

***In vivo* Anti-plasmodial activity of ethanolic leaf extract of *Alstonia boonie* (Ewe ahun) and its effect on Hematological parameters of Swiss albino mice infected with *Plasmodium berghei* NK 65**

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ABSTRACT

Malaria is one of the most killing diseases in the world particularly in tropical countries and is worst in Africa. The study was conducted to determine the effect of ethanolic leaf extract of *Alstonia boonie* (Ewe ahun) on hematological parameters and its anti-plasmodial activities in Swiss albino mice infected with *Plasmodium berghei* NK65. Swiss mice were inoculated intraperitoneally with *Plasmodium berghei* NK65. The mice were grouped into six groups, five per group. Only Group I were not infected with *P.berghei*, Group II and III served as both positive and negative controls respectively, while Group IV, V, and VI were treated with 200, 400, and 800 mg/kg body weight of *Alstonia boonie* leaf extract, respectively. The phytochemical constituents of the extract showed the presence of secondary metabolites like tannin, flavonoids, steroids and saponin. It is clear that *A. boonie* showed marked anti-malarial effects in dose seeming fashion from the percentage parasitaemia computed after carrying out suppressive and curative tests. The curative test showed that only Chloroquine, the standard drug, cleared the parasites by 91.7% at 5mg/kg body weight while the different concentrations of the extract exerted a growth inhibition of 39.31%, 49.19% and 38.54% at 200, 400, 800mg/kg body weight of the extract respectively. The Hematological parameters showed that *A. boonie* had a significant increase ($P<0.05$) in RBC, HGB, HCT and PLT values when compared to negative control. This study showed that *A. boonie* extract slightly suppressed the growth of *P. berghei* NK65 and the extract does possess hematopoietic activity and is not hematotoxic.

KEYWORDS: *Alstonia boonie* (Ewe ahun), Anti-plasmodial activities, Swiss albino mice, *Plasmodium berghei* NK 65 and Hematological parameters.

1.0 INTRODUCTION

Malaria is one of the most killing diseases in the world particularly in tropical countries and is worst in Africa. According to World Health Organization, there were an estimated 247 million episodes of malaria in 2006, with a wide uncertainty interval from 189 million to 327 million cases. Eighty six percent (212 million) were recorded in the African region. Eighty percent of the cases were in 13 countries and over half in Nigeria (WHO, 2008). The problems militating against the effective management of malaria have been enumerated.

The most important problem is that Plasmodial parasites

are resistant to most widely available and affordable drugs like Chloroquine and Fansidar (Kisame, 2005). Secondly, the control of mosquitoes which transmits malaria is made difficult by their resistance to a wide range of insecticides. The third which is the production of fake antimalarial drugs. In Southeast Asia 32% and 53% of artesunate blister packs sampled contained no active ingredients (Newton, 2006). Lastly, most countries in Africa lack the necessary infrastructure and resources to manage and control malaria (WHO, 1994).

A number of traditional herbs have been tested and used in the prevention and also treatment of malaria including Mango leaves, *Artemisia annua* (Madu, 2007), *Picralima nitida*, leaves of *Carica papaya*, *Dialium guineense*, roots and leaves of *Guinensis*, unripe fruit of *Capsicum frutescence* and *Azadirachta indica*. Recent studies indicate that lemon grass can be successfully used to treat drug resistant malarial and typhoid fever (Akininyi,2006).

Plant extracts have been very useful sources of medication for various disease conditions (Gill *et al*, 2002). *Alstonia boonei* De Wild is large deciduous evergreen tree, usually up to 45m tall and 1.2m in diameter. It belong to the family *Apocynaceae* which consists of 40-60 species widely distributed in the continents of Africa, Asia and America (Ojewole,1984 and Iwu, 1993).

Alstonia boonei is known as Ahun in Yoruba, Egbo-ora in Igbo, Ukhu in Edo and Ukpukunu in Urhobo .It is widely distributed in the lowlands and rain-forest areas of Nigeria. Different parts of the plant are employed for the treatment of a variety of ailments in traditional medicine, to treat malaria, fever, insomnia, chronic diarrhea, rhematic pains, as anti-venom for snake bites and in the treatment of arrow poisoning (Koumaglo *et al*,1992, Asuzu, 1991 and Obih, 1985).

2.0 STUDY OBJECTIVES

To investigate the anti-plasmodial activity of ethanolic leaf extract of *Alstonia boonie* (Ewe ahun) and its effect on Hematological parameters of Swiss albino mice infected with *Plasmodium berghei* NK 65.

3.0 MATERIALS AND METHODS

3.1 Collection and identification of plant material

The leaves of *Alstonia boonei* were obtained from Abeokuta in Ogun State, Nigeria with the help of a traditional herbalist. The plant was authenticated by Miss Shokefun a botanist from Lagos State Polytechnic, Ikorodu, SLT Department, Environmental biology Unit.

3.2 Preparation of Plant extract

The leaves were washed with water, air dried under shade in the Biochemistry Laboratory, pulverised to coarse power using blender. Extraction was carried out by dispersing 200g of the grounded *Alstonia boonei* plant material in 1L of 70% ethanol and shaking was done with GFL shaker for 72 hours. This was followed by vacuum filtration and concentrated by rotary evaporator at a temperature not exceeding 40°C. The concentrated extract was dried to complete dryness in an aerated oven at 40°C for 48 hours. The *Alstonia boonei* ethanolic extract was latter stored in a refrigerator at 4°C.

3.3 Phytochemical analysis

Phytochemical analysis for bioactive constituents were carried out on the ethanolic extract of *Alstonia boonei* using standard phytochemical procedures (Harborne (1993), Trease and Evans (1995) and Sofowora (1993).

3.4 Experimental animals

Seven (7) weeks old Swiss mice weighing 22- 32g were obtained from Nigeria Institute of Medical Research (NIMR), Lagos, Nigeria. They were housed in plastic cages with saw dust as beddings; food and water were given *ad libitum*. The mice were used in accordance with NIH Guide for the care and use of laboratory animals; NIH Publication revised (1985) NIPRD Standard Operation Procedures (SOPs).

3.5 Animal grouping for infection and treatment

The parasite *Plasmodium berghei* NK 65 was obtained from Nigeria Institute of Medical Research (NIMR), Lagos, Nigeria (from Dr Aina, O.O). The parasites were kept alive by continuous intraperitoneal inoculation of known amount of the parasite into Swiss mice. Iml of blood was taken from donor mice and diluted with 5ml phosphate buffer; such that 0.1ml contained standard inoculum of 1×10^7 infected red blood cells (Maegraith *et al*, 1952). Thirty acclimatized mice were randomly selected and twenty five Swiss mice were inoculated intraperitoneally from the same source to avoid variability in parasitemia. The mice were randomly distributed into six groups of five per group as shown below:

GROUP I (Normal control group) Healthy Swiss mice

GROUP II (Positive control) = *P.berghei* +5mg/kg b,wt of chloroquine (Standard drug)

GROUP III (negative control) were infected with *P.berghei* received no treatment.

GROUP IV = *P. berghei* + 200mg/kg b.wt of *Alstonia boonei* extract.

GROUP V = *P. berghei* + 400mg/kg b.wt of *Alstonia boonei* extract.

GROUP VI =*P. berghei* + 800mg/kg b.wt of *Alstonia boonei* extract.

3.6 anti-plasmodium studies

3.6.1 Suppressive test

The Peter's 4-day suppressive test against *P. berghei* NK65 infection in Swiss mice was used (Peters, 1965). Adult Swiss mice weighing between 22 to 32g were inoculated by intraperitoneal injection with standard inoculum of *Plasmodium berghei* NK65 with 1×10^7 infected red blood cells. The mice were divided into six groups as shown above and treated for 4 consecutive days with 5mg/kg.b.wt of Chloroquine, 200, 400, and 800mg/kg body weight of *Alstonia boonei* extract orally daily. On day 5 of the experiment, blood was collected from the tail of each mouse and smeared onto microscope slide to make a film.

The blood films were fixed with methanol, stained with Geimsa at pH 7.2 for 10 minutes and examined under the microscope for the presence of parasites. The parasite density was calculated for each group by comparing the parasitaemia in infected control group (Group III) with those of treated mice.

3.6.2 Curative test

The Curative test of *Alstonia boonei* ethanolic leaf extract on another infected Swiss albino mice were carried out according to the method described by Ryley and Peters, 1970. The mice were injected intraperitoneally with standard inoculums of 1×10^7 *Plasmodium berghei* NK 65 infected erythrocytes on the first day (day 0). Seventy two hours later, the mice were divided into six groups of five mice per group as shown above.

The groups were orally treated with 5mg/kg b.wt of chloroquine and *Alstonia boonei* leaf extract (200,400 and 800mg/kg b.wt respectively). The treatment was carried out once daily for 5 days, on each day of the treatment, blood was collected from the mice tail and smeared onto microscope slide to make thin and thick films. The blood films were fixed with methanol, stained with 10% Giemsa at pH 7.2 for 10 minutes and examined microscopically to monitor the parasitaemia level. The parasite density was calculated for each group over a period of six days.

3.7 HEMATOLOGICAL ANALYSIS

The Swiss albino mice in the curative assay groups were sacrificed and their blood were collected in EDTA bottles by ocular puncturing. The blood in the EDTA bottles was assayed using BC -3200 Auto Hematology Analyzer in Lagos University Teaching Hospital (LUTH) in Lagos- Nigeria.

3.8 DATA ANALYSIS

Data analysis was done using the GraphPad prism computer software. Students't'-test and one-way analysis of variance (ANOVA) were used for comparison. A P-value < 0.05 was considered significant.

4.0 RESULTS

The results obtained from the phytochemicals analysis of *Alstonia boonei* extract showed the presence of some secondary metabolite like tannins, saponins, steroids,

flavonoids, protein, reducing sugar and fats and oil. (TableI). The presence of these secondary metabolites in this extract may be responsible for the anti -plasmodial activity of *Alstonia boonei*.

Table I. The phytochemical screening of *Alstonia boonei*. leaf extract.

Phytochemical analysis test	Qualitative abundance
Tannins (Ferric chloride test)	+++
Saponins (Emulsion test)	+
Steroids (Salkowski test)	+
Flavonoids (Ammonium test)	++
Protein (Million test)	+
Reducing Sugar (Fehling solution)	++
Fat and Oil (Stain test)	+

(+) present at low levels, (++) present at moderate levels, (+++) present at high levels.

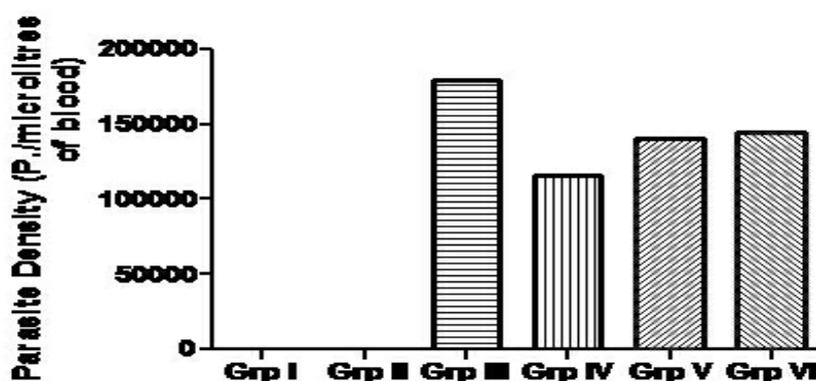


Figure I The effect of ethanolic leaf extract of *Alstonia boonei* on Swiss mice infected with *Plasmodium berghei* NK65 obtained during suppressive test.

Chloroquine, a standard anti-malarial drug used exerted 100% suppression at 5mg/kg body weight. The ethanolic leaf extract of *A. boonei* caused 35.7%, 21.6% and 19.8% suppression in parasitaemia of *P.berghei* infected mice at

200mg/kg, 400mg/kg and 800mg/kg body weight respectively (Figure I). Extract with 200mg/kg of *A. boonei* showed the highest suppression (35.7%) compared to others.

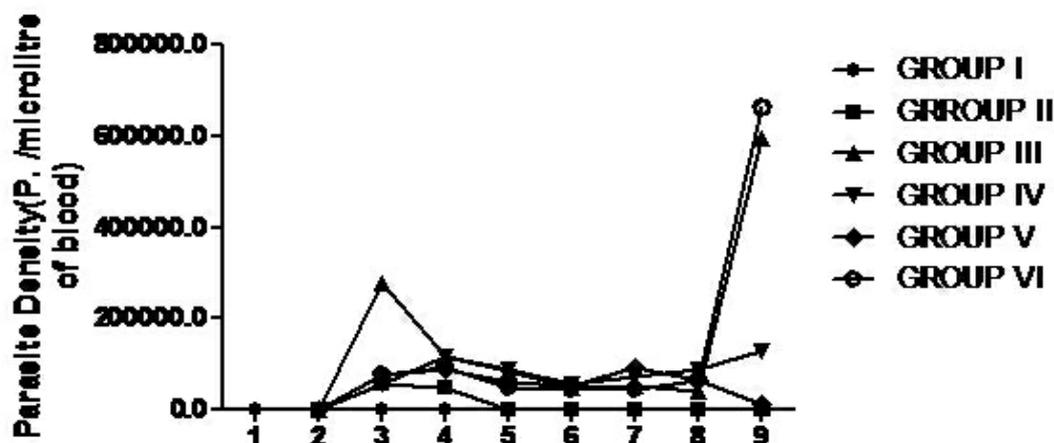


Figure II. The effect of ethanolic leaf extract of *Alstonia boonei* on Swiss mice infected with *Plasmodium berghei* NK65 obtained during Curative test.

The curative test shows that only Chloroquine, the standard drug, cleared the parasites by 91.7% at 5mg/kg body weight while the different concentration of the extract exert a

growth inhibition of 39.31%, 49.19% and 38.54% at 200, 400, 800mg/kg body weight of the extract respectively (Figure II above).

Table II: Curative test showing the effect of ethanolic leaf extract of *Alstonia boonei* and chloroquine on hematological parameters of Swiss albino mice infected with *Plasmodium berghei* NK65.

Hematological Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
WBC (X10 ⁹ /L)	*16.4 ± 0.6	*38.6 ± 0.8	68.7 ± 0.4	*50.1 ± 0.4	*52.2 ± 0.1	*59.1 ± 0.3
PLT (X 10 ⁹ /L)	745 ± 116*	452 ± 58*	386 ± 16	457 ± 58*	481 ± 91*	450 ± 83*
PCT (%)	0.393 ± 0.050	0.278 ± 0.030	0.321 ± 0.020	0.263 ± 0.021	0.280 ± 0.030	0.301 ± 0.020
MPV (fL)	6.1 ± 0.20	7.3 ± 0.2	7.9 ± 0.30	6.7 ± 0.10	7.4 ± 0.20	7.9 ± 0.10
PDW	14.40 ± 0.20	15.10 ± 0.20	15.5 ± 0.3	14.9 ± 0.10	15.20 ± 0.30	15.10 ± 0.10
HGB (g/dl)	*11.90 ± 1.20	*9.20 ± 0.4	4.20 ± 0.30	*6.80 ± 0.02	*6.45 ± 0.15	*6.37 ± 0.13
RBC (X10 ¹² /L)	*7.53 ± 0.25	*5.62 ± 0.3	2.10 ± 0.20	*3.91 ± 0.10	*3.85 ± 0.14	*4.30 ± 0.01
HCT (%)	*39.2 ± 0.80	*35.2 ± 0.50	17.6 ± 1.40	*20.1 ± 1.20	*21.8 ± 0.7	*23.7 ± 1.10
MCV (fL)	66.1 ± 3.40	68.1 ± 2.30	67.3 ± 2.10	67.2 ± 0.30	66.10 ± 0.5	68.2 ± 0.20
MCH (Pg)	17.5 ± 0.7	16.9 ± 1.3	18.5 ± 0.10	18.60 ± 0.80	18.50 ± 0.30	18.30 ± 0.20
MCHC (g/dL)	31.30 ± 0.20	26.50 ± 0.6	27.20 ± 0.65	29.70 ± 0.02	29.50 ± 0.20	28.10 ± 0.10
RDW-CV (%)	18.40 ± 0.40	18.30 ± 0.6	19.90 ± 1.40	18.80 ± 1.30	18.40 ± 1.20	20.70 ± 1.20
RDW-SD (fL)	32.50 ± 0.70	37.60 ± 0.50	47.90 ± 0.30	44.80 ± 1.60	33.20 ± 1.20	44.2 ± 2.30

Values are expressed in mean ± SD of five mice in each group. * indicate Significant difference when compared to negative control (P < 0.05). WBC: White blood cell; PLT: Platelet count; PCT: Plateletcrit ; MPV: Mean platelet volume; PDW: Platelet Distribution Width; HGB: Hemoglobin Concentration; RBC: Red Blood Cell; HCT: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean Cell hemoglobin; MCHC: Mean Cell hemoglobin concentration; RDW-CV: RBC distribution width-coefficient of variation; RDW-SD: RBC distribution width-standard deviation.

The total WBC counts were significantly lowered following chloroquine and extract administration. The mean RBC, HGB, HCT and PLT values of the infected untreated group (group III) were significantly (P < 0.05) lowered when compared to group I and all other groups (Table II above). The other hematological parameters were in-significantly affected by the parasites.

5.0 DISCUSSION

The ethanolic leaf extract of *Alstonia boonei* shows the presence of secondary metabolites like tannin, saponin, steroids, reducing sugar, protein, flavonoids and fats and oil (Table I). The beneficial effect of this plant material has been attributed to the combinations of secondary metabolites and other components present in the plant. It is evident by these findings that *A. boonei* possessed anti-plasmodium activity justifying its usage in the management of malaria. The anti-plasmodial activity may be attributed to steroids, flavonoids and other phytoconstituents present in the extract as confirmed in this study.

Anti-plasmodial screening of plant substances has been shown to be caused by terpenes, flavonoids and alkaloids (Philip and Wright, 1990, Miliken, 1997, Christensen and Kharazmi, 2001). These compounds could be acting singly or in synergy with one another or other component to exert the anti-plasmodial activity observed in this study. Tannins have the ability to bind to proteins in aqueous solution (Makkar, 2003) and have therapeutic value as astringents, since they are able to precipitate protein, through this, they can be used to stop hemorrhage caused by plasmodium species (Elolemy, 1994).

The four day suppressive test is a standard test commonly used for anti-malarial screening and the determination of

percent inhibition of parasitaemia is the most reliable parameter. There were significant decrease (P < 0.05) in parasite density in the treated group compared to the untreated group. The significant decrease in parasitaemia observed was dose dependent. The extract did not cause a significant change (P < 0.05) in mean parasitaemia in mice infected with *Plasmodium berghei* in comparison to chloroquine treated mice.

The ethanolic leaf extract of *A. boonei* caused 35.7%, 21.6% and 19.8% suppression in parasitaemia of *P. berghei* infected mice at 200mg/kg, 400mg/kg and 800mg/kg respectively. while chloroquine, a standard anti-malarial drug used in this research work exerted 100% suppression at 5mg/kg body weight (Figure I). Kiseko *et al*, 2000 showed that when a standard anti-malarial drug is used in mice infected with *P. berghei*, it suppresses the parasitaemia to a non-detectable level.

It is clear that *Alstonia boonei* showed marked anti-malarial effect in dose seeming fashion from the percentage parasitaemia computed after carrying out curative test. The curative test showed that only Chloroquine the standard drug cleared the parasites by 91.7% at 5mg/kg body weight while the different concentration of the extract exert a growth inhibition of 39.31%, 49.19% and 38.54% at 200, 400, 800mg/kg body weight of the extract respectively (Figure II).

Hematological and biochemical indices have been reported to be a reliable parameter for assessment of the health status of animals (Sexena, *et al* 2011 and Ohaeri, 2011). There was a significant increase (P < 0.05) in the WBC count in group III compared to group II and all the groups treated with *A. boonei* extract (Table II). WBC count functions primarily in body defence against foreign bodies and this is often

achieved through leucocytosis and antibody production (Marieb, 1995). Leukocytosis observed in group III may be due to leukemia, bone marrow tumors, tissue damage and inflammatory disease of the mice infected with *P.berghei* NK65. The values were significantly high ($P<0.05$) in the treated group compared to the healthy group because some of the parasites were still present in the animals.

There were thrombocytosis in all the groups that received chloroquine and the extract of *A.boonie* compared to the infected untreated group. Since platelets play an important role in the maintenance of normal homeostasis, this may be that the extract contains some compounds that are capable of causing the release of a thrombopoietin (Erslev,1979).

Thrombocytopenia was observed in all the treated groups (group II and group IV-VI) compared to the normal control group (group I). These signify that the treated groups' mice have haemolytic anemia. There were significant reduction ($P<0.05$) in RBC, HGB and HCT of the untreated infected mice (group III) compared to the group treated with Chloroquine and *A.boonie* extract (group II and group IV-VI). This is an indication of severe anemia in group III. The extract prevented a drastic reduction in HGB, RBC and HCT values, features signifying severe anemic conditions.

This observation is supported by a report stating that anemia is characterized by decreased values of HGB, RBC and HCT (Aleksandro,2009). Lathia and Joshi, 2004 showed that reduction in erythrocyte number or a reduction in the concentration of hemoglobin in each erythrocyte is the major causes of anemia. This also shows that the extract does not have the potential to stimulate erythropoietin release from the kidneys, which is the humoral regulator of RBC production (Polenakovic and Sikole, 1996).

There were significant reduction ($P<0.05$) in RBC, HGB and HCT of the treated infected mice (group II and group IV-VI) compared to the healthy group (group I). This is an indication that some of the mice in these groups (group II and group IV-VI) are anemic. This may be due to the destruction of RBC by the *P. berghei* before or during treatment. There were no significant change ($P<0.05$) in the MCV, MCH, MCHC, PCT, PDW, RDW-CV.and RDW-SD values in the entire experimental groups. In conclusion, the ethanolic leaf of *A.boonie* extract does possess hematopoietic activity and is not hematotoxic.

6.0 CONCLUSION

The result obtained from this study reveals that 200, 400 and 800mg/kg body weight of *Alstonia boonie* ethanolic leaf extract suppresses *Plasmodium berghei* NK 65 and could be used in the management of malaria. The extract does possess hematopoietic activity and is not hematotoxic

6.1 RECOMMENDATIONS FOR FURTHER STUDIES

Further studies are needed in order to isolate and identify the active compounds in the extract of *Alstonia boonie* that is responsible for the anti-plasmodial activity observed in this study.

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