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In vivo antiplasmodial activity of extract and fractions of *Trema orientalis* in *P. berghei*-induced malaria in mice

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ABSTRACT

Objective: To assess the *in vivo* antimalarial potential of various solvent extracts and fractions of *Trema orientalis*.
Methods: In this study, the animal model of antimalarial activity was employed using *Plasmodium berghei*-induced mice. The crude methanol extract was fractionated using vacuum liquid chromatography in the order of increasing polarity using dichloromethane, ethylacetate and methanol. Percentages of parasitemia and clearance were used as indices for antiplasmodial activities. The full blood count was also assayed while the gas chromatography-mass spectrometer analysis of the most potent fraction was carried out to detect the active compounds presenting in it.
Results: Dichloromethane fraction had the least percentage of parasitemia [(0.19 ± 0.07)%] and the highest percentage of clearance [(91.74 ± 8.38)%] at the highest dose used (200 mg/kg body weight) after day 7 relative to the artemisinin control which cleared the parasite after day 3. The ethylacetate fraction showed the least percentage of clearance [(70.52 ± 5.64)%] at the highest dose used (200 mg/kg body weight) after day 7.
Conclusions: The results obtained showed that purification enhanced the antiplasmodial activity of *Trema orientalis* in *Plasmodium berghei*-induced malaria in mice. The antiplasmodial activity of the dichloromethane is a strong indication that the fraction, if purified further, may contain drug candidates for the treatment of malaria in the nearest future.

1. Introduction

Malaria remains a major threat to the health of a large populace in Africa and beyond. The double tragedy of poverty and poor health care delivery in Africa had made a major sector of the population to be at a very high risk of infection and ultimately death from malaria parasite. The major sectors of the population under lethal infections are infants of tender age. Malaria also infects the adults with its death rate higher than that of HIV/AIDS. In recent years, several attempts had been made both for the chemotherapeutic, prophylactic and vaccine-based preventions and treatments for malaria. Although there is no effective vaccine yet discovered and world widely used, there are many candidates in the making. Several attempts including the introduction of gamma radiation attenuated sporozoites and genetically attenuated sporozoites offered plausible breakthrough, but there are concerns about their safety since they are not wholly effective such as the possibility of breakthrough infections especially in immunocompromised individuals. Therefore, chemotherapy remains the most effective approach for the treatment of malaria[1,2].

After the synthesis of chloroquine in 1934, the treatment of malaria received a great achievement until resistance to chloroquine and other quinine drugs were discovered years after[3-5]. Nowadays, phytomedical approach to malaria treatment and other diseases is popular around the world because it is cheap, affordable and efficient. The most recent antimalarial drug, artemisinin, is discovered from *Artemisia annua,* but unfortunately, *Plasmodium falciparum* (*P. falciparum*) has also developed resistance to this drug and because of this, World Health Organization has recommended the withdrawal of oral artemisinin-based monotherapies from the market and the substitution of this monotherapy with artemisinin combinative therapy[6,7]. Several plants such as *Alstonia boonei*

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All experimental procedures involving animals were conducted in accordance to National Institute of Health guide (#85-23, revised in 1985) and approved by the Department of Pharmacology and Toxicology Ethical Committee on Animal Experimentation.

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and *Vernonia amygdalina* have had their antimalarial activities documented and many more are still being screened[8].

In this study, the antiplasmodial activity of the methanol extract and solvent fractions of *Trema orientalis* (*T. orientalis*) stem bark is being documented for the first time. *T. orientalis* is an evergreen tree which belongs to the family Ulmaceae. Apart from its uses in paper production and in the manufacturing of poles, it has been used for medically for the treatment of respiratory, inflammatory and helminthic diseases[9]. Almost every part of the plant is used as medicine in various parts of Africa. The root of *T. orientalis* is used in folk medicine for the treatment of trauma, blood stasis, hematuria and bleeding of intestines and stomach. The stem bark extractions are used to kill intestinal worms and also as anti-dysenteries. The stems and twigs infusion are used to treat fever and toothache[10].

The leaves of *T. orientalis* are mixed with leaves of *Bidens pilosa*, *Citrus aurantifolia* and peels of unripe pineapple. It is boiled and the extraction used in the management of jaundice[11]. The leaves macerated in lemon juice are used as remedy for bronchitis, pneumonia, pleurisy and cough. The leaves decoction of *T. orientalis* is also used as an anti-helminthic medicine for roundworms and hookworms in West Africa, East Africa and some parts of Central Africa and Madagascar. The fruit and flowers are used to prepare infusion that are administered to children as a therapy for bronchitis, pneumonia and pleurisy[12].

In spite of many uses of this plant, there is paucity of information to substantiate the indigenous claim of its antimalarial use. Locally, this plant is normally soaked in water for few days and the extracted aqueous infusion taken orally *ad libitum*. This study seeks to establish the indigenous claim for antimalarial treatment and optimize the antimalarial effect of this plant via the use of different solvent systems to know whether the active principle is water soluble or not.

2. Materials and methods

2.1. Chemicals

All the solvents used in this study were pure and of analytical grade. They were procured from Sigma Aldrich Inc., Steinhelm, Germany. Artemisinin was purchased from Guilin, China. Silica Gel G, a product of Lobal Chemie was purchased from Lobal Chemie Ltd. Mumbai, India. Dimethyl sulfoxide was purchased from Merck Company (Darmstadt, Germany).

2.2. Ethical considerations

Experimental procedures, protocols and animal treatment used in this research work were conducted in accordance with National Institute of Health guide (#85-23, revised in 1985) for the care and use of laboratory animals and approved by the Department of Pharmacology and Toxicology Ethical Committee on Animal Experimentation.

2.3. Plant material and extraction

The stem bark peel of *T. orientalis* was obtained in April, 2015 from a single population of *T. orientalis* growing around Iworoko community, southern region of Ekiti State University, Ado-Ekiti, Nigeria. The specie was authenticated and identified at the Herbarium, Plant Science Department, Ekiti State University, and a specimen was deposited in the Herbarium. This was air-dried, blended and soaked in sufficient methanol for 5 days, decanted and filtered using Whatman filter paper. The filtrate was concentrated under reduced pressure at 40 °C using rotary evaporator (Stuart Brand, United Kingdom).

A known weight of the concentrated, brown residue methanol extract (50 g) was partitioned using vacuum liquid chromatography by adsorbing the extract on 50 g of the silica gel. The vacuum liquid chromatography column was parked with 80 g of the gel using *n*-hexane. The adsorbed sample was applied to the column and eluted successively with *n*-hexane, dichloromethane, ethylacetate and methanol. The obtained fractions were concentrated using the rotary evaporator weighed and kept in the refrigerator until used. The percentage yields of the fractions thus obtained were 0.03%, 1.50%, 2.30% and 93.20% for *n*-hexane, dichloromethane, ethylacetate and methanol fractions, respectively.

2.4. Experimental animals and transfection

One hundred and thirty Swiss albino mice weighing approximately 14 g were obtained from the Preclinical Animal House, University of Ibadan and were transfected using a donor mouse with an inoculum size of 1×10^7 of chloroquine-sensitive strain of *P. berghei* obtained from Institute of Advanced Medical Research and Training, College of Medicine, University of Ibadan, Nigeria. Parasitemia (established infection) was confirmed after 72 h by obtaining blood from the infected animals via a tail cut and smears (thick and thin films were prepared on the slides). The thin film was fixed in absolute methanol and both thick and thin films were stained using Giemsa stain. The thick film was viewed to assess parasite density per field while the thin film was viewed to assess percentages of parasitemia and clearance after treatments. The percentage of parasitemia and percentage of clearance were calculated as follows:

Clearance (%) = Control – Test/Control × 100

Parasitaemia (%) = (Number of infected red blood cells counted/ Total number of red blood cells counted) × 100

2.5. Animal grouping and treatment

Seventy male mice (13–18 g) were randomly assigned to fourteen groups of five animals in each group. The drug candidates were dissolved in dimethylsulfoxide (5% v/v) and three groups for each fraction/extract were treated once daily with 50 mg/kg, 100 mg/kg and 200 mg/kg for methanol extract (ME), dichloromethane fraction (DF), ethylacetate fraction (EF) and methanol fraction (MF). The artemisinin group received a daily dose of 10 mg/kg treatment while the negative control group received an equivalent volume of the vehicle.

2.6. Haematological study

Twenty-four hours after the last administration, the blood samples were collected from the animals and were put into ethylene diamine tetra acetic acid sample bottles .The samples were carefully inverted to avoid blood clot and were submitted for full and differential blood count.

2.7. Gas chromatography-mass spectrometer (GC-MS) analysis of potent fractions

The GC-MS analysis of the most potent fraction was carried out using the Agilent 5975 GC-MS system (Santa Clara, California, United States of America) which is equipped with a DB-1 fused silica column. The carrier gas was hydrogen having a flow rate of 1.0 mL/min.

2.8. Statistical analysis

Percentages of parasitemia and clearance of all groups were monitored and calculated. The standard deviation values were determined and levels of significance were also determined using the univariate Duncan's multiple range test.

3. Results

3.1. Effects of ME of T. orientalis on percentages of parasitemia and clearance

In Figure 1A, artesunate had 0 parasitemia on day 5 while the different doses of the ME of *T. orientalis* had varying percentages of parasitemia with the least observed in the 200 mg/kg body weight and the highest in the 50 mg/kg body weight. The control drug, artesunate, cleared the parasite on day 5 while there wasn't any significant difference between the percentage of parasitemia of the control drug and the 200 mg/kg body weight dose of the ME. Figure 1B shows the parasite clearance by the ME relative to the control drug and the untreated control. Artesunate had the highest parasite clearance (100%) as from day 5 followed by the 200 mg/kg dose of the ME while the least parasite clearance was observed in the groups which were given 50 mg/kg body weight dose. This showed that the antiplasmodial activity of the ME of *T. orientalis* was dose-dependent.

3.2. Effects of the dichloromethane fraction of T. orientalis on percentages of parasitemia and clearance

Figures 2 shows the percentages of parasitemia and parasite clearance in parasitized animals treated with DF of *T. orientalis* methanol stem bark extract. From Figure 2, the 200 mg/kg dose of DF significantly reduced the percentages of parasitemia and

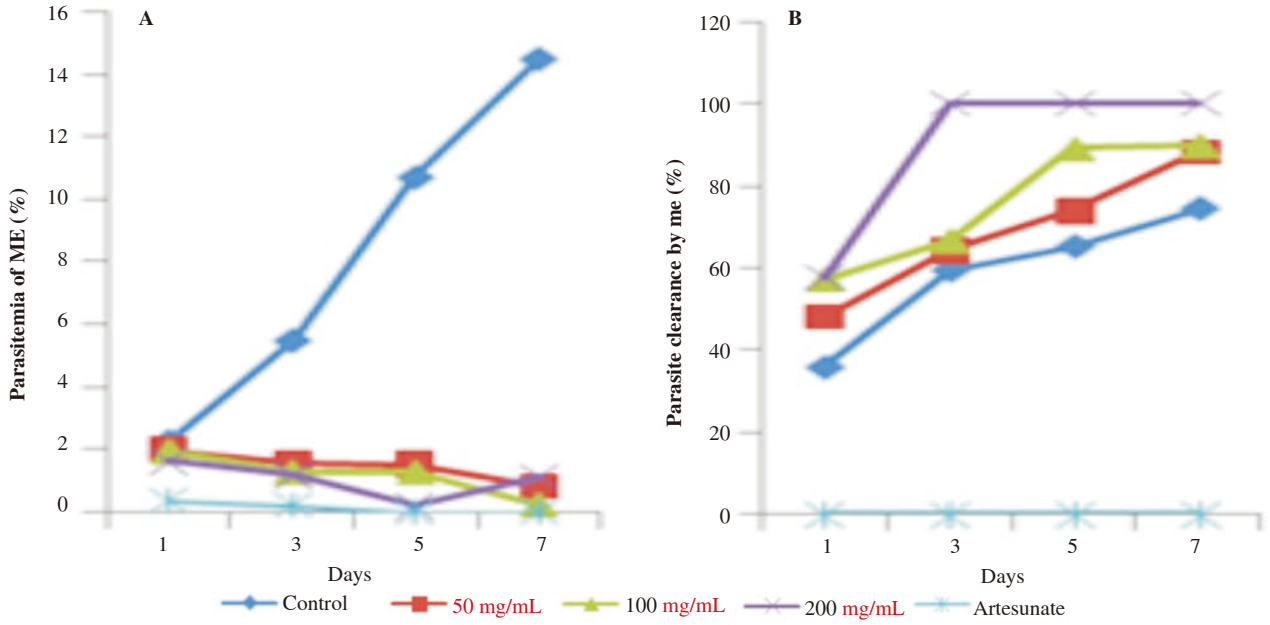

Figure 1. The effects of orally administered ME of *T. orientalis* on percentages of parasitemia (A) and clearance (B).

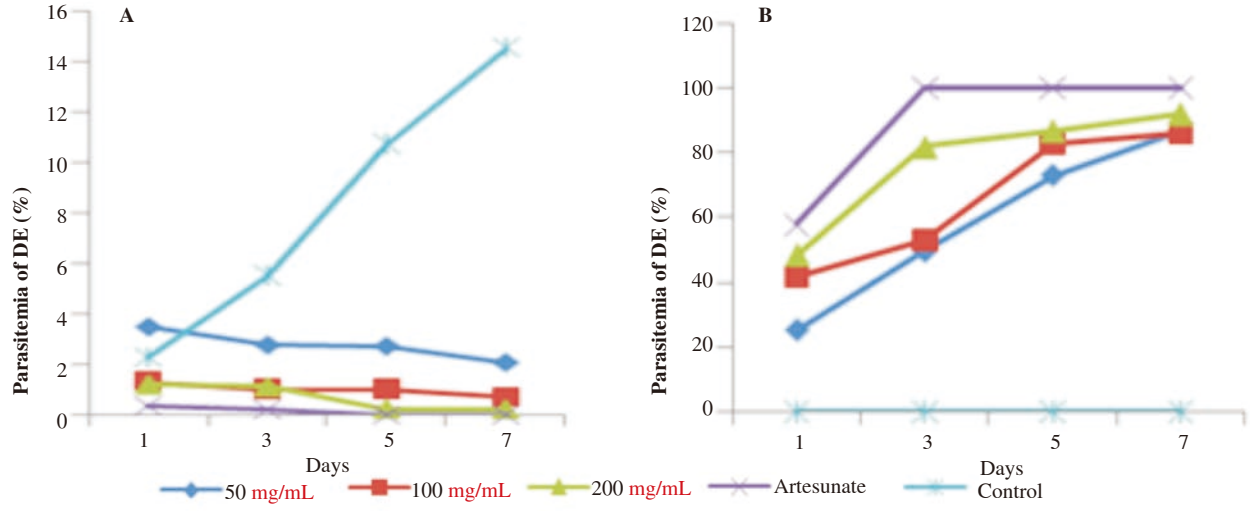

Figure 2. The effects of the DF of *T. orientalis* on percentages of parasitemia (A) and clearance (B).

clearance. On day 7, the percentage parasitemia of the 200 mg/kg dose was nearly zero as the control drug, artesunate.

The EF of *T. orientalis* had the least parasite clearance activity and the highest percentage parasitemia showing that this fraction is not good for malaria treatment (Figure 3). Moreover, there was no significant difference between the parasite clearance and percentage parasitemia of all the doses used .

The antiplasmodial activity of the MF fraction was less than that of the ME. The percentage clearance of the MF fraction was less than that of the ME and the percentage parasitemia of MF was more than that of the ME (Figures 4A and 4B). The curative effect of the MF was found to be dose-dependent while the smallest dose (50 mg/kg body weight) had the highest percentage parasitemia.

It was worthy of note that there were changes in the haematological indices by the extracts of *T. orientalis* in the infected mice. The haematological parameters in vehicle showed in Tables 1–3 formed the bases for comparison for the indices in the treated groups. The group treated with the vehicle showed a sharp decrease in packed cell volume (PCV) when compared with other treated groups. The total white blood cell (WBC) count and some of the differentials (neutrophils and lymphocytes) increased as the dose increased in the extract and fractions used. Monocytes and eosinophils when detected in any of the dose and test drugs used were found to be insignificant as compared to neutrophils and lymphocytes (Tables 1 and 2). However, monocytes and eosinophils were not detected at the highest dose (200 mg/kg body weight) for all the test drugs (Table 3).

Figure 5 showed the mass spectrometry of the compounds presenting in the dichloromethane fraction liable for the antiplasmodial activity. These four compounds (A–D) are the fatty acid methyl esters found in the dichloromethane fraction. The antimalarial effects produced by the dichloromethane fraction in this work are possibly as a result of the synergistic effects by the fraction components.

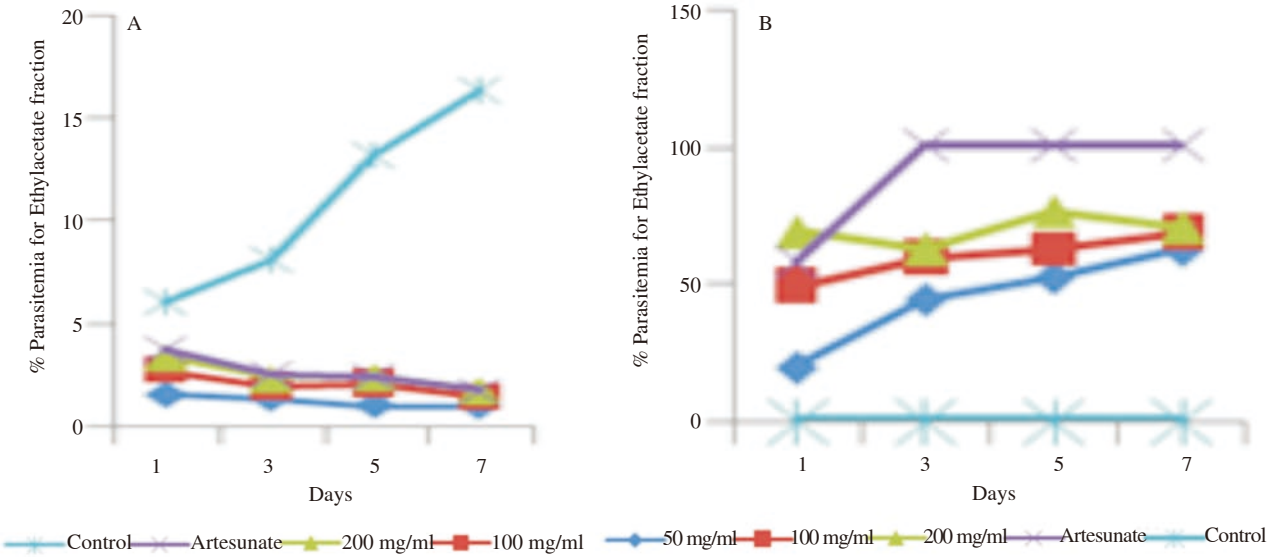

Figure 3: The effects of the orally administered ethylacetate fraction (EF) of T.orientalis on percentage parasitemia and percentage clearance in *P.berghei*-induced malaria in mice. The EF fraction appeared to be the least effective because the 200mg/kg dose had the highest percentage parasitemia and the least percentage parasite clearance of all the fractions and extract used.

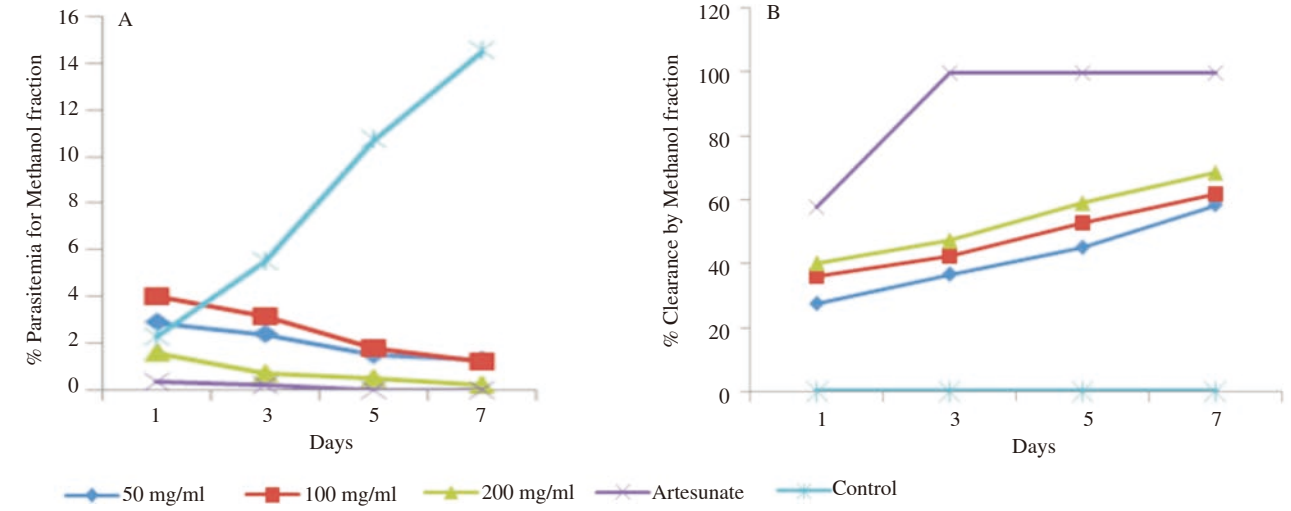

Figure 4: The effects of the orally administered methanol fraction (MF) of *T. orientalis* on percentage parasitemia and percentage cleatrance in *P.berghei*-induced malaria in mice. The antiplasmodial activity of the MF fraction was less than that of the methanol extract (ME). The percentage clearance of the MF fraction was less than that of the ME and the percentage parasitemia of MF was more than that of the ME.

In this study, the results obtained generally showed that the percentage of parasitaemia decreased with increasing dose levels while the percentage of clearance increased with increasing dose levels of the extract, which was an indication that the highest dose exhibited the optimal clearance. The percentage of parasitaemia in artesunate significantly decreased as the experiment progressed and zero parasitemia was obtained on both day 5 and 7. In the same vein, the percentage of clearance of artesunate increased as the experiment progressed and a 100% of clearance was also obtained both on day 5 and 7 when compared to the test drugs. The drug candidates, ME, DF, EF and MF, showed significant clearance when compared with the vehicle, which was an indication that percentage of clearance was observed. However, the results showed that the drug candidates cleared the parasites in dose-dependent manner with the highest clearance noticed in the 200 mg/kg treated groups in all the test drugs.

The overall antiplasmodial activity of the drug candidates showed that the DF had the highest activity at the highest dose, followed by the ME, then the MF and lastly the EF.

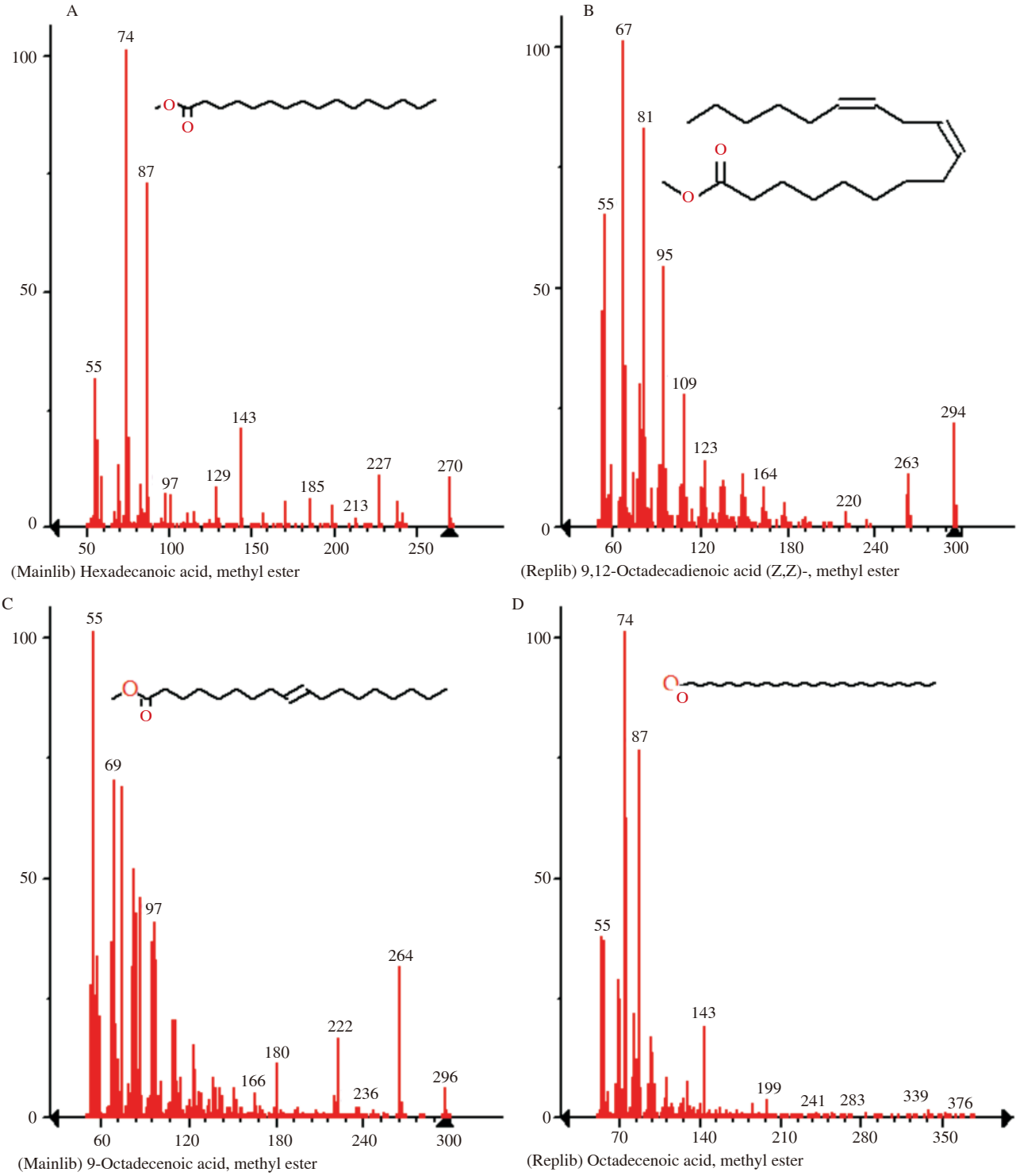

Figure 5. The GC-MS analysis of the most potent fraction, DF, with he fatty acid methyl esters components of the fractions shown in (A) hexadecanoic acid methyl ester (B) 9,12-octadecadienoic acid methyl ester (C) 9-octadecenoic methyl ester and (D) octacosanoic acid methyl ester.

Table 1

The hematological parameter of oral administration of 50 mg/kg body weight of various extracts of *T. orientalis* in mice (%).

Extract	PCV	WBC	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
Dichloromethane	27.0 ± 2.1	8.3 ± 0.9	29.0 ± 2.5	50.0 ± 3.6	1.0 ± 0.0	-	-
EF	10.5 ± 1.6	5.8 ± 0.3	22.5 ± 1.8	36.5 ± 2.4	1.5 ± 0.3	0.1 ± 0.0	-
MF	26.0 ± 2.3	6.0 ± 0.5	25.5 ± 1.2	37.0 ± 1.3	-	-	-
ME	26.5 ± 2.8	6.1 ± 0.3	26.0 ± 1.5	40.5 ± 2.8	0.5 ± 0.1	-	-
Vehicle	09.0 ± 1.1	4.2 ± 0.1	23.0 ± 1.3	37.0 ± 2.0	-	-	-
Artesunate	47.5 ± 3.9	8.4 ± 1.5	37.5 ± 3.3	62.5 ± 5.4	-	-	-

Table 2

The hematological parameters of oral administration of 100 mg/kg body weight of various extracts of *T. orientalis* in mice (%).

Extract	PCV	WBC	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
Dichloromethane	35.0 ± 3.1	10.3 ± 0.8	34.0 ± 2.9	54.5 ± 5.8	01.0 ± 0.0	-	-
EF	18.0 ± 0.9	6.5 ± 0.3	27.0 ± 2.4	43.0 ± 1.4	-	01.0 ± 0.0	-
MF	27.5 ± 2.8	7.0 ± 0.5	28.0 ± 3.0	44.0 ± 1.8	0.5 ± 0.0	-	-
ME	28.0 ± 3.0	7.9 ± 0.3	29.5 ± 3.2	45.5 ± 2.1	01.0 ± 0.0	-	-
Vehicle	09.0 ± 0.6	4.2 ± 0.1	23.0 ± 2.0	37.0 ± 3.2	-	-	-
Artesunate	47.5 ± 4.5	4.4 ± 0.1	37.5 ± 3.3	62.5 ± 5.3	-	-	-

Table 3

The hematological parameters of oral administration of 200 mg/kg body weight of various extracts of *T. orientalis* in mice (%).

Extract	PCV	WBC	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
Dichloromethane	46.0 ± 3.8	17.5 ± 1.6	40.0 ± 2.5	60.0 ± 4.2	-	-	-
EF	20.0 ± 1.7	6.8 ± 0.6	29.0 ± 0.7	47.0 ± 1.9	1.0 ± 0.0	-	-
MF	29.5 ± 2.2	7.2 ± 0.5	30.0 ± 1.1	50.0 ± 2.4	-	-	-
ME	40.0 ± 2.8	8.0 ± 0.3	30.5 ± 2.6	54.5 ± 1.8	-	-	-
Vehicle	09.0 ± 0.4	4.2 ± 0.2	23.0 ± 1.7	37.0 ± 2.1	-	-	-
Artesunate	47.5 ± 2.9	4.4 ± 0.3	37.5 ± 3.1	62.5 ± 2.7	-	-	-

4. Discussion

The antimalarial activity of various tested plants in Africa is an indication that although the treatment method was crude, there were means by which malarial infection was kept at bay before the advent of orthodox medicine. The effectiveness of these plants is a big plus in the management and therapeutic approaches to diseases in Africa in a way that is both accessible and cost-effective. Although an appreciable quantity of the infusion has to be taken before the effect is noticed, this is because the active compound(s) per unit volume of the infusion taken is very small.

The present study investigated the antiplasmodial activities of dichloromethane, ethylacetate, MF and ME of *T. orientalis* for the treatment of malaria infection using 7 days therapeutic model. The determination of percent inhibition of parasitemia is the most reliable parameter[13]. A mean group parasitemia level of less than or equal to 90% of mock-treated control animals usually indicates that the test compound is active in standard screening. The results of the extracts at different doses show that they are capable of reducing the level of parasites in circulation in the curative model. Ojurongbe *et al.*[14] observed that the activities of the extracts were dose-dependent and significantly higher amounts of the crude extracts were required to elicit such activities. This supports the increase in activity observed in the extract as the dose increased in the treated groups, which explains the fact that the crude extract exerted a pronounced activity against the malaria parasite at an optimal dose of 200 mg/kg when compared to artesunate used as standard drugs in the study. In view of this, the extract can be considered to contain some antimalarial active ingredients that could serve as a template for the production of relatively inexpensive antimalarial drugs. It is clear from this results that the percentage of parasitemia of *P. berghei*-infected mice treated with dichloromethane extract of *T. orientalis* changed significantly in comparison to the non-treated infected mice. Moreover, the dichloromethane extract administered at a dose of 200 mg/kg per day for seven days resulted in a significant reduction in percentage of parasitemia, which is a performance that may likely be improved upon if the extract is purified to identify and isolate active ingredients.

It is worthy of note that there were changes in the haematological indices by the extracts of *T. orientalis* in the infected mice. The haematological parameters in vehicle as showed in Tables 1–3 form the bases for comparison for the indices in the treated groups. The group treated with the vehicle showed a sharp decrease in PCV when compared with other treated groups. This showed that a decrease in the blood volume is an indication that the oxygen carrying capacity of the red blood cells will decrease and that the animals might have suffered from haemolytic anemia.

A higher enhancement of haematological indices was observed in the dichloromethane extract of *T. orientalis* as it reversed the observed infection induced by parasite. Similar trend was observed by Adegbolagun *et al.*[15]. This was evident by significant higher PCV, white blood cell, neutrophil and lymphocyte than those obtained form the other extracts in the infected animals. This showed that the dichloromethane extract of *T. orientalis* had a synergistic effect on the rate of parasite clearance of *P. berghei* infection in mice with a significant enhancement of haematological parameters within seven days of administration. This observed significant enhancement of haematological parameters in the infected mice by the seven days administration of the dichloromethane extract of *T. orientalis* showed its possible use in the treatment of malaria.

It is interesting to note that the extract and fractions used in this work increased the percentage of neutrophils at the highest concentration of 200 mg/kg body weight used and that the dichloromethane had the highest percentage as compared with the

control (Tables 1–3). It is likely that the antimalarial activity of the dichloromethane fraction was as a result of the neutrophil priming. Neutrophils are an essential component of the human immune system and they are mobilised to the infection site by host- and/or pathogen-derived components which also prime the host cells for microbicidal activity. Neutrophils bind and digest the invading pathogen via phagocytosis upon the triggering of the production of reactive oxygen species in killing most of the pathogens. Neutrophils have been primed for enhanced adhesion, phagocytosis, production of reactive oxygen species, cytokine secretion, leukotriene synthesis, degranulation and bactericidal activity[16].

The GC-MS analysis of the post potent fraction revealed the presence of methyl esters of fatty acids that served as the drug candidate and had the antiplasmodial activity. Fatty acids and their methyl esters had been found to have antimalarial activity[17]. It had been reported that very long chain fatty acids were non-toxic but inhibited the enoyl-acyl carrier protein reductase, an enzyme that was needed by the *P. falciparum* for the elongation process of its fatty acid during synthesis and also the biosynthesis of type II fatty acid synthase which takes place in the apicoplast of *P. falciparum* could be altered by fatty acids[18,19]. Again, it was also found that the antiplasmodial activity varied with the degree of unsaturation and that the more the unsaturation, the more the antimalarial activity of fatty acids, while the saturated fatty acids exhibited little antiplasmodial effects[20]. The biosynthetic mechanism for fatty acids in the parasite is different from what obtains in the humans, therefore, the fatty acids and their methyl esters are able to have antimalarial activities without harming the human host. Fatty acid methyl esters are also stable when compared with the free acids.

Throughout this experiment, zero parasitemia or hundred percent parasite clearance were not obtained within the seven days period of investigation. This may be as a result of the inability to have adequate dose at the site, short half-life or rapid elimination of the test drugs as compared with the standard drug. Therefore, an improved formulation of the drug candidates is highly required.

We had been able to show, for the first time, that the fatty acid methyl esters from the dichloromethane fraction of the methanol stem bark extract of *T. orientalis* are responsible for the antimalarial activity of the plant. The results suggested that *T. orientalis* showed potential antimalarial activity by its therapeutic clearance and enhanced haematological indices against *P. bergei* parasites. This performance can surely be improved upon in future studies if the extract is purified and the active drug candidates are identified. The extracts have considerably low or no toxicities in experimental mice. This findings support the traditional use of this plant for the treatment of malaria.

Conflict of interest statement

We declare that we have no conflict of interest.

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