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## Ameliorative effect of *Nauclea latifolia* leaf extract on valproic acid-induced neurotoxicity in oxidative stress rats: Role of p53 and S100B.

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### Abstract:

*Nauclea latifolia* leaf (NLL) present with arrays of central nervous system or neuropharmacological effects linked to uncommon display of phytochemical contents. The aim of our study was to evaluate the ameliorative effects of NLL in valproic acid-induced neurotoxic effect in oxidative stressed rats. The extract was prepared by first defatting in n-hexane before extraction with methanol after 72 hour maceration. The extract was subjected to phytochemical screening using standard procedure. The NLL extract obtained was administered in three doses (50, 100, 200 mg/kg) after pre-treatment of the experimental animals with high dose valproic acid (VPA) (500 mg/kg) orally daily by subchronic exposure for 30 days. At the end of the experiment the animals were sacrificed and the prefrontal cortex (PFC) of the cerebrum abstracted and homogenized in ice for biochemical assays. The toxicological assay evaluated the weights changes in animals daily; the biochemical assays estimated the levels of antioxidants and lipid peroxidation; histopathological features were examined and the role of S100B and p53 protein expressions were investigated using histochemistry. The results indicated that intoxication of rats with vPA, induces change in weight in the animals. The three doses of NLL significantly ameliorated VPA-induced neurotoxicity. NLL extract significantly downregulate the lipid peroxidation levels of malondialdehyde (NLL group vs. diseases control group ( $P < 0.001$ ), upregulate the levels of reduced glutathione ( $P < 0.05 - 0.01$ ), glutathione peroxidase ( $P < 0.001$ ), superoxide dismutase ( $P < 0.001$ ), catalase ( $P < 0.05 - 0.01$ ) and glutathione-s-transferase ( $P < 0.05 - 0.001$ ) in the PFC. These study demonstrate the efficacy of NLL in preventing neuronal insults by diminishing the expression of S100B and p53 protein in the PFC. The results of this findings indicate NLL effectiveness in ameliorating subchronic brain damaged induced by VPA by optimization antioxidants parameters, suggesting possible therapeutic potentials in Alzheimer's diseases.

**Keywords:** Neurotoxicity, brain, immunohistochemistry, oxidative stress, *Nauclea Latifolia*, valproic acid

### Introduction

Medicinal plants antioxidants possess multifunctional biological activities targeting the central nervous system (CNS) [1-3].

The biomolecule in these antioxidants might exist as flavonoids or polyphenols, valuable assets for protection against oxidative stress. The public and scientific interest in the utilization of these natural antioxidants continues to grow due to their potentials in mitigating oxidative stress or indeed perceived health-promoting benefits. Oxidative stress is firmly linked to the etiology of many pathology including neurodegenerative diseases (NDDs), [4-6] psychiatric disorders [7] cancers [8] hypertension [9] and diabetes mellitus [10]. Neurodegenerative diseases (NDD) are pathological conditions marked with insidious onset and chronic

progression. A common prototype of NDD is Alzheimer's disease (AD) in which oxidative stress underpinned extensive neuronal death by ATP depletion, axonal degeneration, mitochondrial dysfunction, generation of macromolecules (lipids, proteins, nucleic acids and carbohydrates), impaired proteolysis and autophagy, and marked microglial activation and inflammation [11-13]. Genetic influence by ApoE genotype play significant role in oxidative stress, endoplasmic reticulum stress response; mitochondrial function and immune function [14,15].

Numerous ROS-responsive transcription factors are produced due to the interaction between oxidative stress and A $\beta$  at multiple levels. Increase production and accumulation of A $\beta$  might in part induce the expression of amyloid precursor proteins (APP),  $\gamma$ -secretase and beta-site amyloid precursor protein

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cleaving enzyme 1 (BACE1) [16,17]. Oxidative stress causes a derangement in signaling pathways instigating tau hyper-phosphorylation [6].

Experimentally, oxidative stress can be induced in the laboratory animal by variety of stimuli: physical, chemical, biochemical and toxicological. Drug such as valproic Acid (VPA) has demonstrated increasing evidence as a neuroprotective at low doses [18-20], however, at high concentration numerous intoxicative effects such as neurotoxicity [21] hepatotoxicity [22]. Valproic acid is a widely used drug to ameliorate epilepsy, migraine and bipolar disorders [23]. The mechanism of valproic acid induced neurotoxicity is by VPA-induced hyperammonemic encephalopathy (VHE) [24] which in part leads to irreversible brain damage. Oxidative stress mechanism underlies VPA induced multi-organ toxicity [25-27]. Antioxidants with protective effects against neurodegeneration induced by oxidative stress have been reviewed [28,29].

*Nauclea latifolia* ((syn. *Sarcocephalus latifolius*, Rubiaceae) is frequently called the African pin cushion tree (English) and also known as “African peach”. Other local names are mbong-ibon (Ibibios and Effik), tafashiya or tafiyayaiga (Ibo), ubulinu or oovorolu and egbe yesi or egbesi (Yoruba) in Nigeria. The plant is commonly found in the humid tropical rainforest zone or in Savannah woodlands of West and Central Africa. It is an evergreen multi-stemmed tree growing to about an altitude of 200 m. *Nauclea latifolia* leaf (NLL) is of interest to traditional healers, phytochemist and pharmacologist as it is widely used in different regions of Africa for treating a variety of illnesses, including epilepsy, convulsion, pain and other parasitic infections. In the orthodox medicine of Cameroon, the leaves of *Nauclea latifolia* Smith (Rubiaceae) is used for the treatment of cerebral malaria, behavioral disturbances in mentally-retarded children or central nervous system diseases, such as anxiety, depression and epilepsy [30-32]. A range of bioactive indole alkaloids identified in the tissue of NLL and its neuropharmacological potentials have endeared this plant to the scientific community besides its worldwide scientific highlight following the elucidation of tramadol, a synthetic analgesic from the root of the tree in the 1970s. Pharmacological reports indicate its effectiveness as antipyretic and analgesic [33,34] hypoglycemic [35] and anticonvulsant [36] effects. Its central nervous system effects indicate a role in purinergic neurotransmission [37] exhibited anxiolytic and antidepressant in mice attributed to isolated indole alkaloid, angustine [38,39].

Considering the multifunctional in vitro effects displayed by NLL on numerous pharmacological

targets related to neurodegeneration, epilepsy, pain and purinergic signaling, this study intend to evaluate the ameliorative effect of NLL extract in valproic acid -induced neurotoxicity in oxidative stress rats and to establish potential effects on S100B and p53 protein expression in rat brain prefrontal cortex (PFC).

This study is significant in many respects: it will for the first time demonstrate NLL potentials in attenuating the neurotoxic effect induced by valproic acid commonly used as anticonvulsant therapy; establish its effectiveness in mitigating oxidative stress induced-neurodegenerative diseases with implication for developing novel therapeutic approach in the management of CNS diseases such as Alzheimer’s disease.

The health and management implication of the study is hinged on the two major proteins p53 and S100B proteins investigated which are biomarkers of diagnostic and prognostic significance in clinical conditions such as brain trauma, cancers, ischemia and neurodegenerative, melanoma, inflammatory and psychiatric diseases. The inhibition of p53 as an ideal target to restore neuronal functions and might represent a promising therapeutic approach for amelioration of several neurodegenerative conditions. Molecules investigated to prevent activation of p53-dependent apoptotic cascade potentially may prolong neuronal survival and protect neurons against apoptotic stimuli. Taking together, the probable ability of NLL extract downregulating these aberrant proteins upregulated in several pathological conditions, will definitely bring to fore its therapeutic potentials in management and development of pharmaceuticals for a broad spectrum of pathologies. Further studies will be required for clinical trials of NLL to established pharmacological results of this investigation.

## Materials and Methods

### 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% (Lobal Chemie, Mumbai, India), n- hexane (extrapure 85%) (Lobal Chemie, Mumbai, India), Diethyl ether (Lobal Chemie, Mumbai, India), formalin (Lobal Chemie, Mumbai, India); Avidin Biotin Complex (Boster bioengineering 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 (Labsience, England, UK), centrifuge model 412B (Techmel and Techmel, MI, USA), Water bath (Techmel and Techmel, MI, USA).

### Collection and authentication of plant materials

The stem bark of *Nauclea latifolia* were collected in Uyo, Akwa Ibom state and supplied by Mr. Okon Etefia, a traditional herbalist, attached to Pharmacognosy Department, University of Uyo, Nigeria and 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(a) had already been deposited at Department of Pharmacognosy, University of Uyo, Akwa Ibom State, Nigeria.

### Preparation of *N. latifolia* stem-bark (NLS) extract

The dried *N. latifolia* leaf (NLL) was pulverized to fine particles using mechanical grinder. A 250 g weight of NLL powder was then macerated in 2000 mL of n-hexane for defatting. After 24 hours of maceration, the extract was concentrated using a rotary evaporator and the marc submerged in 2000 mL of methanol. It was macerated for 72 hours while shaking vigorously every 2 hour for 12 hours. Rotary evaporator was used to concentrate the extract and evaporated to dryness on the water bath at 45°C. The percentage yield was then calculated.

### Phytochemical screening

Phytochemical screening of the plant extract was carried at the Pharmacognosy and Phytotherapy Department laboratory, University of Port Harcourt. The bioactive agents screened include: alkaloids, triterpenoids, flavonoids, cardiac glycosides, saponins, and phlobatannins using standardized protocol [40].

### Animals and drug administration

Forty two male Wistar albino rats were used. The animals (150-170 g) were obtained from the animal house, Department of Pharmacology and Toxicology, University of Nigeria, Nsukka. The animals were acclimatized in the University of Port Harcourt Animal House for 14 days under standard laboratory conditions. The animals were randomly selected and housed in pathogen free plastic cages (n=7) per cage under relative humidity (40-55%) and ambient temperature (26°C) and exposed to daily circle of night and day. The rats were provided with pelleted rodent chow (Vital Feeds, Edo state, Nigeria) and water ad libitum and 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/2018/008). The acute toxicity (LD50) of NLL was reported as 850 mg/kg [41] in Wistar albino rats and 1414.2 mg/kg body weight in Swiss albino mice [42]. The dose levels of 50, 100 and 200 mg/kg are the doses chosen in the current study. 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 30 days when administered orally by gavage in the experimental animals. Each one mL contains 200 mg sodium valproate. The animals were divided randomly into six groups with 7 animals per group.

The NLL, valproate and vinpocetin (Cognitol®) (the reference drug) were administered orally per kg of body weight once daily for 30 days. Sodium valproate (500 mg/kg) was administered one hour prior to the administration of the control drugs or extracts respectively for animals in groups 2 to 6. The NLL 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 as follows:

Group 1 (negative control): The animals in this group received 2% Tween 80 in 10 ml/kg distil water.

Group 2 (disease control group) : The animals in this group received sodium valproate followed by 2% Tween 80 in 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, 100 and 200 mg/kg respectively.

Group 6 (reference control): The animals in this group received sodium valproate followed by vinpocetine 25 mg/kg.

The rats were administered valproic acid (500 mg/kg b.w.) and one hour later, either distil water, NLS or vinpocetin was administered adopting standard procedure .

Neurotoxicity percentage (%) was deduced using this formular = 
$$\left[ \frac{VA - W(\text{negative control}) \times 100}{W(\text{negative control})} \right]$$

Neuroprotective activity (%) was calculated as follows:

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

Where, NLL, VA, and W are mean experimental variables estimated in the rats treated with valproic acid (VA) plus NLL (Test groups), valproic acid (VA) only (diseases control group) and distilled water treated animals (W) (negative control) respectively [43].

#### Evaluation of weights

The initial weights of the rats were recorded followed subsequently with daily weights monitored for 30 days and the final weights before sacrificing the animals were recorded. The final body weights and the weights of internal organs such as the brain, the heart, the liver, the lungs, the kidney, the stomach, the spleen, the ovary and the testes were exsanguinated and weighed and the relative organ weights calculated.

#### Preparation of prefrontal brain cortex (PFC) homogenates

All rats were decapitated and the whole brain excised and weighed immediately. The prefrontal cortex was removed and immersed in ice-cooled physiological saline. A 10% homogenate of the PFC was prepared. The homogenate was centrifuged ( $4000 \times g$ , 10 min) at  $-80^\circ\text{C}$  to remove debris and nuclei. The resultant supernatant was stored at  $-80^\circ\text{C}$  for various biochemical assays.

#### Biochemical Assays

Proteins and Malondialdehyde (MDA) levels, as well as reduced Glutathione (GSH), glutathione peroxidase (GSPx), Catalase (CAT), Glutathione-S-transferase (GST), Superoxide dismutase (SOD) was estimated from prefrontal cortex of the rat brain homogenates supernatant as follows:

#### Determination of malondialdehyde (MDA)

Lipid peroxidation was determined by measuring thiobarbituric acids reactive substances (TBARS) method [44]. An aliquot of 0.4 mL of supernatant was mixed with 1.6 mL of Tris-KCl buffer and 0.5 mL of 30% trichloroacetic acid (TCA) added. Finally, 0.5 mL of 0.75% TBA was added and then placed in a boiling water bath for an hour. This was then cooled in ice and centrifuged ( $3000 \times g$ , 10 min). The absorbance of the clear supernatant collected was measured against a reference blank of distilled water at 532 nm and the MDA level estimated [45] method and expressed as nmol of MDA/g of wet tissue using a molar extinction coefficient of the chromophore ( $1.56 \times 10^{-5} \text{ /M/cm}$ ).

#### Reduced glutathione (GSH) level

Sedlak and Lindsay [46] method was used to appraise the level of reduced glutathione. To an aliquot sample (0.2 mL) was added 1.8 mL of distilled water and 3 mL of the precipitating solution (4%

sulphosalicylic acid) and vortexed. The mixture was allowed to stand for approximately 5 minutes and then centrifuged ( $1200 \times g$ , 10 min). To 1 mL of the filtrate was added 4 mL of 0.1 M phosphate buffer and 0.5 mL of DTNB (Ellman's reagent). Similarly a blank was prepared by addition of 4 mL of 0.1 M phosphate buffer, 1 mL of dilute precipitated solution and 0.5 mL DTNB. The solution was incubated at room temperature for 15 minutes and read at 412 nm on a spectrophotometer.

#### Glutathione peroxidase

The level of glutathione peroxidase (GSPx) was estimated by adopting the method [47]. Sodium phosphate buffer mixture (1 mL) was prepared by combining 0.2 mL sodium azide, 0.4 mL of GSH and 0.2 mL of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and 1 mL of 1:10 extract of cell homogenate and made up to 4 mL volume with distilled water. This was incubated for 3 min at  $37^\circ\text{C}$  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 [45]. Tissue homogenates were centrifuged ( $12,000 \times g$ , 10 min) and an aliquot of supernatant was diluted with water to make a 1:10 dilution. The diluted sample (200  $\mu\text{L}$ ) was added carbonate buffer (pH 10.2) and 0.3 mL of 0.3 mmol/L epinephrine was supplemented 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 [48] 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  $\text{H}_2\text{O}_2$ /min. A 0.2 mL of sample was added to 50 mM of phosphate buffer (pH 7.4) containing 100 mM (v/v) of  $\text{H}_2\text{O}_2$  in a total of 1 mL. The reaction mixture was incubated for 2 min at  $37^\circ\text{C}$  and the rate of absorbance change (AA/min) at 240 nm was recorded which indicated the decomposition of  $\text{H}_2\text{O}_2$ . 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 [49]. 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.

#### Histopathology studies

##### Hematoxylin and eosin staining

After 30 days exposure to valproic acid and NLL study, all rats were sacrificed by decapitation. The prefrontal cortex was excised immediately, fixed by 20% phosphate buffered formalin for 48 hrs. Tissues were sectioned using rotary microtome model (Leica Microsystems, Wetzlar, Germany at 6  $\mu$ m and floated in water bath onto charged slides. The sections were dried on hot plate at 60°C for 2 hours. 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 damage and neurodegenerative features such as shrinkage of the neuron, hyperchromasia,

and nuclear pyknosis as revealed by hematoxylin and eosin staining techniques following the procedure of [50]. Cortical Purkinje cells estimation was based on semi-quantitative scale described in previous study was used to assess the extent of neurodegeneration in the PFC with 400 $\times$  magnification.

#### Immunohistochemical examination

Immunohistochemistry assessment of neuronal damage was executed by evaluating the levels of S100B protein and tumor suppressor gene p53 markers. Avidin Biotin Complex (ABC) method also referred to the Avidin biotin immunoperoxidase method as described [51] was adopted. Colorectal cancer cells line known to be positive for p53 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 S100B and p53 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 Glial fibrillary acidic protein (GFAP) and p53. The stained cells without any form of brown colors were scored as negative.

#### Statistical analysis

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

#### Results

##### Effect of NLL on body weight

The effects on body weights are evaluated in Table 1 below. No treatment or intoxication related adverse-effect on body weight was observed from his study.

**Table 1:** Effects of valproic acid intoxication and pretreatment with NLL extract on body weights of experimental rats following continuous oral sub-acute dosing for 4 weeks.

Group	Initial Weight	WK 1	WK2	WK3	WK4	Final Weight
Grp 1	162.6 $\pm$ 23.7	164.8 $\pm$ 23.7	167.3 $\pm$ 25.1	167.9 $\pm$ 18.7	183.6 $\pm$ 30.45	167.9 $\pm$ 18.7
Grp 2	168.3 $\pm$ 6.3	175.8 $\pm$ 2.8	182.8 $\pm$ 10.3	184.4 $\pm$ 17.6	178.1 $\pm$ 38.3	184.4 $\pm$ 17.6
Grp 3	174.5 $\pm$ 26.1	172.4 $\pm$ 36.7	180.1 $\pm$ 27.2	177.7 $\pm$ 34.2	176.1 $\pm$ 32.8	177.7 $\pm$ 34.2
Grp 4	143 $\pm$ 19.2	150.4 $\pm$ 15.4 <sup>ce</sup>	180.1 $\pm$ 27.2	170.6 $\pm$ 13.7	180.4 $\pm$ 14.5 <sup>af</sup>	170.6 $\pm$ 13.7
Grp 5	153.5 $\pm$ 37.6	156.4 $\pm$ 38.8 <sup>ce</sup>	159.7 $\pm$ 33.2	155.6 $\pm$ 32.6	164.7 $\pm$ 26.9	155.6 $\pm$ 12.6
Grp 6	145.7 $\pm$ 6.8	154.3 $\pm$ 9.7	172.8 $\pm$ 17.8	85.2 $\pm$ 16.4 <sup>bf</sup>	190.5 $\pm$ 17.4 <sup>bf</sup>	185.2 $\pm$ 16.4 <sup>af</sup>

**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 ml/kg; **Group 3:** receiving NLL extract (50 mg / kg b.w.) + valproic acid 500 ml/kg; **Group 4:** receiving NLL extract (100 mg / kg b.w.) + valproic acid 500 mg/kg; **Group 5:** receiving NLL extract (150 mg / kg b.w.) + valproic acid 500 ml/kg, **Group 6:** Reference control receiving vinpocetin (30 mg/kg b.w.) + valproic acid 500 ml/kg.

NLL: *Nauclea Latifolia* Leaf

Values presented as mean  $\pm$  standard deviation (n=3-7), <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01, <sup>c</sup>P < 0.001, <sup>e</sup>Values are compared with Grp 2, <sup>f</sup>values are compared with initial weight using one way ANOVA and Turkey Test.

There was significant increase ( $P < 0.001$ ) in the body weights of the animals when compared with the initial weights at the start of the experiment. Comparison of the disease control group with the normal control showed a significant reduction in body weight of the rats during the course of the experiment.

#### Effect of NLL on MDA, antioxidant and protein levels in PFC

The effect of NLL after sub-acute intoxication with valproic acid on lipid peroxidation examined by MDA formation, and antioxidant activity is presented in Table 2.

The result indicated neuronal damage as evidence in statistical significant depression ( $P < 0.05$  -  $< 0.001$ ) of SOD (-35%), GSH (-53%), GST (-57%), GPx (-91%), CAT (-64%) and TP (-9%) compared with the normal control group. Similarly lipid peroxidation was evident by increased formation of MDA (195%) in the intoxicated rats with  $P < 0.001$ . However, sub-acute treatment with NLS extract protected against the neurotoxicity induced by valproic acid. This neuroprotection was demonstrated by the elevation of protein (59.3%, 105.1%, 122.0%), SOD (59%, 68%, 91%), GSH (67%, 84%, 98%), GST (50%, 75%, 100%), GPx (30%, 50%, 100%), CAT (89%, 111%, 122%) and TP (59%, 105%, 122%); and reduction of MDA (25%, 61%, 90%) with NLL 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. Vinpocetine demonstrated neuroprotective activity on antioxidant enzymes with increased in SOD, GSH, GST, GPx, CAT, and TP by 140%, 99%, 100%, 70%, 113%, and 68% but reduced lipid peroxidation in brain prefrontal cortex by 97%.

#### Histomorphological observation of the neurons in PFC

HE staining of neurons loss in the PFC is shown in Figure 1, Plate 1. Rats in the control group have morphological intact and tightly arranged neurons in the PFC (Group 1). Generalized damage are distinctly observed in the PFC treated with VPA (Group 2b). 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 (Group 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 (Group 6). Neurons in the PFC of rats treated with NLL in group 5 almost appear normal with the reference Group and the control Group indicating neuroprotection.

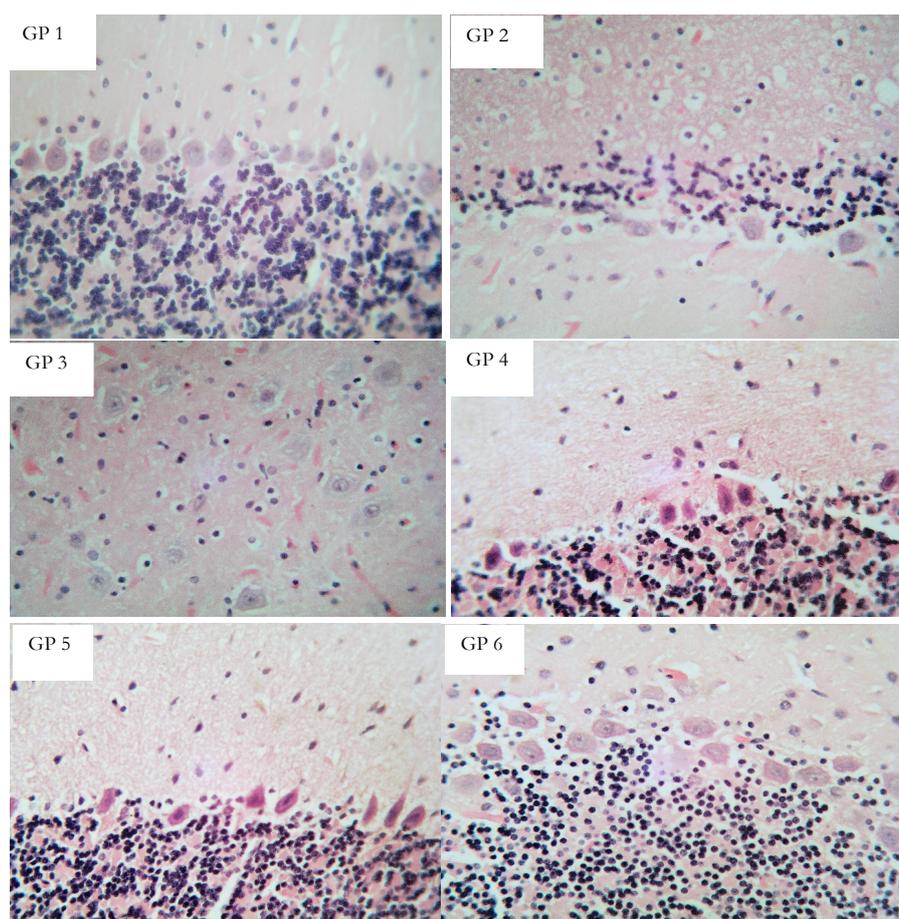
**Table 2:** Effects of valproic acid intoxication and pre-treatment with NLL hydro-methanol extract on the levels of antioxidant and lipid peroxidation following sub-acute dosing for 30 days.

PARAMETERS	GRP1	GRP2	GRP3	GRP4	GRP5	GRP6
SOD	0.63 ± 0.03	0.41 ± 0.07*** (-35%)	0.54 ± 0.02** (59%)	0.56 ± 0.03** (68%)	0.51 ± 0.01** (91%)	0.72 ± 0.02*** (141%)
GSH	1.84 ± 0.09	0.86 ± 0.08** (-53%)	1.52 ± 0.33* (67%)	1.68 ± 0.28NS (84%)	1.82 ± 0.51** (98%)	1.83 ± 0.04** (99%)
GST	0.07 ± 0.004	0.03 ± 0.005** (-57%)	0.05 ± 0.02* (50%)	0.06 ± 0.02*** (75%)	0.07 ± 0.01** (100%)	0.07 ± 0.01* (100%)
GPx	0.11 ± 0.002	0.01 ± 0.004* (-91%)	0.04 ± 0.004NS (30%)	0.06 ± 0.01*** (50%)	0.08 ± 0.003** (70%)	0.08 ± 0.004* (70%)
CAT	0.86 ± 0.13	0.31 ± 0.09* (-64%)	0.8 ± 0.24** (110%)	0.92 ± 0.25* (110%)	0.98 ± 0.27** (122%)	0.93 ± 0.05* (123%)
MDA	0.37 ± 0.05	1.09 ± 0.10* (195%)	0.91 ± 0.06* (25%)	0.65 ± 0.04*** (61%)	0.44 ± 0.04*** (91%)	0.39 ± 0.05*** (97%)
T.P	6.92 ± 0.46	6.33 ± 0.28NS (-9%)	6.68 ± 2.14NS (59%)	6.95 ± 0.69NS (105%)	7.05 ± 0.60NS (122%)	6.73 ± 0.21NS (68%)

**Group 1:** Negative control group 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 NLL extract (100 mg / kg b.w.) + valproic acid 500 mg/kg; **Group 4:** receiving NLL extract (200 mg / kg b.w.) + valproic acid 500 mg/kg; **Group 5:** receiving NLL extract (400 mg / kg b.w.) + valproic acid 500 ml/kg; **Group 6:** Reference control receiving vinpocetine (25 mg/kg b.w.) + valproic acid 500 mg/kg.

NLL: Nuclea Latifolia Leaf; SOD: Superoxide Dismutase; GSH: Glutathione; GST: Glutathione-S-Transferase; GPX: Glutathione Peroxidase; CAT: Catalase; MDA: Malondialdehyde; TP: Total Protein in the prefrontal cerebral cortex.

Values presented as mean ± standard deviation (n=3-5), \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , ns=no significance analysis was done using one way ANOVA and Turkey Test.



**Figure 1:** PLATE 1 x 400 photomicrography of hematoxylin and eosin stained brain tissues showing glial cells and neuronal expression in valproic acid induced neurodegeneration treated with NLL extract. GRP1 shows photomicrographs of negative control showing healthy neurons within the purkinje layer with few apoptotic within molecular layer consistent with the histomorphology of the layer. GRP 2 –disease control shows extensive pyknosis, vacuolation and reduced neuronal population within the granular layer indicating neuronal damage. GRP3 share the same features with GRP2 in rats treated with 50 mg /kg.b.wt of extract. GRP4 shows moderate neuronal population with little apoptotic cells in rats treated with 100 mg/ kg. b.wt of extract compare with control. GRP5 shows mark expression neurons in rats treated with 200 mg/kg.b.wt compared with negative control, GRP6 treated with 30mg/kg.b.wt of Cognitol shows histomorphology consistent with control. Higher doses of extracts reduces neuronal damage by valproic acid.

Histomorphology (plate 1) shows neuronal protection in group GP 5 and GP6 rats treated with 200 mg/kg.b.wt of extract and 25 mg/kg.b.wt of vinpocetin compared with control.

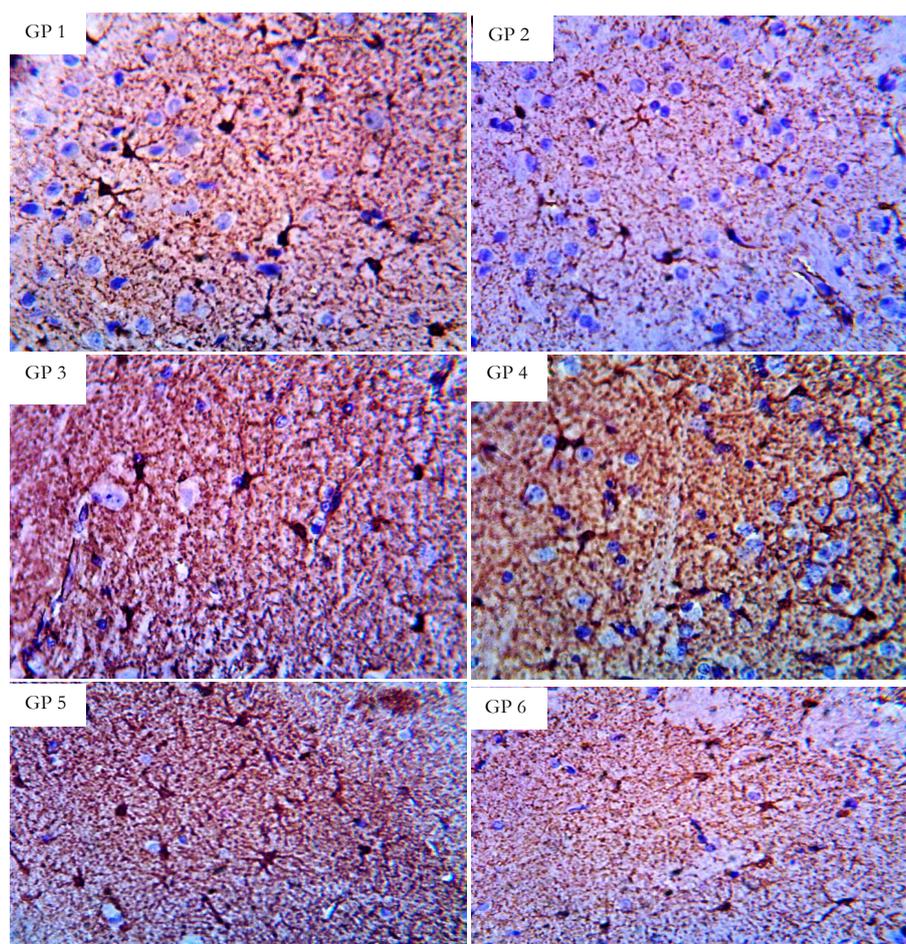
#### **S100 immunohistochemistry in PFC**

Immunohistochemistry labelling of S100B on PFC brain tissues showing glial cells (brownish) expression in valproic acid induced neurodegeneration treated with NLL extract (Figure 1, Plate 2). Glial cells (astrocytes) help to maintain hemostasis and provide support and protection for neurons. A number of positive glial neurons revealing S100B protein expression in PFC is consistent with normal histomorphology is observed in control group (Group1). Increased protein expression of S100B is observed in the diseases control group (Group 2).

Mild protein expression of S100B in PFC of rats treated with 50 mg/kg.b.wt of NLL extract (Group 3). Moderate protein expression of S100B in PFC of rats treated with 100 mg/kg. b.wt of extract (Group 4). Reduced protein expression of S100B and increase proliferation of astrocytes in PFC of rat brain treated with 200 mg/kg.b.wt compared with negative control (Group 5). Similarly the reference control (Group 6) treated with 25 mg/kg.b.wt of vinpocetin demonstrate the reduce protein expression of S100B and astrocytes proliferation. Higher doses of extract lowered neuronal damage. The expression pattern shows the mechanism of protection by the extract which is by astrocytes proliferation.

#### **p53 immunohistochemistry in PFC**

The effect of NLL p53 immunohistochemistry



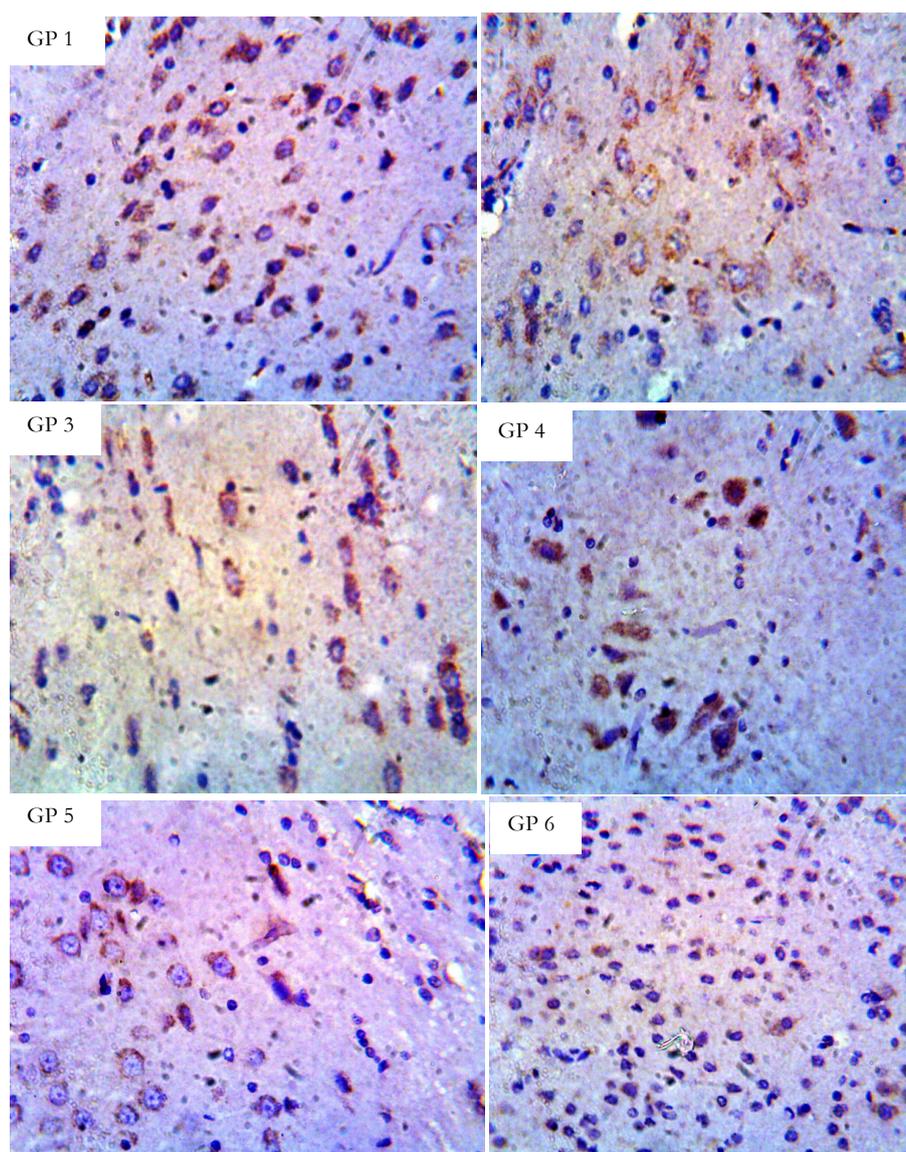
**Figure:2** PLATE 2 x 400 shows immunohistochemistry labelling of S100B on brain tissues showing glial cells (brownish) expression in valproic acid induced neurodegeneration treated with NLL extract. Glial cells (astrocytes) helps to maintain hemostasis and provide support and protection for neurons. GRP1 shows photomicrographs of negative control S100 protein expression in cerebral glia cells consistent with normal histomorphology. GRP 2 (disease control) shows increased expression of S100B. GRP3 shows mild expression in animals treated with 50mg /kg.b.wt of extract. GRP4 shows moderate expression in rats treated with 100mg/ kg b.wt of extract GRP5 shows reduced expression of S100B and increase proliferation of astrocytes in rats treated with 150mg/kg.b.wt compared with negative control and reference GRP6 treated with 30mg/kg.b.wt of Cognitol. Higher doses of extract lowered neuronal damage. The expression pattern shows the mechanism of protection by the extract is by astrocytes proliferation.

is shown in (Figure 3, Plate 3). Few p53 positive neurons were observed in the control group (Group 1). Great number of p53 positive neurons were noted in the PFC of rats treated with VPA indicating generalized p53 positive neurons were over expressed in the disease control rat brain (Group 2). In contrast, administration of NLL in three doses (50, 100, 200 mg/kg) progressively downregulate p53 positive neurons observed in the PFC dose dependently (Groups 3–5). Similarly, vinpocetin 25 mg/kg markedly reduced the number of p53 positive neurons in the PFC (Group 6). Generally the immunohistochemistry reveal that the extract demonstrated neuronal protection by suppression of p53 gene and activation of astrocytes as expressed by S100B immunostain.

## Discussion

Phytotherapy in African traditional medicine plays a significant role in the management of neurotoxic diseases, mainly among populations with very low income. Wide variety of plant species, such as *Annona muricata* Linn, *Annona senegalensis* Pers (Annonaceae), *Bidens pilosa* Linn (Asteraceae), *Bryophyllum pinnatum* (Lam) Oken (Crassulaceae), *Citrus sinensis* (Linn) Osbeck (Rutaceae), *Clerodendron thomsoniae* Balf (Verbenaceae), *Daniellia oliveri* (Rolfe) Hutch and Dalz (Caesalpiniaceae), *Datura stramonium* Linn (Solanaceae) etc. are reported useful for empirical treatment or management of neurological conditions. They are neuroprotective agents and are employed in treatment of neurotoxic diseases [52].

Valproic acid, (VPA) is a double edged-sword



**Figure 3:** PLATE 3 x 400 shows immunohistochemistry labelling of p53 gene. Its expression is directly proportional to its mutation. GP 1 photomicrographs shows expression of p53 on cells of the cerebral tissue. GP 2 shows marked expression of p53 on the neuronal and glia cells of the cerebral cortex when compared with the control indicating disease progression with numerous apoptotic cells. GP 3 and GP 4 shows moderate expressions of p53 on the neuronal and glia cells of the cortex when compared with the control. GP5 and GP 6 Cognitol treated group shows mild and reduced expression of p53. The extract shows neuronal protection by suppression of p53 gene.

drug offering neuroprotection at reduced dose, used as an antiepileptic therapy and mood stabilizer in bipolar mania [53] but present with idiosyncratic neurotoxicity in human [54] and neurotoxicity in experimental animal when used in high concentrations [21]. Valproic acid has been linked with numerous toxicities most serious being hepatotoxicity, neurotoxicity and teratogenicity [55]. VPA induced neurotoxicity has been linked to oxidative stress induced brain damage and neurological impairment [21]. VPA induces oxidative stress by compromising the antioxidant status of the neuronal tissue but the cellular and molecular

mechanisms whereby this branched chain fatty acid-induced neurotoxicity is yet to be decipher [54].

Neurodegenerative disorders are the result of multiple physiopathological processes regulating the neurodegenerative cascade. For this reason, multi-target drug candidates are of great interest for the treatment of such diseases [56,57]. It is known that nature is a rich source of phytochemicals possessing multifunctional properties [58]. Considering the inhibitory effects most alkaloids from *Rubiaceae* family on cholinesterase and monoamine oxidases [38,39]. Their role in enzymatic targets on the CNS

and in neurodegenerative conditions [59] the present worked to evaluate the ameliorative effects of NLL extracts against VPA insults on PFC in the brain of rats. The PFC is involved in a number of higher-order (executive) functions [60] and has significant vulnerability to stress [61] which may be vulnerable oxidative stress. The rationale of the study is to identify potential medicinal plants and CNS potential targets to fight against neurodegeneration.

The ameliorative effect of NLL extract on VPA –induced neurotoxicity study indicate that neuroprotective potentials is achieved in part by the attenuation of oxidative stress, enhancement of acetylcholine system, reduction of histological abnormalities and limiting of cell loss by proliferation of astrocytes in PFC neurons of experimental rats.

This study proffers a supportive role of NLL in therapeutic intervention in neurodegenerative diseases like Alzheimer's disease, Parkinson disease and Amyotrophic lateral sclerosis (ALS). Numerous endogenous antioxidants, SOD, GSH, GST, GPx, and CAT constitute natural body defense system and regulate the balance between the oxidative radicals: reactive oxygen species (ROS) and reactive nitrogen species (RNS) and these natural antioxidants preclude free radical induced insult in the neuronal tissues. Oxidative damage of cellular macromolecules occurs due to downregulation of natural antioxidants and upregulation of ROS/RNS in the neurons. The study shows that NLL upregulated the levels of these antioxidants in the neuronal cells of the PFC dose dependently. The percentage of VPA- induced neurotoxicity on antioxidant profile at PFC range from 35 to 91%. However, the neuroprotection on antioxidant parameters by NLL ranged from 25% to 122% dose dependently. The level of CAT activity was reduced by the VPA subacute treatment in the PFC however similar effects were noticed in the report [21] who observed depression in CAT activity in the cerebellum and cerebral cortex of rats. The subacute therapeutic intervention with NLL indicated dose dependent amelioration alluding to possible neuroprotective potentials.

Tremendous in vitro and in vivo studies have documented the beneficial effects of natural product and their phytochemicals in modulating numerous cell functions related to oxidative stress and/or antioxidant protection [1-3]. Reduced glutathione (GSH) is a major antioxidant and redox regulator and plays an important role in defense against oxidants and electrophiles [55-61]. In this study, the increased production of ROS by VPA caused inactivation of antioxidant enzymes which reflects their consumption on the disease control group

indicating oxidative stress. Assessing the effect of NLL extract showed the attenuation of oxidative stress by the reduction in the antioxidant enzymes SOD, GSH, GPX, CAT and GST.

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 [62]. 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 [21,55]. Lipid peroxidation is a consequence of ROS mediated cell damage. This was seen by the elevated level of malondialdehyde (MDA) in the presence frontal cortex tissue homogenates following VPA intoxication. This corroborates induction of lipid peroxidation by VPA reported [21]. Treatment with NLL extract showed significant dose dependent reduction in lipid peroxidation, the highest dose showing the greatest neuroprotective activity (25%, 65%, and 90% respectively). The level of pressure frontal cortex protein concentration was greatly reduced in the disease control group (-8.5%), there was however a dose dependent 3-, 7- and 11-fold increase in the total protein after treatment with NLL extract progressively from the least dose to the highest dose administered.

This study demonstrates neuroprotective effect of NLL extract on Valproic acid-induced neurodegeneration in the prefrontal cortex of rats. The animals treated with higher doses of NLL extract exhibited reductions in tissue destruction as evidence in hematoxylin and eosin stain. Previous studies had focus mainly on the neuroprotective properties of valproic acid with a few reports on the neurodegeneration like the current work.

Numerous biomarkers have been deployed to investigate neurodegenerative condition. One of such biomarkers is a calcium binding protein, S100B. It has generated prodigious consideration as a biomarker for a multiplicity of diseases. This protein is chiefly expressed in glial cells, astrocytes, oligodendrocytes and Schwann cells and functions through intracellular and extracellular signaling pathways [63]. S100B interacts with p53 in melanomas and down-regulates p53 mediated apoptosis [64]. High levels of S100B have been detected with various clinical conditions such as brain trauma, ischemia and neurodegenerative, inflammatory and psychiatric diseases [65]. The levels of S100B in the blood may function to

predict the progress or the prognosis of many kinds of diseases, such as cerebrovascular diseases, neurodegenerative diseases, motor neuron diseases, traumatic brain injury, schizophrenia, depression, diabetes mellitus, myocardial infarction, cancer, and infectious diseases. S100B should not be simply regarded as a biomarker, as it may also function as therapeutic target for these diseases. Further elucidation of the roles of S100B may formulate innovative therapeutic strategies for multiple diseases [66]. S100B also plays important roles in spatial and fear memory, learning capabilities, epileptogenesis and myocardial functions [67,68]. Oxidative stress activation of microglia may have a role in the pathogenesis of neurodegenerative disorders, as it is over-expressed in anaplastic astrocytomas and glioblastomas [69], and melanomas [70].

The neuroprotective potential of NLL extract of neuroprotective significance on S100B in this study was revealed as increase proliferation of astrocytes as shown in plate 2 of immunohistochemistry labelling. High levels of S100B have been detected with various clinical conditions such as brain trauma, ischemia and neurodegenerative, inflammatory and psychiatric diseases [65]. Besides the brain, S100B is a well-established prognostic marker for melanoma and high serum concentration of S100B correlate with poor prognosis [71]. Animal studies with S100B knock-out or S100B overexpressing transgenic mice revealed that S100B is not an essential protein for life. However, S100B plays important roles in spatial and fear memory, learning capabilities, epileptogenesis and myocardial functions [67,68,72] reported upregulation of S100B levels in more severely ill patients presenting with impaired cerebral functionality underlying with AD. The result in part reflects participation of this protein in the pathogenesis of AD. Indeed, glial cells particularly microglia and astrocytes are able to modulate cerebral plasticity and to protect brain from oxidative insults which in this context result in increase of S100B indicative of astrocytic reaction to neuronal injury (reactive astrogliosis) in AD patients. Once activated, these cells increase the expression of substances that can participate in the excitotoxicity and inflammatory processes that occur during the disease process.

p53, the transcription factor, which demonstrate significant role in DNA damage response, maintenance of the genome integrity and tumor suppression [73] was another significant biomarker evaluated. p53 genes regulate heterogeneous repertoire of biological processes thus any insult on p53 expression and activity generate broad spectrum of disorders: neurodegeneration, cancer and metabolic diseases [74]. One of the bastions in

p53-guided cellular response to various genotoxic challenges is oxidative stress; the appropriate response to oxidative injury relies on p53 ability to sense the intensity of the damage. In general, p53 ensures antioxidative activities in mild oxidative injury to promote cell survival, but if the overall cellular health is seriously compromised (when the antioxidative capacity is greatly surpassed), p53 exhibits prooxidative activity that ends in cell apoptosis.

Previous studies have suggested a role for p53 in neurodegenerative diseases, and have reported neuronal cell death associated with enhanced levels of p53 [75-77]. There is accumulating evidence indicating that p53 is perturbed in the central nervous system in a number of neurodegenerative disorders [78,79]. The study reported neuroprotection potentials of Neuroprotective significance of NLL extract by inhibition of p53 is in line with the report of Chang et al. [62] of the inhibition of p53 as an ideal target to restore neuronal functions. p53 critical involvement in neuronal death in several experimental models and its relationship to pathological processes in neurodegenerative diseases, it is suggested that p53 inhibitors might represent a promising therapeutic approach for amelioration of several neurodegenerative conditions [62,80]. Molecules that prevent activation of p53-dependent apoptotic cascade potentially may prolong neuronal survival and protect neurons against apoptotic stimuli [6,77]. This substantiates the report that concomitant activation of tau hyperphosphorylation by p53 genes could be mitigated if the neurons are co-incubated with a potent antioxidant [81]. Many of the neuroprotective properties observed may in part be attributed to phytochemicals and elucidated isolated molecules. Isolated molecules like strictosamide is reported to mediate CNS depression, while cadambine and 3 $\alpha$ -dihydrocadambine displayed neuroprotective effects in glutamate-induced HT22 cell death [82]. Therefore the observed neuroprotective potential of NLL underpins the armamentarium of yet to be elucidated phyto-constituents.

## Conclusion

Since p53 is underpinned in oxidative stress, a common denominator of neurodegenerative diseases and a genesis in its pathogenesis; with chemical signatures (e.g. free radicals, O<sub>3</sub>) or biological consequences (e.g. DNA damage, mitochondrial dysfunction) is been inhibited by NLL extract in this study, it might represent a promising therapeutic approach for amelioration of several neurodegenerative conditions. The study revealed NLL extract diminution of S100B and p53 protein

expression, neuronal lipid peroxidation, free radical garnering and upregulation of neuronal antioxidants which are effects significant in neuroprotection and oxidative stress mitigation. Further study in standardization and development of NLL extract as a therapeutics for ameliorating neurodegenerative diseases as well as its utilization as an antioxidant agent is in progress in our laboratory.

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