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Hippocampal Immunohistopathological and Blood Morphological Effects of *Nauclea latifolia* Extract and Artemether-Lumenfantrine in Suppressive Malaria Mice Model.

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ABSTRACT

The effect of ethanolic leave extract of *Nauclea latifolia* and Artemether-Lumfantrine was investigated in a suppressive model of malaria on the hippocampus of Swiss albino mice. Mice were infected intraperitoneally with *P. berghei* 1 x 10^7 parasites, and then treated within an hour with Nacl 0.9% 10ml/kg for control; and 5 mg Artemether-Lumefantrine per kg body weight of the mice for test 3 days. Organosomatic index, thick blood smear, parasite density, hippocampal histomorphology and immunolabeling of glial fibrillary acidic protein were assessed. Result show the organosomatic index was not statistically significant ($p>0.05$) when treated groups were compared with control; the parasite density decreased significantly ($p<0.05$) in a dose dependent manner in the extract as well as Artemether-Lumefantrine compared with the control; hyperparasitemia observed in untreated group moderately declined in the extract treated but was completely cleared in Artemether-Lumefantrine group; the histomorphology of hippocampal sections stained with haematoxylin and eosin showed severe neuronal shrinkage and distortion in the untreated groups compared to treated groups, while the immunolabelling of glial fibrillary acidic protein (GFAP) expression indicated reactive astrogliosis by the intensely stained cells in all groups, but marginally reduced at 500 mg/kg of the extract. In conclusion the suppressive malaria model in mice provides evidence that phytochemicals in ethanolic extract of *Nauclea latifolia* leaf moderately reduces *P. berghei* at 1000 mg/kg in slight comparison with Artemether/Lumefantrine at 5 mg/kg, and it offered minimal neuroprotection against the histormorphological distortion of the hippocampus, astrocyte swelling and intense glial fibrillary acidic protein expression due to *P. berghei* infection.

Key words: *Nauclea latifolia*, Artemether-Lumefantrine, *Plasmodium berghei*, Gliosis

INTRODUCTION

Current practice in treating malaria is based on the concept of combination therapy, and Artemether-Lumefantrine is the drug of choice [1]. However, evidence of artemisinin-resistant malaria has been reported [2,3]. Hence, more concerted effort is required towards the possible discovery of new antimalarial therapy and here nature's flora provides huge ethnopharmacological benefits. Bioactive agents from plants are being continuously extracted and is one of the most intensive areas of natural product research today, yet the field is far from being exhausted [4]. Medicinal plants contain substances that can be used for therapeutic purposes, and serves as precursors for the synthesis of useful drugs or nutrients [5].

Nauclea latifolia plant (NL) possess evidence of antiplasmodial activity in the leaves [6,7,8], in the roots [9,10], and in the fruits [11]. Malaria in children has be reported to induce cognitive deficits [12], with the likelihood of affecting the hippocampus. The aim of this study is to investigate the histomorphological effects of ethanolic leaf extract of *Nauclea latifolia compared with Arthemeter-Lumefantrine on* the hippocampus, blood morphology and organo-somatic index in *Plasmodium berghei* infected Swiss albino mice (*Mus musculus*).

MATERIALS AND METHODS

Experimental Animals

Twenty (20) male Swiss albino mice were obtained weighing 20 - 24g from the animal house of the Faculty of Basic Medical Sciences, University of Uyo, Nigeria. Mice were acclimatized for two weeks prior to the start of the experiment in the institution's animal holding room in well ventilated cages and maintained under properly controlled environment with temperature 28±5°C and 12 hourly light/dark cycles. All the animals were allowed access to feed (rat mash; Vital Feeds from Grand Cereals Limited, Jos, Plateau State), and water *ad libitum*. All procedures involving animals in the study conformed to the guiding principles for the care and use of animals in research and training [13] and granted approval by the Department of Anatomy ethical committee, University of Uyo.

Collection and Authentication of Plant Sample
Fresh leaves of *Nauclea latifolia* obtained from the medicinal farm of the Department of Pharmacology and Toxicology, University of Uyo were identified and authenticated by the Curator at the Herbarium of the University of Uyo with specimen voucher number UUH/67 (g) deposited.

Plant Extraction
Fresh leaves of *Nauclea latifolia* macerated in 95% ethanol (Sigma Aldrich St Louis USA) in a flat bottom flask and kept for 72 hours at room temperature. The filtrate of the macerated leaves was concentrated in water-bath at 45 degree Celsius to dryness. Extract residue was weighed and stored in the refrigerator until required for use.

Parasite Inoculation
P. berghei was obtained from National Institute of Medical Research (NIMER), Yaba, Lagos Nigeria and inoculated in mice intraperitoneally at 0.2 ml injection of infected blood containing approximately about 1×10^7 *Plasmodium berghei* parasitized erythrocytes [14].

Drug administration
Artemether-Lumefantrine anti-malarial drug Coartem® Novartis purchased from a reputable pharmacy within the Uyo metropolis was dissolved in distilled water and dosage administered according to method by [15]. The ethanolic extract was dissolved in 20% Tween® 80, and administered via oro-gavage intubation based on body weights using a predetermine dosage at 10% and 20% of the LD_{50} as low and medium respectively, which was relatively safe at 5000 mg using Lorke's method (data not reported here). At end of experiment parasitemia was determined using thick blood smears stained with Giemsa [16]. Stained thick blood smears obtained from the tail vein of the mice was made and viewed under oil immersion at x100 magnification; thereafter images on plates were obtained using a digital microscopic camera, and the parasites counter per field with a hand held counter.

Evaluation of Suppressive Activity (4-day test)
Suppressive activity was used to evaluate the schizontocidal activity of the extract and Coartem® against early *P. Berghei* infection in mice as described by [17]. Twenty mice randomly divided into four groups of five mice each on the first day (DO), were infected with the parasite. Group 1 served 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 which received 5 mg/kg of Coartem® served as the positive control. On the fifth day (D4), thick blood film was made from tail blood. The film was then stained with Giemsa stain to reveal parasitized erythrocytes in the field of the microscope.

Animal sacrifice
Animals were humanely sacrificed by a cocktail injection of xylaxine/ketamine 0.2ml. Once unconscious without response to motor stimulation, intracardiac perfusion of whole body was achieved with the release of buffered saline and buffered formalin respectively via a drip set-up controlled by a valve, and then allowed to fix for at least 72 hours before they were dissected out and dried on a filter paper. Brain specimens were then processed to reveal the structures required for investigation.

Haematoxylin and Eosin Routine Staining
Sections were processed for light microscopy by method as described [18]. Paraffinized tissue was cut at 5 microns thick and processed for immunolabelling of glial fibrillary acidic protein (GFAP) according to method described [19]. Sections were allowed to heat on hot plate for 1 hour, then transfered to xylene, alcohols and water respectively. Antigen retrieval method was performed using citric acid solution pH 6.0 in a pressure cooker for 15 minutes. Sections were exposed to running tap water for 3 minutes. Peroxidise blocker was used on the sections for 15 minutes and then washed for 2 minutes with phosphate buffered saline (PBS) with tween 20. Protein blocker was then performed with Novocastra® protein block for 15 minutes and then washed for 2 minutes with PBS, and incubated with primary antibody e.g., goat anti-Glial fibrillary acidic protein (GFAP) 1 in 100 dilution for 45 minutes, washed in PBS for 3 minutes and later added rabbit anti-goat secondary antibody for 15 minutes all at room temperature. Tissue section was then washed twice with PBS. Polymer was thereafter added and allowed for 15 minutes, washed twice with PBS and then added the diaminobenzidine (DAB) chromogen diluted 1 in 100 with the DAB substrate for 15 minutes, and then washed with water and counterstained for 2 minutes in haematoxylin. Again the tissue section was washed, dehydrated, cleared and mounted in DPX mountant.

Statistical Analysis
The data obtained was expressed as Mean ± Standard Error of Mean (SEM) and further analyzed using one-way analysis of variance (ANOVA) to determine the difference between the experimental groups and the control group, and the post-hoc test (Student-Newman Keuls) for comparison and values ($p<0.05$) was regarded as significant.

RESULTS
The result is as presented in (Tables 1 to 2) and in (Figures 1 to 3). Table 1 shows the effect of ethanolic leaf extract of *N. latifolia,* and Artemether-Lumefantrine on organosomatic index of *P. berghei*-infected mice was not statistically significant ($p>0.05$) when treated groups were compared with the control. Table 2 shows the antiplasmodial effect of treatment on parasite density; which statistically decreased ($p<0.05$) in a dose dependent manner in the extract as well as

Artemether-Lumefantrine compared with the control. Figure 1 (A to D) is the photomicrograph of Giemsa stained thick blood smear of *P. berghei*-infected mice treated with two dose levels of *N. latifolia*, and Artemether-Lumefantrine; 1A show high parasitemia with dark chromatin dots and pink cytoplasm; 1B also show high parasitemia; 1C show low parasitemia, and 1D show no detectable level of parasitemia. Figure 2 shows changes in the histomorphology of paraffinized hippocampal sections stained with haematoxylin and eosin of P. berghei-infected mice treated with N. latifolia and Artemether-Lumefantrine; 2A showed pale staining neuropil but intensely stained neuronal cell bodies, severe neuronal shrinkage, foci pyknosis and minor vacuolations (severely affected); 2B showed pale staining neuropil, moderate neuronal shrinkage, decrease cellular density and minor vacuolation (severely affected); 2C showed pale staining neuropil, foci vascular plugging and vacuolations (moderately affected) and 2D showed pale neuropil with foci instensely stained pyramidal neurons, neuronal atrophy and compact cellular layer with minor loss of cellular density. Figure 3 shows the immunolabelling of glial fibrillary acidic protein (GFAP) expression; 3A widespread and intensely expressed (+++ severely affected); 3B is intensely expressed (+++ severely affected); 3C is intensely expressed (+++ severely affected) and 3D is intensely expressed (severely affected)

Table 1: Changes in Organosomatic Index Post-Treatment with *Nauclea latifolia* ethanolic leaf extract and Artemether/lumefantrine in Suppressive Malaria Mice Model

Treatment Groups (n=5)	Brain Weight (g)	Initial Body weight (g)	Final Body weight (g)	(%) Change in Body weight	Organo somatic Index
Pbb +Normal saline - 10 ml/kg	0.43±0.02	23.00±0.63	24.00±0.71	+4.35	1.78±0.04
Pbb +Ethanolic extract 500 mg/kg	0.40±0.01 ^{NS}	21.20±0.49	22.60±0.68 ^{NS}	+6.60	1.86±0.07 ^{NS}
Pbb +Ethanolic extract 1000 mg/kg	0.43±0.02 ^{NS}	20.80±0.49	21.40±0.68 ^{NS}	+2.89	2.06±0.13 ^{NS}
Pbb +Coartem® - 5 mg/kg	0.42±0.00 ^{NS}	20.00±0.32	20.60±0.51 ^{NS}	+3.00	2.04±0.05 ^{NS}

Coartem® - Artemether-lumefantrine. NS - Not significant compared with control.

Table 2: Parasite Density and Chemosuppression of *Nauclea latifolia* ethanolic leaf extract and Artemether/Lumefantrine in Suppressive Malaria Mice Model

Treatment Groups (n=5)	Parasite Density (Mean±SEM)	(%) Chemosuppression
Pbb + Normal saline - 10 ml/kg	246.00±49.27	
Pbb + Ethanolic extract 500 mg/kg	145.40±24.81*	41.06
Pbb + Ethanolic extract 1000 mg/kg	66.40±19.85*	73.13
Pbb + Coartem® - 5 mg/kg	0.00±0.00*	100

Coartem® - Artemether-lumefantrine; * - $p < 0.05$ compared with control

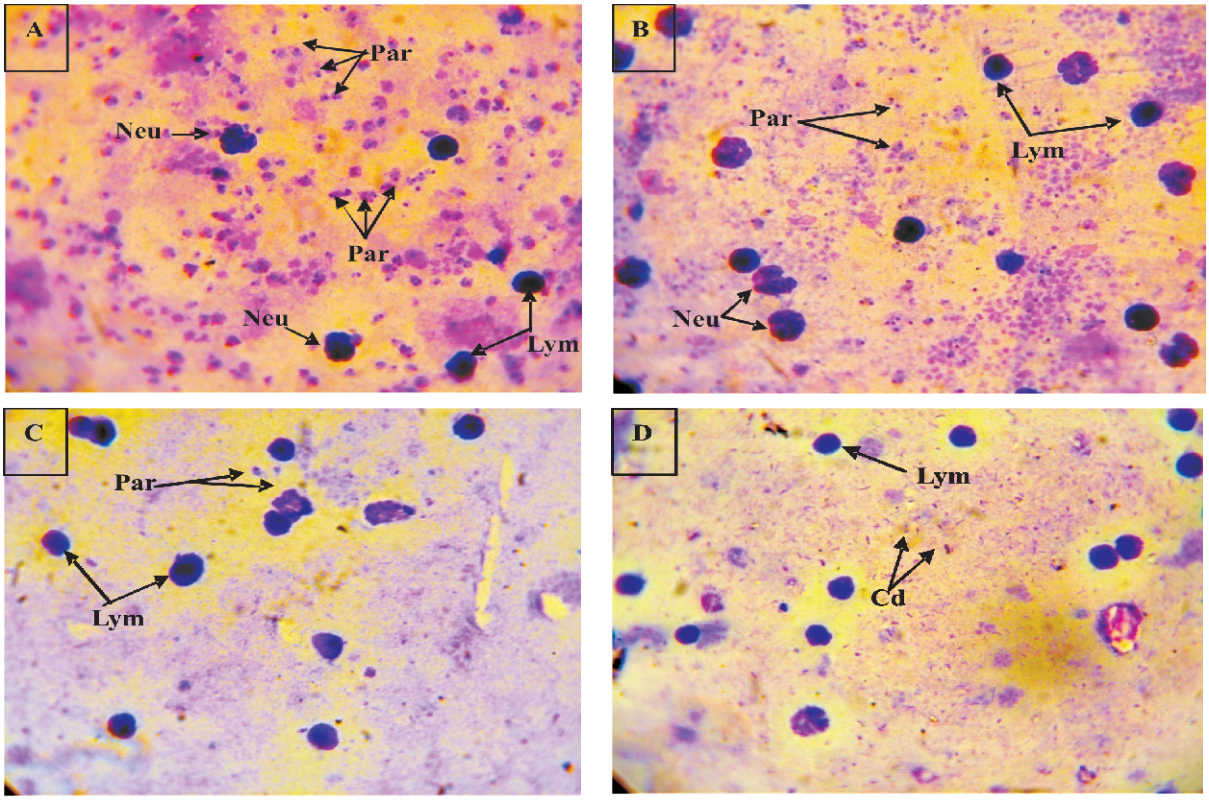

Figure 1: Blood morphological effect of *Nauclea latifolia* ethanolic leaf extract and 5 mg Artemether-Lumefantrine per kg body Swiss albino mice in suppressive malaria model.

Legend: Lym – lymphocyte; Neu – neutrophil; Par – parasite; Cd – cellular debri

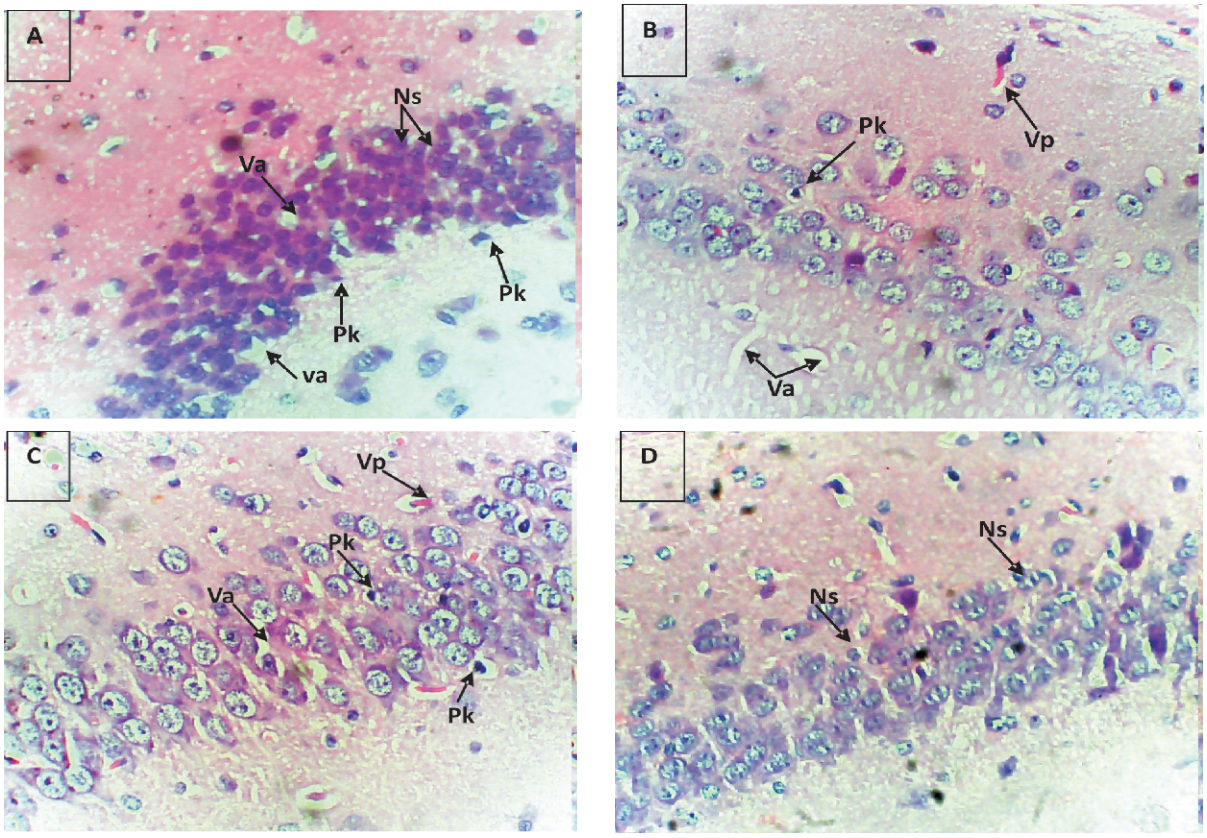

Figure 2: Histological effect of *Nauclea latifolia* ethanolic leaf extract and 5 mg Artemether-Lumefantrine on the Hippocamus of suppressive malaria mice model at x400 (H&E staining).

Legend: Ns – neuronal shrinkage; Pk – pyknosis; Va – vacuolation; Vascular plugging

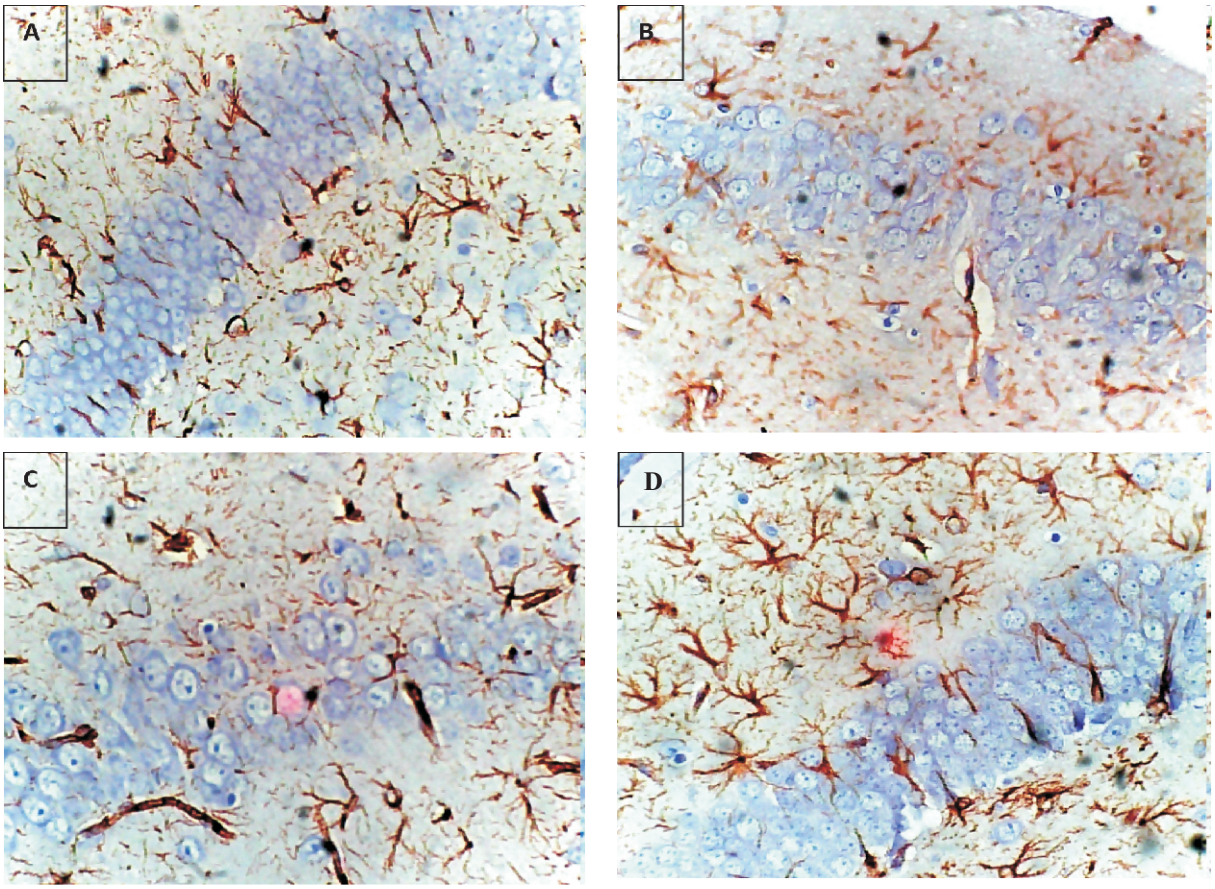

Figure 3: Hippocampal immunolabelling of glial fibrillary acidic protein (GFAP) expression at x400 after treatment with *Nauclea latifolia* ethanolic extract and 5 mg Artemether-Lumefantrine per kg body Swiss albino mice in suppressive malaria model.

DISCUSSION

In this study, Table 1 show that the ethanolic extract and drug did not produce any significant changes in the organosomatic index of *P. berghei*-infected mice compared with control. The changes in parasite density seen in Table 2 and in the morphology of the blood cells in Figure 1 (A to D) corroborates the antiplasmodial activity of NL extract compared with the untreated group, and the total parasite clearance activity of artemether-lumefantrine at 5 mg/kg. Aqueous extract of NL treatment eliminated the *P. berghei* parasites in tissues and protected them from oxidative damage even better than chloroquine treatment did, whose anti-malarial potency also cleared tissue parasites [20]. The results of parasitemia for suppressive activity presented in Table 2 had significantly ($p<0.05$) lower parasite density and percentage chemosuppression in the treated groups compared to the control. Some phytochemical compounds do elevate oxidation, and inhibit the parasite's protein synthesis [21], and may thus ameliorate, and inhibit the oxidative damage induced by the malarial parasite [22]. The photomicrographs of the thick blood smears shown on Figure 1 (A to D) indicates that the parasite density was indeed resolved in dose-dependent manner across the treatment groups, with some groups being slightly/mildly, moderately or severely affected.

In Figure 2 (A to D) the changes in the histomorphology *P. berghei*-infected mice treated with NL and artemether-lumefantrine is presented; the extent of distortion varied in the cytoarchitecture of the neuronal cells of the molecular, pyramidal and polymorphic layers. The untreated group had prominent neuronal shrinkage, pyknosis and lesion signs which diminished in the treated groups. Neuronal shrinkage is and early and recognizable indicator of neuronal degeneration in the hippocampus [23], CA1 neurons are exceptionally sensitive to to oxygen deprivation and the hippocampal pyramidal cells are among first to be affected in a variety of conditions that lead to loss of memory and intellectual functions [24].

Immunolabelling for neuroinflammationin Figure 3 (A to D) revealed that all the groups had intense expression of immunopositivity for glial fibrillary acidic protein (GFAP). GFAP expression correlates closely with rodent cerebral malaria model [25]. Reactive astrogliosis occurs after most forms of CNS injury, including cerebral ischemia and trauma [26].

Perivascular space and astroglial dilatation are evidence confirming of vasogenicedema and cytogenicedema respectively. Though all the groups have severe reactive astrogliosis, there were prominent foci swelling of the astrocytes in the infected untreated group (Figure 3A) compared to the treated groups. Based on the size and duration of CNS injury, astrocytes undergo dramatic changes in gene expression, morphology (hypertrophy), and proliferation [27]. Proliferating reactive astrocytes perform key activities that impact tissue preparation, repair/remodeling, and functional outcome. Specific deletion of proliferating reactive astrocytes after brain injury was shown to prevent repair of the blood-brain barrier and increase immune cell infiltration and neuronal degeneration. Notch 1-STAT3-ETB_R axis connects a signaling network that promotes reactive astrocyte proliferation after brain trauma [28].

In conclusion the phytochemicals in ethanolic leaf extract of *Nauclea latifolia* moderately clears *P. berghei* at 1000 mg/kg in suppressive malaria model when compared with 5 mg Artemether/Lumefantrine, and offered minimal neuroprotection against the histormorphological distortion of the hippocampus, astrocyte swelling and intense glial fibrillary acidic protein expression due to *P. berghei* infection.

Conflict of interest

No conflict of interest declared among the authors.

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