Research Article
Effect of *Acalypha wilkesiana* MuellArg Leaf Extract on the Oxidative Indices, Liver Enzymes and Liver Integrity of Rats Infected with *Plasmodium berghei*

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Abstract: This study investigates the effect of the methanolic extract of *Acalypha wilkesiana* on the oxidative stress caused by *Plasmodium berghei* infection and on the liver of parasitized animals. Phytochemical screening of the extract reveals that it is rich in flavonoids, carotenoids and sterols. Our results showed that liver Malondialdehyde (MDA) significantly ($p<0.05$) increased while Superoxide Dismutase (SOD), (Glutathione peroxide) GSH-P, reduced Glutathione (rGSH) and Catalase (CAT) decreased ($p<0.05$) in the parasitized non-treated rats, evident of increase in lipid peroxidation and oxidative stress following *P. berghei* infection. Extract treatment of the parasitized rats was observed to reverse the effects of the oxidative stress and restore the elevated liver enzymes; on the other hand, extract treatment of the non-parasitized rats increased antioxidant molecule levels, thus increasing their free radicals scavenging activity. The extract was also observed to extend the mean survival time of treated rats and improve malaria symptoms. Histological examination of the liver of treated and non-treated animals reveals that the extract may have a hepato-protective as there were no observable cellular defects. These findings suggest that the extract can be useful in management of oxidative stress to the biological system e.g., during malaria infection.

Keywords: *Acalypha wilkesiana*, antioxidants, lipid peroxidation, oxidative stress, parasitemia, *Plasmodium berghei*

INTRODUCTION

Malaria is caused by infection with protozoan parasites belonging to the genus *Plasmodiun* transmitted by female *Anopheles* species mosquitoes (Cox, 2010). The parasite is carried through the blood stream into the liver where it multiplies (pre-erythrocytic cycle) prior to entering the erythrocytes (erythrocytic cycle) (Rajan, 2009). In malarial studies using rodents, the *Plasmodium berghei* is the model frequently adopted (Sherman, 2008). *P. berghei* shares common characteristics with *P. falciparum* with regards to enzyme activities e.g., protein kinase and phosphoproteins (Augustijn *et al*., 2007). While frequent malaria attacks on the body system may not result to a clinical disease in the short term, it does affect the integrity and function of the liver (Carter and Diggs, 1977). Studies have shown that *P. berghei* infection in rodents, induces liver injury, increases mRNA expression of interleukin-12 (IL-12), protein 40 (p40) as well as IFN-γ, interleukin-4 (IL-4) and IL-10, with consequent increase in NO synthesis (Yoshimoto *et al*., 1998). Elevation of liver enzyme values is an indication of malfunction or diseased state of the organ.

Reactive Oxygen Species (ROS) are generated mainly by the mitochondria; an organelle that make ATP by coupling of respiration generated proton gradient, with the proton driven phosphorylation of ADP (Kowaltowski *et al*., 2009). However, malaria parasites also exert oxidative stress within the parasitized red blood cells of their host, during the conversion of heme (ferroprotoporphyrin) to hematin (ferriprotoporphyrin) and through the activation of the immune system, causing release of ROS (Percário *et al*., 2012). Hematin has the ability to disrupt cell membranes, causing the lysis of the host erythrocyte and the parasite, however, the malaria parasite has an inert protection, since it can detoxify the hematin, which it does by biocrystallization-converting it into insoluble and chemically inert β-hematin crystals (called hemozoin) (Hempelmann, 2007). Apart from the parasite induced ROS, the process of phagocytosis entails that superoxide anion are produced following the formation of the enzyme NADPH-oxidase so that leucocytes are able to engulf the infectious agent (Karupiah *et al*., 2000). The formation of ROS by the various means described is a phenomenon that if not controlled by the host cytoprotective antioxidants and enzymes, or by

exogenous substances could lead to oxidative damage. Oxidative stress can cause cellular injury through the production of peroxides and free radicals and can also disrupt normal mechanisms of cellular signaling.

In humans, oxidative stress is thought to be involved in the development of cancer, chronic lung disease, diabetes, cataract and Alzheimer's disease (Spector, 2000). Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as Glutathione, Superoxide dismutases and Catalases. Superoxide Dismutases (SODs) are a group of closely related enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide. The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases and glutathione *S*-transferases (Wu *et al*., 2004). Catalases are enzymes that catalyse the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor. The net result is that two potentially harmful species, superoxide and hydrogen peroxide, are converted to water (Weydert and Cullen, 2010).

The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, in malaria endemic countries, infection with malaria parasite is so frequent, hence the induction of oxidative stress by it, such that of the three billion people living in such areas, approximately 243 million will develop symptomatic malaria annually (WHO, 2012). Thus, this presents a need for antioxidant supplementation besides anti-malaria therapies, especially among residents in malaria endemic areas.

Antioxidants such as vitamin C, have been shown to provide protection against oxidative stress and hepatoxicity (Adelekan *et al*., 1997; Iyawe and Onigbinde, 2009) Some medicinal plants have been shown to exert anti-oxidative effect and as such has been postulated to be useful as adjuncts in the treatment of malaria; e.g. *Sapium ellipticum*, *Garcinia kola, Carica papaya, Crassocephalum rubens* (Adesegun *et al*., 2008; Oloyede *et al*., 2011; Omoregie and Osagie, 2012). These plants contain essential factors that help the body to cope with the challenges imposed by agents that generate free radicals in the system.

Acalypha wilkesiana MuellArg (copper leaf) is a plant from the family Euphorbiacaece. The genus "*Acalypha*" comprises about 570 species (Riley, 1963), a large proportion of which are weeds while the others are ornamental plants, however, *A. wilkesiana* is an ornamental plant. It is found all-over the world especially in the tropics of Africa. The plant is employed in folk medicine as nutritional supplement and in the treatment of many ailments (Ikewuchi *et al*., 2010; Udobang *et al*., 2010). Also there have been several studies on the phytochemical and elemental constituents of *A. wilkesiana* (Oladunmoye, 2006;

Madziga *et al*., 2010). Unpublished reports from a preliminary ethnobotanical survey in South east, Nigeria reveals that herbalists administer the alcoholic extract of the plant's leaf to patients undergoing treatment for and those who are recovering from malaria or hepatitis (I. Ogbuehi, Unpublished data). Consequently, we became interested in investigating the plant's potential with regards to ameliorating oxidative stress induced by malaria parasite infection. Presently, there are no published studies on its effect on oxidative indices and liver enzymes and liver histology of mice infected with *P. berghei*. Thus the study is designed to evaluate the effect of methanolic extract of *A. wilkesiana* on the oxidative indices, liver enzymes and liver integrity of parasitized rats.

METHODOLOGY

Experimental animals: Albino wistar rats of either sex weighing between 18-25 g were used for the study. They were obtained from the animal house of the Department of Pharmacology, University of Port Harcourt and Rivers State, Nigeria and housed in well ventilated cages. The animals were average of 8 weeks old and were maintained on commercial feeds (Finisher Feed) Top Feeds, Port Harcourt and tap water *ad libitum* for the entire duration of the study. Good hygiene was maintained by constant cleaning and replacement of their beddings from the cages daily.

Plant material: The leaves of *A. wilkesiana* were collected with permission from the gardens in the University of Port Harcourt, Abuja campus and authenticated by a botanist in the department of Plant Science and Biotechnology, University of Port Harcourt. A voucher specimen was deposited at the department's herbarium. The leaves collected were washed and shade dried to constant weight and milled into coarse powder by a mechanical grinder.

Equipments and reagents: Photo Microscope (Olympus, Japan), Syringes (1, 5 mL), oral cannula, Cotton wool, Vitamin C tablets (Emzor Pharmaceuticals), Heparinised capillary tubes, EDTA bottles, Microscopic slides (Olympus, China), Hand gloves, Giemsa stain (Sigma), 70% Methanol (Sigma), Distilled water, Tween 80 (Sigma), Picric acid, 10% tannic solution, Potassium mercuric iodide, Ferric chloride solution, Hydrochloric acid, Chloroform, Sodium hydroxide, Tetraoxosulphate (V1) acid and Dragendorff's reagent.

Preparation of extract and drug: Seven hundred g of the plant material (*A. wilkesiana* leaves) were soaked in 2 L of Methanol (70%) for 72 h, with frequent agitation. Thereafter, it was filtered and the residue (marc) was stored in a dessicator to remove all traces of the solvent and stored for subsequent use. The filtrate

was subjected to solvent recovery using a rotary evaporator 60ºC leaving behind a semi solid extract. The extract was poured in warm into a pre-weighed beaker and allowed to cool in a fume cupboard. The beaker and its content were subsequently weighed and the weight of the dried extract was deduced:

% yield was calculated as:
<u>Weight of dried extract obtained×100%</u>
Weight of plant material used

The extract was then dissolved in Tween-80 and distilled water (Tween-80/water) to give a homogenous solution equivalent to the dose level of 100 mg/mL, given intraperitoneally (i.p.) daily for fourteen days.

Ascorbic acid (Vit C) containing 100 mg/5 mL w/v was diluted with sterile distilled water to achieve the desired concentration, this was then administered [25 mg/kg body weight (b.wt.)] i.p daily for fourteen days.

Phytochemical testing: Testing for the presence of phytochemicals-saponins, tannins, carotenoids, alkaloids, steroids, flavonoids, anthraquinones, cardiac glycosides were carried out using the methods in Trease and Evans (1989).

Preparation of various concentrations of extract: The extract was reconstituted in distilled water to obtain various concentrations of the extract thus: 2 g of extract was reconstituted in distilled water to obtain 100 of a 20 mg/mL solution. A portion of the 20 mg/mL solution was diluted with an equal volume of distilled water to obtain a 10 mg/mL solution. The double dilution procedure was continued to obtain lower concentrations of the extract.

Preparation of inoculum: The rodent malaria parasite, *P. berghei* ANKA strain (chloroquine sensitive) was obtained from the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos. The parasite was maintained in the Malarial Research Laboratory of University of Port Harcourt by serial blood passage from donor rats to normal rats through Intra Peritoneal (IP) inoculation. Parasitemia was assessed by thin blood films made by collecting blood from the cut tip of the tail and this was stained with Giemsa stain. For the test animals, heparinized capillary tubes were used to collect 0.2 mL of blood from the auxiliary plexus of veins of one of the donor rat (parasitemia>35%). The blood was diluted with 5 mL of Phosphate Buffer Solution (PBS) pH 7.1, to give $2×10^7$ Parasitized Red Blood Cells (PRBC) in an injection volume of 0.2 mL (i.p.) (Fidock *et al.*, 2004).

Experimental design: A total of 40 rats (25 surviving parasitized rats and 15 normal rats) were used for the study. They were separated into 8 groups of 5 rats per group as follows:

Group 1: Normal control: Non-parasitized, non-treated rats (nPnT)
Group 2: Positive control: Parasitized, Vitamin C-treated rats (P vit C T)
Group 3: Negative control: Parasitized, non-treated rats. (PnT)
Group 4: Extract vehicle control: Parasitized rats given 0.2 mL of Tween-80. (PTwT)
Group 5: Test group 1: Parasitized rats, given 50 mg/kg b.wt of extract (PeT1)
Group 6: Test group 2: Parasitized rats, given 100 mg/kg b.wt of extract (PeT2)
Group 7: Test group 3: Non-parasitized, rats given 50 mg/kg b.wt of extract (nPeT3)
Group 8: Test group 4: Non-Parasitized rats, given 100 mg/kg b.wt of extract (nPeT4)

The dose of extract was according to the results of a pilot LD 50 studies in rats of both sexes. At the end of the treatment period (14 days), the rats were fasted overnight, sacrificed after ether anesthesia and tissue (liver) and blood were collected for various biochemical estimations. The dose of Vit C given to control animals was 20 mg/kg in accordance to the standard of 5-20 l U/kg per day (Yasunaga *et al.*, 1982). At the end of the treatment period (14 days), the rats were fasted overnight, sacrificed after ether anesthesia and tissue (liver) and blood were collected for various biochemical estimations.

Tissue homogenate preparation and assays: The liver from each animal was excised, rinsed in ice cold 0.25 M sucrose solution and 10% w/v homogenate was prepared in 0.05 M phosphate buffer (pH 7.1) and centrifuged at 12,000×g for 60 min at 4°C. The supernatant was collected and monitored for oxidative stress parameters such as Glutathione (GSH) (Beutler *et al.*, 1963), glutathione peroxidase (Mantha *et al.*, 1993), Superoxide Dismutase (SOD) (Misra and Fridovich, 1972), Glutathione Transferase (GST) (Ellman, 1951), Catalase (CAT) and lipid peroxidation (Chance and Maehly, 1955). Blood samples collected were allowed to clot. Serum was separated by centrifuging at 2500 rpm for 15 min and analyzed for various biochemical parameters such as phosphatase (ALP) (Kind and King, 1954), glutamic alanine aminotransferase (ALT), aspartate Aminotransferase (AST) (Reitman and Frankel, 1957) and total Bilirubin (TB) (Mallay and Evelyn, 1937) and Total Serum Protein (TSP) (Lowry *et al.*, 1951).

Calculation of mean survival time: Test animals were kept and observed for 30 days to determine their mean survival rates.

Statistical analysis: Data was expressed as mean±standard Error of Mean (SEM) and analyzed using Student`s t-test to compare values from experimental and control groups. The data was

analyzed using Analysis of Variance (ANOVA) and the group means were also validated using by Duncan's Multiple Range Test (DMRT). Differences between values were considered significant at p<0.05 and highly significant at p<0.01.

Histopathological examination: Histological analysis was carried out at the Histopathological Department of the University of Port Harcourt, Rivers State, Nigeria. The photomicrographs of the prepared slides were taken at magnification of x100 with a Canon (Melville, NY) Power Shot G2 digital camera.

RESULTS

Phytochemical screening: Preliminary qualitative testing of the phytochemical constituents of *A. wilkesiana* leaves reveals that the plant is rich in flavonoids, carotenoids and plant sterols (Table 1).

Liver function tests: The results from the assay of the liver enzyme activities of the parasitized, non-treated rats showed significant increases (p<0.05) in the values of ALT, AST, ALP, TB and TSP, as compared to the dose dependent reduction in such elevations in the parasitized, treated groups (Table 2).

Oxidative indices: A decline in the activities of intracellular antioxidant enzymes such as SOD, CAT, rGSH and GSH-P and a higher lipid peroxidation level were seen in the parasitized and non-treated groups while a significant (p>0.05) reduction in the lipid peroxidation values and increase in the level of antioxidant molecules were observed in the extract

Table 1: Preliminary qualitative phytochemical screening of *A. wilkesiana*

Phytochemical constituent	Status
Tannins	+
Flavonoids	+++
Steroids	++
Saponins	+
Cardiac glycosides	+
Anthraquinones	-
Alkaloids	+
Carotenoids	++

groups both parasitized and non parasitized as shown in Table 3.

Lipid peroxidation test: There was a significant reduction (p>0.05) in the degree of lipid peroxidation in the test groups who received the extract. However, elevated levels of Malondialdehyde (MDA) were evident in the prepared tissue of the control groups (Table 4).

Mean survival test: All the non parasitized rats used in the study survived the 30- day study period, while the survival rates of the parasitized rats were significantly improved by the administration of the extracts. Parasitized and extract treated groups 50 and 100 mg/kg showed a mean survival time of 19.0±0.00 and 22±0.00 days, respectively as compared to 9.0±0.15, 9.5±0.40 and 13.5±0.50 for the parasitized non treated, parasitized tween-80 treated (vehicle control) and parasitized Vit-C treated (Positive control) respectively (Fig. 1).

Histological analysis: The slides are labeled as follows: A = Non-parasitized, non- treated (Positive

Table 2: Liver function biomarkers in the serum of control and parasitized rats treated with *A. wlikesiana*

Groups	Parameters				
	ALP(μ/L)	ALT(μ/L)	AST (μ/L)	TB (mg/dl)	TSP(mg/dL)
1. nPnT	54.76±0.99[a]	31.98±0.36[a]	77.25±1.34[a]	0.44±0.43[a]	9.27±0.55[a]
2. P vit C T	58.94±0.07[a]	36.58±0.04[b]	82.68±0.12[b]	0.78±0.03[c]	15.35±0.31[b]
3. PnT	100.08±0.17[d]	66.76±0.31[c]	105.15±0.22[c]	1.55±0.30[d]	18.02±0.26[c]
4. PTwT	77.73±0.04[c]	68.84±0.03[c]	106.24±0.11[c]	1.54±0.16[d]	18.32±0.07[c]
5. PeT1	67.89±2.34[b]	33.18±1.57[a]	77.80±1.92[a]	1.23±2.07[d]	16.77±1.62[b]
6. PeT2	59.22±1.77[a]	38.01±1.39[b]	84.15±1.57[b]	0.66±1.29c	12.10±1.64[d]
7. nPeT3	54.34±2.47[a]	36.75±1.05[b]	83.32±1.45[b]	0.48±2.78[b]	9.23±1.87[a]
8. nPeT4	56.95±3.65[a]	37.85±2.57[b]	88.97±1.89[c]	0.47±1.35[b]	9.01±2.33[a]

Values are expressed as Mean±SEM, values not sharing a common superscript letter down a column differ significantly at p<0.05

Table 3: Oxidative indices in control and parasitized rats treated with *A. wilkesiana*

Groups	Parameters			
	SOD	CAT	GSH-P	RGSH
1. nPnT	2.74±0.13[a]	42.47±0.19[a]	18.51±0.07[a]	12.03±0.67[a]
2. P vitC T	3.52±0.62[b]	45.99±0.30[c]	17.91±2.17[a]	10.92±0.55[b]
3. PnT	1.14±0.15[c]	22.02±1.47[b]	12.19±0.89[b]	9.78±3.04[c]
4. PTwT	1.05±0.17[c]	21.98±0.88[b]	12.58±2.27[b]	9.97±0.93[c]
5. PeT1	3.50±1.18[b]	46.98±0.43[c]	17.73±0.18[a]	10.12±0.72[b]
6. PeT2	4.30±0.86[d]	49.27±0.31[d]	21.67±0.54[c]	10.17±1.07[b]
7. nPeT3	3.56±0.33[b]	45.74±0.59[c]	16.75±2.65[d]	12.27±1.01[b]
8. nPeT4	4.44±0.14[d]	45.48±0.55[c]	18.95±1.39[b]	14.56±0.92[d]

Values are expressed as Mean±SEM, values not sharing a common superscript letter down a column differ significantly at p<0.05
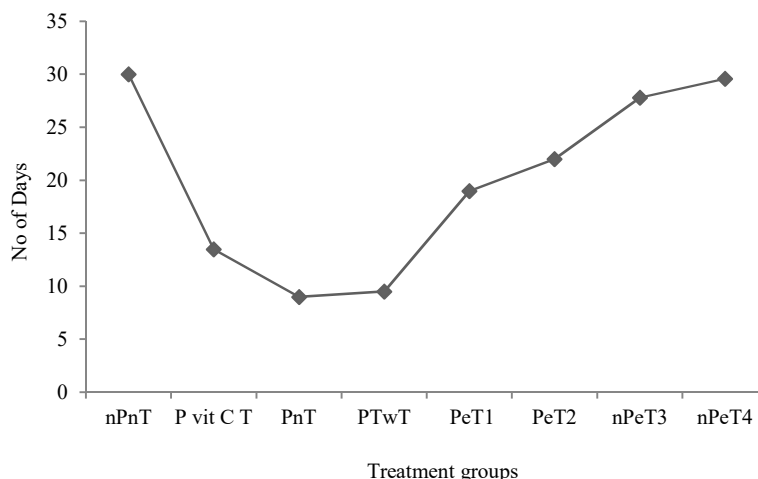
Fig. 1: Mean survival values for control and test animals

Table 4: Changes in the liver lipid peroxidation level of the control and parasitized mice treated with *A. wilkesiana*

Groups	Malondialdehyde (MDA) (Units/g of wet tissue)
1. nPnT	2.19±0.02[a]
2. P vit C T	2.23±0.01[b]
3. PnT	6.86±0.01[c]
4. PTwT	6.77±0.01[c]
5. PeT1	2.42±0.01[b]
6. PeT2	2.26±0.01[b]
7. nPeT3	2.17±0.01[a]
8. nPeT4	2.16±0.02[a]

Values are expressed as Mean±SEM, values not sharing a common superscript letter down a column differ significantly at $p<0.05$

Control), B = Non- Parasitized rats, treated with 100 mg/kg b.wt of extract, C = Parasitized, treated with 100 mg/kg b.wt of extract, D = Parasitized, Non-treated (Negative Control). Slides (A), (B) and (C) shows intact lobular architecture and normal hepatocytes with no evidence of adhesion or inflammation in the cytoplasm, lobular architecture. Slide (D) shows mild inflammation and periportal hepatocyte degeneration.

DISCUSSION

Phytochemicals are plant constituents which provide various biological functions leading to the promotion of health as well as the reduced risk of chronic diseases. Flavonoids and carotenoids are phytochemicals ubiquitously present in plants and scientific studies have reported their antioxidant properties (Jovanovic *et al*., 1994; Paiva and Russell, 1999). The presence of flavonoids and carotenoids in substantial quantities in the methanolic extract of *A. Wilkesiana* may be responsible for the demonstrated anti-oxidant activity of this plant.

Malaria infection affects the liver and can derange enzyme values. Results from the present study, shows that malarial infection can induce changes in liver enzyme and other biomarker activities. These findings

agree with earlier report by Onyesom and Onyemakonor (2011).

Biological system protects itself against the damaging effects of free radicals and activated species by the actions of free radical scavengers and chain terminator enzymes such as SOD, CAT and GPx system (Kurata *et al*., 1993). Glutathione protects the cellular system against the toxic effects of lipid peroxidation. Decreased level of reduced glutathione observed in the serum of *plasmodium* infected rats is an indication of an increased utilization due to oxidative stress. Administration of *A. wilkesiana* in varying doses had a dose dependent effect, as there was a significant ($p>0.05$) difference in results of administration of 50 mg/kg and that of the 100 mg/kg. This suggests that the optimal dose for administration of the extract for its antioxidant effect could be up to 100 mg/kg. Interplay between the different antioxidant enzymes provides protection to the liver cells, hence no cellular damage was observed in the liver of the treated groups (Fig. 2).

Malonaldehyde (MDA) is the major oxidation product of the peroxidation of poly unsaturated fatty acids, thus making an increased MDA level, an important indicator of lipid peroxidation. Thus, it was not surprising that in this study, parasitized non treated rats had high MDA levels when compared with the treated groups. It may be that the extract contributed to the biosynthesis of the antioxidant enzymes thereby increasing free radical scavenging ability and reducing membrane lipid peroxidation.

Our results also lend support to the efficacy of Vitamin C as an antioxidant. Several studies have reported the use and efficacy of ascorbic acid as an anti-oxidant (Adelekan *et al*., 1997; Iyawe and Onigbinde, 2009).

There is evidence that the high levels in total proteins in the parasitized non treated group in comparison to the non parasitized non-treated group is associated with the presence of plasmodial parasites in
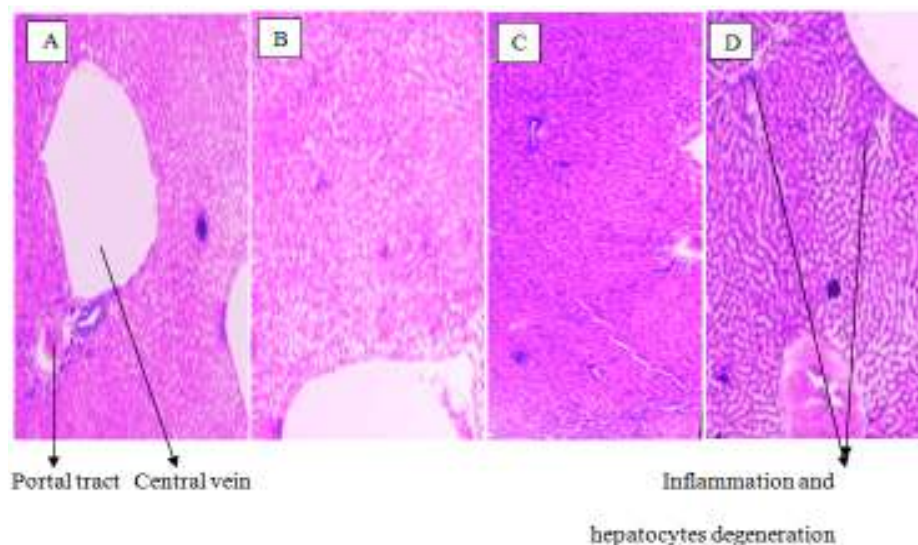
Fig. 2: Photomicrographs of liver of rats (Hematoxylin and Eosin, X 100)

the rats. In a similar study, infection with malaria parasite was reported to result in hyperproteinaemia in rabbits (Orhue *et al.*, 2005). The reversal of increased liver enzymes in the extract treated groups may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. These results agree with the finding that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Wolf, 1999).

The increase in survival time of the animals may also be due to the proposed anti-plasmodial property of the extract (Udobang *et al.*, 2010).

Essentially, the liver section as shown in slide A, B and C (Fig. 2), showed well preserved hepatocytes in the cytoplasm and well demarcated sinusoids. Also, no area of infiltration by inflammatory cells and fatty degenerative changes were observed. These features are indicative of normal hepatic architectural integrity for rats in the positive control group and the parasitized and non-parasitized groups treated with the extract. However, slide D while not showing fatty liver, had signs of necrosis and inflammation.

CONCLUSION

The result of this study justifies the use of *A. wilkesiana* for patients undergoing therapy or recovering from malaria and as a nutritional supplement. It has also established its antioxidant and hepato-protective property. Therefore, we propose that the methanolic extract of the leaves of *Acalypha wilkesiana* could offer protection against oxidative damage and improve liver function in the presence of malaria infection.

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