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Use of Nigerian Medicinal Plants Protected Liver from Injury in *Plasmodium berghei* Infected Mice

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Author's contributions

This work was carried out in collaboration between all authors. Author AJU designed the study with author ANCO, wrote the protocol, and wrote the first draft of the manuscript. Authors UAI, KNA and NAO managed the literature searches; analyses of the study performed the spectroscopy analysis. Authors CPO and MEO managed the experimental process and author AJU identified the species of plant. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

The effects of ethanol leaf extracts of *Spilanthes uliginosa, Ocimum basilicum, Hyptis spicigera* and *Cymbopogon citratus* on mice infected with malaria parasite was investigated. Eighty four (84) swiss mice of both sexes were used for the study. All the mice were passaged intraperitoneally with 0.2 ml parasitized blood suspension and parasitemia assessed by Geimsa stain thin blood films after seventy two hours. The mice were divided into 6 groups namely; A, B, C, D, E and F. Groups B, C, D and E were subdivided into three (3): B_1, B_2, B_3, C_1, C_2, C_3, D_1, D_2, D_3, E_1, E_2 and E_3. Both groups and subgroups contained 6 mice each. The subgroups were treated with the

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extracts of *Spilanthes uliginosa (Sw), Ocimum basilicum, Hyptis spiligera* and *Cymbopogon citratus* each for five (5) consecutive days with 200, 400 and 800 mg/kg body weight via oral intubation daily respectively. The results indicated a general significant ($P<0.05$) decrease in the average body weight of the parasitized untreated mice while the histological photomicrographs showed alterations in the liver architecture of parasitized untreated mice and restorative effects of all the plant extracts and standard drug on the liver architecture of the parasitized treated mice.

Keywords: Liver; malaria parasite; medicinal plants; oxidative stress.

1. INTRODUCTION

Malaria is a significant public health problem in many countries of the world most especially in the tropical and sub-tropical countries where majority bears the burden of the disease [1,2]. It is one of the six most killer diseases in the world today and causes an estimated 200 million infections each year [3,2], with more than 500 thousand deaths annually [4]. Nine out ten of these deaths are found in sub-Saharan Africa, where the disease is estimated to kill one child every 30 seconds [4]. In other areas of the world, malaria had caused morbidity and mortality on a large scale especially in the rural areas of some countries such as Asia and South America [5]. The control of malaria infection over the years, relies largely on two broad strategies; use of chemoprophylaxis and vector control. The alarming rate at which the parasites have developed resistance to currently used malaria control measures and the loss of chemotherapy constitutes the greatest threat to the control of malaria infection [6].

Liver is a target organ and primary site of detoxification and intense metabolism/ transformation of xenobiotics which also happens to be the major site of plasmodium infection [7,8]. The liver is an important organ involved during the hepatic stage of the malaria parasite's life cycle, where malaria sporozoites develop into merozoites [3]. Then, the merozoites are released into blood circulation and enter the erythrocytic stage. Malaria infection has been shown to cause surplus amount of free radical generation which promotes oxidative stress and damage to hepatic cells/architecture [9,10]. However, the role of oxidative stress during malaria infection is still unclear [5]. Some authors suggest a protective role, whereas others claim a relation to the physiopathology of the disease [11]. A recent study has affirmed that the generation of reactive oxygen species (ROS) and nitrogen species (RNS) associated with oxidative stress, plays a crucial role in the development of systemic complications caused by malaria [5]. The generated ROS and RNS by the malaria parasites if not checked by the host enzymic and non-enzymic antioxidants could lead to oxidative damage and there is increasing evidence that the latter contributes to pathophysiology of many diseases.

Several synthetic anti-malaria and antioxidant agents are commercially available; however, there can sometimes have some serious adverse effect [12]. Based on this therefore, it is necessary to search for alternative drugs for the treatment of the disease and to replace currently used drugs with uncertain safety and wholesomeness. The ethno-medicinal use of plants and plant products for the treatment of malaria could be a promising source of wholesome therapy [12]. In fact, the traditional medicine of Africa continent particularly West Africa constitutes an important source for ethnopharmacological investigations [13]. Locally, the following plants: *Spilanthes uliginosa (Sw), Ocimum basilicum, Hyptis spicigera* and *Cymbopogon citratus* have traditionaly been used for the treatment of many diseases including malaria.

This study assessed the effects of plant leaf extracts on the liver histology of mice infected with *Plasmodium berghei* parasite.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

Fresh leaves of *Spilanthes uliginosa (Sw), Ocimum basilicum, Hyptis spicigera* and *Cymbopogon citratus* were collected from Ogboji - Agoutu Ezzagu in Inyaba Development Centre of Ebonyi State, Nigeria. Dr (Mrs) K. C. Nnamani of the Department of Applied Biology of Ebonyi State University, Abakaliki graciously authenticated and identified the plants. Apparently healthy leaves of the plants were removed from plant stalk, rinsed with clean water and shade dried to a constant weight. The dried plant samples were ground to fine powder with grinding machine, packaged in glass jars and stored at 4ºC until analysis.

2.2 Extraction of Plant Materials

Exactly 150 g of powder samples of *Spilanthes uliginosa, Ocimum basilicum, Hyptis spicigera* and *Cymbopogon citratus* were soaked at 25ºC in 500 ml of absolute ethanol (analytical grade, 98%) each for 24 hours. They were filtered using whatman paper into a graduated beaker and exposed to mild heat at 40ºC in water bath to remove the ethanol until solid crude extracts were obtained in variable amounts depending on the plant. The obtained crude extracts were dissolved in normal saline and administered to experimental animals at different concentrations based on body weights.

2.3 Experimental Animals

Eighty four (84) Swiss mice aged 2 months weighing 17-34 g of both sexes were obtained from Chris King Animal Farm of Nnamdi Azikiwe University Awka, Anambra State and transferred to Animal House of Department of Biochemistry, Ebonyi State University, Abakalki. The animals were housed in metal cages under controlled conditions and acclimatized for 7 days under standard environment conditions and fed *ad-libitum* on their normal diets.

2.4 Rodent Parasite (*Plasmodium berghei* NK65)

The rodent parasite was sourced from National Institute for Medical Research (NIMR), Lagos, Nigeria and maintained alive in mice by continuous intraperitoneal passage in mice after every 5 days. The re-infected mice were moved to the Animal House of Department of Biochemistry, Faculty of Biological Science, Ebonyi State University Abakaliki where the study was carried out. Prior to the start of the study, one of the infected mice was kept and observed to reproduce signs of disease similar to human malarial infection.

2.5 Preparation of *Plasmodium Berghei* and Inoculation of Animals

The swiss mice were all inoculated by intraperitoneal (IP) injection with standard inoculums of *Plasmodium berghei* NK 65 with 1×10^7 infected erythrocytes. The *P. berghei* was subsequently maintained in the laboratory by serial blood passage from mice to mice every 5 days. Ten animals at a time were used as infected donors and as parasite reservoir. The donor mice were monitored for signs of infection which included lethargy, anorexia, ruffled appearance, shivering and heat-seeking behavior. Blood was taken from the third day (72 hours) via the cut tip of the tail to confirm level of parasitaemia in the donor mice, using the White Blood Cell (WBC) count method [2]. Blood collected from the tail of the infected donor mice was diluted with phosphate buffer saline pH 7.4 to produce standard inoculums of 0.2 ml containing $1x10^7$ *P. berghei* infected erythrocytes. Each mouse was inoculated intraperitoneally on day 0 with 0.2 ml of infected blood containing 1×10^7 *P. berghei*. Parasitaemia was assessed by thin blood film made by collecting blood from the cut tip of the tail and this was stained with Giemsa stain [12].

2.6 Experimental Design

The mice were injected intraperitoneally with standard inoculums of 1×10^7 *P. berghei* NK 65 infected erythrocytes on the first day. Seventy two hours later, the mice were divided into 6 groups namely; A, B, C, D, E and F of 6 mice each. Groups B, C, D and E were subdivided into three (3): B_1, B_2, B_3, C_1, C_2, C_3, D_1, D_2, D_3, E_1, E_2 and E_3. The subgroups were treated with the extracts of *Spilanthes uliginosa (Sw), Ocimum basilicum, Hyptis spiligera* and *Cymbopogon citratus* each for five (5) consecutive days with 200, 400 and 800 mg/kg body weight via oral intubation daily respectively. Two control groups, A and F were used. The negative control (A) was treated daily with 5 ml/kg normal saline while positive control group (F) was treated with 5 mg/kg body weight of chloroquine. All groups were given water and fed *ad libitum*. On the sixth day, mice were starved overnight, sacrificed and livers were collected for histological analysis.

2.7 Preparation of Liver

The representative samples of livers of mice were excised on day 7 post infection and were fixed in 10% formalin (Sigma, St. Louis, USA) for a minimum of 72h, stained with haematoxylin and eosin and microscopic examination for tissue degeneration was carried out.

2.8 Histopathological Examination of Liver [14]

This involved different method of processing the tissue for microscopic analysis and they include; dehydration, clearing /dealcoholization, wax

impregnation/infiltration, embedding, mounting on the wooden block, microtomy, heamatoxylin and eosin method of staining.

2.8.1 Dehydration

This involved passing the tissues through different grades of alcohol (analytical grade) to remove water. The different grades of alcohol included, 50%, 70%, 90% and 100% for 30 minute each.

2.8.2 Clearing/dealcoholization

The dehydrated tissues were cleared by removing the alcohol from the tissues by immersing them through 3 changes of xylene (Mayer and Beaker, England) for 20 minute each.

2.8.3 Wax impregnation/infiltration

The cleared tissues were impregnated/infiltrated to remove the clearing agent xylene in hot oven (Fisher, England, 175 models) at 60ºC by passing through 3 changes of paraffin wax for 30 minutes each.

2.8.4 Embedding

The infiltrated tissues were embedded with molten paraffin wax (Alex Refined Wax, Egypt) in an embedding mold and allowed to solidify.

2.8.5 Mounting on the wooden block

The paraffin block of tissues was attached to a wooden block with aid of a hot spatula held in between wooden block and paraffin wax. The spatula melts the wax which solidified when the spatula was removed.

2.8.6 Microtomy

The block of tissues was sectioned using rotary microtome (model 1434 England). The tissues were trimmed to obtain the cut surface at 15 µm and sectioned at 5 µm. The sectioned tissue was floated using hot water bath (Fisher, England) at 45ºC to expand and remove folds from the tissue. The section was picked with a clean slide and labeled for staining.

2.8.7 Heamatoxylin and eosin method of staining

The sectioned tissues were dewaxed in xylene for 15 minutes and then, rinsed in descending order of alcohol ranging from absolute to 50% for 2 seconds each. The tissues were washed in 2 changes of water and stained in haematoxylin (M and K Laboratory Chemical, England) for 5 minutes. Thereafter, the tissues were washed again in 2 changes of water, differentiated in 1% acid alcohol and washed again in water and blued in tap water. The tissues were counterstained in eosin (Merck, England) for 2 minutes, cleared in xylene and then mounted in dibutene putrine xylene (DPX) (Trust Chemical, England) and dried for histopathological analysis. The photomicrographs of the relevant stained sections were taken with the aid of a microscope (Olympus, BX3).

2.9 Statistical Analysis

The results obtained were expressed as mean ± S.D of 6 rats in each group. All the avearge body weights were subjected to statistical analysis using ANOVA. Differences between means were regarded significant at P<0.O5.

2.10 Results of Mean Body Weights (g) in Mice Treated with Ethanol Leaf Extracts

The results of the effect of leaf extracts of *S. uliginosa (Sw), O. basilicum, H. spicigera and C. citratus* on the body weights of the mice are showed in Table 1.

3. DISCUSSION

The results of average body weight showed that there was a general decrease in average body weight of the untreated mice on the 7th day relative to those treated (Table 1) and this observation could be attributed to malaria infection. Infection of animals with malaria parasite leads to loss of weight due to loss of appetite or blood quality of the animals [12] as well as generation of reactive oxygen species (ROS) by the parasites inside host erythrocytes [15]. The gain in weight of animals treated with leaf extract may be attributed to the presence of some metabolites found in the plant that reduce the level of malaria parasite and thereby increase appetite of the animals. These results contrasted with those reported by Haruna *et al*. [12]. These observations could be due to ameliorating effect of the plant extracts on acute fluid loss, proteolysis and lipolysis which is usually associated with weight loss in malaria infection [16].

Table 1. Mean body weight (g) of treated and untreated mice

Plant/Dosage (mg/kg)		Initial wt (g)	Final wt (g)
Spilanthes uligonosa (Sw)	A	33.00±1.73[b]	27.67±2.52[a]
	B1	20.00±3.56[a]	19.50±3.42[a]
	B2	20.25±2.87[a]	20.67±6.40[a]
	B3	17.50±4.51[a]	20.67±1.41[b]
	E	20.25±3.40[a]	20.50±1.73[a]
Ocimum basilicum	C1	18.50±4.04[a]	19.00±3.56[a]
	C2	17.75±4.57[a]	21.75±2.22[b]
	C3	21.50±2.38[b]	18.95±4.71[a]
Hyptis spicigera	D1	19.50±2.38[a]	19.25±03.59[a]
	D2	23.50±4.36[a]	22.75±3.86[a]
	D3	19.00±4.83[b]	17.25±4.24[a]
Cymbopogon citratus	E1	33.00±1.73[a]	32.00±2.00[a]
	E2	24.00±1.00[a]	25.00±2.51[a]
	E3	19.33±2.08[a]	19.33±1.52[a]

Liver is a targeted organ and primary site of detoxification as well as the major site of intense metabolism [17]. To this effect, it is therefore prone to various disorders as a consequence of exposure to the toxins of various forms [18,19]. Liver plays very important roles in metabolism to maintain energy level and structural stability of the body [19]. It is also site of biotransformation by which toxic compounds are transformed in less harmful form to reduce toxicity [7].

The histopathological examination of liver sections from parasitized untreated mice (negative control) revealed gross distortion of liver architecture with complete loss of cellular morphology (necrosis) marked by pronounced inflammatory changes (Fig. 1). The liver Damaged observed in parasitized untreated mice may be due to the generation of reactive oxygen species such as superoxide anion and hydrogen peroxide by the *Plasmodium berghei* leading to oxidative damage since liver is the site of action [20]. This observation is in agreement with the results of antiplasmodial, liver enzyme and antioxidant activities of the plants in which there is an increase levels of parasitaemia [2], ALP, AST, ALT and malondialdehyde (MDA) in the blood serum of parasitized untreated mice as well as decrease activities of superoxide dismutase (SOD), catalase (CAT) and glutathione perioxidase (GPX) [20]. The elevation of these parameters indicates liver injury and altered hepatocytic integrity as well as surplus amount of free radical generation. The observations of liver sections in treated mice showed less distortions of hepatic architecture suggesting attempts by the host at restoration of cellular morphology; this might have been aided by administered extracts of *S. uliginosa (Sw), O. basilicum, H. spicigera* and *C. citratus* (Fig. 2a-c, 4a-c, 5a-c and 6a-c*)* as well as chloroquine treated group (Fig. 3). These gross pathological presentations may help explain further the observed significant changes in the activities of enzymes as reported in our previous research [2]. Increased serum tissue enzymes have been associated with organ damage, particularly the liver [20]. The pathological changes in liver among other organs, could explain the increased enzyme concentrations in serum of infected mice. The effects of the extracts could be attributed to the presence of some phytochemicals and vitamins such as saponins, alkaloids, flavonoids, ascorbic acid, beta carotene and tocopherol etc in which the plants contained in variable amounts [2]. The result of the present investigation has shown that the leaf extracts of *S. uliginosa (Sw), O. basilicum, H. spicigera* and *C. citratus* are capable of producing biochemical alterations in malaria induced animals. However, the alterations appear to be more pronounced in infected animals treated with *Spilanthes uliginosa* (Sw) and *Hyptis spicigera.* The reversal of the histological alterations observed in the mice treated with the leaf extracts might be due to antioxidant property of the extract which preserved the hepatic tissue from possible *Plasmodium berghei*– induced oxidative stress which could lead to hepatic damage. The hepatic protective property of the leaf extracts may also be because of its other properties such as anti-inflammatory property which may prevent or inhibit inflammatory hepatic damage and anti-oxidant property thereby reducing the oxidative stress imposed by the *Plasmodium*. It could therefore be concluded that the administration of the extracts restored the damaged liver architecture of the mice caused by malaria parasite, *plasmodium berghei* in a dose dependent manner in some of the plant.

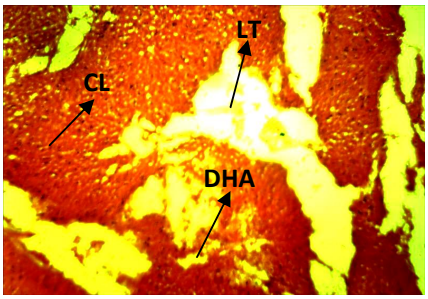

Fig. 1. Photomicrograph (X100) of section of the liver of *Plasmodium berghei* infected mice (5 ml/kg of normal saline) (negative control)
CI: Cell inflammation, DHA: Distortion of hepatic architecture, LT: Loss of tissues

The result of light photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 0 mg/kg of plant extract (5mg/kg of normal saline) showed that the hepatic architectures are distorted (DHA) with cell inflammation (CI) and extensive loss of tissue (LT) in some areas.

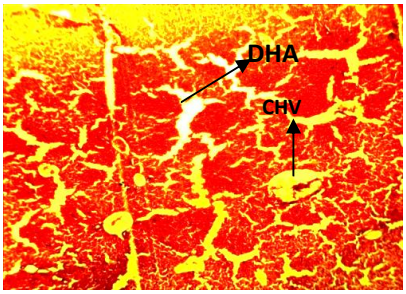

Fig. 2a. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 200 mg/kg of *Spilanthes uliginosa* (Sw)
CHV: Congestion of hepatic vessel, DHA: Distortion of hepatic architecture

The results showed congestion of hepatic vessels (CHV) and distortion of hepatic architecture (DHA).

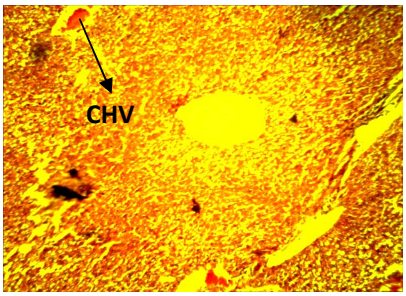

Fig. 2b. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 400 mg/kg of *Spilanthes uliginosa* (Sw)
CHV: congestion of the hepatic vessels

The results showed congestion of the hepatic vessels (CHV) and no hepatic distortion otherwise normal.

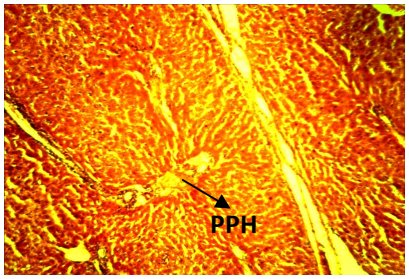

Fig. 2c. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 800 mg/kg of *Spilanthes uliginosa* (Sw)
PPH: Periportal hepatitis

The results showed periportal hepatitis (PPH) otherwise normal.

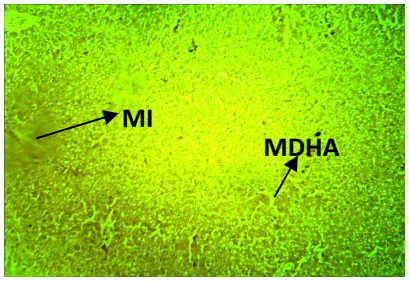

Fig. 3. Photo micrographs (X100) of the liver of *Plasmodium berghei* infected mice treated with 5 mg/kg of Chloroquine
MI: Mild inflammation, MDHA: mild distortion of hepatic architecture

The result showed that there is mild inflammation (MI) and moderate fatty change (MFC) with mild distortion of hepatic architecture (DHA).

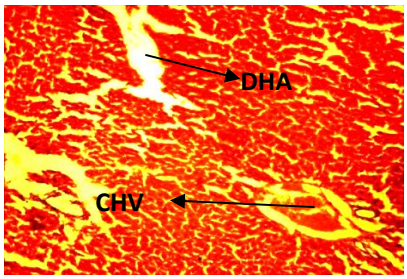

Fig. 4a. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 400 mg/kg of *Ocimum basilicum*

The result of light photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 400 mg/kg of *O. basilicum* showed that the

hepatic vessels are congested (CHV) and the hepatocytes are mildly distorted (DHA).

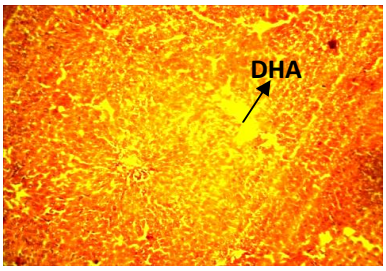

Fig. 4b. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 200 mg/kg of *Ocimum basilicum*

The result of light micrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 200 mg/kg of *O. basilicum* showed moderate infiltration of inflammatory cells (MIIC) and mild distortion of hepatic architecture (DHA).

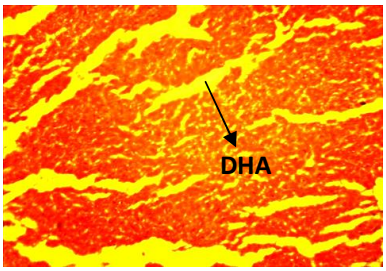

Fig. 4c. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 800 mg/kg of *Ocimum basilicum*
DHA: Distortion of hepatic architecture

The result of light photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 800 mg/kg of *O. basilicum* showed moderate to severe distortion of hepatocytes (DHP) with artifactual separation (AS)

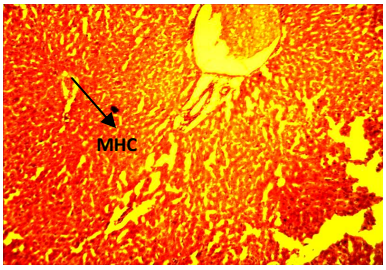

Fig. 5a. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 200 mg/kg of *Hyptis spicigera*
MIIC: Infilteration of inflammatory cells, MHC: Mild hepatic congestion

The result of light photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 200 mg/kg of *H. spicigera* showed that the hepatocytes are normal except mild hepatic congestion (MHC) otherwise normal.

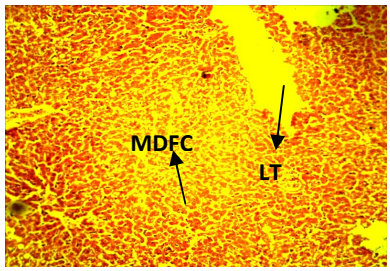

Fig. 5b. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 400 mg/kg of *Hyptis spicigera*
MDFC: Moderate fatty change, LT: Loss of tissues.

The result showed some degree of mild to moderate fatty change (MDFC) with focal loss of hepatic tissues (LT) otherwise normal.

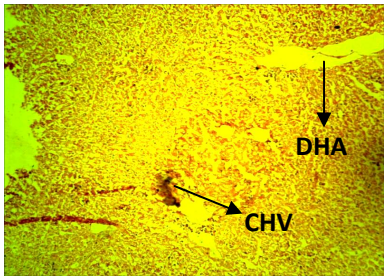

Fig. 5c. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 800 mg/kg of *Hyptis spicigera*
FC: Fatty change, CHV: Congestion of the hepatic vessels, DHA: Distortion of the hepatic architecture.

The result showed distortion of the hepatic architecture (DHA) and moderate congestion of the hepatic vessels (CHV).

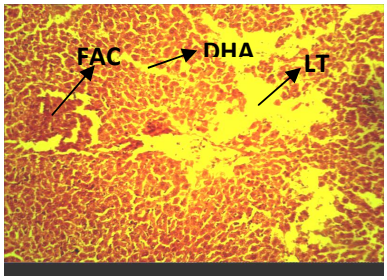

Fig. 6a. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 200 mg/kg of *Cymbopogon citratus*
FAC: Focal area of congestion, DHA: Distortion of hepatic architecture, LT: Loss of tissues

The result of light photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 200 mg/kg of *C. citratus* showed that the hepatocytes are healthy looking with focal area of congestion (FAC) and distortion of hepatic architecture (DHA) with loss of tissues in some areas (LT).

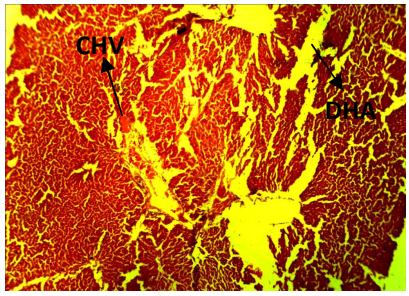

Fig. 6b. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 400 mg/kg of *Cymbopogon citratus*
CHV: Congestion of the hepatic vessels, DHA: Mild distortion of hepatic architecture.

The result showed congestion of the hepatic vessels (CHV) and mild distortion of hepatic architecture (DHA)

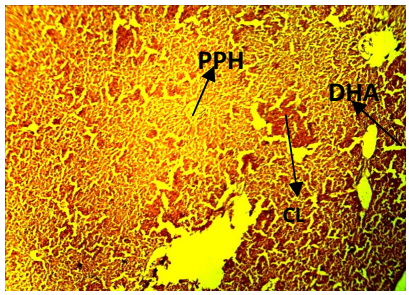

Fig. 6c. Photomicrograph (X100) of the liver of *Plasmodium berghei* infected mice treated with 800 mg/kg of *Cymbopogon citrates*
CH: Clumping of hepatocytes, PPH: Periportal hepatitis, DHA: Distortion of hepatic architecture.

The result showed a generalized clumping of the hepatocyte (CH) and there is perovascular or periportal hepatitis (PPH) round the vessel. There is distortion of hepatic architecture (DHA).

CONSENT

It is not applicable for this study.

STATEMENT OR INFORMATION ON ETHICAL ISSUES

The University does not give ethical clearance for research on experimental animal rather on human trial.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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