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Full-Length Research Article

Ethanol Extract of *Salacia nitida* Root Bark Ameliorates Lipid Peroxidation and Hepatosplenomegaly in *Plasmodium berghei* Malaria-Infected Mice

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Summary: The root bark of *Salacia nitida* L. benth (celastraceae) is used as a remedy for malaria and typhoid fever in the Southern part of Nigeria. This study is designed to evaluate the effect of treatment with ethanol extract from the root bark of *S. nitida* on lipid peroxidation, hepatomegaly, and splenomegaly in *Plasmodium berghei* malaria-infected mice. Thirty *P. berghei*-infected and six uninfected mice were used for the study. 280, 430, and 580 mg/kg b. w/day of ethanolic extract and 4 mg/kg b. w/day of artesunate were administered orally to infected mice in groups B, C, D, and E, while 4 ml/kg b. w/day of physiological saline was given to infected untreated mice in group A and the uninfected untreated mice in group F. Treatments were administered for five days. Levels of malondialdehyde were measured as means of assessing lipid peroxidation. Weights of experimental animals, liver, spleen were recorded, and the length of spleen was taken by planimetry. Animal's liver and spleen-body weight ratios were determined. Results from the study showed a significant decrease in levels of malondialdehyde and a significant increase in body weights. Significant decreases were observed in liver and spleen weights, lengths of the spleen, and organ-body weight ratios of malaria-infected treated mice. This study confirmed anti-plasmodic activity of ethanol extract of *S. nitida* root bark probably via reduced lipid peroxidation and hepatosplenomegaly in *P. berghei*-infected mice, as it is seen in its ability to attenuate lipid peroxidation and hepatosplenomegaly in mice.

Keywords*:* Hepatomegaly, Splenomegaly, Malaria, Lipid peroxidation, Organ-body weight ratio, *Salacia nitida*.

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INTRODUCTION

Malaria is one of the tropical diseases mostly reported worldwide. In 2008, malaria was reported to be responsible for 20% of childhood deaths in Africa (WHO, 2008). Malaria is caused by *Plasmodium* species that are introduced into the host's body by infected female *Anopheles* mosquitos. Malarial schizogony takes place in the liver at the pre-erythrocytic stage with the primary schizonts developing into the merozoites. In the erythrocytic phase, merozoites invade the erythrocytes, which manifest into malaria. Features of malaria infection that appear with haemolysis of erythrocytes include hepatomegaly, fever, malaise, splenomegaly, nausea, weight loss, etc. (Cheesbrough, 2004; Perlmann and Troye-Blomberg, 2007).

Malaria has been associated with hepatosplenomegaly (Cook, 1994). Hepatomegaly results from the congestion of the sinusoids (Bruce-Chwatt, 1978), haemozoin pigments, hepatic sinusoidal infiltrations and dilatation by lymphocytes, and abnormally increases in the number of kuffer cells (Lichtenberg, 1989; Kamal and Rodney, 1996). On the other hand, splenomegaly is due to the presence of cytoplasmic particles, parasites, toxins, and nuclear remnants that are left behind in the spleen as a result of erythrophagocytosis of old and deformed erythrocytes, vascular swellings, depositions of iron, and edema (Buffet *et al*. 2011; Dennis *et al*., 2012; Krucik, 2016), and hyperplasia of the lymphoid tissues (Marsden and Hamilton, 1969).

The production of oxygen free radicals by malaria parasites in the body cause lipid peroxidation, which is the oxidative deteriorations of polyunsaturated fatty acids (PUFA) of cell membrane phospholipids, causes nucleic acid injury and damages to protein structure (Prabha *et al*., 1990; Omodeo-Sale *et al*., 2003). Lipid peroxidation led to the formation of a mixture of lipid hydroperoxide and aldehyde end-products such as malondialdehyde (MDA) (Poli and Parola, 1997; Girotti, 1998), and causes some structural and functional alterations in red cell membranes such as changes in permeability and fluidity, leading to haemolysis (Chen *et al*., 1995), thus making hemoglobin available to the parasites for degradation. Proteolysis of haemoglobin by malaria parasites provide free amino acids for the parasite protein synthesis and haem in which Fe^{2+} is oxidized to Fe^{3+}, and electrons produced reacting with molecular oxygen to form oxygen radicals (Gamboa and Rosenthal, 1996; Francis *et al*., 1997) that lead to oxidative stress and inflammation. As a result of oxidative stress, lipid peroxidation and accelerated aging of erythrocytes occur, thus contributing to anaemia.

The dependence on medicinal plants for the treatment of malaria is gaining more recognition because of the lack of health facilities in the rural areas and the high cost of drugs. *Salacia nitida*, a member of the celastraceae family is one

of the medicines used traditionally to treat malaria in Nigeria. Decoctions of root bark of *S. nitida* are orally taken for treatments of malaria. The root bark of *S. nitida* has been reported to contained alkaloids such as spartein, lunamarine and ribalinidine, tannins, sapogenin, flavonoids such as epicatechin, catechin, rutin, and kaempferol, phenol, phytate, and anthocyanin which are bioactive phytochemicals (Nwiloh *et al*., 2016). The antimalarial activity of the root bark of *S. nitida* has been reported (Nwiloh *et al*., 2017). Therefore, this research is designed to study the amelioration of lipid peroxidation and hepatosplenomegaly in *P. berghei* malaria-infected mice treated with ethanol extract from the root bark of *S. nitida*.

MATERIALS AND METHODS

Chemicals and Drug: Chemical reagents, assay kits, and drug used for the study are of analytical grade. MDA assay kit was provided by Randox Laboratories Ltd (USA) and the antimalarial drug used was artesunate tablet, 50 mg (Artesunat ®) (Mekophar chemical pharmaceutical Joint-Stock Company, Vietnam).

Collection of plant material and preparation of extract: *Salacia nitida* was collected in February 2016, from Diidi farm in Nyogor-Beeri, Khana local government area in Rivers State, Nigeria, and the plant was identified by Dr. N. L. Edwin-Wosu of the Department of Plant Science and Biotechnology, Faculty of Science, University of Port Harcourt, Rivers State, Nigeria (voucher number UPHV-1033). The plants were uprooted and the roots were washed in clean water and air-dried under shade. The barks were removed from the root and reduced to smaller bits with machete onto a clean leather material and air-dried again under shade for one week and pulverized with a grinding machine (corona-16D).

Extraction was done according to the method reported by Nwiloh *et al* (2017), using Soxhlet extractor with 300 g of powder root bark material and 250 ml of ethanol at a temperature of 80^{o}C with a water bath for about 18 hours. The extract was then concentrated with a water bath regulated at 60^{o}C and dry extract stored in a refrigerator regulated at 4^{o}C until required for use. The dosages of ethanol extract from the root bark of *S. nitida* and artesunate used were calculated according to OECD (2000) and WHO (2015).

Experimental animals and Ethics: The study was conducted according to the United States National Institute of Health (NIH) "Guides on Care and Use of Laboratory Animals" (NIH, 1986) and the guidelines on the use of laboratory animals of the University of Port Harcourt (UPH/BCH/AEC/2016/015). Thirty-six (36) healthy Swiss mice of mixed-sex, weighing between 28g - 37g, and randomly selected out of eighty (80) healthy Swiss mice procured from the Department of Pharmacology, College of Medicine, University of Port Harcourt, were used for the study. Physical appearances and feeding behavior of the mice were used to ascertain their health status and were also confirmed malaria-free with rapid diagnostic test strips (Access Bio Inc, NJ, USA). They were housed in plastic cages and maintained under standard environmental conditions of humidity, room temperature of about 26.5 ^{o}C and, 12 hours' light/12 hours' darkness cycle, with free access to animal feed and clean water *ad libitum* for two weeks.

Acute toxicity test: An acute toxicity test was carried out according to Lorke (1983). Initially, twelve mice divided into three groups of four mice each were used. Doses of 100, 200, and 300 mg of the ethanol extract were orally administered to healthy mice in groups 1, 2, and 3 respectively. The mice were monitored for 24 hours for signs of toxicity. No toxicity was observed, so, a second phase was designed with another 12 mice, further divided into another three groups each containing four mice. The mice were orally given 400, 700, and 1000 mg of the ethanol extract and were monitored for another 24 hours for signs of toxicity. The geometric mean of the least dose that killed at least one mice and the highest dose that did not kill any mice was used to calculate the LD_{50} (Akhila et al., 2007).

Inoculation of mice: *Plasmodium berghei* (NK-65) obtained from the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos State, Nigeria, was used for the study. They were contained in five (5) donor mice and maintained at the World Bank-Assisted Malaria and Phytomedicines Research Laboratory, University of Port Harcourt. Before inoculation, levels of parasitaemia in donor mice were determined by cutting the tip of the tail of donor mice with sterile pairs of scissors and blood extruded into a small beaker containing 0.5 ml normal saline. A drop of diluted infected blood from the donor mice was placed on the rapid diagnostic malaria test strips (Access Bio Inc, NJ, USA) and the intensity of colour produced was used to ascertain the level of parasitaemia (Moody 2002; Cheesbrough, 2004; Tarazona *et al*., 2004). During inoculation, all donor mice with higher levels of parasitaemia (Moody 2002; Cheesbrough, 2004; Tarazona *et al*., 2004) were sacrificed by cervical dislocations, and blood was collected by cardiac puncture into a 50 ml beaker, using sterile disposable syringe and needle to avoid any variability in parasitaemia. 0.2 ml of parasitized blood from donor mice were added to 9.8 ml of normal saline. 0.2 ml of the infected diluted blood containing $1x10^{7}$ *P. berghei* infected red blood cells (RBC) obtained from donor mice were injected intraperitoneally into 30 experimental mice on day one (Akuodor *et al*, 2010), and randomly divided into five (5) groups labelled A, B, C, D, and E containing six (6) infected mice each, while another six (6) healthy mice were placed in group F to serve as a reference control.

Treatment of malaria-infected mice: Seventy-two (72) hours after infections, all the *P. berghei* malaria-infected mice were treated by oral administration with 0.2 ml of ethanol extract from the root bark of *S nitida* and artesunate according to the protocol stated below.

Group A - contained six (6) *P. berghei* malaria-infected mice were given 4 ml/kg b. w/day of physiological saline (negative control group, NC),

Group B - contained six (6) *P. berghei* malaria-infected mice were given 280 mg/kg b. w/day of ethanol extract from the root bark of *S. nitida*,

Group C - contained six (6) *P. berghei* malaria-infected mice were given 430 mg/kg b. w/day of ethanol extract from the root bark of *S. nitida*,

Group D - contained six (6) *P. berghei* malaria-infected mice were given 580 mg/kg b. w/day of ethanol extract from the root bark of *S. nitida*,
Group E- contained six (6) *P. berghei* malaria-infected mice were given 4 mg/kg b. w/day of artesunate (positive control group, PC), and
Group F - contained six (6) healthy mice were given 4 ml/kg b. w/day of physiological saline (reference control group, RC).

Treatments were done by 8.00 am once daily for five consecutive days, using oral metal gavage. The mice were also allowed free access to food (grower's marsh) and clean water *ad libitum*. At the end of the treatment period, all the mice were sacrificed by cervical dislocations, and blood was collected by cutting the jugular veins with sterilized steel disposable scalpel (Nwiloh *et al*., 2009; George *et al*., 2012).

Determination of weights of experimental animals: The experimental mice were weighed before and after treatments with a digital analytical weighing balance (Mettler Toledo Type BD6000, Mettler-Toledo GmbH, Greifensee, Switzerland), and the weights obtained were recorded in grams.

Determination of malondialdehyde (MDA): The serum from the blood that were pooled into labeled dry free sample bottles was used for evaluation of MDA. Malondialdehyde was determined according to the method in the manual that accompanies the assay kit by dispensing 0.4 ml of serum into each of the six labeled centrifuge tubes, and the same amount of distilled and deionized water was dispensed into a centrifuge tube labeled as blank. 1.6 ml of Tris-KCl buffer was pipetted into all the tubes, to which was added 0.5 ml of 30% trichloroacetic acid (TCA), and 0.5 ml of 0.75% thiobarbituric acid (TBA). Tubes were placed in a beaker containing ice cubes for about 5 minutes and then centrifuged at 3000 g for 15 minutes. Supernatants were decanted into cuvettes accordingly, and the absorbance read spectrophotometrically at 532 nm against the blank. The MDA was estimated as absorbance of test sample – absorbance of blank/1.56 x 10^5 M^{-1} cm^{-1}, and result obtained expressed as nmol MDA ml^{-1}.

Determinations of hepatomegaly and splenomegaly experimental mice: The sacrificed mice were dissected with t aid of a steel disposable scalpel, and the liver and spleen remov for assessment of levels of hepatomegaly and splenomegaly, usi a pair of scissors and placed on tissue paper. The liver and sple were then weighed with a digital analytical weighing balance, a their weights were recorded in grams. The length of each splee was planimetrically done and recorded in mm. Liver-body weigh (LBWR) and spleen-body weight ratios (SBWR) of experimental animals were calculated according to Lazic *et al*. (2020), using the formula;

Organ to body weight ratio =
Weight of organ (g)/body weight of the animal (g).

Data analysis: Results were expressed as mean values ± standard error of means (SEM). The data obtained were statistical analyzed using one-way analysis of variance (ANOVA) with the SPSS version 22 statistical package. Multiple comparisons were done using Scheffe's post hoc test to compare differences between results. Results were considered significant at 95% confidence level ($p < 0.05$).

RESULTS

Ethanol extract from the root bark of *S. nitida* was safe at the doses administered to experimental mice. The extract was not lethal to mice and no gross behavior or physical changes were observed in the experimental animals.

From Fig.1 below the level of MDA slightly increased in the malaria-infected mice in group A (NC) when compared to healthy mice in group F (RC). There was a decrease in MDA levels in the *P. berghei* malaria-infected mice in groups B treated with 280 mg/kg b. w/day of extract, C treated with 430 mg/kg b. w/day of extract, D treated with 580 mg/kg b. w/day of extract, and those in group E (PC) treated with 4 mg/kg b. w/day of artesunate.

From figure 2 below, it was observed that the body weights of *P. berghei* malaria-infected untreated mice in group A decrease significantly ($p < 0.05$) from day 1 through day 8 when compared to the healthy mice in the reference control group (group F). Weights of infected treated mice in groups B through E decrease significantly ($p < 0.05$) on day 3, and which significantly increased ($p < 0.05$) on day 8 after treatments with 280, 430, 580, and 4 mg/kg b. w/day of extract and artesunate compared to the *P. berghei* malaria-infected untreated mice in group A, and non-significantly ($p > 0.05$) compared with *P. berghei* malaria-infected treated mice in groups B through E on the 3rd day.

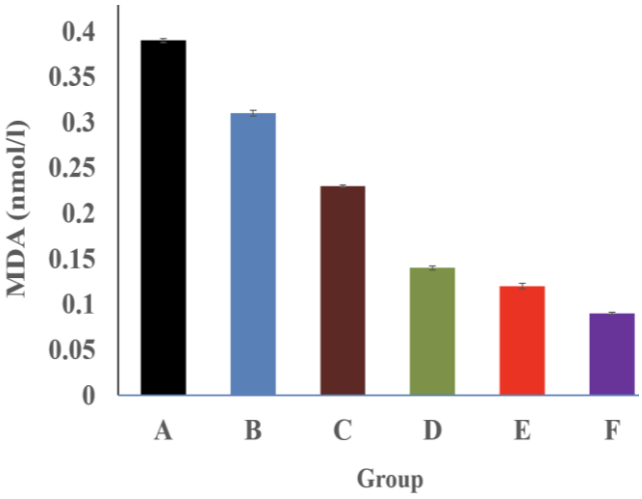

Figure 1:
Effect of ethanol extract of the root bark of *S. nitida* on MDA (nmol/l) in *P. berghei*-infected mice. n = 6. A = 4 ml/kg b. w/day of saline, B = 280, C = 430, and D = 580 mg/kg b. w/day of extract, E = 4 mg/kg b. w/day of ART, and F = 4 ml/kg b. w/day of saline.

The results for weights of liver and spleen in the experimental mice are displayed in figure 3. The figure showed a significant increase ($p < 0.05$) in the weights of liver and spleen in the group A (NC) mice when compared to healthy mice in group F (RC). There was a significant decrease ($p < 0.05$) in mean weights of liver and spleen in the *P. berghei* malaria-infected mice in groups B through E that were treated with different graded doses of extract from *S. nitida* root bark and artesunate.

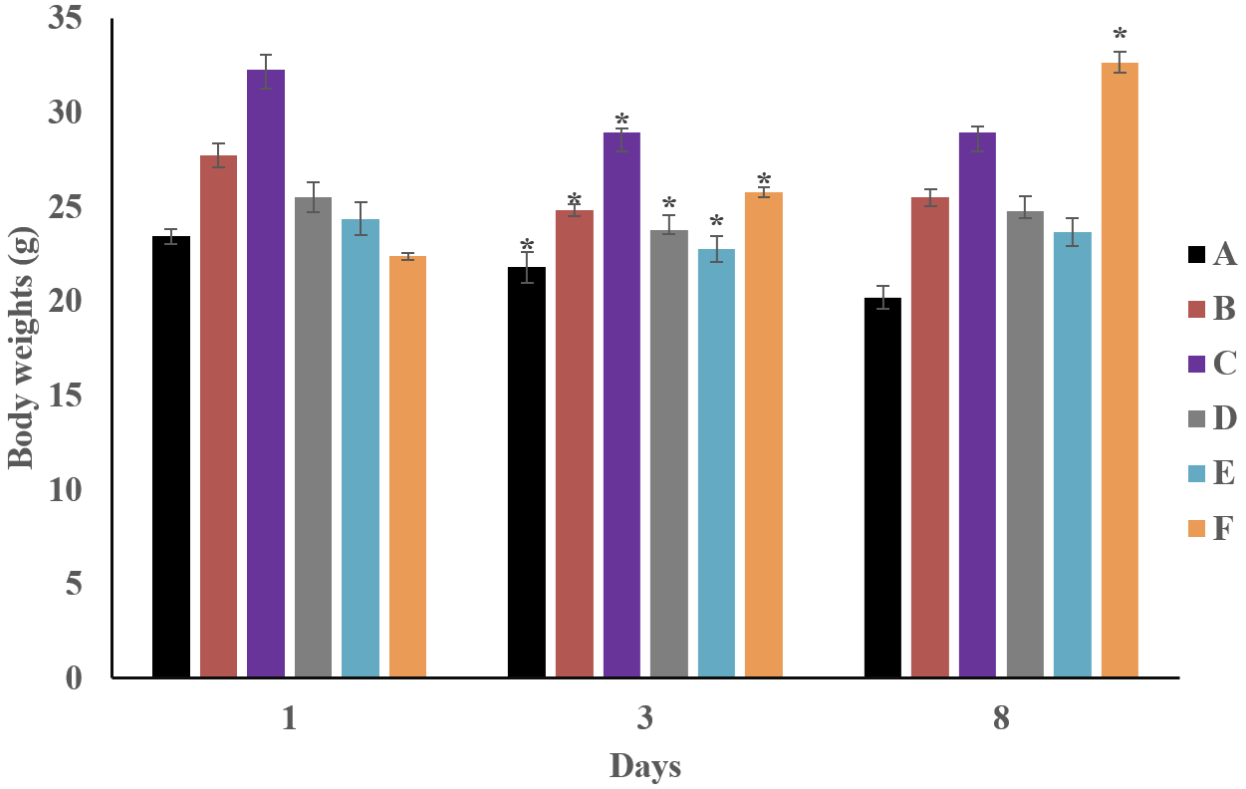

Figure 2:
Effect of ethanol extract from the root bark of *S. nitida* on body weights of *P. berghei* malaria-infected mice; n= 6. A = 4 ml/kg b. w/day of saline, B = 280, C = 430, and D = 580 mg/kg b. w/day of extract, E = 4 mg/kg b. w/day of ART, and F = 4 ml/kg b. w/day of saline; * = values are significant ($p < 0.05$).

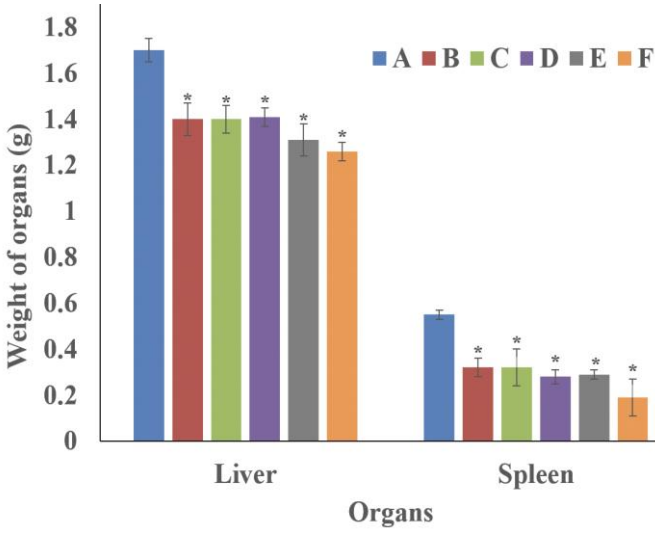

Figure 3:
Effect of ethanol extract from the root bark of *S. nitida* on weights of liver & spleen (g) in *P. berghei* malaria-infected mice; n= 6. A = 4 ml/kg b. w/day of saline, B = 280, C = 430, and D = 580 mg/kg b. w/day of extract, E = 4 mg/kg b. w/day of ART, and F = 4 ml/kg b. w/day of saline; * = values are significant ($p < 0.05$).

Results showing various length of spleens in the experimental mice investigated are shown in figure 4. The mean length of the spleen in malaria-infected mice in the negative control (group A) showed a significant ($p < 0.05$) increase when compared to those of healthy mice in reference control (group F). It was seen also from the figure that there is significant ($p < 0.05$) decrease in the length of spleens in the *P. berghei* malaria-infected mice treated with 280, 430, 580 mg/kg b. w/day of extract in groups B, C, D, and E that were given 4 mg/kg b. w/day of artesunate compared to those of the *P. berghei* malaria-infected untreated mice in group A and the healthy mice in group F.

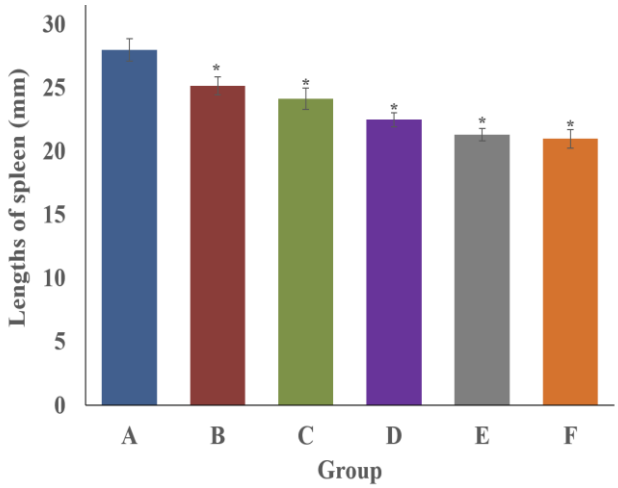

Figure 4:
Effect of ethanol extract from the root bark of *S. nitida* on length of spleen (mm) in *P. berghei* malaria-infected mice; n= 6. A = 4 ml/kg b. w/day of saline, B = 280, C = 430, and D = 580 mg/kg b. w/day of extract, E = 4 mg/kg b. w/day of ART, and F = 4 ml/kg b. w/day of saline; * = values are significant ($p < 0.05$).

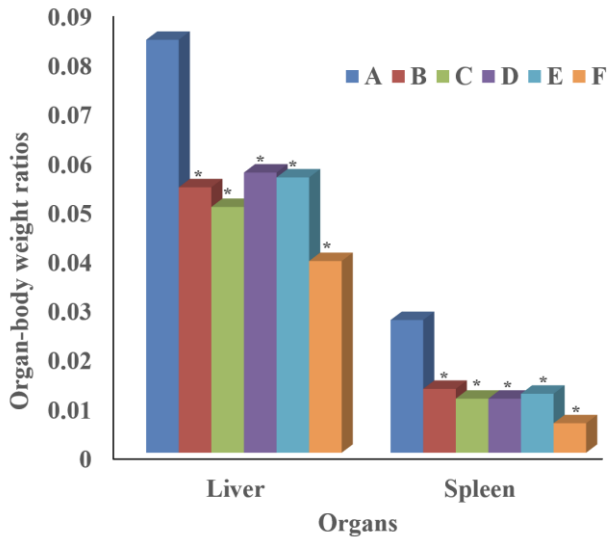

Figure 5:
Effect of ethanol extract of the root bark from *S. nitida* on organ-body weight ratios in *P. berghei* malaria-infected mice; n= 6. A = 4 ml/kg b. w/day of saline, B = 280, C = 430, and D = 580 mg/kg b. w/day of extract, E = 4 mg/kg b. w/day of ART, and F = 4 ml/kg b. w/day of saline; * = values are significant ($p < 0.05$).

It was observed from figure 5, that administration of extract and artesunate to the *P. berghei* malaria-infected mice in groups B through E brought about a significant ($p < 0.05$) decrease in both liver-to-body weight and spleen-to-body weight ratios (LBWR and SBWR) when compared to experimental mice in the negative control (NC) and reference control (RC) groups respectively.

DISCUSSION

The observed non-toxic effect of the extract is an indication that the root bark extract is very safe. Lipid peroxidation is one of the disorders that take place when malaria parasites infect erythrocytes (Omodeo-Sale *et al*., 2003), and malondialdehyde (MDA) is the biomarker of lipid peroxidation. The observed increase in MDAs in the malaria-infected untreated mice in group A might be due to the activities of malaria parasites in the RBCs, initiated by free radicals, and decreased antioxidant activity that resulted in inflammations of cell membranes (Kiklugawa *et al*., 1984). This increase could also be used as a biomarker in severe malaria diagnosis. It is also noticed that the increase in MDAs was ameliorated by the administration of ethanol extract from the root bark of *S. nitida*. The presence of phytochemical compounds like flavonoids, tannins, phenols, phytates, and anthocyanins with antioxidant and anti-inflammation activities, in the *S. nitida* root bark extract might be implicated in this study (Santos *et al*., 1998; Seeram and Nair, 2002; Okwu, 2004; Han *et al*., 2007; Buzzini *et al*., 2008; Oomah *et al*., 2008). Rutin and kaempferol, which are present in the extract (Nwiloh *et al*., 2016), are inhibitors of lipid peroxidations (Husain *et al*., 1987; Robak and Glyglewski, 1988). Also, to be implicated are the quinoline alkaloids present in the root bark extract, which are known metal chelators and antioxidants (Fernandez-Bachiller *et al*., 2010a).

The results obtained for the effect of ethanol extract from *S. nitida* root bark on body weights of experimental mice are in agreement with the report of Dikasso and colleagues (2006a), working with *Asparagus africanus*. Weight loss is one of the features of malaria and may be due to a reduced appetite for food (Perlmann and Troye-Blomberg, 2007).

Hepatomegaly and splenomegaly are also features of malaria (Cook, 1994). The observed increase in mean weights of liver and spleen, and length of the spleen in group A mice are shreds of evidence of liver and spleen enlargement (hepatomegaly and splenomegaly). Hepatomegaly observed in the group A mice might be due to sinusoidal infiltration with lymphocytes and congestion, and abnormally increase in the number of kuffer cells due to malaria parasites (Bruce-Chwatt, 1978), while the splenomegaly might also be due to the presence of cytoplasmic particles, malaria parasites, oxidized haemozoin and nuclear remnants left behind in the spleen as a result of erythrophagocytosis of old and deformed parasitized erythrocytes (Buffet *et al*. 2011; Dennis *et al*., 2012; Krucik, 2016). This finding is in tandem with the reports of Lawson and colleagues (1969), and that of Lacelle (1970), that abnormal erythrocytes encounter difficulty in traversing the cords and sinuses of the spleen, which result in congestion in the spleen. Splenomegaly also accelerates the clearance of old erythrocytes (Wyler *et al*., 1981; Looareesuwan *et al*., 1987). Hepatosplenomegaly in this study might also be caused by inflammation resulting from malaria. The dark-red colour and hardness of the liver and spleen might be due to inflammations and accumulation of malaria pigments in capillaries and sinusoids (Bruce-Chwatt, 1978). Enlargements of liver and spleen observed in this work are indications of hepatomegaly and splenomegaly as a result of *P. berghei* parasitized and damaged erythrocytes, anaemia, hepatic sinusoidal congestions, and splenic vein thrombosis (Buffet *et al*. 2011). The results obtained also showed that treatments of malaria in the infected mice in groups B through E, with ethanol extract from the root bark of *S. nitida* and artesunate, ameliorated the hepatosplenomegaly.

The organ-to-body weight ratio is another useful index for assessing the toxic effect of substances on the body (Nirogi et al., 2014; Lazic *et al*., 2020), that may cause diseases. The increase in liver-to-body weight and spleen-to-body weight ratios (LBWR and SBWR) recorded in this work for the *P. berghei* malaria-infected untreated mice in group A (NC) are indications of malaria infections in these mice. The decreased in LBWR and SBWR that was seen in the *P. berghei* malaria-infected treated mice in groups B through E, as a result of treatments with ethanol extract from the root bark of *S. nitida* and artesunate also supported the fact that the extract is antimalarial and anti-inflammatory in actions. These actions might be due to the presence of some pharmacologically bioactive compounds with antimalarial, antioxidant, and anti-inflammatory activities in the root bark of *S. nitida* extract. Hepatomegaly and splenomegaly were resolved once the infection was cleared, an observation that is in tandem with the reports of Greenwood (1987), Sowunmi *et al*. (2001), and Vennervald *et al*. (2005). Therefore, ethanol extract from the root bark of *S. nitida* ameliorates lipid peroxidation and hepatosplenomegaly in *P. berghei*-induced malaria-infected mice, which explains the reason for its common use traditionally in Southern Nigeria, for treatment of malaria.

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