography packed with silica gel. The column was eluted with n-Hexane, chloroform, ethylacetate and methanol successively to obtain hexane fraction (HF), chloroform fraction (CF), ethylacetate fraction (EF) and methanol fraction (MF), respectively. These were concentrated using the rotary evaporator. The solvent-free fractions were stored in the refrigerator until used. The HF, CF and EF were insufficient for *in vivo* analysis and therefore, not considered for use in this work.

2.4 Experimental Animals

Ninety-five Swiss albino mice (13-15 g) were obtained from Institute of Advanced Medical Research and Training (IAMRAT). The animals were kept in cages and acclimatized for two weeks prior to the commencement of the experiment with free access to food and water. The animals were infected with *P. berghei* (chloroquine sensitive *NK65 strain*) with an inoculums size of 1×10^7 parasite load at Institute of Advanced Medical Research and Training (IAMRAT), College of Medicine University of Ibadan, Nigeria.

2.5 Antiplasmodal Activity of the Plant Extract and Fraction

2.5.1 Curative study

The method of established infection as described by Ryley and Peters [9] was used to assess the antiplasmodial activity of the extract and fraction obtained from *A. difformis.* Male Wistar mice were infected intraperitoneally by injecting them with malaria infected erythrocytes from a donor mouse with an inoculums size of 1×10^7. After the experimental mice were infected, parasitemia was confirmed after 72 hours. Parasitized animals were then grouped and treated orally with 100, 200 and 400 mg/kg bw of ME and MF. Artesunate was used as control drug at 10 mg/kg bw. We use artesunate as a standard drug for our curative study because it is effective against uncomplicated malaria. Parasitized untreated control (MCT) received the vehicle only.

2.5.2 Prophylactic study

The animals were pretreated with the oral administration of ME and MF at 100,200 and 400 mg/kg bw) for seven days prior to their intraperitoneal infection with infected erythrocytes from a donor mouse with an inoculums size of 1×10^7. After infection,

treatment was continued and slides were collected at two-day interval until the seventh day. Daily dose of 10 mg/kg bw of Sulfadoxine-pyrimethamine (SP) was used as positive control. Different drugs were used for curative and prophylactic experiments because the drugs have proved potency for the conditions they were used for.

2.5.3 Survival study

To assess the survival rate of mice that had malaria and were treated with the drug candidates, the same set of animals used for both curative and prophylactic studies were used except that this group did not contain normal unparasitized control.

2.6 Determination of Hematological Parameters

Hemoglobin concentration, the Packed Cell Volume (PCV) and red blood cell count were determined as described by Jain [10], and white blood cells were counted using hematocytometer.

2.7 Tissue Preparation for Histopathology

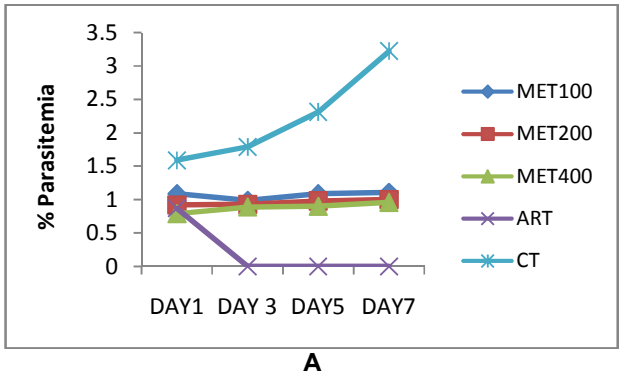
Tissue histopathology was done for liver. They were placed in 10% formalin for about five days for proper fixation, dehydrated by ascending grades of isopropyl alcohol for an hour. The dehydrated organs were cleared in xylene and transferred into two changes of liquid paraffin wax. The tissue sections were stained in Ehrlich's hematoxylin for eight minutes, washed in 10% aqueous eosin, incubated and mounted for photomicrography.

2.8 Statistical Analysis

Results were analysed statistically by using GraphPad Prism (5.0 version) by using Analysis of Variance (ANOVA) followed by Tukey post-test. Statistical significance was set at $p<0.05$.

3. RESULTS

Fig. 1 shows the percentage parasitemia of the animals treated with both ME and MF. It was observed that both ME and MF showed an insignificant reduction in parasite levels relative to the standard drug: ME did not show any significant difference between the treated groups. It further showed that the dosage level did not have any effect. Parasite clearance was recorded by ART on day 3. Fig. 1 further showed the percentage clearance of the malaria parasite as a measure of potency of the drugs against the disease. It could be observed that both ME and MF did not have significant parasite percentage clearance compared with the control drug.



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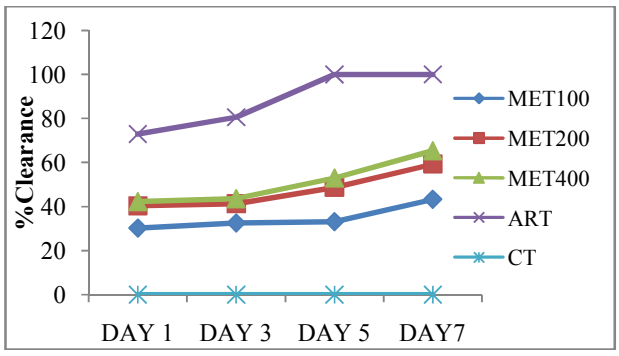


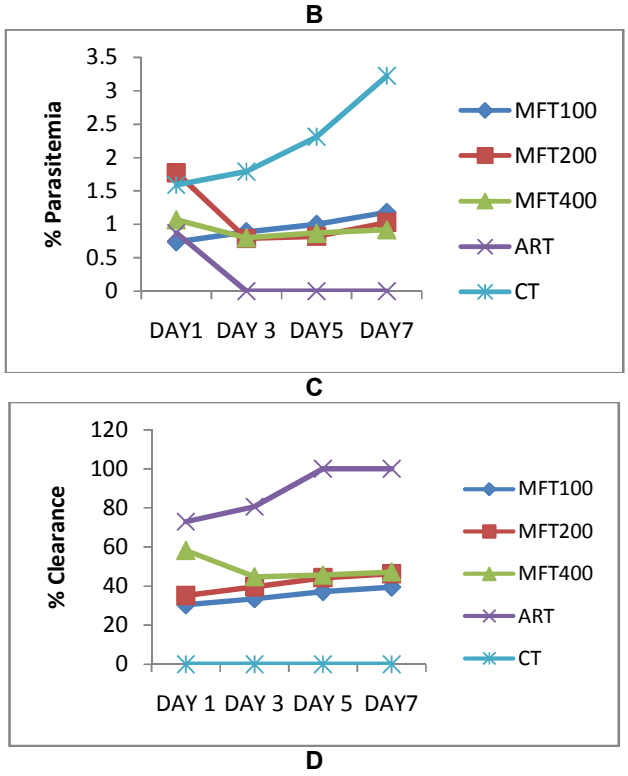


Fig. 1. Effects of ME (Fig. 1a and 1b) and MF (Fig. 1c and 1d) of *A. difformis* on the percentage parasitemia and clearance of *P. berghei* infected malaria in mice

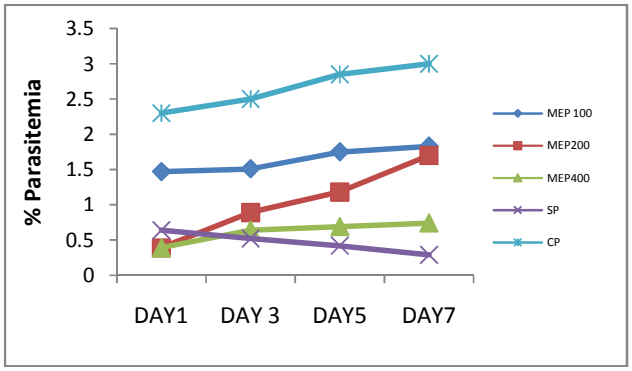
In order to assess the chemopreventive effects of the ME and MF, we pretreated the animals before experimental infection with the malaria parasite. The results obtained showed that the pretreatment of the animals with the extract and fraction did not show any significant suppression in parasite invasion of the Red Blood Cells (RBC) compared with SP. Similarly, the percentage parasite clearance expressed in Fig. 2 showed that both ME and MF did not show any significant parasite clearance compared with SP.

Fig. 3 shows the Packed Cell Volume (PCV) of *P. berghei*-infected mice after seven days of treatment. The PCV improved insignificantly in a dose-dependent manner for ME. The PCV of MF also increased insignificantly in a dose-dependent manner. UTA had the highest PCV when compared with the ART-treated group. The PCV for ME and MF in the prophylactic groups decreased in a dose-dependent manner. None of the animals survived in the group treated with MEP 400 mg/kgbw. These results indicated that long-term exposure to the ME and MF may be toxic. Fig. 3 further shows that WBC values for ME and MF decreased significantly compared to SP, ART, and UTA.

The histopathology (Fig. 4) of the liver revealed that *A. difformis* was toxic both at low and high dosage levels used in this work. There were pathological lesions noticed in the liver of the animals treated with extracts and fraction of methanol. Fig. 5 shows the survival rate of the animals in each group at the end of the experiment. This result shows that the survival is dose and drug dependent. This is so because mice treated with lower doses survived better than those exposed to higher doses. Again, mice treated with ART and SP survived better than those treated with the crude ME and MF.

4. DISCUSSION

In tropical Africa, malaria still remains a major health challenge for both young and old due to so many factors: the increase in the multidrug resistance for which there is need for a constant change in drugs because of their reduced efficacy, poor health care delivery in which the curative and preventive medicine approaches still remain underdeveloped and the high cost of Western medicines.



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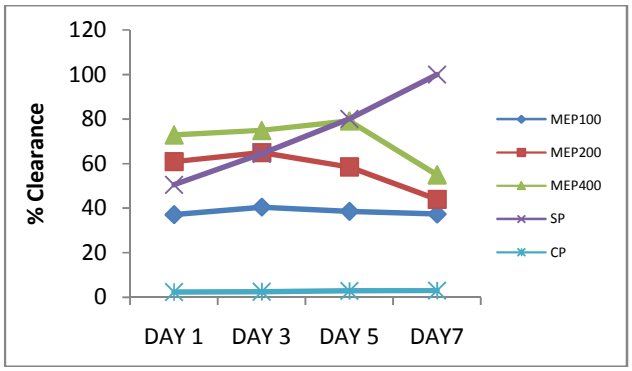

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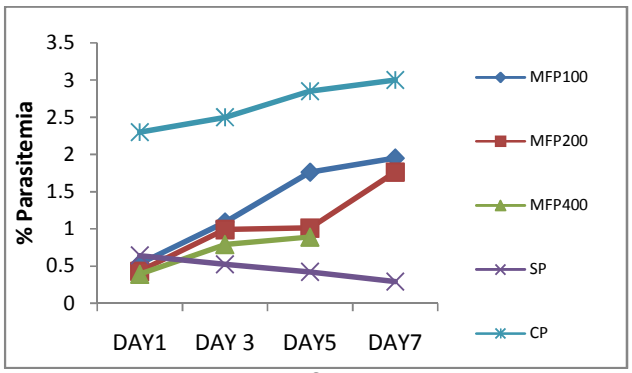

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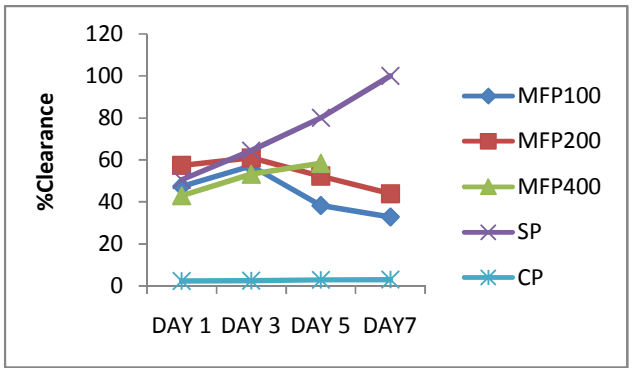

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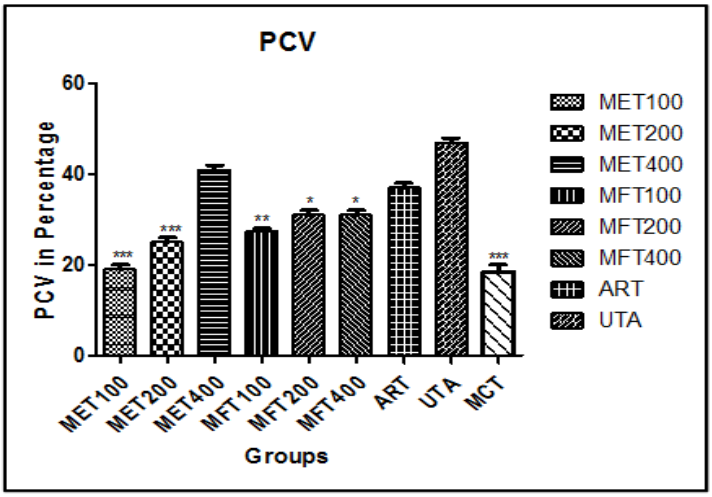
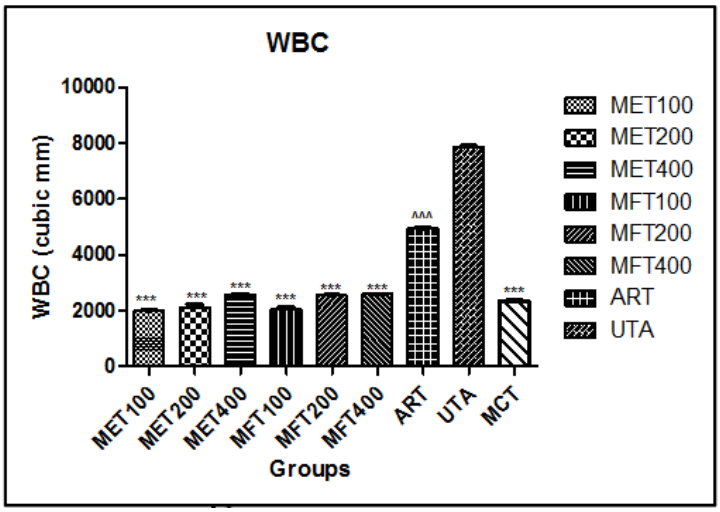
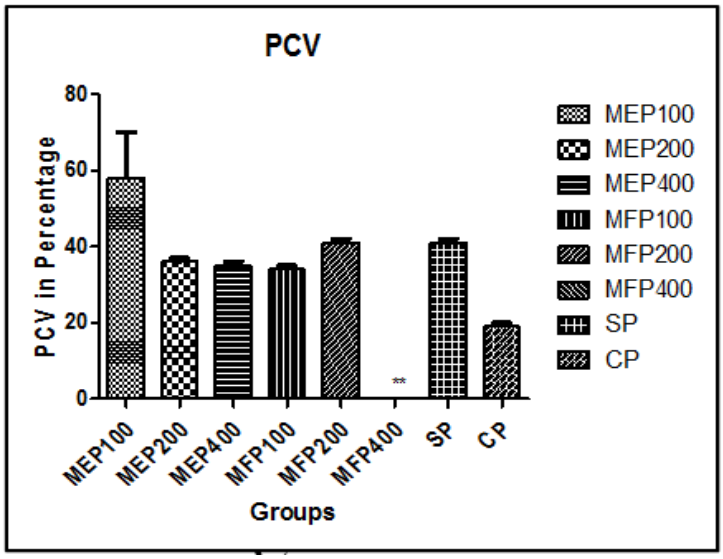
Fig. 2. Prophylactic effects of ME (Fig. 2a and 2b) and MF (Fig. 2c and 2d) of *A. difformis* on the percentage parasitemia and clearance of *P. berghei*- infected malaria in mice.


PCV
PCV in Percentage
60
40
20
0
MET100
MET200
MET400
MFT100
MFT200
MFT400
ART
UTA
MCT
Groups

A


WBC
WBC (cubic mm)
10000
8000
6000
4000
2000
0
MET100
MET200
MET400
MFT100
MFT200
MFT400
ART
UTA
MCT
Groups

B


PCV
PCV in Percentage
80
60
40
20
0
MEP100
MEP200
MEP400
MFP100
MFP200
MFP400
SP
CP
Groups

C

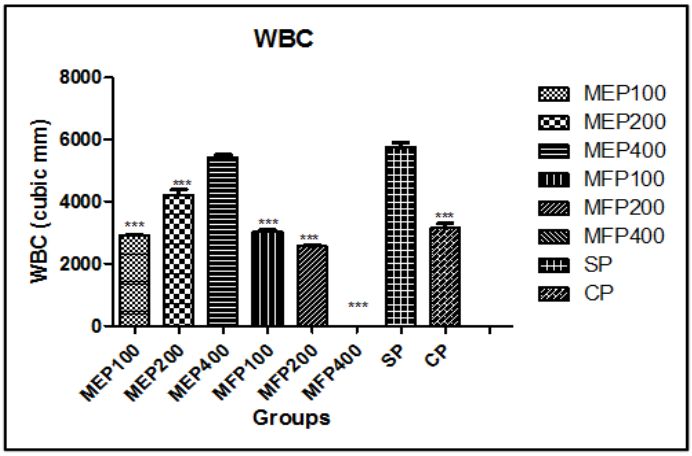


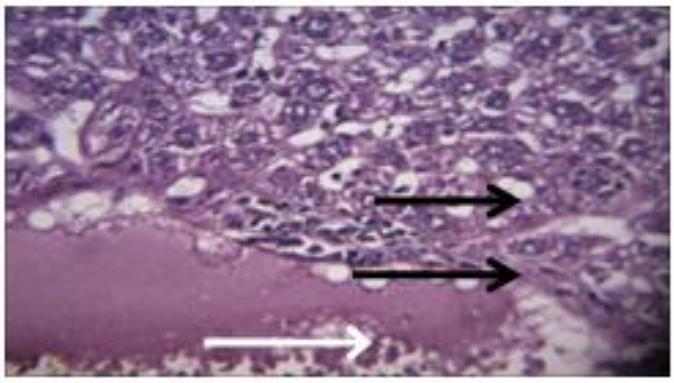
Fig. 3. Effects of oral administration of ME and MF of *A. difformis* on the PCV and WBC of both therapeutic (Fig. 3a and 3b) and prophylactic (Fig. 3c and 3d) treatments. Fig. 3a shows a significant decrease (* p<0.0001, **p<0.0001 and *p<0.0001 vs unparasitised control (UTA)) in the PCV values. Parasitized untreated (MCT) group had the least PCV. Control drug, artesunate (ART) compared favorably with the UTA. Fig. 3c shows that there was no significant difference when the controls (Sulfadoxine-pyrimethamine (SP) and untreated control (CP)) were compared with the treated groups. WBC values decreased significantly (***p<0.0001) among the treated groups for ME and MF compared with ART. Again, WBC in ART- treated group increased significantly (^^^p<0.0001) when compared with MCT. In contrast, WBC values decreased as the dose increased in the MF groups.**

In view of these facts, phytomedicine remains a major source of hope for the treatment of diseases especially malaria. Although, some of these herbs used for the treatment of malaria have promising curative potentials, it is advisable to subject these herbs to scientific proofs to substantiate their folkloric use. In this way, plants that have folkloric antimalarial potentials that failed the scientific proofs of this claim can be removed from the local pharmacopeia. In Nigeria and Republic of Benin, *A. difformis* root tuber is used for the treatment of malaria. We therefore present the curative and chemopreventive antimalaria efficacy of the root tuber of *A. difformis* in animal models.

Previous experiments showed that different parts of *A. difformis* are employed for the treatment of several ailments in Africa and the tuber has been listed in the African pharmacopeia as an antimalarial drug. Traditionally, *A. difformis* has been used as diuretic, antidiabetic, anti-tuberculosis, antimalarial, anticancer as well as oral and anal lesions [8,11]. In this study, we evaluated the antimalarial activity of ME and MF of the tuber of *A. difformis*. Analysis and comparison of the trends of parasitemia among ME and MF of the tuber of *A. difformis.* treatment groups and the negative control did not appear to demonstrate the antimalarial potency of the plant. The parasitemia levels in all the groups for the extract and fraction seem to increase with increasing number of day, although the increase was not significant. This finding is suggestive of the fact that *A. difformis* may have no significant antiplasmodial effect when compared with artesunate. Similar results were obtained in *in vitro* models by Bero et al. [8]. The antiplasmodial activity of *A. difformis* was tested on mice because of the susceptibility of the animal to the chloroquine-sensitive strain of the parasite.

We have also shown in this study that there were no significant increases in the PCV of the animals treated with both ME and MF as compared with the negative control. This is against the claim that *A. difformis* has therapeutic effect as an antimalarial. The low PCV in the negative control group may be indicative of hemolytic anemia. The erythrocytic stage of malaria parasite affects hemoglobin, in which the globin part of the blood meal is converted to harmless hemozoin thus lowering the oxygen-carrying capacity of the red blood cell. The oxidative damage of the RBC membrane coupled

with an increasing population of the malaria parasite per unit cell increase the cells' susceptibility to hemolytic lysis. The etiology of severe malarial anemia can include a number of distinct as well as overlapping features, including lysis of infected and uninfected red blood cells [12], splenic sequestration of red blood cells, [13], and chronic transmission of malaria in holoendemic regions. The degree of parasitemia is typically an indicator of malaria disease severity. Therefore, high level of parasitemia can certainly result in massive lysis and clearance of red blood cells, resulting in profound anemia [14]. This study also showed that there was a reduction in the WBC of the animals across the groups. This suggested that the extract and the fraction may have the ability to reduce white blood cell production, creating a possible case for leucopenia.



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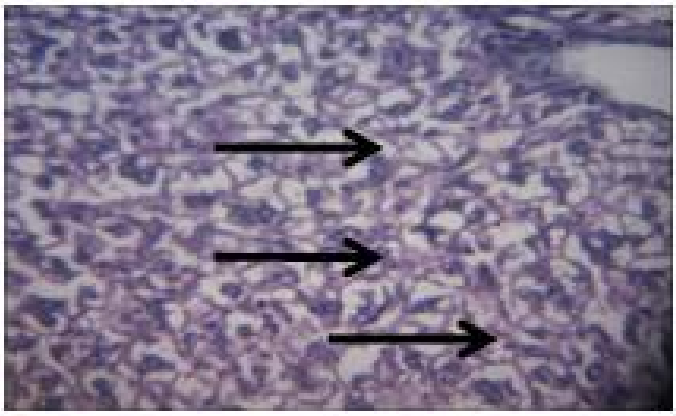

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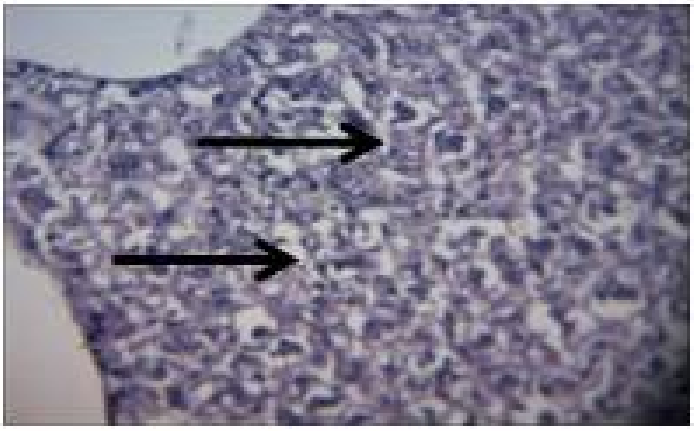

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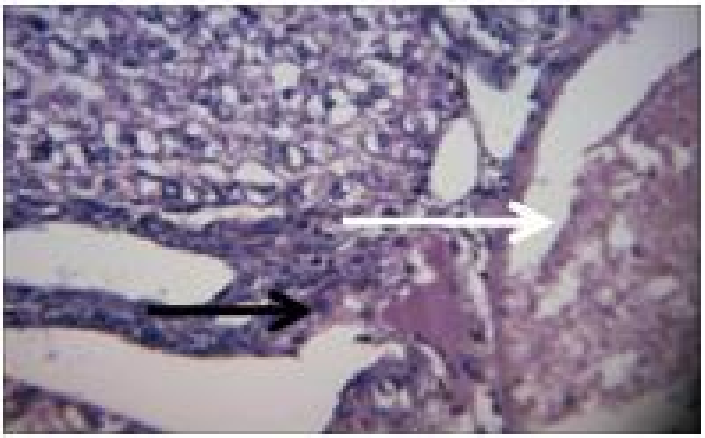
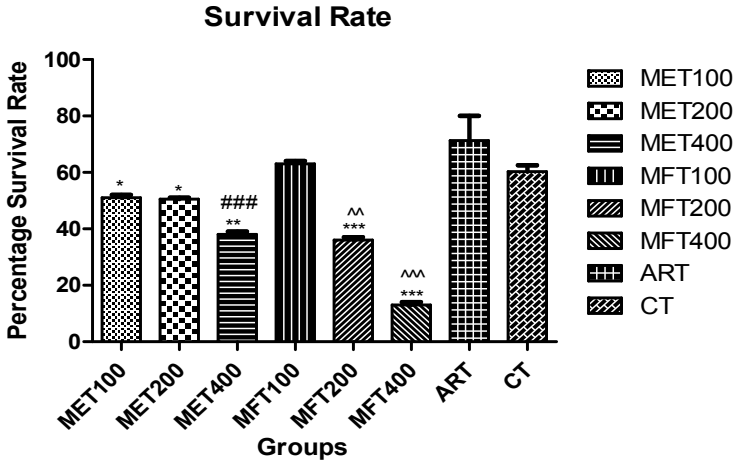


D

Fig. 4. Liver tissue section of the 400mg/kg bw for ME of *A. difformis* showing a marked portal and central venous congestion (WHITE arrows) and, mild periportal cellular infiltration (black arrows). Mild to moderate diffuse vacuolar degeneration was observed (Figure 4a). Figure 4b shows a liver tissue section of a mouse treated with 400mg/kg bw of MF of *A. difformis* showing a severe diffuse vacuolar degeneration and necrosis of hepatocytes. Fig. 4c shows liver tissue section of the ART-treated group showing a severe diffuse vacuolar degeneration and necrosis of hepatocytes. Few inflammatory cells were seen (short arrows). Fig. 4d shows a liver tissue section of SP-treated animal showing a severe portal congestion, with moderate periportal cellular infiltration. There is a diffuse vacuolar degeneration of hepatocytes (X400)

Death from malaria occurs from the complications of the infection: cerebral manifestations leading to coma and a severe and refractory anemia leading to hypoxia and cardiac problems. It was not surprising to observe that there was a dose-dependent increase in PCV as ART, a standard drug, also caused an increase in PCV because malaria parasite which could cause hemolysis had been cleared. There was therefore, a direct proportionality between parasite clearance and PCV increase since the schizonts are implicated to cause hemolysis, and any drug with schizonticidal effect is expected to boost the volume of red blood cell in the animal. This phenomenon thus explains the direct proportionality in the pathogenesis of malarial anemia, such as erythrocyte lysis and phagocytosis and sequestration of parasitized red blood cells.



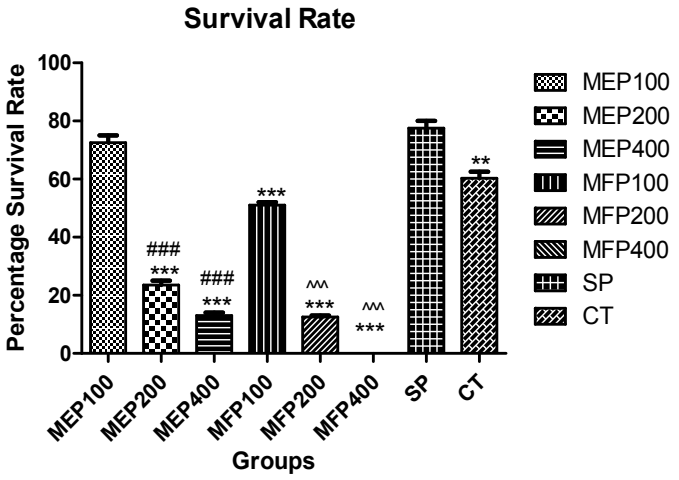


Fig. 5. Percentage Survival rates of the animals treated with the drug candidates (MET and MFP). The survival of the group treated with the control drug (ART) is significantly higher ($p<0.0001$) than the groups treated with either methanol extract or fractions (ART vs MET and MFT, ***$p<0.0001$) while there was a significant difference also among the dosage groups (MET 100 vs MET 400, ###$p<0.001$) and MFT 100 vs MFT 400 (^^^$p<0.0001$)). Similarly, there was a significant difference between the prophylactic control drug, SP and the methanol extract and fraction (*$P<0.0001$) which were also more toxic at high doses. Similar difference was noticed between the extract and fraction at different dosage levels (MEP 100 vs MEP 400, ###$p<0.0001$) and MFP 100 vs MFP 400 (^^^$p<0.0001$). No animal survived under the MFP 400 group.**

This study also showed the prophylactic effect of ME and MF of *A. differmis* root tuber on *P. berghei*-infected mice. In this study, the negative control showed a significant increase in the percentage parasitemia when compared with the standard drug, SP showed a significant decrease in percentage parasitemia, and it had zero percent parasitemia from day three to day seven. However, the test drugs (ME and MF) did not have a significant reduction in percentage parasitemia in all the doses used.

The pathophysiology of the liver tissue revealed that the root tuber extract of *A. differmis* is highly toxic both at low and high dosage as a severe diffuse vacuolar degeneration and necrosis of hepatocytes were seen in the tissues of the animals treated with this extract. The damage caused to the liver tissues of the animals in the prophylactic groups was even more severe when compared to the therapeutic groups due to the duration of treatment to which these test groups were exposed to the drug candidates. This suggested that long-term exposure of animals to this plant extract is highly dangerous to their health.

Oxalate and cyanide had been reported as part of the antinutrient compounds found in the root tubers of *A. differmis* [15]. Oxalate chelates metals especially calcium and are capable of causing renal failure via stone formation. *A. differmis* had been used in agriculture for the control of pest [16] and when the tubers are to be included in animal feed, it is first boiled probably to degrade oxalate and any antinutrient that can be found in it. The histopathology of the liver of animals treated with 400 mg/kg bw of the extract showed both portal and central venous congestion and severe vacuolar degeneration with accompanying necrosis of the hepatocytes in liver tissues of animals treated with 400 mg/kg bw of the fraction.

Interestingly, we also noticed the toxic effects of the standard antimalarial drugs ART and SP on the liver tissues when compared with the normal untreated animal liver tissue. A severe diffuse vacuolar degeneration and necrosis of hepatocytes and few inflammatory cells were seen in both liver tissues. This result corroborates a study carried out by Farombi et al. [17] where it was reported that some antimalarial drugs induce oxidative stress and altered enzymatic and non- enzymatic antioxidant defense in the host which is exacerbated in *P. falciparum*-infected patients administered with antimalarial drugs. Therefore, prolonged usage

of antimalarial warrants caution and supplementation with dietary antioxidant regimen.

Both curative and prophylactic treatments of malaria seek to improve the survival rate to hundred percent and the mortality rate to zero. A drug candidate that achieves less than this gives room to seek for a better drug or an optimization of potent and existing ones. The results obtained in this study showed that the survival rate is reduced in both curative and prophylactic studies and also among the ME and MF of *A. difformis* used in this study. As the dose increased, survival rate decreased, meaning that the ME and MF of *A. difformis*, are toxic leading to more death of animals. MF of *A. difformis* is more toxic than ME of *A. difformis* and since MF is more polar than ME, the conventional extraction method via an aqueous infusion may be toxic. Interestingly, no animal survived the highest dose of the fraction used after the experiment. The overall survival rate showed that animals used for the curative studies survived better than the pretreated ones because the latter took the fraction for a longer time than the former. The survival rate was also higher in the SP group better than the ART group. Again, groups treated with ME of *A. difformis* survived better than their MF counterpart both in the curative and prophylactic groups.

5. CONCLUSION

It is concluded that *A. difformis* root tuber may be hepatotoxic both at low and high dosages. Different extraction methods or processing of the extracts that would not predispose the animal models to cytotoxicity but probably reveal other phytomedical use of this plant will be of advantage.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Rules guiding animal studies in accordance with National Institutes of Health (NIH, Maryland, USA) principles of Laboratory Animal Care and use were strictly followed in this experiment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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