Journal of Pharmaceutical Research International

19(2): 1-10, 2017; Article no.JPRI.36236
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

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

A. A. Imam[1*], M. K. Atiku[1], I. U. Muhammad[1], M. D. Ezema[1], A. J. Alhassan[1], A. Idi[1], A. Mohammed[2], H. Abdullahi[3] and I. Alexander[4]

[1]Department of Biochemistry, Faculty of Basic Medical Sciences, Bayero University, P.M.B. 3011, Kano, Nigeria.
[2]Department of Biochemistry, College of Medical Sciences, Abubakar Tafawa Balewa University, P.M.B. 0248, Bauchi, Nigeria.
[3]Department of Biochemistry, Faculty of Basic Medical Sciences, Northwest University, Kano, Nigeria.
[4]Department of Biochemistry, Faculty of Natural Sciences, Caritas University, Amorji-Nike, P.M.B. 01784, Enugu, Nigeria.

Authors' contributions

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

Article Information

DOI: 10.9734/JPRI/2017/36236
Editor(s):
(1) Barkat Ali Khan, Department of Pharmaceutics, Gomal University, Dera Ismail Khan, Pakistan.
Reviewers:
(1) Voravuth Somsak, Walailak University, Thailand.
(2) Misael Chinchilla, Universidad de Ciencias Médicas (UCIMED), Costa Rica.
(3) Kikakedimau Nakweti Rufin, University of Kinshasa (UNIKIN), Democratic Republic of the Congo.
Complete Peer review History: http://www.sciencedomain.org/review-history/21575

Original Research Article

Received 19th August 2017
Accepted 30th September 2017
Published 26th October 2017

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 vitro* antimalarial activity of different solvents extract (aqueous, methanol, ethyl acetate, chloroform and hexane) of *Alstonia boonei* against NK-65 Chloroquine sensitive *Plasmodium berghei* infected mouse blood. The antimalarial activity of the extracts was determined by calculation of percentage parasitaemia. GCMS analysis was conducted for the detection of possible bioactive compound(s) in the most active extract(s). Results showed that all the extracts had intrinsic antimalarial properties that were

**Corresponding author: E-mail: aaimam.bch@buk.edu.ng;*
Email: ibrahimmuhd@yahoo.com;

dose dependent. Furthermore, there was a significant increase ($p<0.05$) in mean percentage anti-malarial activity of all the extracts when compared with the placebo (normal saline) and standard anti-malarial drugs Chloroquine and ACT, with aqueous extract showing highest antiplasmodial activity of 95.24% at 2000 µg/ml. GCMS results revealed the presence of 3-Nitrophthalic acid and coumarine 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 vitro; Plasmodium berghei; 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 was collected from Okpuje community, Northwest of Nsukka LGA of Enugu State (co-ordinates $6^{0}30^{1}N7^{0}30^{1}E$). 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). It was washed, shade dried (25°C) and ground into powder. Two hundred (200 g) of sample was measured and transferred into each of the flasks containing 1000 cm^3 (1 litre) of methanol, hexane, ethyl acetate, 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 using Muslin fabric. The filtrates were concentrated under vacuum using a rotary evaporator (Buchi R210),

then measured and stored in screw capped vials under room temperature at the Postgraduate laboratory of Department of Biochemistry, Bayero University Kano.

2.3 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 Arora and Arora [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.

2.4 Preparation of Culture Media (RPMI 1640)

The media, Roswell Park Memorial Institute (RPMI) 1640, was prepared by dissolving 1.04 g of RPMI 1640 in 100 ml of distilled water. This was autoclaved at 121°C for 15 minutes as instructed by the manufacturers to stabilize the media. It was then allowed to cool. After cooling 40 µg/ml of Gentamycin Sulphate was added to sterilize the media. The media was then supplemented with about 5 ml of serum obtained from apparently healthy rabbits and thus, was kept ready for the *in vitro* antimalarial study.

2.5 Preparation of Working Concentrations for the *in vitro* Study

An electronic digital balance (Model FA 2104A) manufactured by Gulfex Medical and Scientific Company, England was used to measure 20 mg (0.02g) of each of the extracts and then dissolved in 2 ml of DMSO (Dimethyl Sulphonic Oxide) as stock solution in separate sterile bottles. Using serial doubling dilution, four different concentrations (500 µg/ml, 1000 µg/ml, 2000 µg/ml and 5000 µg/ml) of each extract were prepared and transferred into separate EDTA bottle.

2.6 Determination of *in vitro* Antimalarial Activity of Crude Stem Bark Extracts of *A. boonei* on Infected Blood Cells of Albino Mouse

Blood sample with 30% parasitaemia (as determined) was centrifuged at 2500 rpm for 15 minutes. After centrifugation, the supernatant (plasma) was discarded while the sediments (erythrocytes) were further centrifuged with normal saline at 2500 rpm for 5 minutes. The supernatant was then discarded and the erythrocytes were suspended in normal saline. Blood from two healthy white rabbits was used to enrich the media for the growth of the parasites. Equal volume of each of the aqueous, methanol, ethyl acetate, hexane and chloroform extract solution (0.5 ml) and the culture media (earlier prepared) were transferred into test tubes and labeled accordingly for the respective doses of 500 µg/ml, 1000 µg/ml, 2000 µg/ml and 5000 µg/ml. For each concentration of the extract, 0.1 ml of the malaria positive erythrocytes was added and shaken gently to ensure even distribution of the erythrocytes. The test tubes (flat bottomed) were transferred into a bell jar containing burning candle. The cover of the bell jar was then replaced until the flame of the candle stopped burning. This supplied about 95% nitrogen, 2% oxygen and 3% carbon dioxide. The whole set up was transferred into an incubator maintained at 37°C for 24 - 48 hours. A control group consisting of culture media plus positive erythrocytes and antimalarial drugs, chloroquine and ACT (positive control) were incubated along with the test concentrations. The same procedure was taken for normal saline as negative control.

After 24 hours of incubation, a thin smear from test tube in each was made on clean glass slides and fixed in absolute ethanol and then stained with Giemsa's stain. Each smear was observed under microscope using oil immersion to count the number of infected erythrocytes. The same procedure was repeated after 48 hours of incubation to determine the activity. The activity of each of the tested samples was calculated as the percentage elimination of the parasites after incubation of 24 and 48 hours using the formula below and the result were tabulated according to Mukhtar et al. [12].

$$\% \text{ Parasitaemia} = \frac{\text{Number of infected cells}}{\text{Total number of cells}} \times 100$$

$$\% \text{ elimination (Activity)} = \frac{N_c}{N_x} \times 100$$

Where:

N_c= Total number of cleared RBC
N_x= Total number of parasitized RBC

3. RESULTS

Fig. 1 shows the result of the *in vitro* anti-malarial activities of aqueous, methanol, ethyl acetate chloroform and hexane extracts of *Alstonia*

boonei at lower concentrations (500 µg/ml and 1000 µg/ml). A significant difference ($p<0.05$) in mean percentage activity of the extracts, with the aqueous extract showing the highest anti plasmodial activity of 87.37% at 1000 µg/ml while ethyl acetate showed lowest anti-plasmodial activity of 47.95% at the same concentration.

The result of *in vitro* anti-malarial activities of the aqueous, methanol, hexane, ethyl acetate and chloroform extracts of *Alstonia boonei* at higher concentration (2000 µg/ml and 5000 µg/ml) is shown in Fig. 2 The result showed a significant increase in mean percentage activity ($p<0.05$) of the solvent extracts when compared with the negative control (Normal saline). Aqueous extract has highest anti-plasmodial activity of 95.24% at 2000 µg/ml which decreases with increase in concentration, while Methanol extract has lowest activity of 33.88% at 2000 µg/ml but increased with increase in concentration.

Fig. 3 Fig. 4 and Fig. 5 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.

4. DISCUSSION

From the *in vitro* study carried out, it was observed that the anti-malarial activity of the extracts was dependent on the concentration of the extract to a certain level. At a very low concentration (500 -1000 µg/ml), the activity was reduced, at a moderate concentration (1000 – 2000 µg/ml) the activity was significant while at a much higher concentration (5000 µg/ml), the activity dropped in most extracts (Figs. 1 and 2). From this study, aqueous extract showed highest percentage activities of 95.24% at 2000 µg/ml and 87.37% at 1000 µg/ml. Chloroquine and ACT had their highest activities of 77.02% and 79.55% respectively at 5000 µg/ml. All the extracts showed good anti-malarial activity when compared with negative control (normal saline). These findings supports the work of Ogbuehi and Ebong [13]. According to their survey research, eleven (11) plant species were frequently used for the treatment of malaria in the South East Nigeria, *Alstonia boonei* (Egbu) inclusive. In addition, studies by Olajide et al. [14] and Chime et al. [15] showed that *Alstonia boonei* is rich in phytochemical compounds such as alkaloids and flavonoids with antimalarial properties.

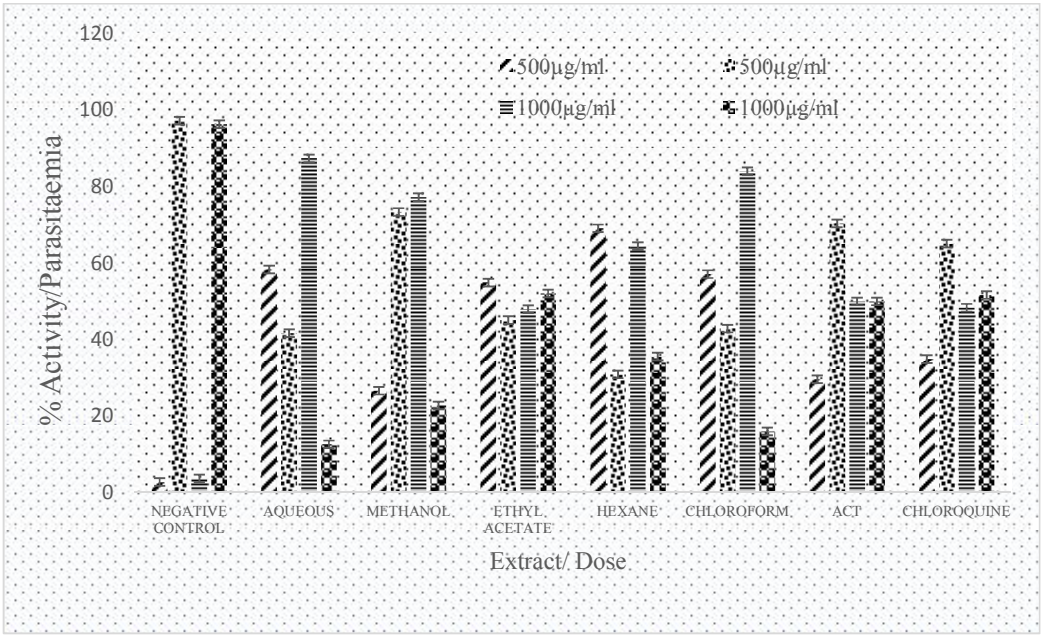
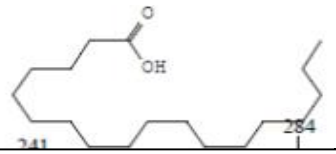
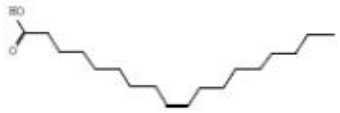
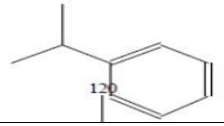
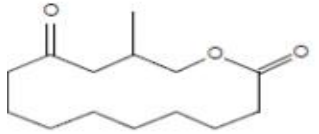
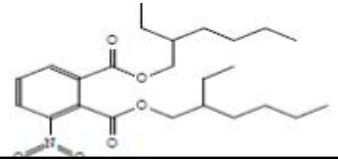
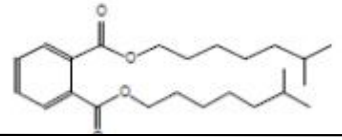
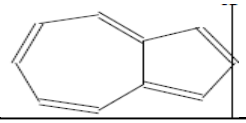
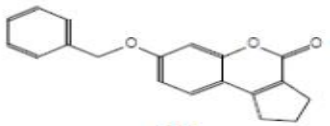
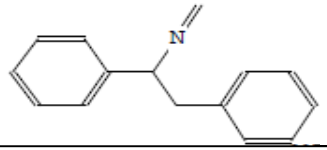


Fig. 1. Result of the *in vitro* antimalarial activities of aqueous, methanol, hexane, ethyl acetate and chloroform the extracts of *A. boonei* at lower concentration (500 and 1000 µg/ml)

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	$C18H_{34}O_2$	
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	32	25.184	13.60	$C_{24}H_{37}NO_6$	
1,2-Benzenedicarboxylic acid, diisooctyl ester	32	25.184	13.60	$C_{24}H_{38}O_4$	

Compound	Peak#	R.Time	Height%	Molecular Formula	Structure
Chloroform extract Bicyclo[5.3.0]decapentaene (Azulene)	2	6.852	2.51	$C_{10}H_8$	
Coumarine	9	24.053	9.21	$C_{19}H_{16}O_3$	
Naphthalene	2	6.852	2.51	$C_{10}H_8$	
1,2-Diphenyl-1-isocyanoethane	2	13.672	3.51	$C_{15}H_{13}N$	

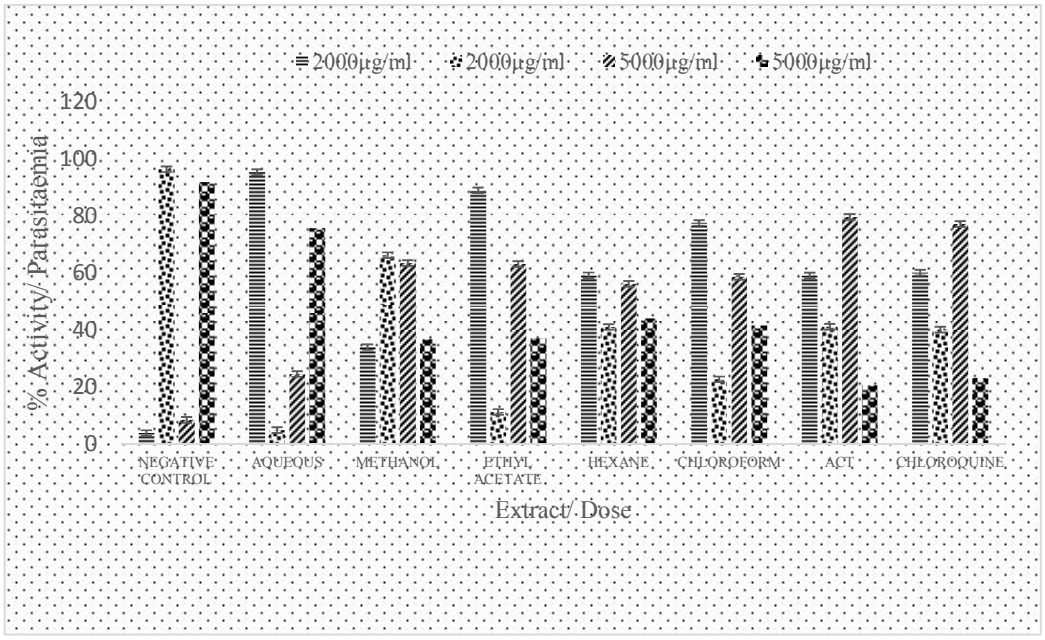


Fig. 2. Result of the *in vitro* antimalarial activities of aqueous, methanol, hexane, ethyl acetate and chloroform extracts of *A. boonei* at higher concentrations (2000 and 5000 µg/ml)

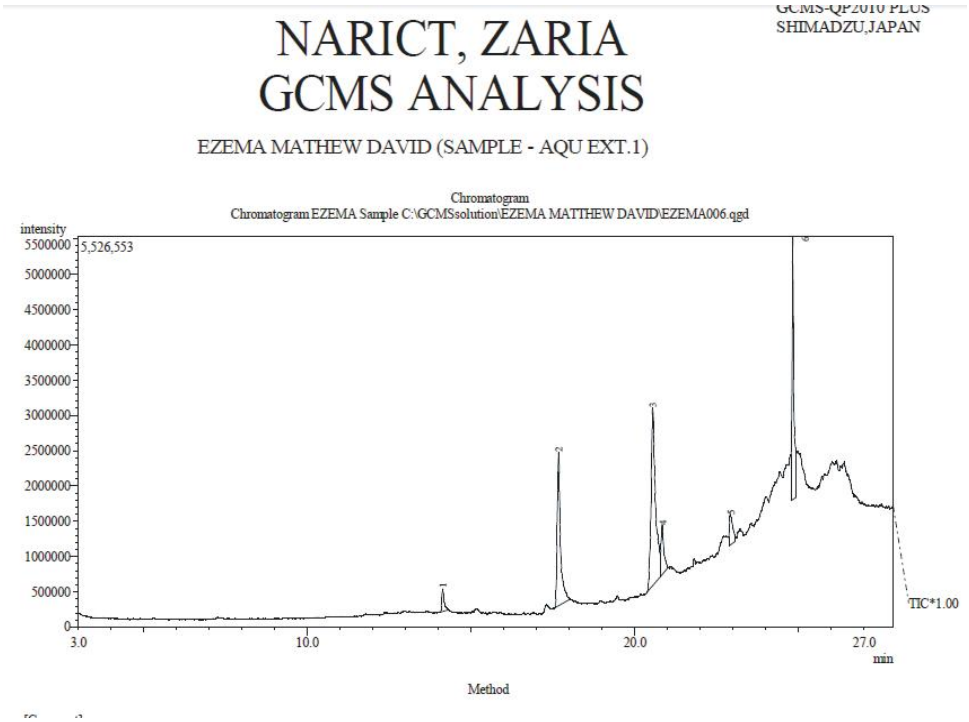


Fig. 3. GCMS chromatogram of aqueous extract

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 such as 3 – Nitrophthalic acid and coumarine

and with $Fe^{(II)}$ of hemoglobin and protein alkylation involving compounds such as Malononitrile, pheny propane dinitrile (1 - Methylethenyl; Benzene acetonitrile, 2 - cyano. Denisov [16] in his study has proved that the intramolecular oxidation of these compounds

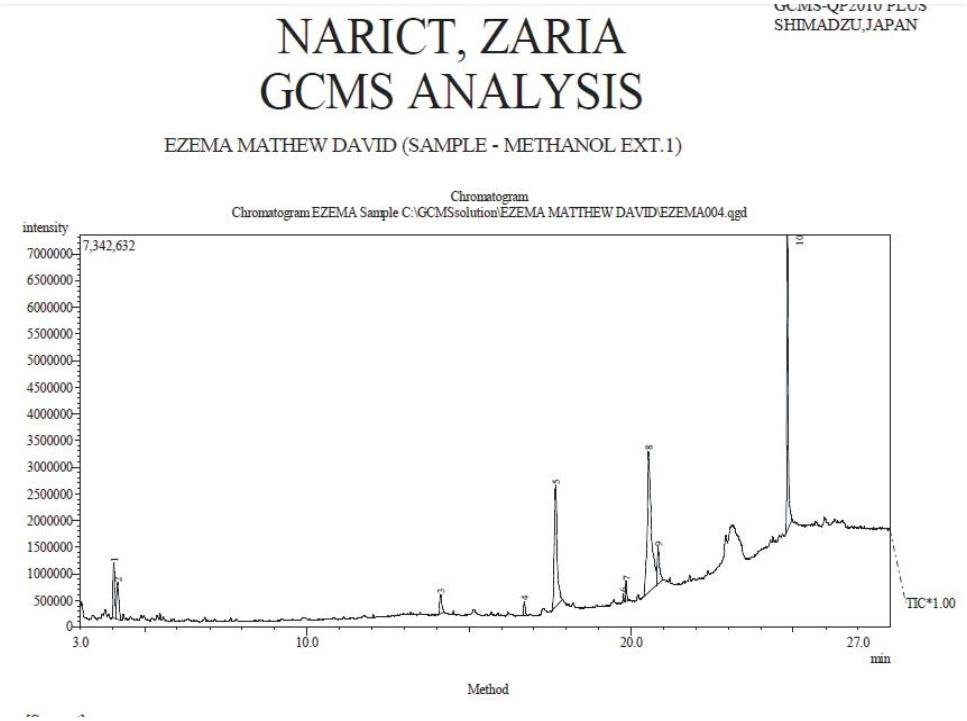


Fig. 4. GCMS chromatogram of methanol extract

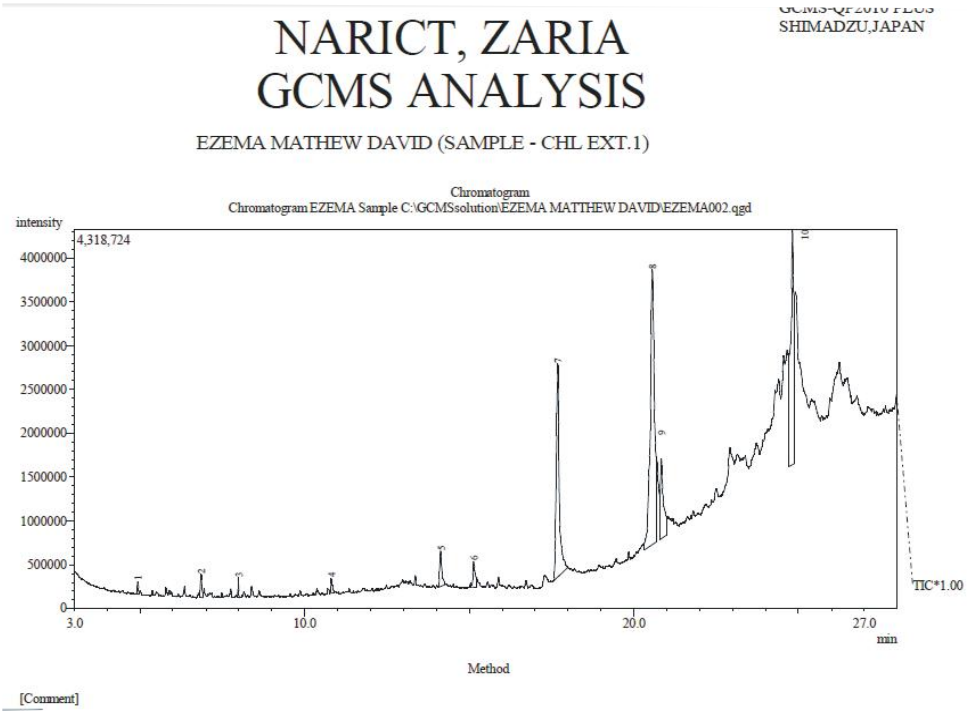


Fig. 5. GCMS chromatogram of chloroform extract

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. Francisco et al. [17] also reported that coumarines form complexes with Fe and Ni in exerting their anti-parasitic activity.

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). 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, and study has shown that the peroxide reacts with intraparasitic iron to form free radicals, carbocations, or other reactive species [18]. Therefore the single electron reduction of the peroxy group with ferrous ion is essentially responsible for the antimalarial activity of the compounds [19]. 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 mouse blood 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. Further *in vivo* studies of the extracts as well as completes characterization of the extract(s) are therefore recommended.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. WHO. World Malaria Report. 2016;1-2. Available:http://www.who.int/malaria/publications/world-malariareport-2016/report/en/
2. Federal Ministry of Health. Annual review of malaria situation in Nigeria 2014; 2015.
3. WHO. Malaria Fact Sheet N'95'; 2014. (Retrieved 2015-08-11)
4. Greenwood BM, Bojang K, Whitty CJM, Targett GAT. Malaria. The Lancet. 2005; 365:1487-1898.
5. WHO. Regional office evaluating the safety and efficacy of herbal medicines. Manila. 2005;3-5.
6. Caraballo H. Emergency department management of mosquito borne illness: Malaria, dengue and west Nile virus. Emergency Medicine Practice. 2014; 16(5):23-31.
7. Onori E, Majori G. Recent acquisition in chemotherapy and chemophylaxis of malaria. Ann 1st Super & Sanita Publ. 1998;25(4):659-673.
8. Wongsrichanalai C, Wernsdofer WH, Pickard AL, Meshnick SR. Epidemiology of drug resistant malaria. Lancet Infectious Disease. 2002;2:209-218.
9. Elujoba AA, Odeleye OM, Ogunyemi CM. Traditional medicine development for medical and dental primary health care delivery system in Africa. African Journal of Traditional Medicine. 2005;21:46-61.
10. Abosi AO, Raseroke BH. *In vivo* antimalarial activity of *Vernonia amygdalina.* Journal of Biomedical. Science. 2003;60:89-91.
11. Arora DR, Arora BB. Medical parasitology (3rd Ed.). CBS Publishers and Distributors PVT Ltd. New Delhi, India. 2010;71-85: 248.

12. Mukhtar MD, Bashir M, Arzai AH. Comparative *in vitro* studies on antiplasmodial activity of some Nigerian and foreign brands of chloroquine formulations marketed in Kano. African Journal of Biotechnology. 2006;5(24): 2464-2468.
13. Ogbuehi IH, Ebong OO. Traditional medicine treatment of malaria in Onitsha, South East Nigeria. Greener Journal of Medical Science. 2015;5(1):1-18.
14. Olajide AO, Awe SO, Makinde JM, Ekhelar AI, Olusola A, Morebise O. Studies on the anti-inflammatory, antipyretic and analgesic properties of *Alstonia boonei* stem bark. Journal of Ethnopharmacology. 2000;71:179–186.
15. Chime SA, Ugwoke EC, Onyishi IV, Brown SA, Onunkwo GC. Formulation and Evaluation of *Alstonia boonei* stem bark powder tablets. Indian Journal of Pharmaceutical Science. 2015;75(2):226-230.
16. Denisov E. An important role of intramolecular free radical reactions in antimalarial activity of artemisinin and its analogs. Organic and Biomolecular Chemistry. 2011;9(11):4219-25.
17. Francisco CA, Valquinia FL, Adelia VV, Maria RM, Regina MP. New coumarin complexes of Zn, Cu, NI and Fe with anti-parasitic activity. Polyhedron. 2015; 101(24):165-170.
18. Eva AI, Andrea P, Rachid S. Novel arylalkylamine compounds exhibits potent selective anti-parasitic activity against *Leishmania major*. Bio Org Med Chem. 2015;25(22):5315-5320.
19. Liang X, Fang W. Medicinal chemistry of bioactive natural products. John Wiley & Sons Inc. Publication. New Jersey. 2006; 221-238.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/21575