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Nauclea latifolia Ethanolic Leaves Extract Moderately Hepatoprotects, but Decreases Periodic Acid-Schiff and Up-regulates Cytokeratin-7 Expression in Prophylaxis Malaria

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Abstract: Some herbal antiplasmodial plants have not been investigated for their hepatotoxic and glycogen lowering effects in experimental malaria models. We investigated the histochemical and immunohistochemical effects of ethanolic leaves extract of *Nauclea latifolia* (NL) an antiplasmodial plant on the liver of experimental prophylactic malaria mice. Twenty (20) mice (20-24 g) were grouped after acclimatization as follows: group A administered normal saline for 3 days, then inoculated with *Plasmodium berghei* (Pb); group B received 500 mg per kg body weight extract for 3 days then infected with Pb; group C received 1000 mg per weight extract for 3 days then infected with Pb; group D received 5 mg per kg body weight Artemether/lumefantrine (AL) for 3 days then infected with Pb. Extracts and drug were administered orally via oro-gavage needle. Inoculums of Pb at $1x10^6$ were injected intraperitoneally and were subsequently monitored for 72 hrs, then fasted over night, and humanely sacrificed with liver tissues excised and processed for light microscopy. Result of routine hematoxylin and eosin stain in Figure 1 revealed that group A had the most heptocellular distortions and inflammation with prominent hyperplasia compared to extract and drug treated groups (groups B - D); in figure 2 periodic acid-Schiff (PAS) showed that group A had moderate PAS expression of glycogen stores; extract groups had depleted glycogen stores compared to group D – the AL group had more glycogen stores. In figure 3 the cytokeratin-7 showed that groups A - C had moderate positivity, while group D was mild/lower. In conclusion NL ethanolic leaf extract is moderately hepatoprotective in a dose dependent manner, but decreased periodic acid-Schiff (PAS) expression of glycogen granules and moderately up-regulated cytokeratin-7 in experimental prophylaxis malaria mice.

Keywords: Hepatocytes, Malaria, Prophylaxis, Periodic acid-Schiff, Cytokeratin-7

Background of the Study

Malaria estimates indicate that 212 million cases occurred globally in 2015, leading to 429 000 deaths, most of which were in African children under the age of 5 (WHO, 2016) and artemther-lumefantrine (AL) is the drug of choice especially in African countries for the treatment of *falciparum* malaria (WHO, 2010). A three-day regimen of AL was efficacious and generally well tolerated in infants weighing <5 kg with uncomplicated *P. falciparum* malaria (Tiono *et al.*, 2015).

Several species of plants are routinely applied in traditional medicine as prophylactics or curative agents for many diseases in Africa (Farombi, 1993). *Nauclea latifolia* (NL) leaves were cited at 50% frequently by traditional healers as possessing antiplasmodial activity (Igwe *et al.*, 2012). *Nauclea latifolia* SM (Rubiaceae) extract exhibited the least activity in the assay with an IC_{50} value of 478.9 mg/ml compared to Chloroquine (Ajaiyeoba *et al.*, 2004), and has been reported to inhibit the erythrocytic cycle of *Plasmodium falciparum* (Akubue and Mittal, 1982).

Hypoglycaemic and antihyperglycaemic potentials of the aqueous and ethanolic extracts have been reported to be comparable to that of glibenclamide at a dose of 1mg/kg (Gidado *et al.*, 2008), and 250 mg/kg body weight of ethanol extract and fractions at same dose of *Nauclea latifolium* leaves possess hypoglycemic activity (Effiong *et al.*, 2013). Yet there is no available study on the glycogen expression in

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experimental malaria models treated with this widely used plant.

Glycogen is especially abundant in the liver where it may constitute as much as 7% of the wet weight, these hepatocytes glycogen is found in large granules, which are themselves clusters of smaller granules composed of single, highly branched glycogen molecules with enzymes responsible for the synthesis and degradation of glycogen (Nelson and Cox, 2008). The liver is the major site of drug metabolism, where it is converted from fat-soluble to water-soluble substances created in the urine or bile, a process mediate by group of mixed enzymes (Kumar and Clark, 2012).

Most histopathological Departments in Nigeria do not routinely perform special stains (Orah *et al.*, 2016). The period acid-Schiff (PAS) stain is useful for identifying glycogen (Krishna, 2013) and liver biopsy has become established gold standard for the diagnosis and staging of liver diseases (Saleh and Abu-Rashed, 2007). This study was designed to histologically investigate the expression of glycogen and cytokeratin-7 in the liver of experimental prophylaxis malaria mice.

Methodology

Experimental Animals

Twenty (20) male Swiss albino mice weighing 20 - 24g were obtained from the Faculty of Basic Medical Sciences animal house, University of Uyo, Nigeria. Acclimatization was for two weeks before the start of the investigation at the institution's animal holding room, in well ventilated mice cages and maintained under controlled environmental conditions of temperature 25 ± 5^{o}C and 12 hour light/dark cycle. Animals were fed with rat mash (Vital Feeds from Grand Cereals Limited, Jos, Plateau State), and had access to water *ad libitum.* This study conformed to the guide for the care and use of laboratory animals (National Institute of Health, 2011), approved by the Department of Anatomy Ethical Committee, University of Uyo.

Plant Collection, Identification and Authentication

A batch of *Nauclea latifolia* fresh leaves was obtained from the medicinal farm of Pharmacology and Toxicology Department, University of Uyo, identified and authenticated by the Curator at the Herbarium of Department of Pharmacology and Toxicology, University of Uyo with specimen deposited and voucher number UUH/67 (g) obtained.

Ethanolic Extraction

Fresh leaves of *Nauclea latifolia* were macerated in 95% ethanol (Sigma Aldrich St Louis USA) in a flat bottom flask was kept for 72 hrs at room temperature and filtered, the filtrate was concentrated to dryness in a water-bath at 45^{o}C, the yield was calculated and the extract stored in the refrigerator for later use.

Malaria Prophylaxis Evaluation and Experimental Design

Prophylaxis was studied via the repository activity of the ethanolic plant extract and Coartem® (Artemether/Lumefantrine) as described by Peters (1965) with modification (data obtained for parasitemia was published elsewhere). Five mice were randomly divided into four groups each; Group 1 acted as the negative control and received 10 ml/kg of normal saline (0.9% Nacl), group 2 received 500 mg/kg of ethanolic leaf extract, group 3 received 1000 mg/kg of ethanolic leaf extract and group 4 received 5 mg/kg of Coartem®. Administration of the extract and drug was for three consecutive days (D0 – D2). On the fourth day (D3) the mice were inoculated with *P. berghei.*

Parasite Inoculum

P. berghei was obtained commercially from National Institute of Medical Research (NIMER), Yaba, Lagos Nigeria in three host mice, and each mouse was inoculated intraperitoneally with 0.2 ml of infected blood containing about 1x10^{6} *Plasmodium berghei* parasitized erythrocytes. Preparation was by determining both the percentage parasitaemia and the erythrocytes count of the donor mouse and diluting the blood with isotonic saline in proportions indicated by both determinations (Odetola and Basir, 1980).

Experimental Treatments

Coartem® Novartis (Artemether/lumefantrine) was purchased from a reputable pharmacy within Uyo metropolis, was utilized for the study at dose according to method by Olorunnisola and Afolayanin (2011). *Nauclea latifolia* ethanolic leaf extract was dissolved in 20% Tween® 80 and administered orally based on body weights and in accordance to 10% and 20% of LD_{50} for low and medium dosage respectively.

Animal sacrifice and tissue processing

Xylazine and ketamine cocktail was injected to mice at 0.2 ml, when animals lost consciousness and unresponsive to tail compression, alcohol pad was used to sterilize the trunks, mid line insertion made, then intracranial perfusion via cardiac puncture with buffered saline to flush out the blood for 1 min via left ventricle, and released circulating fluid through right atrium, and later buffered formalin was released via a drip-set controlled by a valve until mice tail was

stiff, after that brains were dissected out, dried on a filter paper, weighed and fixed in 4% paraformaldehyde for immunohistochemistry. Plastic embedded brain sections were also processed for light microscopy by method as described (Cardiff *et al*., 2008). The immunolabelling of glial fibrillary acidic protein (GFAP) according to method described (Faddis and Vijayan, 1988; O'Callaghan and Sairam, 2005) was cut at 5 µ thick. Sections were allowed to heat on hot plate for 1 hr, and then transferred to xylene, alcohols and water respectively. Antigen retrieval method was performed using citric acid solution pH 6.0 in a pressure cooker for 15 mins. Sections were exposed to running tap water for 3 mins. Peroxidise blocker was used on the sections for 15 minutes and then washed for 2 mins with phosphate buffered saline (PBS) with tween 20. Protein blocker was carried out with Novocastra® protein block for 15 mins and then washed with PBS for 2 mins, and incubated with primary antibody, monoclonal mouse anti-glial fibrillary acidic protein (GFAP) by DAKO 1:100 dilution for 45 mins, washed in PBS for 3 minutes, and added rabbit anti-mouse secondary antibody for 15 mins all at room temperature. Tissue section was then washed 2x with PBS. Polymer was thereafter added and allowed for 15 mins, washed 2x with PBS and then added the diaminobenzidine (DAB) chromogen diluted 1:100 with the DAB substrate for 15 mins, and washed with water, and counterstained with haematoxylin for 2 mins. Tissue section were washed again, dehydrated, cleared and mounted in DPX mountant.

Results

Histological findings

In figure 1 is shown routine H&E staining which revealed that group A (placebo treated and infected) had prominent cellular infiltrations (hyperplasia) and diffuse inflammation around the portal triad, with signs of eosinophilic cytoplasm, darkly stained nuclei and hypertrophied hepatocytes (inference - severely affected); group B (500 mg per kg *Nauclea latifolia* extract, then infected with *P. berghei*) had a moderate hyperplastic cells with pale looking cytoplasm (inference - moderately affected); group C (1000 mg per kg *Nauclea latifolia* extract, then infected with *P. berghei*) had mild to moderate hyperplasia, polymorphic nuclei and eosinophic cytoplasm (inference - mildly affected); group D (treated with 5 mg per kg artemether/lumefantrine, then infected) had near normal hepatocyte cytoarchitecture (inference - appears normal).

Histochemical effect presented in figure 2 (special staining with periodic acid-Schiff (PAS)) for glycogen store expression indicates that group A (placebo treated and infected) had moderate expression; group B had little or no PAS expression; group C had mild to moderate PAS expression; group D (treated with artemether/lumefantrine, then infected) had high PAS expression.

Immunohistochemical expression of cytokeratin – 7 in the experiment indicates that group 1 had moderate expression; group 2 and 3 had moderate expression while group 4 had mild expression

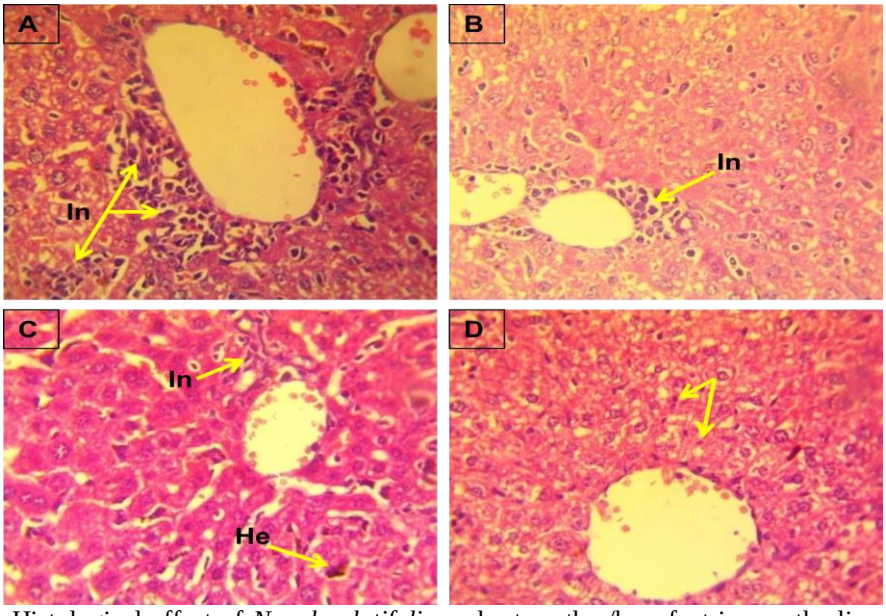

Figure 1 (A - D) Histological effect of *Nauclea latifolia* and artemether/lumefantrine on the liver of experimental prophylaxis malaria mice

He – Hemozoin, In – Inflammation with hyperplasia,

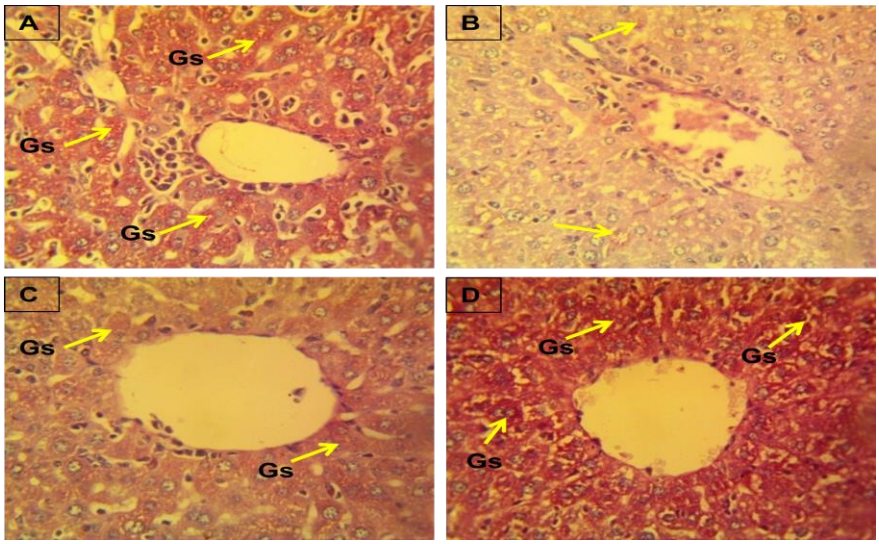

Figure 2 (A - D) Histochemical effect of *Nauclea latifolia* and artemether/lumefantrine on liver of experimental prophylaxis malaria mice stained with Periodic acid-Schiff at x400 mag.
Gs – Glycogen stores

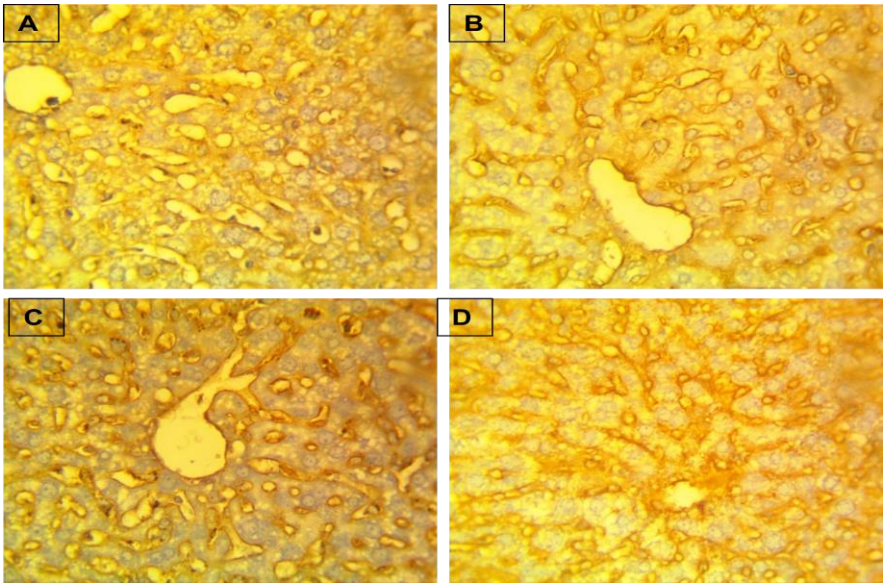

Figure 3 (A - D) Cytokeratin-7 expression in liver of experimental prophylaxis malaria mice following administration of *Nauclea latifolia* and artemether/lumefantrine at x400 mag.

Discussion

Malaria infections may cause vital organ dysfunction and death, and severe malaria is defined by clinical or laboratory evidence of vital organ dysfunction and nearly all deaths from severe malaria are due to infection with *Plasmodium falciparum* (WHO, 2012). Common histopathological findings of the liver in *P. falciparum* malaria include reactive Kupffer cells, retention of haemozoin pigment and minimal PRBC sequestration (Whitten *et al.*, 2011; Rupani and Amaraparkar, 2009). An ultra-structural study reported an association between high PRBC load in the liver of malaria pigments with jaundice, hepatomegaly and liver enzyme elevation (Prommano *et al.*, 2005). Experimental prophylaxis malaria in figure 1 group A (placebo treated and infected) had prominent cellular derangements and distortions signs of severe infection and diffuse inflammation which without prolong treatment can lead to mortality. Visible macroscopic changes (stiffness, viscosity and fracture toughness) are very strongly correlated with the increase in collagen accumulation, and decrease in deposited glycogen at the microscopic level. Also the largest changes in mechanical and histological properties occur after the first 11 – 17 h of preservation (Yarpuzlu *et al.*, 2014).

Figure 1 group B (500 mg per kg *Nauclea latifolia* extract, then infected with *P. berghei*) had a moderate hyperplastic cells with pale looking cytoplasm; while this distortion decreased in group C (1000 mg per kg *Nauclea latifolia* extract, then infected with *P. berghei*) and not detected in group D (treated with 5 mg per kg artemether/lumefantrine, then infected). Edagha *et al*., 2014 reported that at dose of 1000 mg/kg body weight of mice, ethanolic leave extract of NL was hepatoxic in non-infected healthy mice, and no mortality at dose up to 5000 mg/kg in acute toxicity study, and but exerted erythropoietic and hepatoprotective effects in *Plasmodium berghei*-infected Swiss mice which confirms the finding in this study. Antioxidant and antiplasmodial activities of aqueous extract of leaf of NL at 200 and 300 mg/kg body weight of Swiss mice infected with *P. berghei* has been reported (Onyeasom *et al*., 2015). In this study figure 1D showed AL prophylaxis had no hyperplastic cells, with no signs of inflammation and normal appearing hepatocytes.

After conventional tissue preparation of the liver using HE stain, glycogen is usually removed from the hepatocytes, thus the hepatocytes are diffusely swollen with pale cytoplasm and accentuated cell membranes, frequently displaced nuclei to cell periphery, the sinusoids are compressed by swollen hepatocytes and glycogenated nuclei and giant mitochondria (Julian *et al*., 2015). Glucose production after 16 h of fasting was 15 % higher in malaria patients than in control and the relative decrease in glycogenolysis is independent of the absolute rate of glycogenolysis and the regulation of glycogenolysis during fasting seems not preferentially dictated by glycogen content but driven by the necessity to maintain glucose output and euglycemia (Sprangers *et al*., 2004). Histochemical effect presented in figure 2 (special staining with periodic acid-Schiff (PAS)) for glycogen store expression indicates that group A (placebo treated and infected) had moderate expression; group B had little or no PAS expression; group C had mild to moderate PAS expression; group D (treated with artemether/lumefantrine, then infected) had high PAS expression. Therefore bioactive principles in the extract might be responsible to the marked decline in the levels of glycogen granules stained by PAS. Intracellular accumulation of excessive glycogen in the liver occurs as a manifestation of metabolic dysfunction or drug-induced toxicity in humans or animals, and traditional method for detecting intracellular glycogen utilizes periodic acid-Schiff (PAS) reaction, with or without diastase digestion (Gates *et al*., 2016), and PAS distribution of glycogen is usually reasonably uniform. Pathological excessive glycogen accumulation in hepatocytes is characterized by hepatomegaly and transient elevation in liver transaminases (Julian *et al*., 2015).

Immunohistochemical structural elements of the liver can be studied be studied using keratin 7 antibody (Lopez-Panquera, 2013). Intensity of Cytokeratin – 7 expressions in figure 3 indicates that group A had moderate expression; group B and C had moderate expression while group D had mild expression. The bioactive molecules of this extract might be responsible for the alteration in the protein expression. Many drugs impair liver function, these drugs are usually classified as being either predictable (dose-related) or non-predictable (not dose-related). However there exist some overlap and at least six mechanisms involved in liver damage; (i) disruption of intracellular calcium homeostatsis, (ii) disruption of bile canalicular transport mechanism, (iii) formation of non-functioning adducts (enzyme-drug) which then, (iv) presents on the surface of the hepatocyte as new immunogens (attacked by T cells), (v) induction of apoptosis, (vi) inhibition of mitochondrial function, which prevents fatty acid metabolism and accumulation of both lactate and reactive oxygen species (Kumar and Clark, 2012)

Conclusion

In this study, NL-ethanolic leaf extract offered moderate hepatoprotection in a dose dependent manner in experimental prophylactic malaria mice model, but markedly decreased periodic acid-Schiff (PAS) glycogen expression in the liver, as well as moderately up-regulated cytokeratin-7 expression when compared to *Plasmodium berghei* infected group and AL-treated groups, therefore dose and duration of the extract in prophylactic malaria must be with caution.

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