Neurohistological and immunohistochemical effects of prophylactic ethanolic leaf extract of *Nauclea latifolia* and Artemether/Lumefantrine on the hippocampus of *Plasmodium berghei*-infected mice

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The prophylactic effect of ethanolic leaf extract of *Nauclea latifolia*, widely used in herbal malarial treatment, was compared against standard drug Coartem® (Artemether/Lumefantrine) in *Plasmodium*-infected mice was investigated, and thereafter analysed for organosomatic index, parasitemia, histomorphological and immunohistochemical changes in the hippocampus. Twenty male mice about 6 to 8 weeks weighing 20 to 24 g were allotted into four groups of five mice each. Group 1 served as control received placebo; group 2 received extract 500 mg/kg; group 3 received extract 1000 mg/kg; and group 4 received 5 mg/kg of Artemether/Lumefantrine. Extract and drug administrations were performed for 3 days, and thereafter mice were infected with 10⁶ of *Plasmodium berghei* parasites, and monitored for 4 days, after which the experiment was terminated. Thick blood smear were prepared from lateral tail vein, then under anesthesia via a cocktail of xylazine and ketamine, intracardiac perfusion was performed first with phosphate buffered saline to clear systemic blood, and then 4% paraformaldehyde to fixed brain for routine histology and immunohistochemistry. Result indicates organosomatic index was not statistically significant, parasitemia in the treated groups were significantly (p<0.05) decreased compared to control and were corroborated in the photomicrographs of respective blood morphology, however, histologically there was moderate to severe distortion of the hippocampus across the groups, but glial fibrillary acidic protein expression, a marker for neurotoxicity indicated that group 4 had the most immunolabelling intensity compared to other test groups. In conclusion, the prophylactic ethanolic extract of *N. latifolia* and Artemether/Lumefantrine cleared parasitemia also seen in the blood morphology, and low dose *N. latifolia* plausibly has better safety and hippocampal toxicity profile with decreased neuronal shrinkage and distortions, with a more down regulated glial fibrillary acid protein than higher extract doses and Artemether/lumefantrine.

Key words: Malaria, hippocampus, neuronal perturbation, Artemether/Lumefantrine, *Nauclea latifolia*.

INTRODUCTION

The staggering number of malaria cases and mortality from malaria infection requires consistent efforts at actionable plans to mitigate the trajectory, as according to World Health Organization (WHO) Malaria Report (2015)
the global estimated number of malaria cases was 214 million, and the number of deaths was about 438000, and percentage-wise the WHO African Region had the most number of cases at 88% and the most number of deaths at 90%, far higher than other WHO regions and this has remained so for several decades now. The current practice in treating malaria is based on the concept of combination therapy, and Artemether/Lumefantrine is the drug of choice (WHO, 2010). Effects of drugs on biochemical targets possibly precede manifestation of morphologic endpoints as chemically induced neurodegeneration is evident by different patterns of neuronal cell death, gliosis, swollen or destroyed axons, or destruction of the myelin sheath. In vitro studies of brain stem cells show that they were selectively sensitive against artesiminisin, in contrast to cells from other brain regions, such as the cortex (Schmuck et al., 2002).

Researchers with interest in natural products have intensified their efforts toward scientific evaluation of traditional medicines (Taheri et al., 2012), and numerous claims abound on the potency and the use of these plants which require further authentication to establish their scientific bases and efficacy especially in the management of certain diseases in rural communities (Builders et al., 2012). Some ethnic tribes in Akwa Ibom State, Nigeria, drink the leaf decoction of Nauclea latifolia (NL) for the prevention and cure of malaria (Udobre et al., 2013). N. latifolia have been reported to possess antiplasmodial activity (Ajayioboa et al., 2004; Asase et al., 2005; Adzu et al., 2013). Still little research data exist on the neurotoxicity of these antimarial bio-chemicals in vivo studies especially their prophylactic impact.

Neuronal cell damage in the sector CA1 of the hippocampus is common in severe and cerebral malaria especially brain-derived neurotrophic factor (BDNF) levels in the hippocampus relate with memory impairment (Comim et al., 2012; Kihara et al., 2009). This study was to investigate the neurohistomorphological effects of prophylactic ethanolic leaf extract of N. latifolia compared with Arthemether-Lumefantrine on the hippocampus, blood morphology and organo-somatic index of Plasmodium berghei infected Swiss albino mice (Mus musculus).

MATERIALS AND METHODS

Experimental animals

Twenty (20) male Swiss albino mice were obtained weighing 20 to 24 g from the animal house of the Faculty of Basic Medical Sciences, University of Uyo, Nigeria. The animals were acclimatized for two weeks before the start of the investigation at the institution’s animal holding room, in well ventilated mouse cages and maintained under controlled environmental conditions of temperature 25 ± 5°C and 12 h light/dark cycle. 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 this study conformed to the guide for the care and use of laboratory animals (National Institute of Health, 2011) and granted approval by the Department of Anatomy ethical committee, University of Uyo.

Collection and authentication of plant sample

Fresh leaves of N. latifolia were harvested from the medicinal farm of Pharmacology and Toxicology Department, University of Uyo were identified and authenticated by the Curator at the Herbarium of Department of Pharmacology and Toxicology, University of Uyo with specimen and voucher number UUH/67 (g) deposited.

Plant extraction

Fresh leaves of N. latifolia macerated in 95% ethanol (Sigma Aldrich St Louis USA) in a flat bottom flask and kept for 72 h at room temperature. The macerated leaves were filtered and the filtrate concentrated in water-bath at 45°C to dryness. Extract was weighed and stored in the refrigerator until required for use.

Evaluation of prophylactic activities

The repository activity of the extract and Coartem® (Artemether/Lumefantrine) was assessed by using the method described by Peters (1965). The mice were randomly divided into four groups of five mice each. 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 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 berghei. The parasitaemia level was assessed by blood smears 72 h later.

Parasite inoculation

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 10⁶ P. berghei parasitized erythrocytes. This was prepared 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 a popular ACT purchased from a reputable pharmacy within the Uyo metropolis was dissolved in distilled water and treatment performed according to method by Olorunnisola and Afolayanin (2011). N. 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 LD₅₀ for low and medium

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Table 1. Prophylactic effect of Artemether-Lumefantrine and Nauclea latifolia on organosomatic index in Plasmodium-infected mice.

<table>
<thead>
<tr>
<th>Treatment groups (n=5)</th>
<th>Brain weight (g)</th>
<th>Initial body weight (g)</th>
<th>Final body weight (g)</th>
<th>Change in body weight (%)</th>
<th>Organosomatic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water - 10 ml/kg</td>
<td>0.36±0.01</td>
<td>21.40±0.87</td>
<td>20.60±0.75</td>
<td>-3.74</td>
<td>1.74±0.05</td>
</tr>
<tr>
<td>Ethanolic extract 500 mg/kg</td>
<td>0.41±0.01NS</td>
<td>23.00±0.71</td>
<td>22.80±0.58NS</td>
<td>-0.87</td>
<td>1.84±0.09NS</td>
</tr>
<tr>
<td>Ethanolic extract 1000 mg/kg</td>
<td>0.40±0.01NS</td>
<td>24.60±0.51</td>
<td>26.60±0.40*</td>
<td>+8.13</td>
<td>1.52±0.06NS</td>
</tr>
<tr>
<td>Coartem® - 5 mg/kg</td>
<td>0.42±0.00NS</td>
<td>23.20±0.66</td>
<td>24.20±1.11NS</td>
<td>+4.31</td>
<td>1.66±0.09NS</td>
</tr>
</tbody>
</table>

Coartem® - Artemether-lumefantrine; Values are expressed as Mean ± SEM; n=5; NS: Not significant compared with control; *p<0.05 compared with control.

Table 2. Prophylactic effect of Artemether-lumefantrine and N. latifolia on parasitemia in P. berghei-infected mice.

<table>
<thead>
<tr>
<th>Treatment groups (n=5)</th>
<th>Parasite density (Mean±SEM)</th>
<th>Chemosuppression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water - 10 ml/kg</td>
<td>280.80±51.43</td>
<td>-</td>
</tr>
<tr>
<td>Ethanolic extract 500 mg/kg</td>
<td>110.00±26.14***</td>
<td>60.61</td>
</tr>
<tr>
<td>Ethanolic extract 1000 mg/kg</td>
<td>57.00±21.45***</td>
<td>79.69</td>
</tr>
<tr>
<td>Coartem® - 5 mg/kg</td>
<td>0.00±0.00***</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Coartem® - artemether-lumefantrine; ***p < 0.001 compared with control.

dosage, respectively. Meanwhile, Giemsa stained blood smears obtained from the tail vein of the mice was made and viewed under oil immersion at ×100 magnification at end of experiment to determine parasitemia by direct enumeration (WHO, 2000) and images were obtained with a digital microscopic camera.

Animal sacrifice and tissue processing

Xylazine and ketamine cocktail was injected to mice at 0.2 ml, when completely unconscious, alcohol pad was used to sterilize the trunks, mid line insertion performed, thereafter intracranial perfusion via cardiac puncture with buffered saline to flush out the blood for 1 min via left ventricle, and right atrium pierced to release circulating fluid, and then buffered formalin was released via a drip-set controlled by a valve until mice tail was stiff, and then brains dissected out from the skull, 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., 2014). 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 microns thick. Sections were allowed to heat on hot plate for 1 h, 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 min. Sections were exposed to running tap water for 3 min. Peroxidase blocker was used on the sections for 15 min and then washed for 2 min with phosphate buffered saline (PBS) with tween 20. Protein blocker was carried out with Novocastra® protein block for 15 min and then washed with PBS for 2 min, and incubated with primary antibody, monoclonal mouse anti-gial fibrillary acidic protein (GFAP) DAKO 1 in 100 dilution for 45 min, washed in PBS for 3 min, and added rabbit anti-mouse secondary antibody for 15 min all at room temperature. Tissue section was then washed twice with PBS, Polymer was thereafter added and allowed for 15 min, washed twice with PBS and then added the diaminobenzidine (DAB) chromogen diluted 1 in 100 with the DAB substrate for 15 minutes, and washed with water, and counterstained with haematoxylin for 2 min. Tissue section were washed again, dehydrated, cleared and mounted in DPX mountant.

Statistical analysis

Data obtained in the study were expressed as mean ± standard error of mean (SEM) and 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 was regarded as significant at (p<0.05).

RESULTS

Table 1 shows that brain weights of test groups were not significantly changed compared control, the final body weight of the ethanolic extract of NL was significantly increased compared to the control, but the organosomatic index of tests groups compared to control was significantly unchanged. Table 2 shows a statistically significant decrease in the parasite density of the ethanolic extract groups compared to control at a dose dependent level, and Artemether-Lumefantrine proved to possess the most of chemosuppression, and these is evident in the micrographs of the blood morphology in Figure 1A to D. Figures 2A to D is the histomorphology of the hippocampus; Figure 2A shows partially dark stained neurons with hypochromatic parenchyma (inference – severely affected); Figure 2B shows neuronal hypertrophy with disperse neuronal shrinkage (inference – severely affected); Figure 2C shows prominent neuronal atrophy, polymorphic neurons with few foci pyknosis (inference – severely affected); 2D has a
Figure 1. (A-D) Prophylactic effect of ethanolic leaf extract of *Nauclea latifolia* and 5 mg Artemether-Lumefantrine per kg body weight of *Plasmodium* infected mice on blood morphology.

reduced cellular layer with few atrophic neurons and dispersed vacuolations (inference – moderately affected). Immunohistochemical expression of glial fibrillary acidic protein as shown in Figure 3A to D; groups 1, 3 and 4 indicate severe immunoreactivity with the exception of group 2 which had moderate immunolabelling intensity, however group 4 (Figure 3D) had the most severe neurotoxic presentation.

DISCUSSION

Acute toxicity study of the ethanolic leaf extract of *N. latifolia* did not cause mortality at up to 5000 mg/kg body weight of mice, and hence can be claimed to be relatively safe (Gidado et al., 2011). The organosomatic indices (OSI) of this prophylactic study in Table 1 was not significantly (p>0.05) changed when treated groups (2 to 4) compared to the control (infected untreated). This OSI outcome may not be unconnected with the sub-acute duration of the experiment. However, final body weights was significantly increased (p<0.05) when ethanolic leaf extract 1000 mg/kg group was compared with all the groups. A dose-dependent reduction in body weights of rats administered root extract of *N. latifolia* compared with the control group has previously been reported (Odey et al., 2012). Organosomatic indices are described as the ratios of organ to body weights; measured organ in relation to body mass can be directly linked to toxic effects of chemical on target organ (Giulio and Hinton, 2008). Plants that contain bioactive compounds like alkaloids, flavonoids and triterpenoids may in part contribute to their plasmocidal activity and therefore explain their mechanism of action (Okokon et al., 2012). Curative malaria studies have shown evidence that aqueous leaf extract *N. latifolia* treatment at 200 and 300 mg/kg body weight respectively eliminated the *P. berghei* parasites and protected against oxidative damage in liver and brain tissues even better than chloroquine (Onyesom et al.,
2015), while ethanolic leaf extract of *N. latifolia* via its phytochemical bioavailability decreases *P. berghei* in dose dependent manner, offers moderate neuroprotection to hippocampus of infected mice, when compared to hippocampal H&E findings of 5 mg/kg Artemisinin/Lumefantrine treated group which showed severely distorted neurons (Edagha et al., 2017), but mildly expressed GFAP, a down-regulating marker of the neuroinflammatory protein which may suggest GFAP level declined prior to slightly improved neuronal morphology seen in the respective H&E group, whereas in this prophylactic study the reverse is now reported, in which hippocampal H&E showed in Figure 2D that 5 mg/kg Artemisinin/Lumefantrine presented mild neuronal distortion, but a moderately expressed GFAP suggestive of an underlying trauma and/or infection, perhaps inducing an immunologic response required to overcome the circulating parasite.

Nucleic acid base pairing of *Plasmodium* parasite chelate with flavonoids (Lui et al., 1992), and triterpenes like quassinoïds are potent protein inhibitors (Liao et al., 1976). Oxidative damage induced by the malarial parasite is ameliorated by this bioactive activity (Alli et al., 2011). Parasitemia outcome following prophylactic activity as shown in Table 2 indicated significantly (p<0.001) lower parasite density and percentage chemosuppression in the treated groups compared to the control, suggesting that the antiplasmodial activity of *N. latifolia* ethanolic leaf extract is based on the antioxidant and plasmocidal effects of its bioavailable phytochemicals (Alli et al., 2011). A dose dependent antiplasmodial activity in the stem-bark of aqueous and fractions of *N. latifolia* extracts has been reported to be based on their phytochemicals (Ettebong et al., 2015).

Figure 2. (A-D) Prophylactic effect of ethanolic leaf extract of *Nauclea latifolia* and Artemether/Lumefantrine on hippocampal histomorphology in *Plasmodium*-infected mice. PK: Pyknosis; Ns: neuronal shrinkage; Hz: hemozoin; A: astrocytes.
Photomicrographs of the thick blood smears as shown in Figure 1A to D corroborates the antiplasmodial activity of the plant extract.

Neurotoxicity is the study of the undesirable consequences that develop in the central nervous system or peripheral nervous system or both after an organism is exposed to a neurotoxic agent during development or adulthood (Bolon and Graham, 2011), and can present as aberrations in neural structure (toxicological neuropathology) or function (including altered behaviour, biochemistry, cognition, or impulse conduction), or both (Bolon and Graham, 2011). Haematoxylin and eosin (H&E) is a cell body-specific stain and it is quite valid to reach a conclusion of neurotoxicity when using such staining tool, (Switzer and Butt, 2011). Results from the H&E staining of the hippocampal neuronal cell bodies presented for the prophylactic activity as shown in Figure 2A indicates severe cellular distortion of the hippocampal architecture; Figure 2B had moderate morphological changes with inflammation and neuronal shrinkages being common and neuronal shrinkage is an early and easily recognizable indicator of neuronal degeneration in the hippocampus (Bonde et al., 2002). The large pyramidal cells in area CA1 are exceptionally sensitive to oxygen deprivation and die after few minutes without a supply of fresh arterial blood, and pathologists call the area CA1, Sommer’s sector. The hippocampal pyramidal cells are among the first to be affected in a variety of conditions that lead to loss of memory and intellectual functions (Kiernan, 2009). The hippocampus as shown in Figure 2D showed depletion of pyramidal cells (lower neuronal density). The low dose of the ethanolic extract at 500 mg/kg was perhaps not sufficiently capable of ameliorating the neurotoxic effect of the P. berghei. Although at 1000 mg/kg of the extract of N. latifolia moderately affected the hippocampus with the intact cell layers indicating an agonistic effect perhaps due to the prophylactic protection of the extracts against the neurotoxic P. berghei via a dose dependent antioxidant constituents in the treatment. Antioxidant nutrients of
plants have the potential to scavenge for free radicals in the system and neutralize them before they do any damage to cells. *N. latifolia* could be a potential source of pharmacologically active natural products and/or for development of neutraceuticals (Egbung et al., 2013), especially from the rich source of flavonoids, alkaloids and tannins. The result of glial fibrillary acidic protein (GFAP) as shown in Figure 3A to D revealed moderate to severe immunoreactive astrogliosis. GFAP expression can be regarded as a sensitive and reliable marker that labels most, if not all, reactive astrocytes that are responding to CNS injuries (Sofroniew and Vinters, 2010) and reactive astrogliosis is prominent in most CNS infections. Since the hippocampus is the principal brain structure for learning and memory acquisition, GFAP up-regulation may support hypothesis of learning and memory impairment.

In this study, prophylactic Artemether-Lumefantrine ensured a more significant decline in parasitemia load compared with dose dependent ethanolic leaf extract of *N. latifolia*, although Artemether/Lumefantrine treated group showed a moderate hippocampal GFAP immunopositivity and neuronal distortion mainly shrinkage with presence of hemozoin in the hippocampal CA1 region in *Plasmodium* infected mice stained with H&E similar with extract treated groups, so the prophylactic herbal or Artemether/Lumefantrine clears parasitemia but only mildly protects the hippocampus at higher extract doses after *P. berghei* infection in mice.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

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