Oxidative Stress, GFAP and NFT Activities in Prefrontal Cortex of Rats Treated with *Musanga cecropioides* (Urticaceae) Stem-Bark Extract in Sodium Valproate-Toxicity

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Abstract

Valproic acid (VPA) is a foremost drug in the management of epilepsy and several kinds of seizures. Cellular toxicities concerns of VPA arising from the treatment of diseases have received global attention. Oxidative stress underpins VPA-induced neurotoxicity and is caused by free radicals in the brain tissue. The aim of our investigation was to evaluate the protective effects of *Musanga cecropioides* (MCS) against VPA-induced toxicity in prefrontal cortex rat brain in vivo model of neurotoxicity. Oxidative stress such as lipid peroxidation (LPO), glutathione reductase (GSH), glutathione peroxidase (GPx), glutathione transferase (GST), catalase (CAT), superoxide dismutase. Besides, the expression of glial fibrillary acidic protein (GFAP) and neurofibrillary tangles (NTs) neurological markers were appraised. The levels of oxidative stress markers, LPO, GSH, GST, GPx, CAT, and SOD were significantly (P < 0.05 - 0.001) elevated. Valproate exposure significantly produces alteration of the activities of the glutathione metabolizing enzymes such as glutathione-S-transferase, glutathione peroxidase, LPO and the expression of GFAP and NTs tangles. Post-treatment with MCS reversed the VPA-induced effects in brain tissue preparation of rat and mitigated the expression of GFAP and NTs. Based on the protective and antioxidant action of MCS and abrogation of the expression of GFAP and NTs in the PFC of the brain, we propose that MCS possess potential safe active compounds that could be considered effective therapeutic approach in attenuating the adverse effects of VPA-induced neurotoxicity.

**Keywords:** *Musanga cecropioides; Sodium Valproate; Oxidative Stress; Glia Fibrillary Acidic Proteins; Neurofibrillary Tangle; Prefrontal Cortex; Immunohistochemistry*

Introduction

Reactive oxygen species (ROS) play an important role in physiological functions [1]. Upregulation of reactive species, especially reactive oxygen (ROS) and nitrogen (RNS) species in addition to the imbalance in the body’s antioxidant enzyme systems will results in destruction of cellular structures, lipids, proteins, and genetic materials such as DNA and RNA [2]. Moreover, the effects of reactive species on mitochondria and their metabolic processes eventually cause a rise in ROS/RNS levels, leading to oxidation of mitochondrial proteins, lipids, and DNA. Oxidative stress has been considered to be linked to the etiopathology of many diseases, including neurodegenerative diseases (NDDs) such as Alzheimer diseases, Amyotrophic lateral sclerosis, Friedreich’s ataxia, Huntington’s disease, Multiple sclerosis,
and Parkinson's diseases. In addition, oxidative stress causing protein misfold may turn to other NDDs include Creutzfeldt-Jakob disease, Bovine Spongiform Encephalopathy, Kuru, Gerstmann-Straussler-Scheinker syndrome, and Fatal Familial Insomnia [1,3-7].

Oxidative stress has been proven to be involved in VPA-induced toxicity [8,9]. Valproic acid (VPA) is broad spectrum antiepileptic drug used for the management of idiopathic and symptomatic generalized seizures, neurological and psychiatric illness [10-12]. Diverse molecular and cellular events underlie different types of seizures [13].

Though valproate offer protection in some neurological disease investigated [14-16], other reports indicated that valproate - induced neurodegeneration in both cultured neuronal cells and experimental animals [17-20]. Intrapertoneal valproate injected rats reveal the decimation of the proliferation of hippocampal neurons and cognitive impairment [17,21] and clinically relevant doses of VPA therapy administered to neonatal mice and rats exhibited widespread apoptotic neurodegeneration in several brain regions [22,23]. Teratogenic activity in humans induced by VPA has been established [24,25]. Besides, neural tube defects and increases the incidence of children with autism spectrum disorder (ASD) were reported when taken during pregnancy [26,27]. Free radicals generation and concomitant oxidative stress was reported in developmental neurotoxicity observed with valproate therapy [28] and caspase-dependent apoptosis [18,29]. In the brains of autistic children the histomorphological changes observed confirmed that VPA induces programmed cell death (PCD) [30-32]. Though, the pathways involved in the VPA-induced neurotoxicity is still poorly understood [33]. Caspase-independent PCD was also reported [34] but its exact mechanism is unknown. Valproate induced cascades of deleterious events contributing to neuronal cell death via atypical calpain-dependent necroptosis pathway [35,36]. The valproic acid activated calpain-dependent necroptosis cascade is recognized as the principal player in apoptosis [37-39].

Glia fibrillary acidic proteins (GFAP) play important physiological roles in brain functions. The presence of GFAP indicates astrocytes orependymal differentiation. Glia fibrillary acidic protein (GFAP) is an intermediate filament (IF) III protein exceptionally found in astrocytes and a main type of glial cells in the central nervous system [40,41]. In traumatic brain and spinal cord injuries and stroke, GFAP and its metabolites are quickly released into biological fluids, presenting them as strong candidate biomarkers for such neurological disorders. Intermediate filament (IF) in astrocytes is a crisis-command center coordinating cell responses in situations connected to cellular stress, which is a central component of many neurological diseases [40,41]. Activation of astrocytes has been implicated in the pathogenesis of a variety of neurodegenerative diseases and astrocyte-mediated pathways as targets for drugs treating the root causes of the pathology [42,43]. The activated astrocytes secrete several neurotropic factors necessary for neuronal survival; it is believed that rapid and severe activation of astrocytes causes secretion of neurotoxic substances that augments/initiates an inflammatory response, leading to neuronal death and brain injury. Thus, the injury then causes astrocyte to be express can enhanced the level of glial fibrillary acid protein (GFAP) expression which is considered a marker protein for astrogliosis [42,43]. Activation of astrocytes is characteristic morphological hallmarks and has important functional consequences in situations such as stroke, trauma, epilepsy, Alzheimer's disease (AD) and other neurodegenerative diseases [44,45]. Neurofibrillary tangle or protein (NFP) is mostly commonly known as a primary hall marker of Alzheimer’s disease [45]. Their presence is also found in numerous other diseases known as tauopathies. These neurofibrillary tangles are also found in the brain of normal aged humans. Similarly, neurofibrillary changes are also found in Guan-Parkinsonism dementia complex, mongolism, post encephalitic Parkinsonism and tuberous sclerosis [46].

The reference drug, vinpocetine, utilized in this study, is an apovincaminic acid ethyl ester, a semi-synthetic derivative of vincamine, an alkaloid extract derived from the periwinkle plant (Vinca minor). Vinpocetine is accessible as a prescription pharmaceutical worldwide; it, is indicated for management of cerebrovascular and cognitive disorders [47]. It is usage as dietary supplements underpin advocated use for claims of cognitive enhancement [48-52].

Finally, the medicinal importance of Musanga cecropioides has been extensively reported in M. cecropioides subchronic toxicity [53], anti-hyperglycemic effect [54], hepatoprotective effect [55], inhibition of edema [56] and antihypertensive agent [57].

Here, we investigated for the first time the neuroprotective effect of MCS extract on VPA - induced neurotoxicity in rat brain PFC. The relationship of several important molecules such as lipid peroxidation (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH), glutathione peroxidase (GSP), glutathione-S-transferase (GST), glial fibrillary acidic proteins (GFAP) and neurofibrillary proteins (NFP). The significant downregulation of LPO and GFAP, NFP protein expression and upregulation of enzymatic and

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non-enzymatic antioxidants alterations by MCS extract reveal its potential health benefits and probable exploitation as pharmaceuticals for mitigating VPA- induced toxicity in anticonvulsant therapy and management for neurodegenerative diseases.

Materials and Methods

Drugs, Chemicals and equipment

Sodium valproate (Epilim, Sanofi, France), Vinpocetine (Cognitol, Tyonex, Nigeria) both purchased from Luckpharm Pharmacy Int'l Ltd (Nigeria), Rivers State, Nigeria. n-hexane (extrapure 85%) (LobalChemie, Mumbai, India), Methanol 99.8% (LobalChemie, Mumbai, India), formalin (LobalChemie, Mumbai, India), Diethyl ether (LobalChemie, Mumbai, India); Avidin Biotin Complex (Bosterbioengenering limited, Wuhan, China). The equipment utilized includes: rotary evaporator (Shenke® R-205, ShangaiShenshun 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).

Collection and authentication of plant materials

Musanga cecropioides stem-bark was collected and authenticated by, a taxonomist (Dr. Oladele Adekunle) attached to the Forestry Department at university of Port Harcourt from the University of Port Harcourt campus, Nigeria. The Herbarium specimen with voucher number UUPH 2001 had already been deposited at Department of Pharmacognosy, University of Uyo, Akwa Ibom State, Nigeria.

Preparation of Musanga cecropioides stem-bark (MCS) extract

The dried M. cecropioides stem-bark (MCS) was pulverized to fine particles using mechanical grinder. A 500g weight of MCS 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 to be 15.7%.

Phytochemical screening

Phytochemical screening of the M. cecropioides extract was executed 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 [58].

Animals and drug administration

Forty two male Wistar albino rats were used. The animals (180 - 200g) 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, Onitsha, Anambra State, Nigeria) and water ad libitum and animals were allowed unfettered access to water and food. The experimental protocol was in line with the 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).

Rationale for dose selection (Valproic acid)

Low doses of VPA such as 200 mg/kg, s.c. (mice) [59], 350 mg/kg, i.p. (rat) [60] and 400 mg/kg (mice) [59,61] have been utilized for experimental investigations. Moderate doses of VPA such as 500 mg/kg and 600 mg/kg (i.p. /s.c.) [62-66] were used in mice. High dose of VPA (800 mg/kg) was also used [67]. Animal studies report a significant amount of VPA (900 µg/ml) in the maternal plasma within 1h of VPA administration [63,65]. Although majority of the reports followed 500 mg/kg [68], therefore this dose was utilize in the present 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 28 days when administered orally by gavage in the experimental animals [68]. Each one mL contains 200 mg sodium valproate. The animals were divided randomly into six groups with 7 animals per group.

**Rationale for dose selections (Vinpocetine)**

The oral median lethal dose (LD$_{50}$) of vinpocetine in rats (strain not specified) is approximately 500 mg/kg [69]. Toxicity of vinpocetine was investigated by [69] in a multiple studies in rats (strain not specified). Report indicated that subchronic gavage exposure to vinpocetine at doses between 25 and 100 mg/kg elevated salivation, liver, and thyroid weights especially with the highest dose. But with intraperitoneal injection of 5 or 25 mg/kg for 3 months, mortality was recorded. In a similar report chronic gavage exposure, at doses between 25 and 100 mg/kg, no adverse effects were stated [69]. Since the lowest dose commonly used is 25mg/kg, this those was utilize in the current study. Oral bioavailability of vinpocetine in rats was 52% suggesting extensive first pass metabolism [70].

**Experimental doses (MCS, valproate and vinpocetine)**

The acute toxicity (LD$_{50}$) of MCS indicated that at dose above 5 g/kg did not show any sign of toxicity as reported in previous publication [53]. Therefore, three dose levels, 100, 200 and 400 mg/kg were utilized for this investigation.

The MCS, valproate and vinpocetine (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 extracts respectively for animals in groups 2 to 6. The MCS extract (100, 200, 400 mg/kg) and vinpocetine 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 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 MCS extract 100, 200 and 400 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 distilled water, MCS or vinpocetin was adminis-
tered adopting standard procedure [68].

Neurotoxicity percentage (%) was deduced using this formula:

$$\text{Neurotoxicity percentage} = \frac{\text{MCS, VPA, and } W}{\text{mean experimental variables estimated in the rats treated with valproic acid (VPA) plus MCS (Test groups), valproic acid (VPA) only (diseases control group) and distil water treated animals (W) (negative control) respectively}}$$ [43].

**Tissues processing**

All rats were sacrifice killed 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 centrifuge (4000 × g, 10 min) at -80°C to remove debris and nuclei. The resultant supernatant was stored at -80°C for various biochemical assays.

**Oxidative stress determinations**

**Lipid peroxidation (Malondialdehyde, MDA):** Lipid peroxidation was examined by the thiobarbituric acids reactive substances (TBARS) method [71]. 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 × g, 10 min). The clear supernatant absorbance was measured against a reference blank of distilled water at 532
nm and the MDA level estimated by Todorova, et al [72] method and expressed as nmol of MDA/g of wet tissue using a molar extinction coefficient of the chromophore (1.56 × 10^{-5} /M/cm).

**Reduced Glutathione (GSH):** Sedlak and Lindsay [73] method was used to evaluate 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% sulphasaliclyc acid) and vortexed. The mixture was allowed to stand for approximately 5 minutes and then centrifuged (1200 × g, 10 min). To 1 mL of the filtrate was added 4 mL of 0.1M phosphate buffer and 0.5 mL of DTNB (Ellman’s reagent). Similarly, a blank was prepared by addition of 4 mL of 0.1M 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 using by adopting the method of Rotruck, et al [74]. Sodium phosphate buffer mixture (1 mL) was prepared by combing 0.2 mL sodium azide, 0.4 mL of GSH and 0.2 mL of hydrogen peroxide (H₂O₂) 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 minutes at 37°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 Mmol/L) were added. The change in color was assayed at 412 nm. Similar treatments were administered to aliquots of the standard solutions.

**Catalase (CAT):** Catalase level was evaluated according to the method of Clairborne [75] with slight modifications. The method is based on the ability of catalase in the sample preparation to split hydrogen peroxide which and can be measured spectrophotometrically at 240 nm. One unit of catalase equals the amount of protein that converts one micromole H₂O₂/min. A 0.2 mL of sample was added to 50 mM of phosphate buffer (pH 7.4) containing 100 mM (v/v) of H₂O₂ in a total of 1 mL. The reaction mixture was incubated for 2 minutes at 37°C and the rate of absorbance change (AA/min) at 240 nm was recorded which indicated the decomposition of H₂O₂. Activities were calculated using the molar extinction coefficient of H₂O₂ at 240 nm. All samples were measured in quadruplicates.

**Superoxide dismutase (SOD):** Superoxide dismutase (SOD) level was evaluated using the auto-oxidation technique [76]. Tissue homogenates were centrifuged (12, 000g for 10 minutes) and an aliquot of supernatant was diluted with water to make a 1:10 dilution. The diluted sample (200 mL) 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 minutes.

**Glutathione-S-transferase (GST):** Glutathione-S-transferase level was determined in accordance with the method of Habig, et al [77]. 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.

**Proteins determination:** Protein content estimation was determined from PFC of rat brain tissues by the modified method of Lowry assay [78] using bovine serum albumin (BSA) standard.

**Histopathology**

**Hematoxylin and eosin staining**

After 28 days exposure to valproic acid and MCS, all rats were sacrificed by decapitation. The prefrontal cortex was excised immediately, fixed by 20% phosphate buffered formalin for 48hrs. 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°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

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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 Oyinbo, et al [79]. Cortical Purkinje cells estimation was based on semi-quantitative scale described in previous study [79] was used to assess the extent of neurodegeneration in the PFC with 400× magnification.

**Immunohistochemistry of GAFP and NFP**

**Tissue preparation**

The abstracted prefrontal cortex which was excised is fixed in 4% formaldehyde in 0.01 mmol/L phosphate buffer and embedded in paraffin. Tissues were sectioned 3 µm using rotary microtome model (Leica Microsystems, Wetzlar, Germany). Slice were mounted on a clean slide and toasted at 60°C for 2 hrs. Treatment with dimethyl benzene was instituted for 20 minutes. Slices were sequentially treated with 95%, 80% and distilled water until transparency of slide was achieved.

**StreptAvidin- Biotin Complex (SABC) Histochemistry**

Immunohistochemistry assessment of neuronal damage was executed by evaluating the levels of glial fibrillary acid protein (GFAP) and neurofibrillary protein (NFP) biomarkers. The tissue sections were placed in 0.3% hydrogen peroxide (H₂O₂ in phosphate buffer solution (PBS) for 30 minutes, then pre-incubated with normal goat serum to prevent nonspecific binding at 4°C for 30 minutes after which further incubation with a primary polyclonal rabbit anti-GFAP or -NFT (1:1000; Sigma) antibody at 37°C for 1 hr or overnight at 4°C. The tissue slices were washed thrice for 10 minutes with PBS; the sections were then incubated for 1 hr at room temperature with their corresponding horseradish peroxidase conjugated anti-species secondary antibody (polyclonal goat anti-rabbit IgG) immune-labelled against GFAP or NFT.

Antibody immunoreactivity or Immunoreactions were observed under an optical microscopes described by Oboma, et al. [80] was adopted. Cells with specific brown colors in the cytoplasm, cell membrane or nuclei depending on the antigenic sites were considered to be positive for glial fibrillary acid protein (GFAP) and neurofibrillary protein (NFP). The stained cells without any form of brown colors were scored as negative.

**Statistical analysis**

The Graph pad Prism 5.1 was used for data analysis by one-way analysis of variance (ANOVA). Multiple comparisons among groups were made according to the Turkey’s test. *P* values < 0.05 were considered significant. Data are represented as expressed as Mean ± standard deviation (SD).

**Results**

**Effect of MCS on MDA, antioxidant and protein levels**

The effect of MCS after sub-acute intoxication with valproic acid on antioxidant activity is presented in table 1. The result indicated that VPA treated rats exhibited increased level of neuronal damage and oxidative stress witnessed by statistical significant depression (*P* < 0.05 - 0.001) of SOD (47%), GSH (61%), GST (84%), GPx (90%), CAT (54%) and TP (9%) compared with the normal control group. However, sub-acute treatment with MCS extract protected against the neurotoxicity induced by valproic acid. This neuroprotection was demonstrated by the elevation of SOD (121%, 124%, 189%), GSH (70%, 115%, 102%), GST (67%, 135%, 98%), GPx (78%, 60%, 95%), CAT (57%, 81%, 76%) and TP (59%, 105%, 122%); and reduction of MDA (89%, 101%, 98%) with MCS treatment at 100 mg/kg, 200 mg/kg and 400 mg/kg body weight respectively when compared with the disease control group. Vinpocetine demonstrated neuroprotective activity on antioxidant enzymes with increased in SOD, GSH, GST, GPx, CAT, and TP by 86%, 83%, 128%, 65%, 86%, and 68% but reduced lipid peroxidation in brain prefrontal cortex by 96%.
Histomorphological observation

Haematoxylin Eosin staining of neurons loss in the PFC is shown in figure 1. Rats in the normal control group have morphological intact and tightly arranged neurons in the PFC (Group 1). Generalized damage is distinctly observed in the PFC treated with VPA (Grp 2). Rats in grp 2 shows that valproic acid induced neurodegeneration by prompting 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 (Grps 3, 4 and 5) treated with three different doses (100, 200, 400 mg/kg, respectively, and reference group (Group 6) treated with vinpocetine, 25 mg/kg. Neurons in the PFC of rats treated with MCS (400mg/kg) in group 5 almost appear normal with the reference group and the normal control group indicating neuroprotection.

Table 1: Effects of valproic acid intoxication and post-treatment with MCS extract and vinpocetine (25 mg/kg) on antioxidant enzymes of experimental rats following continuous oral sub-acute dosing for 28 days.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Grp1</th>
<th>Grp2</th>
<th>Grp3</th>
<th>Grp4</th>
<th>Grp5</th>
<th>Grp6</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>0.59 ± 0.15</td>
<td>0.31 ± 0.11** (47%)</td>
<td>0.65 ± 0.12** (121%)</td>
<td>0.77 ± 0.14** (164%)</td>
<td>0.84 ± 0.04** (189%)</td>
<td>0.55 ± 0.03* (86%)</td>
</tr>
<tr>
<td>CAT</td>
<td>0.78 ± 0.10</td>
<td>0.36 ± 0.05* (54%)</td>
<td>0.60 ± 0.16** (57%)</td>
<td>0.70 ± 0.08** (81%)</td>
<td>0.68 ± 0.07** (76%)</td>
<td>0.72 ± 0.08* (86%)</td>
</tr>
<tr>
<td>GSH</td>
<td>1.46 ± 0.28</td>
<td>0.57 ± 0.29* (61%)</td>
<td>1.19 ± 0.15** (70%)</td>
<td>1.59 ± 0.29* (115%)</td>
<td>1.48 ± 0.39* (102%)</td>
<td>1.31 ± 0.19* (83%)</td>
</tr>
<tr>
<td>GPx</td>
<td>0.96 ± 0.03</td>
<td>0.10 ± 0.01* (90%)</td>
<td>0.77 ± 0.33** (78%)</td>
<td>0.62 ± 0.36** (60%)</td>
<td>0.92 ± 0.05** (95%)</td>
<td>0.66 ± 0.38* (65%)</td>
</tr>
<tr>
<td>GST</td>
<td>0.23 ± 0.05</td>
<td>0.05 ± 0.03* (84%)</td>
<td>0.32 ± 0.32 (67%)</td>
<td>0.76 ± 0.12** (135%)</td>
<td>0.15 ± 0.02 (98%)</td>
<td>0.40 ± 0.20 (128%)</td>
</tr>
<tr>
<td>MDA</td>
<td>0.36 ± 0.14</td>
<td>0.90 ± 0.13** (150%)</td>
<td>0.42 ± 0.19** (99%)</td>
<td>0.35 ± 0.05** (101%)</td>
<td>0.37 ± 0.17** (98%)</td>
<td>0.38 ± 0.05** (96%)</td>
</tr>
</tbody>
</table>

Table 1: Effects of valproic acid intoxication and post-treatment with MCS extract and vinpocetine (25 mg/kg) on antioxidant enzymes of experimental rats following continuous oral sub-acute dosing for 28 days.

Group 1: Negative control receiving 10 ml/kg b.w. 2% Tween 80; Group 2: Diseases control group receiving 10 ml/kg b.w. with 2% Tween 80 + valproic acid 500 ml/kg; Group 3 receiving MCS extract (100 mg/kg b.w.) + valproic acid 500 ml/kg; Group 4 receiving MCS extract (200 mg/kg b.w.) + valproic acid 500 ml/kg; Group 5 receiving MCS 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 ml/kg. MCS = Musanga cecropioides stem-bark, SOD=Superoxide dismutase, S=Catalase, GSH=Glutathione Reductase, GPx=Glutathione peroxidase. MDA: Malondialdehyde, Values presented as mean ± standard deviation (n = 4 - 7); *P < 0.05, **P < 0.01, ***P < 0.001. ^Values are compared with the negative control group, Values are compared with the diseases control group; all values were compared using one way ANOVA and Turkey Test.

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Histochemical Evaluation

Glial fibrillary acid protein (GFAP) immunohistochemistry

Immunohistochemistry labelling of GFAP on PFC brain tissues showing glial cells (brownish) expression in valproic acid induced neurodegeneration AND treated with MCS extract (Figure 2). Glial cells (astrocytes) help to maintain hemostasis and provide support and protection for neurons. A number of positive glial neurons revealing GFAP protein expression in PFC is consistent with normal histomorphology. As observed in control group (Grp 1). Increased protein expression of GFAP is observed in the diseases control group (Grp 2). Mild protein expression of GFAP in PFC of rats treated with 100 mg/kg-b.wt of MCS extract (Grp 3). Moderate protein expression of GFAP in PFC of rats treated with 200 mg/kg b.wt of extract (Grp 4). Reduced protein expression of GFAP and increase proliferation of astrocytes in PFC of rat brain treated with 400 mg/kg b.wt compared with negative control (Grp 5). Similarly, the reference control grp 6 treated with 25 mg/kg b.wt of vinpocetine demonstrate the reduce protein expression of GFAP 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.

Figure 2: Photomicrograph showing immunohistochemistry labelling of glial fibrillary acid protein (GFAP) expression (x 400). Gp1 shows photomicrograph of GFAP expression within the cerebellum. The Astrocytes processes are stained brown and are unremarkable consistent with normal brain tissue. GP 2 section shows moderate expression of GFA protein compared to the control consistent with neuronal inflammation. Gp 3 section shows mild expression of the Glia fibrillary acid protein on the astrocytes processes. Gp 4 section shows moderate expression of the astrocytes processes. Gp 5 section shows mild expression of GFAP while Gp 6 section shows moderate expression of GFAP. Gp 3 and Gp 5 exhibited higher neuronal protection with Gp 5 competing strongly with compared to the reference drug (Gp 6).

Neurofibrillary protein (NFP) immunohistochemistry

The effect of MCS ON NFP immunohistochemistry is shown in figure 3. Few NFP positive neurons were observed in the control group (Grp 1). Great numbers of NFP 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 (Grp 2). In contrast, administration of MCS in three doses (100, 200, 400 mg/kg) progressively downregulate NFP positive neurons observed in the PFC dose dependently (Grp 3 - 5). Similarly, vinpocetine 25 mg/kg markedly reduced the number of NFP positive neurons in the PFC (Grp 6). Generally, the immunohistochemistry reveal that the extract demonstrated neuronal protection by suppression of NFP and activation of astrocytes as expressed by NFP immunostain.
Oxidative Stress, GFAP and NFT Activities in Prefrontal Cortex of Rats Treated with *Musanga cecropioides* (Urticaceae) Stem-Bark Extract in Sodium Valproate-Toxicity

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Discussion

Reactive oxygen species (ROS) are involved in numerous cellular events, as well as second messengers in the activation of several signaling pathways leading to the stimulation of transcription factors, mitogenesis, gene expression, and the induction of apoptosis, or programmed cell death [81]. Cellular oxidative stress occurs as a result of the imbalance between pro-oxidants and antioxidants [82]. Currently available, scientific evidence suggest that oxidative stress may result in cell damage and even cell death [83].

Animal study showed that prenatal VPA exposure leads to the oxidative stress in the adulthood [84]. Oxidative stress may lead to rapid change in the antioxidant system such as the dropping of the cellular endogenous antioxidant glutathione [85,86]. Accumulating evidence intimates that oxidative stress caused by free radicals contributes to the pathogenesis of VPA-induced nephron-toxicity [9]; behavioral disorders [8]; hepato-toxicity [87]; neuro-toxicity [88]. Valproate is endowed with numerous several neurochemical and neurophysiological mechanisms underpinning its broad spectrum antiepileptic efficacy [33]. Its mechanism of anticonvulsant effect was challenged due to its side-effects and HDAC-dependent and-independent toxicity [29,89,90].

Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress [91,92], which plays a role in heart diseases, neurodegenerative diseases, cancer and in the aging process [93]. This concept is supported by increasing evidence indicating that oxidative damage plays a role in the development of chronic, age-related degenerative diseases, and that dietary antioxidants oppose this, thus lowering the risk of disease [94]. Antioxidants are substances that when present in low concentrations, compared to those of an oxidisable substrate significantly delay or prevent its oxidation [95].

Valproic acid induces oxidative stress in the present study as shown in table 1. There are numerous evidences which support the deteriorating action of free radicals, which can lead to cognitive ageing and CNS disorders [96]. The study indicated that sodium valproate
intoxication significantly reduces the level of all the antioxidant enzymes (SOD, CAT, GSH, GPx and GST) and markedly upregulated lipid peroxidation dose dependently in experimental rats. However, the post-treatment of MCS extract at all doses upregulated all the antioxidant enzymes and down regulated lipid peroxidation. The percentage of VPA-induced neurotoxicity on antioxidant enzymes profile range from 47 to 84% and the lipid peroxidation was as high as 150%. However, the neuroprotection offered by MCS for the antioxidant enzymes range from 121 - 189% (SOD), 57 - 81% (CAT), 70 - 115% (GSH), 60 - 95% (GPx), 67 - 135% (GST); while the alleviation of lipid peroxidation range from 89 - 101% (MDA). Non-enzymatic serum GSH is a sensitive biomarker of the antioxidant status was markedly increased, it plays a pivotal defensive role against oxidative insults as an endogenous scavenger of free radicals. GSH is considered crucial in cellular function and viability [97]. Alteration of glutathione metabolizing enzymes, GST, GPx, and Glutathione reductase (GSSH) as part of the armamentarium of antioxidant defense system has been elucidated [98].

Similarly, lipid peroxidation was significantly \( (P < 0.001) \) evident by increased formation of MDA (150%) in the intoxicated rats. The augmentation in LPO observed in our study may be as a result of elevated ROS production or decimation in the status of antioxidants and membrane fluidity [99]. Understandably, MCS diminished oxidative stress marker LPO by scavenging ROS/RNS and MCS extract could underpin a beneficial action against LPO, providing additional compensatory mechanism for restoring cellular integrity and protection against free radicals-induced damage [8,100]. Probably, LPO is regarded as an excellent biomarker of oxidative stress because it is the most singular largely considered product produced by free radicals [101]. It is mediated by oxygen free radicals and is thought to be significant cause of destruction and damage to cell membranes and suggested to be a causative factor to the development of VPA-mediated tissue damage [8,102]).

Moreover, enzymatic antioxidants such as SOD and CAT were depleted significantly \( (P < 0.01 - 0.001) \). Similarly, glutathione was reduced significantly \( (P<0.001) \). Taking together, all these exemplify exacerbation of oxidative stress that provoke impairment or damage to nervous tissues and development in the VPA exposed rats. The disequilibrium in the action of ROS/RNS production and scavenging system result in oxidative stress and concomitant brain insults. The susceptibility of brain tissues to oxidative stress has been demonstrated by Cechetti., et al [103]. Both ROS/RNS cause neuronal damage including lipid peroxidation and optimizes the fluidity of membrane disrupting essential biochemical and physiological function as well as down-regulating oxidized phospholipids and cholesterol [103-105]. Therefore, the level of glutathione is depleted in oxidative stress as observed in the VPA-intoxicated rats.

Conflicting results also exist with respect to the effect of VPA on the levels of glutathione metabolizing enzymes or non-enzymatic antioxidants. All glutathione metabolizing enzymes may serve as antioxidants [9]. The neurotoxicity of VPA was established by a significant \( (P < 0.05) \) declined in the activity of GST in prefrontal tissue of rat brain. GST is an all-important multifunctional enzyme, which plays a role in toxic electrophiles detoxification and catalyzing their conjugation with GSH [106]. The subacute supplementation of MCS along with VPA in PFC of brain tissue of rats restore significantly \( (P < 0.05) \) the diminished GST level only at MCS 200 mg/kg and vinpocetine, 25 mg/kg. The findings of this study did not corroborate earlier published reports that the concentration of GSH is upregulated during xenobiotic intoxication [107,108], as VPA-induced significant down regulation of GST in this investigation. However, protection against the VPA-induced neurotoxicity was achieved through repeated supplementation of antioxidant, MCS. The present study confirms that subacute exposure of MCS significantly abrogated neuronal VPA-oxidative stress induction. Besides, GPx is selenium-containing enzymes, which contribute a noticeable role in the reduction of hydrogen peroxide \( (H_2O_2) \) and hydroxide and elimination of excess free radicals to non-toxic products [109].

In our results, VPA treatment showed a significantly \( (P < 0.01) \) increase in activity of GPx in PFC tissues of the brain. But MCS extract administration was able to reverse significantly \( (P < 0.05 - 0.001) \) the activities of GPx in PFC cells of VPA exposed rats. The restoration might in part be due to alteration of substrate (GSH) availability [110]. Reduced glutathione (GSH) is produced from the activity of Glutathione reductase (GR) which is an enzyme responsible for the reduction of oxidized glutathione (GSSG) to GSH. The activity of GR directly influence the protection and reparation of sulphydryl (-SH) protein under oxidative stress [111]. In our study, VPA treatment showed increased activity of GSH in kidney. It may be due to the protection from oxidative stress formed by over production of free radical [112]. The significant prevention in alteration in the activity of GR in PFC can possibly be attributed to its antioxidant effect. Antioxidant enzymes most times become inactive due to VPA exposure, which is due to direct binding of VPA to their active sites [113]. In the present study, the

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Alteration of the activities of the antioxidant enzymes probably reflect the inability of the antioxidant defense to overwheled the influx of ROS on VPA exposure. Sub-chronic MCS extract supplementation along with VPA markedly restored the levels of antioxidant enzymes and non-enzymatic antioxidants, which might be due to the ability of MCS to diminish the accumulation of free radical generation during VPA exposure. The role of SOD as an antioxidant is to convert superoxide to hydrogen peroxide, thereby protecting against the pervasive harmful effects of superoxide.

Apart from oxidative stress, many and varied neurotoxic insults occur such as the proliferation and hypertrophy of astrocytes and the accumulation of neurofibrillary proteins (NFP). The hallmark to these responses are creation of imbalance between the endogenous antioxidant and oxidants moiety (upregulation of oxidants and down regulation of antioxidants); heightened expression of the major intermediate filament protein of astrocytes, glial fibrillary acidic protein (GFAP) and formation of aggregates of hyperphosphorylated proteins also called neurofibrillary tangles respectively. These are aetiopathological biomarkers of neurodegenerative disease mechanisms and are foici for therapeutic target [1,6,40-45,114]. This study observed VPA - induced elevation in the expression of NFT and GFAP which were diminished by subacute supplementation with antioxidant, MCS extract for 28 days. The down-regulation of VPA - mediated increased of NFT and GFAP in experimental animals by administration of MCS extract thereby in part alludes to its neuroprotective potential.

The VPA - induced astrogliosis and increased neurofibrillary protein activity in PFC tissue of experimental rat model is shown in figure 2 group 2. Astroglialosis is a prominent neuropathological feature of many diseases of the cerebral tissue [115,116]. However, evidence indicates that the role played by gliosis in pathological situations may not be restricted to its “housekeeping” function but may also include actions that significantly and actively contribute to the demise of neurons, especially in neurodegenerative diseases like Parkinson disease. Interestingly, several lines of evidence demonstrate that gliosis may actually exert very different effects in the diseased brain, because, depending upon the situation, it may mediate either beneficial or harmful events [117]. The gliosis in the present work was evident with neuronal loss evidences in the haematoxylin and eosin stain. Also [117,118] reported that the density of GFAP-positive astrocytes appears to be inversely related to the magnitude of dopaminergic neuronal loss across the different main dopaminergic areas of the brain in PD post mortem samples as it is obtainable in the present animal model. Furthermore [119] reported that there is neuronal expression of GFAP in patients with Alzheimer pathology in the human hippocampus. Surprisingly, the GFAP^1 immunopositive cells observed in AD patients were primarily neurons, although GFAP^1-expressing neurons were mainly restricted to AD and DS patients [119].

Hol., *et al.* [119] provide strong evidence that GFAP is expressed in neurons that are not ghost tangles, since the GFAP antibodies stain neurons in which a nucleus is visible. Furthermore, the presence of GFAP mRNA in neurons proves that the GFAP immunostaining observed in the neurons is indeed the result of GFAP expression [119]. In another study by Kamphuis, *et al.* [2014] aimed at characterizing the expression pattern of different GFAP isoforms in normal human hippocampal tissue and in conditions with AD-related gliosis, the number of GFAP^1-expressing astrocytes significantly increased during the progress of AD with lesser effects of gender and age. The *in vitro* studies showed that treatment of AD rats with Ab1e42 oligomers and fibrils increases the number of GFAP^1-positive cells, suggesting that elevated Ab level is a causative factor of neuronal injury [120].

Besides, neurofibrillary protein activity was higher in the disease group compared to control and treated group and this is in line with findings by Irwin., *et al.* [121], study that define the regional distribution of acetylated K280 immunoreactive tau pathology in Alzheimer’s disease, corticobasal degeneration and progressive supranuclear palsy. Acetylated-tau was present throughout all stages of Alzheimer’s disease pathology but was more prominently associated with pathological tau epitopes in moderate to severe-stage cases. Acetylated K280 also co-localized with N- and C-terminal specific anti-tau epitopes, indicating it is present in neurofibrillary tangles prior to subsequent tau truncation [121].

The reference drug, vinpocetine, lowered neurofibrillary protein and glial fibrillary acid protein activity in rats exposed to valproic acid thereby reducing neuronal loss compared to the disease group. Vinpocetine has been shown to exert a brain neuroprotective effect by a combined action on cerebral circulation, brain metabolism and rheological properties of the blood. This boost in the cerebral metabolism thus enhances both oxygen and glucose utilization and consequently improving cerebral functions and protection even in conditions of hypoxia and ischemia [122]. Vinpocetine has been shown to inhibit a cyclic GMP phosphodiesterase, an inhibition which enhances cyclic GMP levels in the vascular smooth muscle, leading to reduced resistance of cerebral vessels and increase of cerebral flow [123].

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The highest concentration of MCS extract demonstrated high neuroprotective potentials compared with the reference drug in the present animal model. The MCS extract was able to lower the tau tangles formation by preventing apoptosis of neuron. Tangles formation has been reported as hallmark of neuronal degeneration and significant acetylated-tau pathology in a distribution pattern similar to hyperphosphorylated-tau [121]. Similar findings have been reported for conformational and truncation tau epitopes that are thought to represent intermediate stages of tangle progression [124]. The study also showed that MCS extract reduces GFAP expression collaborating with other reports that neuronal degeneration initiates glial cell regeneration by triggering various growth factors that result in increased astrocytes activity [118,120]. These results suggest that GFAP persist within degenerating astrocytes in valproic brain injury and of purkinje neurons to accentuate excitotoxic damage. An effect that was abrogated by MCS extracts supplementation. Antioxidants like MCS protect biological targets against ROS, therefore, they have been considered as attractive therapeutic potential to counteract ROS-mediated neuronal damage [125].

Conclusion

The present study confirmed that VPA - mediated oxidative stress compromised the antioxidants status of the brain PFC. Subsequently, the treatment with VPA along with MCS extract mitigated this effects. This study is evident that MCS extract could be utilized in the management of VPA-induced neurotoxicity and lowered VPA - induced oxidative stress, tau formation and gliosis. The neuroprotective effect observed is greater than that of vinpocetine, a standard drug, known to decrease neuronal death thus suggesting a neuroprotective effect by the MCS extract at this concentration. However, further investigation is needed to unravel the mechanism of MCS abrogation of VPA-induced neurotoxicity.

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No conflict of interest declared.

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