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Nauclea latifolia stem-bark extract protects the prefrontal cortex from valproic acid - induced oxidative stress in rats: Effect on B-cell lymphoma and neuron specific enolase protein expression

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Abstract

Nauclea latifolia stem-bark (NLS) is widely utilized for broad spectrum pathologies. The study evaluated the protective effects of NLS extract supplemented along with valproic acid on oxidative stress markers, and neuron specific enolase (NSE) and B cell lymphoma 2 (bcl-2) protein expressions in prefrontal cortex of rats. The NLS extract was administered in three doses (50, 100, 200mg/kg) after pre-treatment of the experimental animals with high dose valproic acid (VPA) (500 mg/kg) orally daily by subacute exposure for 28 days. The rats were sacrificed and the prefrontal cortex (PFC) of the brain abstracted and homogenized in ice for biochemical assays to estimate the levels oxidative stress markers; histopathological examination to reveal the histomorphorgical changes and immunohistochemistry to examine effect on NSE andbcl-2protein expressions. The findings revealed significant ($P <$ 0.05 – 0.001) elevation of oxidative stress induced by VPA. The NLS extract supplementation mediate significant elevation of the levels of reduced glutathione ($P<$ 0.05 – 0.01), antioxidant enzymes [glutathione peroxidase ($P<$ 0.001), superoxide dismutase ($P <$ 0.001), catalase ($P <$ 0.05 – 0.01) and glutathione-s-transferase ($P <$ 0.05 – 0.01)] and significant decline of lipid peroxidation marker, malondialdehyde, (NLS group *vs* diseases control group ($P <$ 0.001). The NLS extract studied down-regulated NSE at the prefrontal cortex preventing neuronal damage but no effect on bcl-2 protein expression. We conclude that NLS extract has potential to mitigate VPA induced neurotoxicity by obliterating oxidative stress and down regulating NSE expression, effects demonstrating probable therapeutic role in neurodegenerative diseases.

Keywords: *Nauclea latifolia* stem-bark; Valproic acid–induced neurotoxicity; Oxidative stress; Prefrontal cortex; Neuron specific enolase; Immunohistochemistry

1. Introduction

The prefrontal cortex (PFC) of the brain is involved in a number of executive functions [1] and worst still has demonstrable vulnerability to stress [2] most especially oxidative stress. Oxidative stress is a pathophysiological disequilibrium in the balance between oxidants and antioxidants which favors the preponderance of the oxidants species [3] and decreased production of antioxidants [4]. Free radicals (FRs) are molecules with one or more unpaired electrons in the valence shell [4, 5, 6], therefore extremely unstable and reactive with other moieties [6, 7]. The FRs may be oxygen or nitrogen based radicals and are ubiquitously encountered in biological systems. The reactive oxygen species (ROS) are superoxide, hydroxyl and peroxyl radicals, and non-radicals such as hydrogen peroxide, hypochlorous acid and ozone. Reactive nitrogen species (RNS) include nitrogen based radicals such as nitrogen

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dioxide, nitric oxide radicals and peroxynitrite, while products of nitric oxide and superoxide via inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase are and non-radicals [4]. Elevation in O_2^- production or inadequacy in antioxidant system, result in hydrogen peroxide (H_2O_2) accumulation. Hydrogen peroxide is produced in response to extracellular responses such as cytokines, neurotransmitters, peptide growth factors and hormones, and this affects protein function. In pathological conditions, H_2O_2 reacts with Fe^{2+} via Fenton reaction to produce highly reactive OH^-, an important intracellular messenger under physiological conditions [8].

Endogenous sources of ROS include aerobic respiration, β-oxidation of fatty acids, microsomal cytochrome P_{450} metabolism of xenobiotics, stimulation of phagocytosis by pathogenesis of lipopolysaccharides, arginine metabolism and tissue specific enzymes. The exogenous sources include pollutants, toxins such as heavy metals like mercury, lead, cadmium, and other exotoxins like anticancer drugs, anesthetic's and analgesics. Other exotoxins include those of bacteria, yeast, viruses and parasites origin; trauma, radiation, electromagnetic fields, alcohol, cigarette smoke, stress, allergens, cold, excessive exercise, dietary factors such as excess sugar, unsaturated fats and fried oils, malnutrition and various disease states are potential sources [6].

Free radicals, ROS, have well-defined functions and are important in many physiological reactions [6]. The generation of ROS is responsible for sustained cellular function with beneficial effects on the organisms. Many cellular biochemical activities such as signal transduction, gene transcription and regulation of soluble guanylate cyclase activity all involve oxygen radicals. Nitric oxide acts as signaling molecule to regulate tone of vascular smooth muscle besides proliferation of leukocyte adhesion, platelets aggregation, thrombosis and hemodynamics [5]. Nitric oxide plays an important role in neurotransmitter release [8]. Exacerbation in free radicals production in excess of antioxidants capacity, underpin oxidative damage and stress to biomolecules such as lipids, proteins and DNA [5, 6, 9, 10].Lipid peroxidation affects many cellular components but occurs primarily in membranes associated with polyunsaturated fats leading to alteration in cell membrane fluidity and permeability eventually damaging the membrane [6]. The peroxidation of lipid membranes raised the concentration of lipid peroxides at the expense of oxygen and unsaturated lipids resulting in further local damage and at close and distant sites to the initial area of insults. The products of advanced peroxidation are numerous including malondialdehyde (MDA), used as a marker for lipid peroxidation [6].Oxygen radicals may directly attack DNA at the sugar moiety, phosphate or purine and pyrimidine bases with indirect actions which may be mediated by the elevation of intracellular calcium (Ca^{2+}) ions. The interaction of free radicals with DNA instigates structural alterations in DNA leading to DNA damage [6]. Oxidants species may also cause mutation to DNA leading to carcinogenesis [4, 5, 11, 12].Free radical denature proteins and render them inactive especially proteins with amino acids containing sulphydryl moiety [5, 13].Tissue injury and eventual cell death could occur due to oxidative stress. This oxidative stress phenomenon is implicated in many diseases including cardiovascular diseases, atherosclerosis, diabetes, rheumatoid arthritis, post ischemic perfusion injury, myocardial infarction, cancer, inflammatory diseases, stroke and septic shock, aging and other degenerative diseases in humans [5]. The brain and neuronal tissues are most vulnerable to ROS because of active oxygen demand. Metabolisms of excitatory neurotransmitters are sources of oxidative stress in the brain through the production of ROS. The ROS produced attack glial cells and neurons leading to neuronal damage [5]. The damage caused by ROS is thought to mediate necrosis and apoptosis [5, 6]. In neurodegenerative diseases, mitochondrial dysfunctions and excitotoxicity in addition to apoptosis have been reported as the main aetiopathological factors. Damaged mitochondrial and activated microglia acts as ROS reservoirs. This is aggravated by preponderance of high level of fatty acids easily susceptible to lipid peroxidation sustained by reduced antioxidant capacity are responsible for the high sensitivity of the brain to oxidative damage compared to other tissues [5].

Enzymatic and non-enzymatic antioxidant systems are essential for cellular response to oxidative stress under physiological condition. Antioxidants are classified as exogenous (natural or synthetic) or endogenous compounds and are responsible for achieving an oxidant/antioxidant balance. The antioxidants are responsible for removal of free radicals, scavenging ROS or their precursors and inhibition of ROS formation or indirectly by chelation of metal ions needed for catalysis of ROS generation [14].Endogenous antioxidants include bilirubin, thiols (glutathione (GSH), lipoic acid, N-acetyl cysteine, NADPH and NADH, ubiquinone and uric acid as well as the enzymatic antioxidants superoxide dismutase (SOD), catalases (CAT), thioredoxin and glutathione reductase (GR) and glutathione peroxidases (GPx) which are involved in detoxification of ROS and lipid peroxides [15, 16, 17]. Superoxide dismutase variants (SOD1, SOD2, SOD3) located in cytoplasm, mitochondria and extracellular compartments respectively catalyze the dismutation of $·O_2^-$ into oxygen (O_2) and H_2O_2. Further catalysis of H_2O_2 into oxygen and water is achieved by catalase in the in peroxisomes and cytoplasm [10]. Exogenous antioxidants include dietary vitamins such as ascorbic acid (vitamin C), tocopherols/tocotrienols (vitamin E), betacarotene and other carotenoids (e.g. lycopene and lutein), polyphenols (e.g. flavonoids, flavones, flavonol's and proanthocyanidins) and metal binding proteins (e.g.

albumin, ceruloplasmin, metallothionein, ferritin, myoglobin, transferrin) with free radical scavenging activity [15, 18].

Biomarkers of lipid peroxidation include various aldehydes like malondialdehyde (MDA), 4-hydroxynonenal and thiobarbituric acid reactive substances (TBARS) derived from disintegration of conjugated diene hydroperoxides and other unstable substances produced by lipid peroxidation. Oxidized LDL (Ox LDL), a product of oxidized phospholipids released from the brain to circulation is also a suggested biomarker of oxidative injury [3].Biomarkers of antioxidant include antioxidant enzymes that vary on conditions of oxidative stress include GPx, SOD, CAT and other non-enzymatic antioxidants GSH, uric acid, vitamin A,C,E etc.[3].

Oxidative stress produced major changes apart from aging to the nervous system. The alterations to the tissue and cells might result in loss of nerve cells, neurofibrillary tangles, and neuritic plaques and cell death. Aging is associated with alteration in neurotransmitter concentration, the synthetic enzymes of these neurotransmitters, loss of neurons, their myelin sheath, slowing down impulse conduction and the formation of specific pathological beta-amyloid plaques and neurofibrillary tangles and oxidative damage to the brain [19].

Sodium valproate is used for the treatment and management of seizure disorders (absence seizures, tonic-clonic seizures (grand mal), complex partial seizures and the seizures associated with Lennox-Gastaut syndrome, mania, and prophylactic treatment of migraine headache, bipolar and other schizoaffective disorders [20, 21, 22]. Valproic acid dissociates in the gastrointestinal (GI) tract to valproate ion which binds to and inhibits GABA transaminase. The anticonvulsant mechanistic activity is by elevated brain concentrations of GABA achieved by inhibiting GABA catabolism or block GABA reuptake into glia and nerve cell endings. The drug act by suppressing repetitive neuronal firing through inhibition of voltage sensitive sodium channels. Valproate (VPA) has evidence as a neuroprotective at low doses [23, 24, 25] however at high doses most common toxicities include: hepatotoxicity [26]; haematotoxicity [27]; nephrotoxicity [28]; neurotoxicity [29], bone marrow suppression [26]; teratogenicity and developmental toxicity [30] and Valproate-induced hyperammonemic encephalopathy (VHE) [31].Valproate-induced hyperammonemic encephalopathy (VHE) is an unusual complication characterized by decreasing level of consciousness, focal neurological and cognitive deficits[32], and increased seizure frequency [33]. This is a form of toxicity peculiar to valproate which eventually leads to brain damage [9].Valproate treatment leads to deficiency in carnitine and also results in diminished mitochondrial function with inhibition of the urea cycle in the liver as metabolism of ammonia occurs primarily via the urea cycle. Another possible enzyme deficiency is ornithine transcarbamoylase, another enzyme of the urea cycle [34].

Varieties of agents have been identified for neuroprotection and over four hundred has been described [9]. One of such agents is vinpocetine, a natural vincamine alkaloid derived from Periwinkle seeds (*Vinca minor*). It has been shown to improve cognitive function and short term memory in both animals and humans and has also been found to be useful in cerebrovascular disease. It is remarkably safe and also possesses antioxidant activity against free radicals and toxic substances [35, 36]. Vinpocetine acts by increasing blood circulation and cerebral metabolism in the brain. This has been demonstrated by the effect of vinpocetine in improving chronic hypoperfusion [37, 38]. Increased cerebral metabolism might be induced through enhanced cerebral flow, increased oxygen and glucose consumption in the brain and increased ATP production [36].

The mechanism by which vinpocetine enhances spatial memory is thought to be by modulation of cholinergic functions specifically increasing firing rate of certain cells in the cholinergic pathway [39]. Several mechanisms involved in vinpocetine improvement of memory deficit and blood flow include inhibition of phosphodiesterase enzyme (PDE) enhancing second messenger signaling; inhibition of kB kinase (IKK)/ nuclear factor – kappa (NF – kB) and extracellular signal related kinase (ERK) reducing the inflammatory responses in vascular smooth muscle; and enhancing the structure of dendritic spines improving memory retrieval in mild to moderate psychodromes and cognitive enhancement. Inhibition of calmodulin dependent PDE type 1 may lead to increased cAMP and cGMP and may be responsible for increased cerebral circulation and decreased platelet aggregation [35]. Neuroprotective action of vinpocetine is related to the inhibition of operation of voltage dependent neuronal sodium Na^+ channels resulting in dose dependent decrease in evoked extracellular Ca^{++} ions in striatal nerve endings [40, 41] through blockade of excitotoxicity and attenuation of neuronal damage induced by cerebral ischemia or reperfusion.

Nauclea latifolia is an evergreen multi stemmed tree growing to an altitude of 200 m. It is commonly known as pin cushion tree and African peach in English and sold by the trade name opepe in Nigeria [28, 42, 43]. Locally, in South-South of Nigeria, it is known as itu by the Itsekiris in Edo state and mbomibong by the Ibibios and Effik in Akwa-Ibom state. In Hausa, Northern Nigeria it is known as Tafashiya or tafiyayaiga and uche by the Igedes in Benue state, Nigeria. In Ibo, South Eastern Nigeria it is known asubulinu or ovoroilu and egbeyesi or egbesi in Yoruba, South West Nigeria

[43, 44]. Ethnomedicinal usage includes hypertension and diabetes [44]. In Northern Nigeria, cold infusion of the stem bark is used as diuretic and anthelmintic. It is used as mouth antiseptic as chewing stick, as remedy against gastric pain and tuberculosis [43, 45]. Usage as antimalarial, antipyretic and aphrodisiac, wound healing activity and as a vermifuge have been reported [46]. Almost all parts of the plant are useful in disease treatment. Frequency of usage in ethno-medicine is: roots >stem> bark > leaf [44]. Pharmacological assays have confirmed effectiveness as anti-infective agent in malaria treatment [46, 47], antipyretic, anti-inflammatory and antinociceptive [48, 49], anthelminthic agent [50, 51], anticonvulsant and anxiolytic [52], antidiabetic [53] and anti-hypertensive [54]; antibacterial [55] and anti-trypanosomal [56]; wound healing [46] and antidiarrheal activity [57].

Oxidative stress and its concomitant effects are susceptible to modification by antioxidants form medicinal plants. Therefore we investigated the effects of 28 days supplementation of NLS on VPA-induced neurotoxicity at the PFC on oxidative stress markers, its effects on bcl-2 and enolase protein expression in experimental rats. The data generated is anticipated to elucidated potential mechanism of NLS as therapeutics for management of VPA –induced toxicity, and plausible role as neurodegenerative therapy.

2. Material and methods

2.1. Drugs, chemicals and equipment

Sodium valproate (Epilim, Sanofi, France), Vinpocetine (Cognitol, Tyonex, Nigeria) both purchased from Sicone Pharmacy (Nigeria) Limited, Rivers State, Nigeria. Methanol 99.8% (Loba Chemie, Mumbai, India), *n*- hexane (extrapure 85%) (Loba Chemie, Mumbai, India), Diethyl ether (Loba Chemie, Mumbai, India), formalin (Lobal Chemie, Mumbai, India); Avidin Biotin Complex (Boster bioengenering limited, Wuhan, China). The equipment utilized includes: rotary evaporator (Shenke® R-205, Shangai Shenshun Biotechnology Co. Ltd, China), analytical balance model AR323 CN (Ohaus Corp. Pine Brook, NJ, USA), auto-hematology analyzer model MY-B002B (Maya Medical Equipment Limited, Shanghai, China), Spectrophotometer model SM-23 D (Surgifield Medical, England, UK), scientific weighing balance model TH 600 (Labscience, England, UK), centrifuge model 412B (Techmel and Techmel, MI, USA), Water bath (Techmel and Techmel, MI, USA).

2.2. Plant collection and extract preparations

Plants stem bark from *Nauclea latifolia* were collected in Uyo, Akwa Ibom state and supplied dried by Mr. Okon Etefia, a traditional herbalist, attached to Pharmacognosy Department, University of Uyo, Nigeria. The plant was authenticated by Dr. Oladele Adekunle, a taxonomist attached to the Forestry Department at university of Port Harcourt, Nigeria. The Herbarium specimen with voucher number UUPH 20(c) had already been deposited at Department of Pharmacognosy, University of Uyo, Akwa Ibom State, Nigeria.

The dried stem bark of *N. latifolia* was reduced to powder mechanically. A 250g weight of stem bark powder was then submerged in 2000 mL of *n*-hexane and agitated vigorously every 12 hours. After 24 hours of maceration, the extract was concentrated using a rotary evaporator and the marc submerged in 2000 mL of methanol. This was macerated for 72 hours while shaking vigorously every 12 hours. Rotary evaporator was used to concentrate the extract obtained after which it was evaporated to dryness on the water bath at 45 °C. The percentage yield was then calculated.

2.3. Phytochemical screening

Phytochemical screening of the plant extract was carried at the Pharmacognosy and Phytotherapy Department laboratory, University of Port Harcourt. The tests for presence of phytochemicals were executed following standard procedures [58, 59].

2.4. Animals study

Forty male Wistar albino rats (160 – 180 g were obtained from the animal house, Department of, University of Nigeria Nsukka. The animals were acclimatized in the animal house for 14 days under standard laboratory conditions before commencement of the experiment. The animals were randomly selected and housed in pathogen free plastic cages (n = 7) per cage under relative humidity (45 – 55%) and ambient temperature (26.5 ºC) and exposed to daily circle of night and day. The rats were provided with pelleted rodent chow from Vital Feed® (Grand cereals Limited Onitsha, Anambra State, Nigeria) and water *ad libitum*. The animals were allowed unfettered access to water and food. The experimental protocol was in line with Guide to the Care and Use of Animals in Research and Teaching (NIH, 1996) and institutional guideline for care and use of animals for experiment as specified in the University of Port Harcourt Animal ethics committee approval (No. UPHAEC/2018/011).

2.4.1. Experimental protocol

Sodium valproate or valproic acid (VPA) brand Epilim® formulated as 300 mL syrup was used to induce neurotoxicity at a dose 500 mg/kg daily for 28 days when administered orally by gavage in the experimental animals [60]. Each one mL contains 200 mg sodium valproate. The animals were divided randomly into six groups with 7 animals per group. The NLS, valproate and vinpocetin (Cognitol®) (the reference drug) were administered orally per kg of body weight once daily for 28 days. Sodium valproate (500 mg/kg) was administered one hour prior to the administration of the control drugs or NLS extract respectively for animals in groups 2 to 6. The NLS extract (50, 100, 200 mg/kg) and vinpocetin 25 mg/kg were solubilized in 2% Tween 80 (Polysorbate 80). The experimental groups utilized for the study are indicated below.

- Group 1 (Negative control): The animals in this group received 2% Tween 80 with 10 mL/kg distil water.
- Group 2 (Disease control group): The animals in this group received sodium valproate followed by 2% Tween 80 with 10 mL/kg distil water
- Group 3 to 5 (Test groups): The animals in these groups received sodium valproate (500 mg/kg) followed by the NLS extract 50 mg/kg, 100 mg/kg and 200 mg/kg respectively.
- Group 6 (Reference control): The animals in this group received sodium valproate followed by vinpocetine 25 mg/kg. The procedure adopted followed the methodology of Niaraki et al. [60].

The formula below was used by Singh et al. [61] to calculate the level of neurotoxicity (%) induced by xenobiotics (VPA) and neuroprotective in percentage induced by NLS extract.

Neurotoxicity percentage (%) was deduced using this formula

$$= \left[\left(\frac{\text{VA} - \text{W (negative control)}}{\text{W(negative control)}} \right) \times 100 \right]$$

Neuroprotective activity (%) was calculated as follows:

$$\text{Neuroprotective activity (\%)} = \left[1 - \left(\frac{\text{NLS} - \text{W}}{\text{VA} - \text{W}} \right) \right] \times 100$$

Where, NLS, VA, and W are mean experimental variables estimated in the rats treated with valproic acid (VA) plus NLS (Test groups), valproic acid (VA) only(diseases control group) and distil water treated animals (W)(negative control) respectively.

2.4.2. Blood and tissue collection

The rats were anaesthetized with diethyl ether and blood was obtained by dissecting the jugular vein with a sharp surgical blade. All the rats were decapitated and the whole brain excised and weighed immediately. The prefrontal cortex was removed divided into two portions; one was   immersed in ice-cooled physiological saline; and 10% homogenate of the PFC was prepared. The homogenate was centrifuge (4000 × g, 10 min) at -80 ºC to remove debris and nuclei. The resultant supernatant was stored at -80 ºC for measurement of oxidative stress markers. The other portion of PFC abstracted for histopathological and immunohistochemistry evaluation.

2.4.3. Oxidative stress markers measurement

Oxidative stress biomarkers were evaluated in brain prefrontal cortex (PFC) tissues homogenate.

Reduced glutathione (GSH) level

Sedlak and Lindsay [62] procedure was used to estimate the level of reduced glutathione. An aliquot of the sample (0.2 mL) was added to 1.8 mL of distilled water and 3 mL of the precipitating solution (4% sulphosalicylic acid) and mixed. The mixture was allowed to stand for approximately 5 minutes and then centrifuged at 1200 g for 10 minutes. A 1mL of the filtrate was added to 4 mL of 0.1M phosphate buffer and 0.5 mL of DTNB (Ellman's reagent) was finally added. A blank was prepared with 4 mL of 0.1M phosphate buffer, 1 mL of dilute precipitated solution and 0.5 mL of DTNB. The solution was kept at room temperature for 15 minutes and read at 412 nm on a spectrophotometer.

Glutathione peroxidase

The level of glutathione peroxidase (GSPx) was evaluated following the method adopted by Rotruck et al.[63]. Sodium phosphate buffer mixture (1 mL) containing 0.2 mL sodium azide, 0.4 mL of GSH and 0.2 mL of hydrogen peroxide (H_2O_2) and 1 mL of 1:10 extract of cell homogenate were combined and made up to 4 mL volume with distilled water. Tubes were incubated for 3 min at 37 ºC before 0.5 mL 10% TCA was added to terminate the reaction. To estimate the remaining glutathione constituent, DTNB reagent (1 mL) and phosphate solution (0.3 mol/L) were added. The change in color was assayed at 412 nm. Similar treatments were administered to aliquots of the standard solutions.

Estimation of superoxide dismutase level

Superoxide dismutase (SOD) level was estimated using the auto-oxidation technique [64]. Tissue homogenates were centrifuged at 12,000g and an aliquot of supernatant was diluted with water to make a 1:10 dilution. The diluted sample (200 mL) was supplemented with carbonate buffer (pH 10.2); 0.3 mL of 0.3 mmol/L epinephrine was added to the mixture and mixed by inversion swiftly. All solutions used were freshly prepared. Two and half milliliter of buffer, 0.3 mL of epinephrine (substrates) and 0.2 mL of distilled water were in the reference cuvette. The change in absorbance was read at 480 nm read at 30 s intervals for 2.5 min.

Determination of catalase level

Catalase level was determined according to the method of Clairborne [65] with slight modifications. The method is based on the ability of catalase in the sample preparation to split hydrogen peroxide which can be measured spectrophotometrically at 240 nm. One unit of catalase equals the amount of protein that converts one micromole H_2O_2/min. A 0.2 mL of sample was added to 50mM of phosphate buffer (pH 7.4) containing 100 mM (v/v) of H_2O_2 in a total of 1 mL. The reaction mixture was incubated for 2 min at 37ºC and the rate of absorbance change (AA/min) at 240 nm was recorded which indicated the decomposition of H_2O_2. Activities were calculated using the molar extinction coefficient of H_2O_2 at 240 nm. All samples were measured in quadruplicates.

Determination of glutathione-S-transferase level

This was determined according to the method of Habig et al. [66]. The principle is based upon the relatively high activity of GST with 1-chloro-2,4-dinitrobenzene (CDNB) as the second substrate. When CDNB is conjugated with reduced GSH, its absorption maximum shifts to a longer wavelength. The absorption increase at the new wavelength of 340 nm provides a direct measurement of the enzymatic reaction. The medium for estimation was prepared with reduced GSH 0.1M, CDNB 20 mM and 0.1 M phosphate buffer and the reaction was allowed to run for 60 seconds each time before the absorbance of the medium containing the sample was read against the blank at 340 nm. The absorbance was measured using spectrophotometer.

Determination of malondialdehyde (MDA)

Lipid peroxidation was determined by measuring the o-barbituric TBARS formation according to the method of Ohkawa and Ohishi [67]. An aliquot of 0.4 mL of supernatant was mixed with 1.6 mL of Tris-KCl buffer to which 0.5 mL of 30 % trichloroacetic acid (TCA) was added. A 0.5 mL of 0.75% TBA was then added and placed in a boiling water bath for 1 hour. This was then cooled in ice and centrifuged at 3000 g. The clear supernatant was collected and the absorbance measured against a reference blank of distilled water at 532 nm. The MDA level was calculated according to the method of Todorova et al. [64] and expressed as nmol of MDA/g of wet tissue using a molar extinction coefficient of the chromophore (1.56×10^{-5} /M/cm).

2.5. Histopathological studies

2.5.1. Hematoxylin and eosin staining

Following 28day'sexposures to valproic acid along with NLS supplementation, the rats were all sacrificed by cervical dislocation. The prefrontal cortex was abstracted immediately, fixed by 20% phosphate buffered formalin for 48hrs. The tissues were sectioned using rotary microtome model (Leica Microsystems, Wetzlar, Germany) at 6μm and floated in water bath onto charged slides. The sections were dried on hot plate at 60 ºC for 2hours.The tissue were further processed by dehydrating with ascending grades of alcohol, cleared in two changes of xylene and embedded in molten paraffin wax and sectioned using rotary microtome, mounted on glass slide and stained with hematoxylin and eosin. Ultra-sections from each group were examined by light microscopy for tissue injury and neurodegenerative features such as shrinkage of the neuron, hyperchromasia, and nuclear pyknosis as exposed by heamatoxylin and eosin staining techniques following the methodology of Oyinbo et al. [68]. Cortical Purkinje cells estimation was based on

semi-quantitative scale described in previous study [68] was used to assess the extent of neurodegeneration in the PFC with 400× magnification.

2.5.2. Immunohistochemical examination

Immunohistochemistry assessment of neuronal damage was executed by evaluating the levels of neuron specific enolase (NSE) protein and B cell lymphoma 2 (bcl-2) markers. Avidin Biotin Complex (ABC) procedure referred to also as the Avidin biotin imuunoperoxidase method described by the methodology of Oboma et al. [69] was utilize. Colorectal cancer cells line known to be positive for NSE were used as positive control while negative control was omission of the primary antibody. Appropriate negative controls for immunostaining were prepared by eliminating the primary antibody step for NSE and bcl-2 using micropolymer detection kit from Abcam (ab80436). Cells with specific brown colors in the cytoplasm, cell membrane or nuclei depending on the antigenic sites were considered to be positive for both NSE protein and bcl-2. The stained cells without any form of brown colors were scored as negative.

2.6. Statistical analysis

The data analysis was done by Graph pad Prism 5.1 using one-way analysis of variance (ANOVA) and expressed as Mean ± SD. Multiple comparison among groups were made according to the Turkey's test. *P* values <0.05 were considered significant.

3. Results

3.1. Effect on antioxidants and lipid peroxidation

The effect of NLS after sub-acute intoxication with valproic acid on lipid peroxidation examined by MDA formation, and antioxidant activity is presented in Fig 1 below. The result indicated neuronal damage as evidence in statistical significant depression (P< 0.05 - 0.001) of GSH (35%), GST (63%), GPx (71%), CAT (67%), TP (18%) and SOD (67%) when compared with the normal control group. Similarly lipid peroxidation was evident by increased formation of MDA (204%) in the intoxicated rats with P <0.001. However, sub-acute treatment with NLS extract protected against the neurotoxicity induced by valproic acid. This was demonstrated by elevation of SOD (97%,121%, 127%), GSH (60%, 75%, 87%), GST (all 80%), GPx (80%, 80%, 100%), CAT (54%, 66%, 63%) and TP (134%, 147%, 158%); and reduction of MDA (102%, 113%, 118%) with NLS treatment at 50 mg/kg, 100 mg/kg and 200 mg/kg body weight respectively when compared with the disease control group. Dose dependent neuroprotective activity was seen in GSH, GPx, TP and MDA only. Reference control group showed substantial expression of vinpocetine's neuroprotective activity on antioxidant enzymes. This was evident as it increased SOD, GSH, GST, GPx, CAT, and TP by 118%, 40%, 180%, 100%, 67%, and 146% and reduced lipid peroxidation in brain prefrontal cortex by 100%.
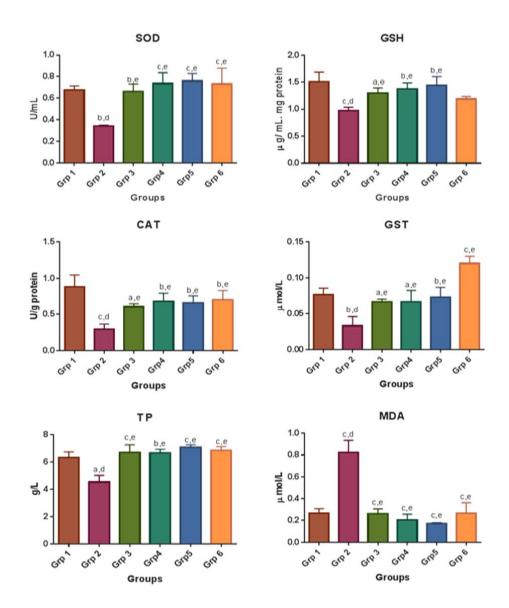
Figure 1: Effect of valproic acid intoxication and post-treatment with NLS extract on antioxidant profile from brain homogenate of prefrontal cortex of experimental rats following continuous oral sub-acute dosing for 4 weeks

Group 1: Negative control receiving 10 mL/kg b.w. 2% Tween 80;Group 2: Diseases control group receiving 10 mL/kg b.w. 2% Tween 80 + valproic acid 500 mg/kg; Group 3 receiving NLS extract (50 mg / kg b.w.) + valproic acid 500 mg/kg; Group 4 receiving NLS extract (100 mg / kg b.w.) + valproic acid 500 mg/kg; Group 5 receiving NLS extract (200 mg / kg b.w.) + valproic acid 500 mg/kg, Group 6: Reference control receiving vinpocetine (25 mg/kg b.w.) + valproic acid 500 mg/kg. NLS = *Nauclea latifolia* stem-bark; SOD: superoxide dismutase; GSH: glutathione; GST: glutathione S transferase; GPx: glutathione peroxidase; CAT: catalase; MDA: malondialdehyde; TP: total protein. Values presented as mean ± standard deviation (n = 3 – 7); [a]$P <$ 0.05, [b]$P <$ 0.01, [c]$P <$ 0.001 [d]Values are compared with the negative control group, [e]Values are compared with the diseases control group; all values were compared using one way ANOVA and Turkey Test.

3.2. Histomorphological evaluation

HE staining of neurons loss in the PFC is shown in Fig 2. Rats in the control group have morphological intact and tightly arranged neurons in the PFC (Grp 1). Generalized damage is distinctly observed in the PFC treated with VPA (Grp 2). Rats in The valproic acid induce neurodegeneration by inducing apoptosis. Neuronal cells in this group alone show numerous cells with pyknosis and vacuolation symbolic of cell death. The population of neurons evaluated semi quantitatively was significantly reduced in the disease control group (Grp 2) compared with the test groups (Groups 3, 4 and 5) treated with three different doses (50, 100, 200 mg/kg) respectively, and reference group (Grp 6). Neurons in the PFC of rats treated with NLS in group 5 almost appear normal with the reference grp and the control grp indicating neuroprotection. Histomorphology (Fig 1) shows neuronal protection in group Grp 5 and Grp 6 rats treated with 200 mg/kg b. wt of NLS extract and 25 mg/kg b. wt of vinpocetine compared with control.
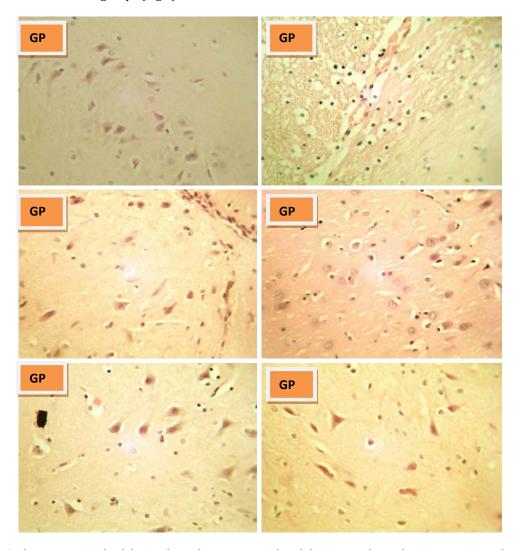
3.3. Immuno histochemistry

3.3.1. Neuron specific enolase (NSE) immune histochemistry

Immunohistochemistry labelling of NSE on PFC brain tissues showing glial cells (brownish) expression in valproic acid induced neurodegeneration treated with NLS extract (Fig 3). Glial cells (astrocytes) help to maintain hemostasis and provide support and protection for neurons. A number of positive glial neurons revealing NSE protein expression in prefrontal cortex while the dendrites processes are stained brown and are unremarkable consistent with normal brain tissue (Group1). Moderate expression of NSE compared to the control consistent with neuronal damage diseases control group (Group 2). The Group 3, Group 4, Group 5 and Group 6 sections were all negative for neuron specific enolase immunohistochemistry stain. Extract administered showed neuroprotection at all concentration compared with the reference drug.

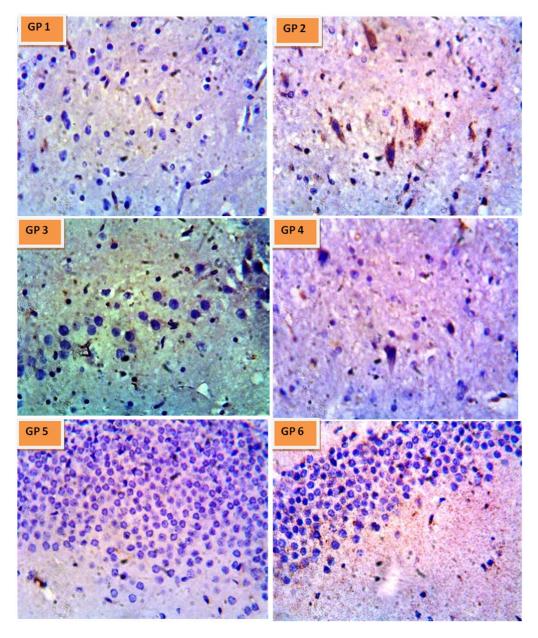*3.3.2. B-cell lymphoma 2 (bcl-2) immunohistochemistry*

This method was employ to localize and labelled the bcl-2 tissue protein. Sections showed negative or under expression for bcl-2 in all the groups (Fig 4).

Figure 2 Photomicrograph of the prefrontal cortex stained with heamatoxylin and eosin staining technique to demonstrate tissue morphology in adult rat's brain exposed to valproic acid and treated with different concentration of NLS extract and reference drug (x 400)
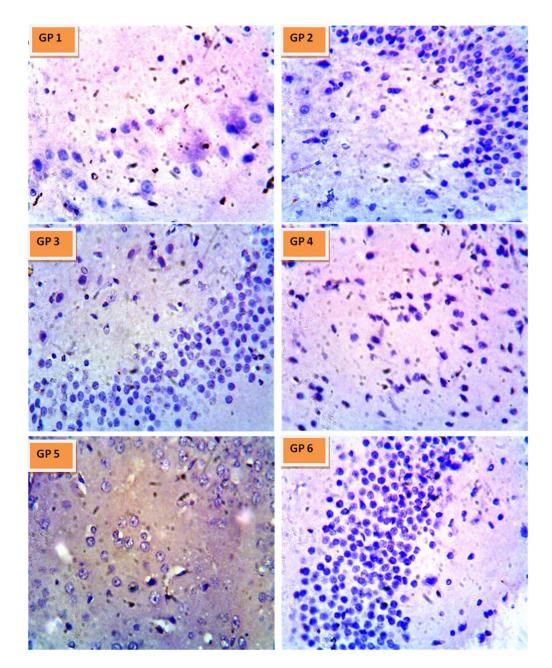
Grp 1 shows the substantia nigra displaying normal neuron with cell bodies and nuclei. Grp 2 shows numerous neurons with pyknotic nuclei. Nuclear condensation is a hall mark of cell death. Histology shows vacuolation in some

areas of the sections. Grp 3 and Grp 4 shows mild reduction in neuronal loss in adult's rats treated with the extract .Grp 5 and Grp 6 shows normal neurons population and morphology compared with the control. High concentration of the extract show high neuronal protection compared with the reference drug. Semi quantitative method and histological examination was used to determine neurodegeneration. Nuclei of the neuron stain blue-black while cytoplasm stained pink.

Figure 3 Photomicrograph showing immunohistochemistry labelling of Neuron specific enolase (NSE) expression in adult rats brain PFC exposed to valproic acid and treated with different concentration of NLS extract and reference drug(x 400)

Grp1 shows photomicrograph of NSE expression within the prefrontal cortex. The dendrites processes are stained brown and are unremarkable consistent with normal brain tissue. Grp 2 section shows moderate expression of NSE compared to the control consistent with neuronal damage. The Grp 3, Grp 4, Grp 5 and Grp 6 sections were all negative for neuron specific enolase immunohistochemistry stain. Extract administered showed neuroprotection at all concentration compared with the reference drug

Figure 4 Photomicrograph showing immunohistochemistry labelling of B-cell lymphoma (bcl-2) expression pattern in adult rats brain PFC exposed to valproic acid and treated with different concentration of NLS extract and reference drug (x 400)

This method was employ to localize and labelled the bcl-2 tissue protein. Sections showed negative or under expression for bcl-2 in all the groups.

4. Discussion

Valproic acid induces oxidative stress by compromising the antioxidant status of the neuronal tissue leading to oxidative stress [70]. Exposition of a new approach on valproic acid induced hepatotoxicity includes its involvement in lysosomal membrane mediated leakiness and cellular proteolysis [71]. Oxidative stress is recognized as a promoter of myriad of pathological disorders due to pro-oxidant compound accumulation [72]. Considering lipid-rich organs such as the brain, ROS may lead to lipid peroxidation, consequently impairing neurotransmitter signaling, which further

aggravates neuronal functions [73, 74]. Brain cells are particularly sensitive to oxidative damage due to their high oxygen uptake; therefore, the regular consumption of antioxidants is important concerning neuroprotection [75, 76].

Although brain tissue contains endogenous antioxidants capable of ROS reduction, some enzymes also contribute to promote redox homeostasis. However, the depletion of this antioxidant system by ROS/RNS results in oxidative-related neurotoxicity [75, 76]. Numerous investigations into therapeutic strategy to mitigate oxidative stress induced neurodegeneration by the use of antioxidants abound in literature [77, 78]. The goal of neuroprotection is to limit neuronal dysfunction after injury and maintenance of integrity of cellular interactions in the brain which result in undisturbed neural function.

Medicinal natural products are regarded as the major antioxidant sources in medicine due to the high reducing power of many plant secondary metabolites [71]. In Nigeria, NLS extract is employed for variety of uses [40 - 57] in ethnomedicine. Therefore we investigated potential ameliorative effects of NLS extract on valproic acid induced oxidative stress and neurotoxicity in rats and its effect on NSE and bcl-2 protein expression.

In this study, the increased production of ROS by VPA caused inactivation of antioxidant enzymes which reflects their impingement on the disease control group indicating oxidative stress. The assessment of the effect of NLS extract ability in attenuating oxidative stress markers were evaluated by assessing the levels of SOD, GSH, GPX, CAT and GST. Reduced glutathione (GSH) is a major antioxidant and redox regulator and plays an important role in defense against oxidants and electrophiles [26]. Free radical generation is implicated as the foundation of abundant neurological and neurodegenerative disorders such as ischemia-reperfusion, seizure, Parkinson's and Alzheimer's disease and antioxidant therapy have been known to protect against such CNS insults [3]. The results obtained in this study are in agreement with other studies which demonstrated that free radical scavenging property of medicinal plants is majorly responsible for protection against oxidative stress induced pathologies and dysfunction caused by VPA [26, 29]. GSH plays a key role in cellular function and viability [79]. In this study valproic acid mediated downregulation of GSH by 35% in diseases control group, and VPA supplementation along with NLS dose dependently and significantly increased neuroprotection by upregulating GSH to 60%, 75%, and 87% with increasing doses of NLS extract by 50, 100 and 200 mg/kg respectively as against 40% protection offered by vinpocetine. The results do not corroborate other report of elevation of GSH during xenobiotic intoxication [80, 81]. GST is reported as a multifunctional enzyme, which plays an important role in the detoxification of toxic electrophiles by catalyzing the conjugation of these electrophiles with GSH [82]. The intoxication with VPA increased neurotoxicity of this biomarker by 63%; however posttreatment with increasing dose of NLS resulted elevated neuroprotection by 80% across all concentration of NLS extract but the effect of vinpocetine was more than double as it offer 180% neuroprotection. GPx is a selenium-containing enzyme, described to play a significant role in the reduction of H_2O_2 and hydroxide and removal of excess free radicals to non-toxic products [83]. The result of investigation revealed that VPA-induced a declined of GPx by 71%; this was elevated following supplementation along with NLS from 80 to 100% with increase dosing from 50 – 200 mg/kg and vinpocetine by 100%. SOD which converts superoxide to hydrogen peroxides and water and CAT which convert hydrogen peroxide to water and oxygen were both diminished in VPA induced intoxication; however the supplementation of NLS extract overturn diminished CAT and SOD resulting in significant increase in percentage neuroprotection. Lipid peroxidation is a consequence of ROS mediated cell damage. This was seen by the elevated level of malondrialdehyde (MDA) in the frontal cortex tissue homogenates following VPA intoxication. This corroborates induction of lipid peroxidation by VOA done by Morsy et al. [29]. Treatment with NLS extract showed significant dose dependent reduction in lipid peroxidation, the highest dose showing the greatest neuroprotective activity (102%, 113%, and 118% respectively). The level of pressure induced by frontal cortex protein concentration was slightly reduced in the disease control group (18%), there was however a dose dependent increase in the total protein after treatment with NLS extract progressively from the least dose to the highest dose administered. Taking together, with the ability of NLS to ameliorate the effects of VPA – induced neurotoxicity its potential mechanism might in part include boosting antioxidant status of the neuronal tissue leading to mitigation of oxidative stress [70], inhibition of VPA-induced lysosomal membrane mediated leakiness and cellular proteolysis [71].

Haematoxylin and eosin used to demonstrate tissue morphology revealed that high concentration of NLS extract exhibited neuroprotective potentials compared with the reference drug in the present animal model by preventing further loss of neuron. Immunohistochemistry using neuron specific enolase (NSE) equally demonstrated the effect of valproic acid on the brain and the neuroprotective effect of the extracts. Isgro et al. [84] reported NSE as a specific biomarker for neurons and NSE provides quantitative measures for brain damage and it can improve the diagnosis and prognosis of intracranial hemorrhage, ischemic stroke and seizures. The present study revealed positive expression of NSE in the group treated with valproic compared with treatment group demonstrating neuronal damage and negative expression. This is in line with previous studies, Sahu et al. [85] who reported that destruction of

neurons leads to the release of NSE from the cytoplasm and dendrites of neuron into the cytosol leading to increased NSE estimation in neurodegeneration.

Additionally, Wijnberger et al. [86] reported the significant use of NSE in amniotic fluid, cerebrospinal fluid, umbilical cord and neonatal blood as neuronal marker and predictor of brain damage in the newborn especially after preterm labor. Also, according to Haque et al. [87] upon stimulation, NSE can move to cell surface and contribute to different pathologies such as injury, inflammation, autoimmunity and cancer. Cell-surface expression of enolase can be detected on activated macrophages, causing extracellular matrix degradation, production of pro-inflammatory cytokines and invasion of inflammatory cells in the sites of inflammation. NSE is found in the cytoplasm and dendrites of neurons and neuroendocrine cells [88]. It is a sensitive marker of neuronal damage in the central nervous system (CNS). Changes in membrane integrity as a result of neuronal injury can cause leakage of protein such as NSE from cytosol into extracellular space. Higher concentrations of NSE have been detected in the gray matter of adult brains, while lower levels of NSE are reported in the white matter. Thus, NSE could be a biochemical marker to estimate neuronal damage in brain lesions. The effect of NLS extract downregulating NSE protein expression indicate potential role in modulating brain damage.

Bcl-2 is expressed in the nervous system and its targeted gene disruption leads to a profound loss of peripheral neurons, including motor neurons, sensory neurons and sympathetic neurons as reported in the present study. The disruption of the bcl-2gene leads to a reduced expression and therefore a loss of its proapoptotic function, leading to an increase loss of neurons. This may be due to the low basal level of bcl-2 in the adult brain in comparison to its high expression in mature peripheral neurons. However, the expression of bcl-2 is altered in many acute and chronic models of neuronal cell death and neurodegeneration and remains a focus of treatment strategies. The expression of bcl-2 has been shown to be up-regulated via activation of the phosphoinositol 3-kinase (PI3-K)/Akt pathway [89]. This is also in line with the negative expression of bcl-2 observed across all groups in the present studies. Histone deacetylases (HDAC), along with histone acetyl-transferases (HAT), modulate gene expression by decreasing or increasing gene expression, respectively. HDACs and HATs act in concert with one another, maintaining homeostasis during normal cellular functions [90]. During abnormal states, however, this imbalance is tilted in favor of HDAC activity, resulting in increased oxidative stress and inflammation [91, 92, 93]. The NLS extract has putative biomolecules elucidated from phytochemical screening which might in part interact at various pharmacological targets to modulate VPA- induced protein expression.

5. Conclusion

This study demonstrates that NLS extract exhibit significant anti-oxidative effects against valproic acid induced neurotoxicity. The result confers to an extent a neuroprotective role on NLS extract as it demonstrate potentials to reverse the neurotoxicity induced by sodium valproate. Recent studies confirmed the potential source of natural antioxidants in the extract. The investigation indicate a potential therapeutic effect of NLS extract with prospect of development into new drug for management neurodegenerative diseases.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest declared.

Statement of ethical approval

The animal study was approved by the Institutional Animal Care and Use Committee with approval code (No. UPHAEC / 2018 / 092) in April 2018. The procedures adopted for animal studies were in accordance with the "Guide and Care and Use of Laboratory Animals" National Research Council, 2011) and study was executed with stringent compliance with Food and Drug Administration Good Laboratory Practice Regulation, 1987).

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How to cite this article

Nwidu LL and Oboma YI. (2019). *Nauclea latifolia* stem-bark extract protects the prefrontal cortex from valproic acid - induced oxidative stress in rats: Effect on B-cell lymphoma and neuron specific enolase protein expression. GSC Biological and Pharmaceutical Sciences, 7(1), 44-61.