Journal of Natural Sciences Research www.iiste.org
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.7, No.22, 2017

Effects of Aqueous and Chloroform Stem Bark Extracts of Alstonia boonei on Liver Function Indices of Plasmodium Berghei Induced Albino Mice

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Abstract
In a preliminary research, authors reported that solvents extracts of *Alstonia boonei* possess strong antimalarial activity against NK-65 Chloroquine sensitive *Plasmodium berghei* infected mice with aqueous extract having the highest decrease in mean percentage parasitaemia. This research was therefore aimed at evaluating the effects of most active stem bark extracts (aqueous and chloroform) of *Alstonia boonei* on liver function indices of *Plasmodium Berghei*-induced mice. A total of 42 albino 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. They were then randomly divided into 7 groups of 6 mice per cage. Group 1 served as normal control, Groups 2 served as negative control (malaria infected but untreated), group 3 were administered with Chloroquine, groups 4 and 5 animals were administered with aqueous extract at a dose of 150 and 250mgkg-1 per day for four weeks, Groups 6 and 7 animals were administered with chloroform extract at a dose of 150 and 200mgkg-1 per day for four weeks. On the 29th day, the mice were euthanized and blood sample was collected and centrifuged for analysis of Liver function indices (AST, ALT, ALP, DB, TB, TP and ALB), the animals were dissected and liver tissues were collected for histological analysis. A significant ($p<0.05$) increase in mean serum of ALT, AST, ALP, and total bilirubin was observed in both negative and positive control compared to normal control. On the other hand, a significant ($p<0.05$) decrease in mean serum of ALT, AST, ALP and total bilirubin was seen in extracts administered groups compared to negative control. Histopathological examination of the liver showed unremarkable liver architecture with a vein containing red blood cells and some malarial pigments and parasites in infected but untreated group (negative control) while no malarial pigment or parasite was seen in either the normal control group or groups administered with extracts, confirming the antimalarial activity of the plant extract.
Keywords: *Alstonia boonei,* toxicity, malaria, liver Function Indices and histopathology.

1. Introduction

Liver is the largest and heaviest internal organ of the body weighting about 1.4 – 1.6 kg, it is a soft, reddish-brown triangular organ with two lobes, averaging about the size of an American football in adults (Guyton and Hall, 1996). Liver performs more than 500 different functions which includes fats, proteins and carbohydrates metabolism. The liver also metabolizes all drugs and other foreign bodies hence, plays a major role in homeostasis (Tortora and Grabowski, 2011).

Malaria is a disease caused by injection of plasmodium parasite into the human body due to bite of certain species of female anopheles mosquitoes. The infective forms (sporozoites) of one or more of at least four different species of plasmodium invade the liver and subsequently red blood cells giving rise to periodic shivering, pyrexia and sweating (WHO, 2014). Malaria infection develops via two phases: One that involves the liver (exo - erythrocytic phase) and another that involves red blood cells (erythrocytic phase). The liver is an important organ involved during the hepatic stage of the malaria parasite's life cycle. When an infected mosquito pierces a person's skin to take a blood meal, sporozoites in the mosquito's saliva enter the bloodstream and migrate to the liver where they infect hepatocytes. There the sporozoites multiply asexually and asymptomatically for a period of 8 – 30 days and develop into merozoites. The merozoites rupture their host cells and are then released into the circulation and enter the erythrocytic stage. In the erythrocytic stage, parasitized red blood cells (PRBCs) become sequestered in small blood vessels. The degraded hemozoin pigment is then engulfed by local tissue macrophages such as kupffer cells and alveolar macrophages (Parnpen *et al.*, 2014). Common histopathological findings of the liver in *Plasmodium falciparium* malaria include reactive kupffer cells, retention of hemozoin pigment and minimal PRBC sequestration.

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 (WHO, 2016). 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 (Federal Ministry of Health, 2015).

Alstonia boonei is one of the species of Alstonia plant. It is a very large, deciduous, tropical-forest tree belonging to the dogbane family, Apocynaceae. It is native to tropical West Africa, with a range extending into Ethiopia and Tanzania. Its common name in the English timber trade is cheese wood, pattern wood or stool wood). In Nigeria, it is locally called "Egbu/Egbu Ora" in Igbo language and "Ahun" in Yoruba language. The tree also finds many uses in folk medicine. Various pharmacological studies have been carried out on this plant, it has been reported to possess antipyretic, analgesic and anti-inflammatory properties (Olajide *et al.,* 2000), anti-malarial (Ojewole, 1984), Immuno-stimulant property (Taiwo *et al.,* 1998), antipsychotic and anxiolytic effect (Elisabetsky and Costa-Campos, 2006). The stem bark of *Alstonia boonei* is locally used in South Eastern Nigeria for the treatment of malaria in the region. This research was therefore carried out to evaluate the effects of aqueous and chloroform extracts of *Alstonia boonei* (Alstonia Plant) on Liver Function Indices of *Plasmodium Berghei*-Induced Mice.

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 $6^{0}30^{1}N7^{0}30^{1}E$). 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). The stem bark was washed, shade dried and ground to powder. 200g of sample was measured and transferred into each of the flasks containing $1000cm^3$ (1 liter) of 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 Whatman No. 1 filter paper (after initial use of clean cheese cloth). The filtrates were concentrated under vacuum using a vacuum rotary evaporator, then measured and stored in screw capped vials under room temperature. The volume of extracts to be administered was calculated according to dose and weight of the experimental animal using the relation below (Muhammad *et al.,* 2016).

$$\text{Volume of extract (ml) to be administered} = \frac{\text{Weight of animal (kg) x dose (mg/kg)}}{\text{Concentration of extract (mg/ml)}}$$

2.3 Experimental animals

Healthy albino mice (weighing 16-20g) 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 Infecting and screening of the mice with Malaria Parasite

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 1.0×10^5 infected RBC per 0.2ml 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. 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, 2010). The existence of *Plasmodium 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.

2.5 Effects of aqueous and chloroform stem bark extracts of Alstonia boonei on liver function indices of Plasmodium Berghei-induced mice

A total of 42 albino mice were randomly divided into 7 groups of 6 mice per cage. Group 1 served as normal control, Groups 2 served as negative control, group 3 were infected with malaria and administered with chloroquine, groups 4 and 5 animals were infected mice and administered with aqueous extract at a dose of 150 and 250mgkg-1 per day for four weeks, Groups 6 and 7 animals were infected and administered with chloroform extract at a dose of 150 and 200mgkg-1 per day for four weeks. On the 29th day, the mice were euthanized and

blood sample was collected and centrifuged for analysis of Liver function indices (AST, ALT, ALP, DB, TB, TP and ALB), the animals were dissected and liver tissues were collected for histological analysis. The biopsies of kidney of the mice used in the research were fixed with 10% neutral suffer formalin, dehydrated with ascending grade of alcohol, cleared with toluene, infiltrated with molten paraffin wax. The microtome sections were stained with haematoxylin and eosin staining technique. Histopathology examination was carried out by the method of Auwioro (Auwioro, 2010).

2.6 Statistical Analysis

All data were expressed as mean ± standard deviation. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test after investigating the data for normality using Shapiro-Wilk test and for variances homogeneity to be sure that the data are normally distributed and variances would be homogenous using GraphPad Instat3 Software version 3.05 Differences of $P < 0.05$ were considered to be significant (GraphPad, 2000).

3. Results

Table 1 present the Liver function indices (AST, ALT, ALP, DB, TB, TP and ALB) of *Plasmodium berghei* infected mice administered with aqueous and chloroform extracts of *Alstonia boonei* after four weeks of extracts Administration. A significant ($p<0.05$) increase in mean serum of ALT, AST, ALP, and total bilirubin was observed in both negative (malaria infected but untreated) and positive (malaria infected, treated with Chloroquine) control compared to normal control. On the other hand, a significant ($p<0.05$) decrease in mean serum of ALT, AST, ALP and total bilirubin was seen in extracts administered groups compared to negative control.

Table 1: Liver function indices (AST, ALT, ALP, DB, TB, TP and ALB) of *Plasmodium berghei* infected mice administered with aqueous and chloroform extracts of *Alstonia boonei* after four weeks of extracts Administration

Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	ALB(g/dl)	T/P(g/dl)	T/BIL(mg/dl)	D/BIL(mg/dl)
Normal Control	31.87±2.99^{a}	30.66±3.08^{a}	233.09±10.02^{a}	3.63±0.17^{a}	8.26±1.04	1.53±0.13^{a}	3.44±0.19
Negative Control	60.86±3.24^{abcde}	120.60±4.24^{abcde}	759.15±13.02^{abcdef}	3.00±0.18	9.07±2.03	3.18±0.03^{abcdef}	4.19±0.18
Chloroquine	62.53±1.02	125.92±2.09	1007.17±45.16^{b}	2.72±0.02^{abcdef}	9.17±2.03	3.65±0.05^{b}	4.27±0.32
AQ 150mg/kg	39.16±4.05^{b}	67.54±1.03^{b}	452.8±0.06^{c}	4.75±0.52^{e}	8.04±2.04	1.38±0.01^{c}	3.15±0.11
AQ 250mg/kg	31.62±3.02^{c}	33.86±2.12^{c}	435.52±0.34^{d}	5.86±0.12^{d}	8.20±2.07	1.17±0.09^{d}	3.06±0.16
CHL 150mg/kg	35.81±4.02^{d}	41.12±4.01^{d}	363.23±0.03^{e}	4.67±0.32^{e}	7.62±1.02	1.51±0.01^{e}	3.43±1.03
CHL 250mg/kg	30.68±3.01^{e}	36.47±3.04^{e}	340.72±0.19^{f}	5.73±0.91^{f}	8.55±0.02	1.41±0.01^{f}	3.13±1.93

Values are presented as Mean ± standard deviation, (n = 6). Value with the same superscripts in a column are significantly different compared to each other ($P<0.05$). ALT = Alanine aminotransferase; AST = Aspartate aminotransferase; ALP = Alkaline phosphatase; ALB = Albumin; T/P = Total protein; T/BIL = Total bilirubin; D/BIL = Direct Bilirubin; AQ = Aqueous extract; CHL= Chloroform extract.

3.1 Histopathological examination of liver tissues

Plate 1 shows a photomicrograph of section of liver showing normal cellular architecture with distinct hepatic cells, sinusoidal space and a central vein, Plate 2 shows photomicrograph of Negative control (Infected but untreated group) showing unremarkable liver architecture with a vein containing red blood cells and some malarial pigments and parasite. Plate 3 shows photomicrograph of Positive control (Group treated with Chloroquine) showing areas of severe fibrosis and inflammation, No malarial pigment or parasite was seen. Plate 4 shows photomicrograph of liver of group VI (administered with 150 mg/kg of aqueous extract) showing areas of mild fibrosis with no malarial pigment or parasite seen, while Plate 5 shows photomicrograph of liver of group V (administered with 250mg/kg of aqueous extract), section showing unremarkable liver architecture with vein containing red blood cells, with no malarial pigment or parasite seen. Plate 6 shows photomicrograph of liver of group VI (administered with 150 mg/kg) section shows unremarkable liver architecture with vein containing red blood cells. No malarial pigment or parasite seen. While Plate 7 shows photomicrograph of liver of group VI (administered with 250 mg/kg) section shows unremarkable liver architecture with vein containing red blood cells. No malarial pigment or parasite seen.

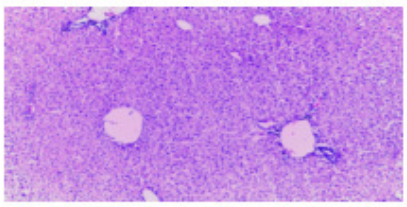

Plate 1: shows a photomicrograph of section of liver showing normal cellular architecture with distinct hepatic cells, sinusoidal space and a central vein

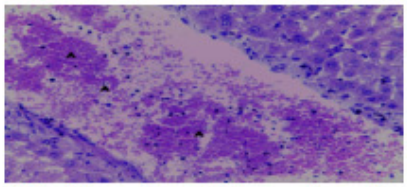

Plate 2: section of liver of negative control (Infected but untreated group) showing areas of fibrosis and inflammation with malarial pigment and parasite seen

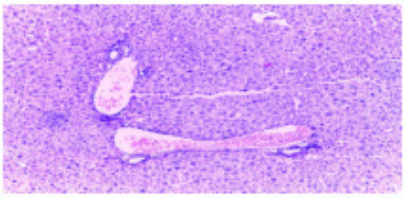

Plate 3: section of liver of positive control (Group treated with Chloroquine) showing areas of fibrosis and inflammation with no malarial pigment or parasite seen

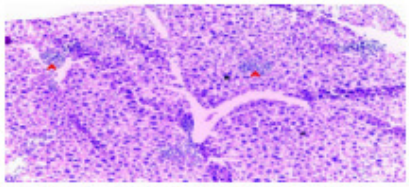

Plate 4: section of liver of group VI (administered with 150 mg/kg of aqueous extract) showing areas of mild fibrosis with no malarial pigment or parasite seen

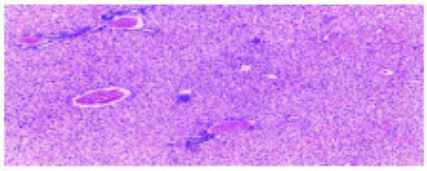

Plate 5: section of liver of group V (administered with 250mg/kg of aqueous extract) showing unremarkable liver architecture with vein containing red blood cells, with no malarial pigment or parasite seen

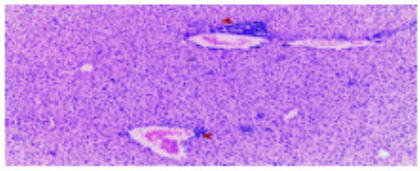

Plate 6: section of liver of group VI (administered with 150 mg/kg of chloroform extract) showing unremarkable liver architecture with vein containing red blood cells. No malarial pigment or parasite seen.

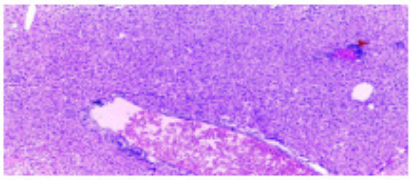

Plate 7: section of liver of group VI (administered with 250 mg/kg of chloroform extract) showing unremarkable liver architecture with vein containing red blood cells. No malarial pigment or parasite seen.

4. Discussion

Liver damage is a serious disease characterized by disturbances in normal functions of the liver. It is clinically

diagnosed by determining the serum concentration of liver enzymes (ALT, AST and ALP). These enzymes are non-plasma specific enzymes and were reported to reach higher than normal levels in the blood when there is necrosis of the parenchymal cells of the liver as in viral or toxic hepatitis, with ALT being the most specific liver injury marker and a more selective liver parenchymal enzyme (Muhammad *et al.,* 2015). Alkaline phosphatase (ALP) test is also used to detect bone disorders. In conditions affecting the liver, damaged liver cells release increased amounts of ALP into the blood. This test is often used to detect blocked bile ducts because ALP is especially high in the edges of cells that join to form bile ducts. If one or more of them are obstructed, then blood levels of ALP will often be high (Sherlock and Dooley, 2002).

The result showed a significant ($p<0.05$) increase in mean serum of ALT, AST and ALP in negative control compared to normal control, This could be due to infection by the malaria parasite that undergo developmental stage in the liver cell. Upon administration of the extracts, a steady fall in marker enzymes was observed in a dose dependent pattern with aqueous extract possessing the highest hepato-curative ability against *plasmodium berghei* induced liver damage. On the other hand, an increase in mean serum of ALT, AST and ALP was seen in Chloroquine administered group compared to negative control. The observed increase may be as a result of infection of the liver by the malaria parasite during the exo-erythrocytic cycle stage.

The significant increase ($P<0.05$) in serum total protein and the non-significant increase in albumin and conjugated bilirubin (Direct bilirubin) of negative control compared to normal control could be due to onset the liver damage which trigger the released of newly synthesized albumin and conjugated bilirubin in addition to challenging immune system. These indices (total protein, albumin and direct bilirubin) are good indicators of chronic liver damage rather than acute and their values are expected to be lower (Hou *et al.,* 2011).

Bilirubin is an orange yellow pigment, a waste product primarily produced by the normal breakdown of haem, a component of haemoglobin. It is ultimately processed by the liver to allow its elimination from the body. A bilirubin test is used to detect an increased level in the blood which helps to determine cause of jaundice or diagnose conditions such as liver disease, hemolytic anemia and blockage of bile duct. Result of total bilirubin showed that there is elevated total bilirubin in negative control and Chloroquine groups than normal while low total bilirubin level is seen in treated groups. This can be attributed to haemolyticanaemia caused by the invasion of the parasite on the RBC (Parnpen *et al.,* 2014).

Our earlier findings of *in vitro* and *in vivo* antimalarial studies of the extracts revealed elevations in parasitaemia levels of malaria parasite in infected but untreated mice and converse reductions in parasitaemia levels upon treatment of infected mice with the extracts. In this study, malaria infected but untreated animals (Negative Control) and positive control (malaria infected and treated with Chloroquine) showed pronounced elevations in serum levels of ALT, AST, ALP, and total bilirubin when compared with the normal control group (uninfected mice) indicating negative impacts of malarial and Chloroquine on the liver. Similarly when compared to the negative control group (infected but untreated), a significant decrease ($P<0.05$) in the mean serum levels of ALT, AST, Total protein and direct bilirubin was observed suggesting that the extracts have hepatocurative effects in addition to the observed anti-malarial activity. This observation which is similar to that reported by Onwusonye *et al* (2014) serve as more evidence in support of the continued use of the herb in malaria treatment.

One possible mechanism for the hepatocuration may be due to their antioxidant properties, which could counteract the toxic effect of malaria parasite. The bark was said to contain flavonoids, phenolic and triterpenoids which may bind to and scavenge the free radicals by the parasite, thus preventing microsomal lipid and protein peroxidation which is thought to be the cause of liver damage by *plasmodium berghei* (Chime *et al.,* 2015).

5. Conclusion

This research concludes that aqueous and chloroform extracts of *Alstonia boonei* possess strong hepatocurative activity against *Plasmodium berghei* liver damage with aqueous extract having the highest activity. Therefore in view of the increasing concern for the resistance of the malarial parasites to available drugs and safety of traditional medicines used in treatment of malaria. The results of this study offers a scientific basis for the traditional use of this indigenous plant in the treatment of malaria. However further studies are recommended for the effect of the extracts on other organs.

Ethical approval

All authors hereby declare that Principle of laboratory animal care and ethical guidelines for investigation of experimental pain in conscious animals were observed during experimentation (Zimmermann, 1983; NIH, 1996).

Competing interests

Authors have declared that no competing interests exist.

Consent

It is not applicable.

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