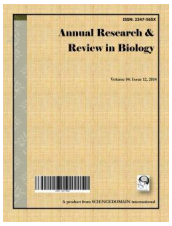

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In vivo Antimalarial Activity of Solvents Extracts of *Alstonia boonei* Stem Bark and Partial Characterization of Most Active Extract(s)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AAI, MDE and MKA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors IUM, AJA and AI and managed the analyses of the study. Authors HA and AM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Alstonia boonei, a plant locally called 'Egbu' in South Eastern Nigeria is used traditionally in the treatment of malaria in the region. This research was carried out to evaluate *in vivo* antimalarial activity of different solvents extract (aqueous, methanol and chloroform) of *Alstonia boonei* against NK-65 Chloroquine sensitive *Plasmodium berghei* infected mice. A total of 84 mice were inoculated with *Plasmodium berghei* and left for 7 days for optimum parasitaemia development after which they

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were screened for malarial parasites using thin blood film. They were then randomly divided into 12 groups of 7 mice per cage. Group 1 serves as negative control, Groups 2-4 animals were administered with aqueous extract at a dose of 150, 250 and 500 mgkg-1 per day for nine days,Groups 5-7 animals were administered with methanol extract at a dose of 150, 250 and 500 mgkg-1 per day for nine days while Groups 8-10 animals were administered with chloroform extract at a dose of 150, 250 and 500 mgkg-1 per day for nine days. Group 11 and 12 were administered with chloroquine and ACT respectively. The antimalarial activity of the different plant extracts was assessed using thin blood films after 3 days, 6 days and 9 days of the extract administration. Results showed that all the extracts had intrinsic antimalarial properties that were both dose dependent and duration dependent. It showed a significant difference ($p<0.05$) in mean percentage activity and percentage parasitaemia of the extracts when compared with placebo (distilled water), Chloroquine and ACT, with methanol showing highest activity (99.68%) on day 3 at a dose of 500mg/kg followed by aqueous extract (99.03%) at a dose of 250 mg/kg. GCMS results revealed the presence of Di-n-Octyl phthalate; 3-Nitrophthalic acid, bis – (2, ethylhexyl-ester) and Bis – (3, 5, 5-trimethylhexyl) phthalate as possible bioactive compounds presents in the extracts. The present study demonstrated that *Alstonia boonei* possess strong antimalarial activity with aqueous extracts possessing the highest activity. Thus, supporting the traditional use of the plant for the treatment of malaria.

Keywords: Antimalarial; Alstonia boonei; in vivo; Plasmodium berghei and GCMS.

1. INTRODUCTION

Malaria is the most important of all the tropical diseases in terms of morbidity and mortality. The global tally of malaria in 2015 was 212 million new cases and 429 000 deaths [1]. Across Africa, millions of people still lack access to the tools they need to prevent and treat the disease. Funding shortfalls and fragile health systems restrict access to life-saving interventions and jeopardize the attainment of global targets. In Nigeria, malaria is endemic throughout the country, accounting for up to 60% outpatient visits to health facilities, 30% childhood mortality and 11% maternal deaths [2]. Malaria is a vector borne disease, caused by protozoan parasites of the genus *Plasmodium*. It is transmitted from the blood of an infected person and passed to a healthy human by a female anopheles mosquito bites [3]. It is the most important human parasitic infection [4], with threats to lives in Sub-saharan Africa [5]. The disease is commonly found in tropical and sub-tropical Africa and Southeast Asia [6]. Malaria chemoprophylaxis especially in chloroquine resistant *P. falciparium* areas has become a real problem. The attempts to secure protection under these circumstance with the utilization of amodiaquine, the combination of sulfadoxine/ pyrimethamine (Fansidar), sulfalene/ pyrimethamine (Metakelfin), or pyrimethamine/ dapsone (meloprim), halfan, halofantrin with or without chloroquine had to be abandoned or to be used with caution in view of the severe complications following the weekly administration of these drugs [7]. Anti-malarial drug resistance, particularly *Plasmodium falciparium* resistance has been a major setback in the fight against malaria and its attendant complications [8]. Plants have been the basic sources of sophisticated traditional medicine systems for thousands of years and were instrumental to early pharmaceutical drug discovery and industry [9]. An appreciable level of studies has been done on African traditional medicinal plants. However, in Nigeria, particularly in the South Eastern part, studies on the extraction and perhaps, testing of the effects of these herbal extracts on malarial parasites have been minimal. In other words not much have been scientifically proved of the antimalarial activity of indigenous Alstonia Species in Okpuje community. Therefore the aim of the study is to investigate the antimalarial activities of Alstonia plant *(Egbu)* used in the traditional treatment of malaria in Okpuje Community in the South Eastern part of Nigeria.

2. MATERIALS AND METHODS

2.1 Malaria Parasite

The Malaria Parasite, NK-65 Chloroquine sensitive *Plasmodium berghei* used in the experiment was obtained from the Malaria Research Laboratory, Department of Pharmacology, Obafemi Awolowo University, Ile-Ife, Osun state, Nigeria. The parasitized mice were also placed under standard laboratory condition at the Animal house of the Department of Biological Science, Bayero University, Kano.

2.2 Preparation of Plant Extract

The stem bark of Egbu plant (*Alstonia boonei*) was collected from Okpuje community, Northwest of Nsukka LGA of Enugu State (co-ordinates $6°30^{1}N7°30^{1}E$). The plant was identified and authenticated at the Herbarium of Plant Biology Department; Bayero University, Kano and was given a voucher number of (BUK/HAN/0258). The stem bark was washed, shade dried and ground to powder. 200 g of sample was measured and transferred into each of the flasks containing 1000 cm^3 (1 liter) of methanol, chloroform and distilled water. The contents of the flasks were shaken and top covered with aluminum foil and kept for 72 hours (3 days). The herb-water mixtures were shaken daily to ensure proper extraction [10]. After 72 hours the extracts were filtered. The filtrates were concentrated under vacuum using a vacuum rotary evaporator, then measured and stored in screw capped vials under room temperature at the Postgraduate laboratory of Department of Biochemistry, Bayero University Kano. Working concentration of 50 mg/ml was periodically prepared by dissolving 5 g of the extract in 100 ml of distilled water.

2.3 Experimental Animals

Healthy albino mice (weighing 16-20 g) were purchased from the Animal house of the Department of Pharmaceutical Science, University of Jos and then kept under standard laboratory condition at the Department of Biological Science, Bayero University Kano for about two weeks for proper acclimatization.

2.4 Screening of the Infected Mice for Malaria Parasite/Inoculation

Preparation of blood film, drying of blood film, staining of the malaria parasite and microscopy were carried out by the method of [11]. The existence of *P. berghei* schizogonic phases (young and mature trophozoite and schizonts stages) in erythrocytes were confirmed by microscopic examination of thin blood smears, hence the % parasitaemia in each mouse. At the end of the acclimatization period, each of the experimental animals (84 albino mice) to be used for the *in-vivo* studies was inoculated with parasitized donor erythrocytes containing plasmodium parasites. Approximately $6.3x10^5$ infected RBC per 0.2 ml of blood was inoculated to each mouse used for the study. To avoid variability in parasitaemia, all the animals were infected from the same source.

2.5 Preparation of Working Concentrations for *in vivo* Study

The earlier prepared extract was used and volume of extracts to be administered was calculated according to dose and weight of the experimental animal using the relation below [12].

Volume of extract (ml) to be administered =

$$\frac{\text{Weight of animal (kg) x dose (mg/kg)}}{\text{Concentration of extract (mg/ml)}}$$

2.6 Experimental Protocol

A total of 84 mice were inoculated with *Plasmodium berghei* and left for 7 days for optimum parasitaemia development after which they were screened for malarial parasites using thin blood film. The percentage parasitaemia was determined. They were then randomly divided into 12 groups of 7 mice per cage. Group 1 serves as negative control, Groups 2-4 animals were administered with aqueous extract at a dose of 150, 250 and 500 mgkg-1 per day for nine days, Groups 5-7 animals were administered with methanol extract at a dose of 150, 250 and 500 mgkg-1 per day for nine days while Groups 8-10 animals were administered with chloroform extract at a dose of 150, 250 and 500 mgkg-1 per day for nine days. Group 11 and 12 were administered with chloroquine and ACT respectively.

The antimalarial activity of the different plant extracts was assessed using thin blood films after 3 days, 6 days and 9 days of the extract administration. The smear was prepared using well-labeled and properly cleaned slides [13]. Blood samples were collected from the tail vein end of each animal using sterile lancet. After the thin smear, the dry blood was allowed to dry and then fixed with absolute ethanol and subsequently stained with Giemsa for one hour. They were then washed with buffer, pH 7.0 to differentiate [14]. The slides were allowed to dry and then microscopically examined using ×100 magnification in oil immersion with model Olympus Microscope.

In each of the day 3, 6 and 9 screening period, the average number of both infected (uncleared) RBC as well as total RBC were counted and the

percentage parasitaemia and Activity were calculated in both the experimental and control groups.

$$\% \text{ Parasitaesmia} = \frac{N_X}{N_T} \times \frac{100}{1}$$

N_X = Average number of parasitized RBC
N_T = Average number of both parasitized and cleared RBC.

The percentage activity was determined by subtracting the percentage parasitaemia from 100 (i.e N_c x100/N_T).

3. RESULTS

Result of the *in vivo* anti-malarial activities of the extracts after 3 days of administration is presented in Fig. 1. The result showed a significant increase ($p<0.05$) in the mean percentage activity of the extracts in a dose dependent pattern when compared with the negative control (distilled water). Chloroform extract had the highest activity of 99.06% at 150 mgkg^{-1}, this was followed by aqueous extract with an activity of 99.03% at 250 mg/kg. Methanol extract has the least activity of 99.68% at a dose of 500 mg/kg. Chloroquine and ACT also had a comparative significant activities of 85.11% and 85.64% respectively.

Fig. 2 shows that the result of the *in vivo* anti-malarial activities of the extracts after 6 days of administration. It showed a significant increase ($P<0.05$) in the mean percentage activity of the extracts when compared with result of Day-3. Although this result confirmed that the *plasmodium* parasite is chloroquine sensitive by its activity (93.76%,) all the extracts (aqueous, methanol and chloroform) showed better activities of 94.69%, 98.11% and 95.08% respectively at dose of 150 mg/kg.

The result of the *in vivo* anti-malarial activities of the extracts of Alstonia plant after 9 days of administration is presented in Fig. 3. Though there is significant difference ($P<0.05$) in the level of percentage activity of the extracts at varying doses, with aqueous and chloroform extracts showing better activity of 96.80 at 150 mg/kg and 94.13% at 500 mg/kg. However both aqueous and chloroform extract showed unusually zero (0%) activity at 250 mg/kg and 150 mg/kg respectively.

Fig. 4, Fig. 5 and Fig. 6 shows the GCMS chromatogram of most active aqueous, methanol and chloroform extracts respectively. The spectra shows the presence of long chain fatty acids (stearic acid, oleic acid, myristic acid, erucic acid, (1-5), alkylhalide and Di-n-Octyl Phthalate in the aqueous extract, while Nonanol, psi-cumene, oxacyclotetradecane-2,11-dione,13-methyl Di-n Octylphthalate and n-Butyric acid 2-ethylhexylester were detected in the methanol extract and finally Azulene, Myristic acid/Stearic acid (5,7,8), Oleic acid/ E-9 Tetradecenoic acid were detected in chloroform extract.

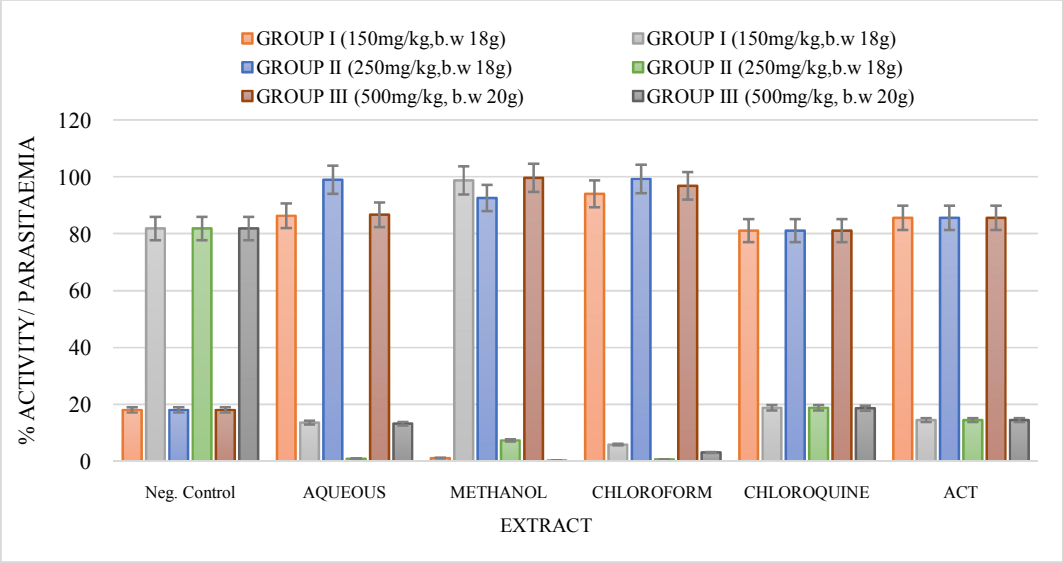


Fig. 1. Effect of oral administration of aqueous, methanol and chloroform extracts on percentage parasitaemia after 3 days of administration of extracts

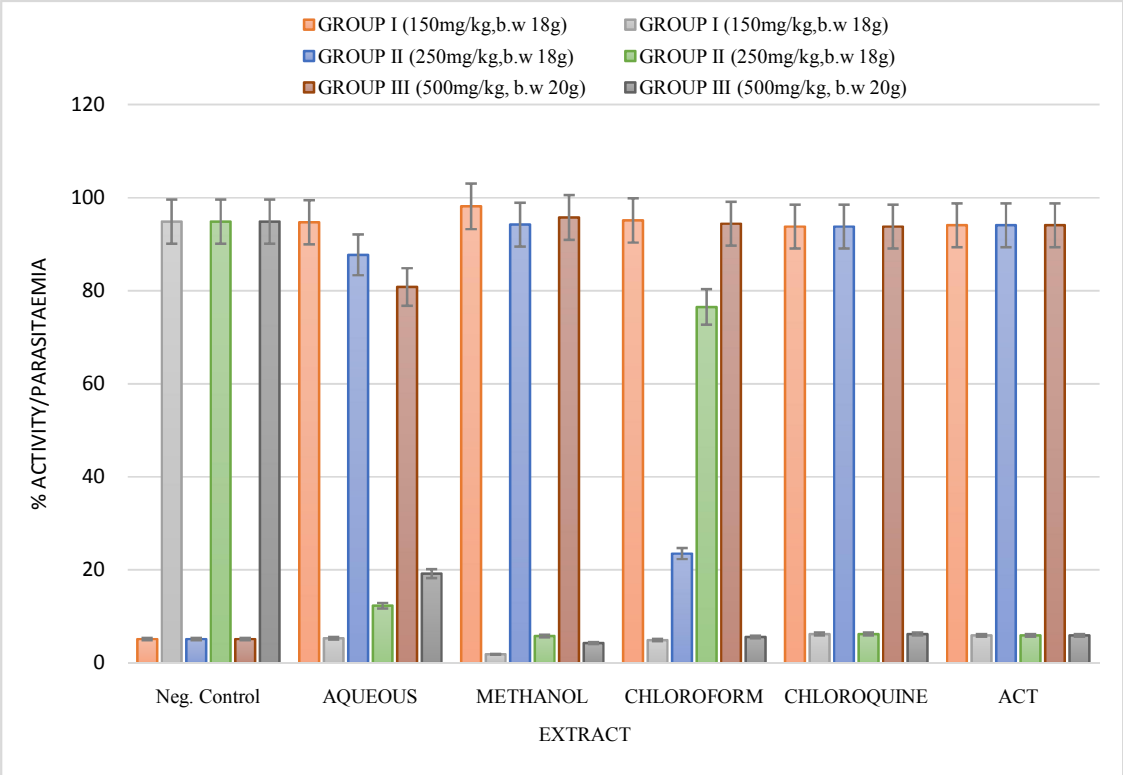


Fig. 2. Effect of oral administration of aqueous, methanol and chloroform extracts on percentage parasitaemia after 6 days of administration of extracts

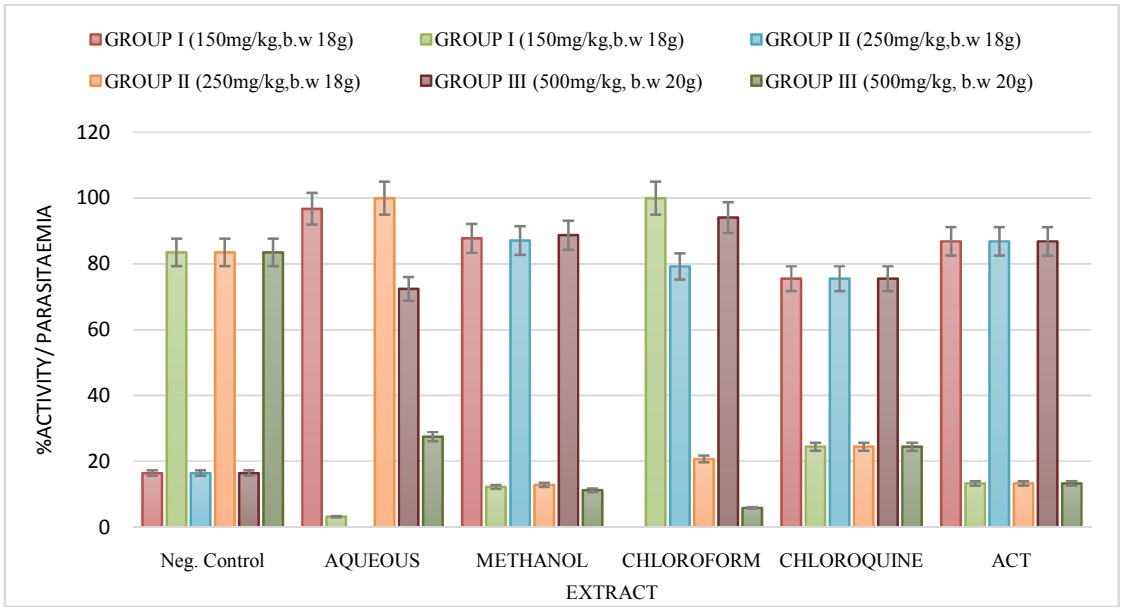


Fig. 3. Effect of oral administration of aqueous, methanol and chloroform extracts on percentage parasitaemia after 9 days of administration of extracts

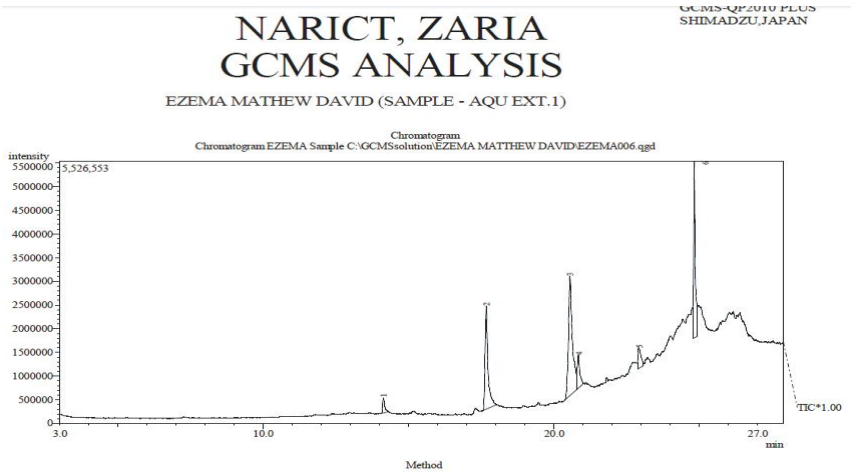


Fig. 4. GCMS chromatogram of aqueous extract

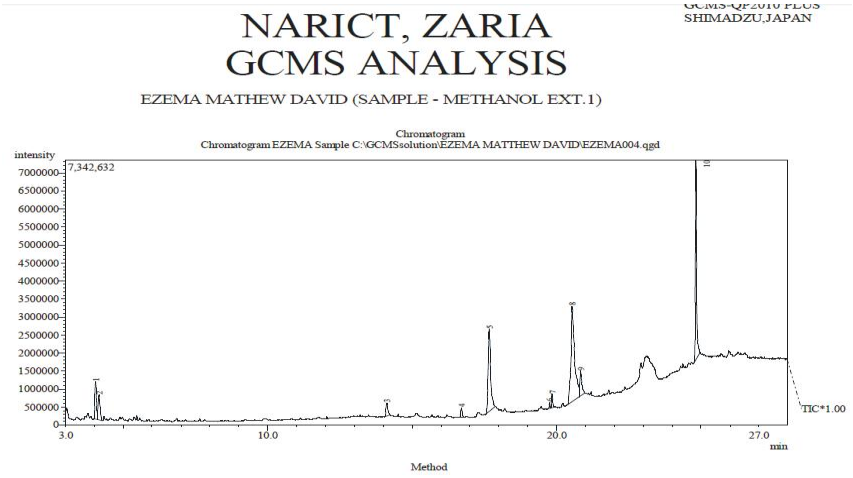


Fig. 5. GCMS chromatogram of Methanol extract

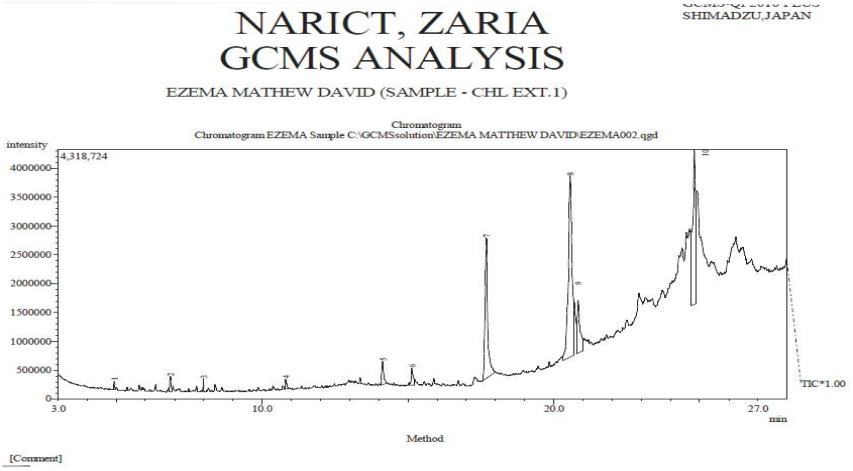
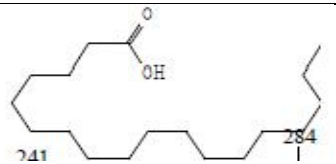
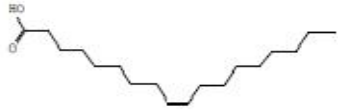
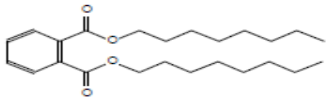
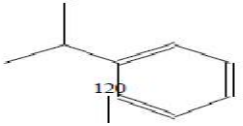
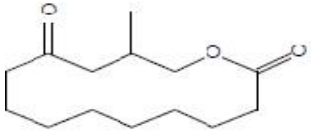
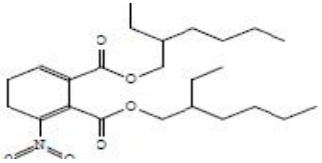
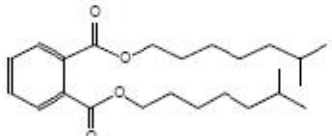
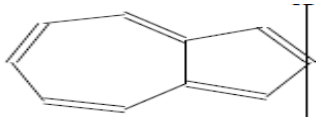
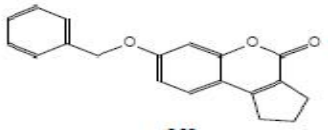
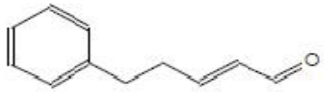
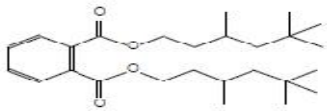
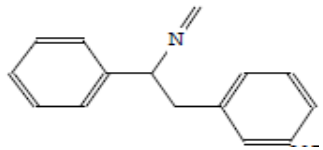


Fig. 6. GCMS chromatogram of Chloroform extract

Table 1. Summary of some compounds identified in potent fractions of aqueous, methanol and chloroform extracts by GCMS techniques

Compound	Peak#	R. time	Height%	Molecular formula	Structure
Aqueous extract Octadecanoic acid (Stearic acid)	2	17.675	21.99	$C_{18}H_{36}O_2$	
9-Octadecenoic acid (Z)- (Oleic acid)	3	20.543	25.51	$C18H_{34}O_2$	
Di-n-octyl phthalate	6	24.825	37.69	$C_{24}H_{38}O_4$	
Methanol extract Benzene, (1-methylethyl)- (cumene)	2	4.157	4.92	C_9H_{12}	
Oxacyclotetradecane-2,11-dione, 13-methyl-	8	20.546	18.76	$C_{14}H_{24}O_3$	
3-Nitrophthalic acid, bis-2-ethyl-hexyl ester	32	25.184	13.60	$C_{24}H_{37}NO_6$	

Compound	Peak#	R. time	Height%	Molecular formula	Structure
1,2-Benzenedicarboxylic acid, diisooctyl ester	32	25.184	13.60	$C_{24}H_{38}O_4$	
Chloroform extract Bicyclo[5.3.0]decapentaene (Azulene)	2	6.852	2.51	$C_{10}H_8$	
1H-Cyclopentano[c]coumarine,2,3-dihydro-7-benzyloxy-	9	24.053	9.21	$C_{19}H_{16}O_3$	
2-Pentenal, 5-phenyl-	9	24.053	9.21	$C_{11}H_{12}O$	
Bis-(3,5,5-trimethylhexyl) phthalate	10	24.827	31.71	$C_{26}H_{42}O_4$	
1,2-Diphenyl-1-isocyanoethane	2	13.672	3.51	$C_{15}H_{13}N$	

4. DISCUSSION

The results of the *in vivo* study indicated that aqueous, methanol and chloroform extracts of stem bark of *Alstonia boonei* displayed a very good activity against *P. berghei* malaria parasite when given at doses of 150, 250 and 500 mgkg-[1], respectively (Figs. 1- 3). The comparative analysis indicated that the extracts showed statistically significant difference ($P< 0.05$) on Day 3, Day 6 and Day 9 compared to the negative control (distilled water) and positive control. Higher activities were observed with 250 mgkg-[1] of aqueous extract and chloroform extracts (99.03% and 99.29%). Similar trend occured with 500 mgkg-[1] of methanol (99.68%) and with 150 mgkg-[1] of chloroform (99.06%) on Day 3. This high activity on Day 3 was similarly reported with the roots of Enyim Ocha (*Salacia nitida*), Ovoro ilu (*Nauclea latifolia*) and stem bark of Erumeru (*Enantia chloranthia Oliv*) against *P. berghei* on Day 4 [15]. The overall result of Day 3 evaluation of activity showed that methanol extract showed highest activity of 99.68% at a dose of 500 mgkg-[1] followed by aqueous extract (99.03%) at a dose of 250 mgkg-[1] (Fig. 1). This significant antimalarial activity could be attributed to the presence of alkaloids, flavonoids, cardiac glycosides and triterpenoids which have antimalarial properties [16].

On Day 6 *in vivo* result, methanol extract also showed highest level of inhibition of the parasite (98.11% activity) at 150 mgkg-[1], followed by chloroform (95.08%) at the same dose, both of which are significantly higher than chloroquine (93.76%) at the same concentration (Fig. 2). It is worthy to note that the extracts showed a comparatively lower activity here than in Day 3.

On Day 9 the consistency of the activities of the extracts reduced. At a dose of 150 mgkg-[1] aqueous and methanol extracts had 96.80% and 87.78% activity while that of chloroform was strangely 0%. At a higher dose of 250 mgkg-[1] it was aqueous that had 0% while methanol and chloroform had 87.13 and 79.26. (Fig. 3). Therefore the overall result of the *in vivo* studies revealed a higher reduction in mean percentage activity as the days progressed. It also revealed that the extracts of *Alstonia boonei* had better anti-malarial activity than ACT and Chloroquine. For instance, Chloroquine (10 mgkg-1) had its highest activity 93.70% on Day 6 (Fig. 2). Result of the comparative efficacy of the different doses of the extracts for the period of evaluation of antimalarial activities showed that aqueous had the better consistent percentage activity than methanol and chloroform for the periods tested. Chloroform had no activity on day 9 (Fig. 3). However prolonged or inconsistent administration of the extract reduces the level of activity and encourages re -infestation by the parasite.

Pharmacological studies carried out on this plant product by Olajide et al. [17] and Ojewole [18] also showed that the extract of *A. boonei* possesses antimalarial, antipyretic, analgesic and anti-inflammatory properties. In addition, it was discovered that the plant extract also has antihelmintic, diuretic, spasmolytic and hypotensive properties [19], Immuno-stimulant property [20], antipsychotic and anxiolytic effect [21], and reversible antifertility effect [22]. The stem bark of *A. boonei* is used in traditional medicine to treat fever, painful micturition, insomnia, malaria and chronic diarrhea, rheumatic pains, as anti- venom for snake bites and in the treatment of arrow poisoning [23].

The antimalarial activities of *Alstonia boonei* may be linked with the presence of compounds identified in the aqueous, methanol and chloroform fractions through GCMS technique. Two main reaction mechanisms of these active compounds linked with the anti-malarial activities are: generation of the free radical by compounds like Di-n-Octyl phthalate; 3 – Nitrophthalic acid, bis-(2, ethylhexyl-ester); Bis-(3,5,5-trimethylhexyl) phthalate; 2,3-Epoxycarane; Oxacyclo tetradecane -2, 11-dione; 1H - Cyclopentano (c) coumarine and 2, 3-dihydro-7 benzyloxyl with $Fe^{(II)}$ of hemoglobin and protein alkylation involving compounds such as Malononitrile, pheny propane dinitrile -1-Methylethenyl, Benzene acetonitrile, 2-Cyano. Denisov [24] in his study has proved that the intramolecular oxidation of these compounds proceeds as a cascade of consecutive free radical reactions with the formation of hydroperoxide groups. The later decompose via reactions with the $Fe^{(II)}$ complexes generating free radicals. Among the radicals formed, the hydroxyl radical was proved to play the key role, a correlation between the yield of hydroxyl radicals n(OH) and antimalarial activity of compounds was also observed by Denisov who demonstrated that there was a strong dependence of the antimalarial effectiveness of a drug on the chemical structure [25].

These compounds may attack the parasite at its intra-erythrocytic asexual stage. At this stage the parasite is mainly living in the red blood cell and takes haemoglobin as its nutritional resources. It digests haemoglobin and leaves free heme, which is then polymerized to polyheme (hemozoin) [26]. However the mature human red blood cell has no nucleus but the parasite does Over 95% iron in the human body exists as heme in the red blood cell. When these bioactive compounds permeate the membrane of the red cells, and reach the nucleus of the parasite, the peroxide (peroxy) segment of the compounds react with $Fe^{(II)}$ which could clear the DNA of the parasite. This is why the extract is only toxic to the parasite and not to the normal red blood cell. It is known that the peroxide bond is essential for activity [27], and study has shown that the peroxide reacts with intraparasitic iron to form free radicals, carbocations, or other reactive species. Therefore the single electron reduction of the peroxy group with ferrous ion is essentially responsible for the antimalarial activity of the compounds [28]. Their cumulative studies were significant in justifying the traditional use of the stem bark extract of *Alstonia boonei* for the treatment of malaria.

5. CONCLUSION

This research concludes that solvents extracts of *Alstonia boonei* (Egbu) possess strong antimalarial activity against NK-65 Chloroquine sensitive *Plasmodium berghei* infected mice with aqueous extract having the highest decrease in mean percentage parasitaemia. Therefore in view of the increasing concern for the resistance of the malarial parasites to available drugs, the results of this study offers a scientific basis for the traditional use of this indigenous plant in the treatment of malaria.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that Principle of laboratory animal care and ethical guidelines for investigation of experimental pain in conscious animals [29] were observed during experimentation [30].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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