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Vernonia ambigua Alters the Expressions of some Antioxidant Enzymes, Pro-Inflammatory Cytokines, and Acetylcholinesterase in the Cerebellar Cortex of Rats

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ABSTRACT

The therapeutic effects of medicinal plants are often attributed to their recognised and scientifically validated phytochemical constituents. However, literature is rare on the effects of *Vernonia ambigua* (VA), a documented medicinal plant, on the expression of antioxidant enzymes, cytokines, and neurotransmitters. This study aimed to examine the relative gene expression of cerebellar catalase (rCCAT), glutathione peroxidase-1 (rCGPx-1), tumour necrosis factor-alpha (rCTNF-α), interleukin-1 beta (rCIL-1β), and acetylcholinesterase (rCAChE) in male Wistar rats after the ingestion of an ethanol leaf extract of VA (ELEVA). The 200-220 g rats were divided into four groups, each containing five rats. The control group received feed and water, while test groups ELEVA (E)1, E2, and E3 received 100 mg/kg, 200 mg/kg, and 400 mg/kg of body weight of ELEVA, respectively, for 14 days. On the 15th day, portions of fresh cerebellar tissues were collected for total ribonucleic acid quantification and analysis using a spectrophotometer. Complementary deoxyribonucleic acid was produced using reverse transcriptase kits. rCTNF-α was significantly increased ($p<0.05$) in E1, E2, and E3, while rCGPx-1 and rCCAT were significantly increased and decreased, respectively, in E3. No significant difference ($p>0.05$) in rCIL-1β was observed in the test groups. The rCAChE in E1 and E2 were significantly reduced. ELEVA demonstrated concentration-dependent effects that reduced rCCAT and rCAChE but increased rCGPx-1 and rCTNF-α. Consequently, ELEVA may decrease AChE activity, offering potential therapeutic benefits while posing a risk of overstretched inflammatory responses. Additionally, the antioxidant effects of ELEVA may rely on elevated rCGPx-1, as it reduced rCCAT.

Keywords

PCR, Transcriptase, Ethanol leaf-extract, Purkinje cells

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INTRODUCTION

The mammalian body undergoes continuous, tightly regulated complex cellular activities crucial for sustaining life. During these cellular processes, several reactive oxygen species (ROS) are produced (Hendrix *et al.*, 2020), which help maintain normal cell function within a physiological range by acting as vital signalling molecules (Hong *et al.*, 2024). These ROS, described as natural by-products of cellular metabolism, primarily form within the cells' powerhouse during energy production (D'Ascenzo and Colussi, 2025) and include superoxide, hydroxyl radical, and hydrogen peroxide (H_2O_2) (Nandi *et al.*, 2019). Paradoxically, when ROS levels exceed physiological limits, leading to difficulties in their elimination and subsequent accumulation (Olufunmilayo *et al.*, 2023) and cellular dysfunction, it is termed oxidative stress (Dash *et al.*, 2025).

Oxidative stress is also characterised by decreased activity of antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Mbah Ntepe *et al.*, 2020), along with a reduction in other antioxidants such as ascorbate, α-tocopherol, and carotene (Philips *et al.*, 2020). Oxidative stress results in the release of pro-inflammatory cytokines, such as tumour necrosis factor α (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) (Muzio *et al.*, 2021). It also affects the activity of acetylcholinesterase (Goschorska *et al.*, 2018).

Oxidative stress disrupts the normal functions of brain tissue (Lee *et al.*, 2020), and regions most susceptible to its deleterious effects include the hippocampus, amygdala, prefrontal cortex, and cerebellum (Wang and Michaelis, 2010). Among these brain regions, the cerebellum stands out, and it is often referred to as the little brain (Amore *et al.*, 2021). The cerebellum has been shown to possess functions beyond its traditionally recognised roles of balance and voluntary muscle control (Phillips *et al.*, 2015). Other reported functions include emotional learning (Ciapponi *et al.*, 2023), pain processing (Li *et al.*, 2024) and cognition (Zhang *et al.*, 2023). Despite its small size, the cerebellum contains a rich reserve of neurons, constituting about 80% of the brain's neurons (Roostaei *et al.*, 2014), estimated to be around 69.03 ± 6.65 billion in humans (Azevedo *et al.*, 2009). This substantial neuronal population portrays the cerebellum as a site of significant and highly active metabolic activities necessary to meet its diverse functional demands and the consequent production of ROS.

Furthermore, emerging scientific evidence supports the notion that medicinal plant products exert protective effects by scavenging ROS and enhancing antioxidant levels (Ashafaq *et al.*, 2023). Additionally, medicinal plants are utilised in managing central nervous system (CNS)-related conditions, such as Alzheimer's disease (Gregory *et al.*, 2021), migraines (Bavarsad *et al.*, 2023), and multiple sclerosis (Breijyeh *et al.*, 2021). The aforementioned medicinal remedies have been linked to phytochemical constituents, or bioactive compounds, establishing medicinal plants as an excellent repository of therapeutic agents (Rabizadeh *et al.*, 2022). Notable plants recognised for their medicinal values within the genus Vernonia include *V. amygdalina* (Asante *et al.*, 2024), *V. cinerea* (Theja and Nirmala, 2024), *V. guineensis* Benth (Nnanga *et al.*, 2022), *V. auriculifera* (Haile *et al.*, 2022), *V. kotschyana* (Vasincu *et al.*, 2022), *V. ambigua* (VA) (Maroyi, 2020) and *V. hymenolepis* (Gelata *et al.*, 2024).

The genus Vernonia, named after the renowned British botanist William Vernon, comprises over one thousand species distributed across North America, South America, Africa, Asia, and Australia (Toyang and Verpoorte, 2013). Generally, the genus is utilised for treating skin infections, gastrointestinal infections, bacterial infections, gynaecological diseases, respiratory conditions, snake bites, and insect bites (Kiplimo, 2016).

Specifically, VA contains phytochemical constituents, such as saponins, tannins, alkaloids, and flavonoids (Kunle and Egharevba, 2009), which confer it with antibacterial (Aliyu *et al.*, 2011), antimalarial, antioxidant (Builders *et al.*, 2011), and fertility-promoting abilities (Igwe *et al.*, 2024). VA has also been used in recipes for treating epilepsy in Fongo-Tongo, in the Western Region of Cameroon, for over two decades (Noumi and Fozi, 2003).

However, studies examining the roles of VA on the complex interplay between the expressions of specified antioxidant enzymes, pro-inflammatory cytokines, and acetylcholinesterase within the CNS remain limited. Furthermore, some of these plants are known to be poisonous (Kavitha *et al.*, 2023), raising safety concerns despite their reported benefits to humans and animals. This caution emphasises the need for scientifically based scrutiny before confirming their safe use. This study aimed to investigate the impact of administering ethanol leaf extract of VA (ELEVA) on the relative gene expressions of cerebellar catalase (rCCAT), glutathione peroxidase-1 (rCGPx-1), tumour necrosis factor-alpha (rCTNF-α), interleukin-1 beta (rCIL-1β), and acetylcholinesterase (rCAChE).

MATERIALS AND METHODS

Assembling of Plants for Identification and Extraction

Fresh plants of VA still bearing their flowers and intact roots were obtained from Ochudo City, Abakaliki Local Government Area, Ebonyi State, Nigeria, for identification. A taxonomist in the Applied Biology Department of Ebonyi State University handled the identification process. A voucher (herbarium) number assigned to the plant was EBSU-H-1100. After identification, a large quantity of the plant was collected, and their leaves were defoliated and dried at room temperature. This is followed by pulverisation of the dried leaves using a mortar and pestle. The resultant powdered VA, weighing 190 g, was subsequently stored in an air-tight container. A mixture of the powdered leaves was constituted by mixing 5 mL of absolute alcohol {(Guangdong Guandgua Chemical Factory Co. Ltd, CAS: 7778-80-5) M.P. 1069°C B.P.1689° D2.66; Lot number 20100408} with 1 g of VA in an air-tight container. The mixture was stirred every 6 h, left tightly covered with the lid of the container and kept at room temperature for 72 h. A double-folded sieve cloth with fine pores and two Whatman filter papers No. 1 were used for the filtration of the mixture, with the former being the first and the latter serving in the second filtration procedure. To concentrate the resultant ethanol-containing filtrate, it was placed in a water bath whose temperature was set at 40ºC to facilitate the evaporation of the ethanol. A sticky texture of the extract was obtained, which was weighed (21.6 g) using an electronic weighing balance. The stock solution of the ELEVA for this study was constituted following the procedure stated in the Organisation of Economic Cooperation and Development (OECD) adopted on 16th October 2008 and corrected on 30th June 2022 (OECD, 2022) and refrigerated to maintain its efficacy.

Study Procedure

A total of twenty male Wistar rats obtained from the Animal Research Facility of the Faculty of Basic Medical Sciences of Ebonyi State University were used for this experiment. The rats were housed at a room temperature of 25 ± 2 °C (natural 12:12 h light/dark cycle) and acclimatised for two weeks. The rats were placed in cages that allowed adequate ventilation and fed regularly during the periods of acclimatisation and the study proper. Before administering the crude ethanol leaf extract of VA, rats (weight range 200-220 g) were randomly grouped into four, each comprising five male Wistar rats. The control group received feed and water only. Groups E1, E2 and E3, which were the test groups, received 100 mg/kg, 200 mg/kg and 400 mg/kg of ethanol leaf extract of VA, respectively. This study lasted for 14 days, during which each rat received the extract orally and once daily with the aid of an orogas-

tric tube attached to the end of a 2 mL insulin syringe. On the 15th day of the experiment, the animals in each group were sacrificed using cervical dislocation. The entire brain was first excised from the skull of rats to reveal the entire cerebellum. This step was immediately followed by the careful excision of a small portion of the cerebellar cortex and placing the same in an Eppendorf tube containing fixative for the relative gene expression study.

Total Ribonucleic Acid (RNA) Isolation

The seclusion of total RNA from the excised cerebellum of each rat was performed according to the procedure explained by Omotuyi *et al.* (2018). The various cerebellar tissues (cortex) from each rat were homogenised in cold TRIzol reagent (Zymo Research, USA, Cat: R2050-1-50, Lot: ZRC186885) at 4°C. Total RNA was segregated in trichloromethane (BDH Analytical Chemicals, Poole, England Cat: 10076-6B) using an Abbott Laboratories centrifuge (Model: 3531, Lake Bluff, Illinois, United States) set at 15,000 revolutions per minute for 15 min. The next stage was the precipitation of RNA, which was achieved by utilising the same volume of isopropanol (Burgoyne Urbidges and Co, India, Cat: 67-63-0) as the RNA obtained from the clear supernatant. This stage was followed by rinsing the RNA pellet twice in a solution of ethanol and nuclease-free water, constituted in the ratio of 7:3 {70 mL of absolute ethanol (BDH Analytical Chemicals, Poole, England Cat: 10107-7Y) and 30 mL of nuclease-free water (Inqaba Biotec, West Africa, Lot no: 0596C320, code: E476-500ml)}. The pellets went through air drying and dissolution. While the former lasted for 5 min, the latter occurred in 1 mM of sodium citrate at a pH of 6.4 (also called RNA buffer).

Complementary Deoxyribonucleic Acid (cDNA) Conversion:

Before initiating the conversion to cDNA, the quantity, as well as the quality of the total RNA, were estimated with A260/A280 (A=absorbance), read with a Jenway UV-VIS spectrophotometer (model 6305, UK), and recorded. The decontamination of RNA from DNA was conducted using DNAse I treatment (NEB, Cat: M0303S) according to the instructions of the producer. RNA was transformed to cDNA in a 2 µL solution holding 100 ng of decontaminated RNA, using the M-MuLV reverse transcriptase Kit (M-MuLV RT, NEB, Cat: M0253S) in a 20 µL final volume (2 µL, N9 random primer mix; 2 µL, 10X M-MuLV buffer; 1 µL, M-MuLV RT (200 U/µL); 2 µL, 10 mM deoxynucleotide triphosphate; 0.2 µL, RNase Inhibitor (40 U/µL) and 10.8 µL nuclease-free water). Apart from the inactivation of M-Mulv reverse transcriptase that was conducted at 65°C for 20 min, these stepwise procedures were conducted at room temperature.

Polymerase Chain Reaction (PCR) Amplification and Agarose Gel Electrophoresis

The PCR amplification for the estimation of the genes under scrutiny was conducted following the procedure specified: PCR amplification was carried out in a 25 µL volume reaction mixture holding 2 µL cDNA (40 ng), 2 µL primer (100 pmol), 12.5 µL Ready Mix Taq PCR master mix (One Taq Quick-Load 2x, master mix, NEB, Cat: M0486S) and 8.5 µL nuclease-free water. Initial denaturation that took place at 95 °C, which lasted for 5 min, was followed by 20 cycles of amplification that involved denaturation at 95 °C and annealing, each lasting for 30 sec, initial extension at 72 °C for 60 sec, and a final extension at 72 °C for 10 min. In all experiments, there were inclusions of negative controls where the reaction mixture lacked cDNA. The amplicons were resolved on a 1.5% agarose gel (Cleaver Scientific Limited: Lot: 14170811) using tris (hydroxymethyl) aminomethane (Tris)-borate-EDTA buffer at pH 8.4 (Tris - RGT reagent, China, Lot: 20170605; Borate - JHD chemicals, China, Lot 20141117).

Data Handling

The data obtained from this study were analysed using the Social Package for Statistical Sciences version 25. Descriptive statistics were presented as mean ± Standard Error of Mean (SEM). A Tukey post hoc test was used to compare each relative gene expression. The significance level in this study was set at $p < 0.05$.

RESULTS

Relative Gene Expression of Selected Cerebellar Oxidative Enzymes

rCCAT

The mean rCCAT of male Wistar rats in the control, E1, E2, and E3 groups were 0.97 ± 0.05, 0.96 ± 0.08, 0.72 ± 0.07, and 0.64 ± 0.06, respectively. The mean rCCAT of test groups E1 ($p = 0.999$) and E2 ($p = 0.087$) showed no significant difference compared to the control group. The mean rCCAT of test group E3 ($p = 0.026$) was significantly lower than the control group (Fig. 1).

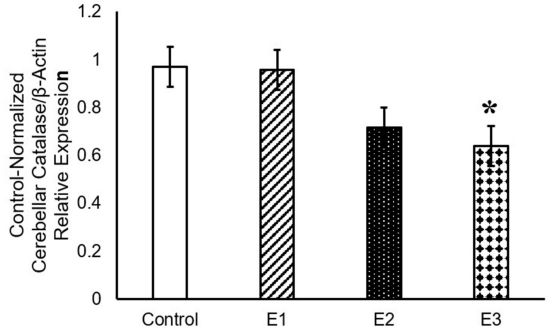

Fig. 1: The mean relative gene expression of cerebellar rCCAT. The control group received only feed and water. The E1, E2, and E3 groups received 100 mg/kg, 200 mg/kg, and 400 mg/kg body weight of ELEVA, respectively. * significant different compared to the control at $p < 0.05$

rCGPx-1

The mean rCGPx-1 of male Wistar rats in the control, E1, E2, and E3 groups were 0.91 ± 0.03, 1.02 ± 0.06, 0.66 ± 0.03, and 2.09 ± 0.11, respectively. The mean rCGPx-1 of test groups E1 ($p = 0.641$) and E2 ($p = 0.102$) was lower and of no significant difference when compared to the control group. The mean of rCGPx-1 of male Wistar rats in the test group E3 ($p = 0.0001$) was significantly higher when compared to the control group (Fig. 2).

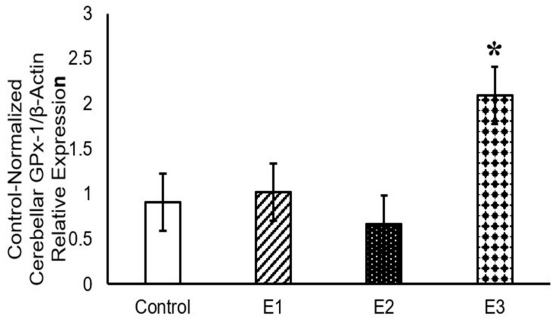

Fig. 2: The mean relative gene expression of cerebellar glutathione peroxidase-1 (rCGPx-1). The control group received only feed and water. The E1, E2, and E3 groups received 100 mg/kg, 200 mg/kg, and 400 mg/kg body weight of ELEVA, respectively. * significant different compared to the control at $p < 0.05$

Relative Gene Expression of Selected Cerebellar Cytokines

rCTNF-α

The mean rCTNF-α of the control, E1, E2, and E3 groups were 1.09 ± 0.05, 2.49 ± 0.03, 1.72 ± 0.10, and 2.42 ± 0.06, respectively. The mean rCTNF-α of E1 ($p = 0.0001$), E2 ($p = 0.001$) and E3 ($p = 0.0001$) were significantly higher when compared to the control group (Fig. 3).

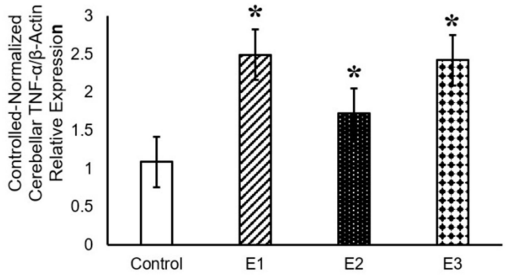

Fig. 3: The mean relative gene expression of cerebellar tumour necrotic factor alpha (rCTNF-α). The control group received only feed and water. E1, E2, and E3 groups received 100 mg/kg, 200 mg/kg, and 400 mg/kg body weight of ELEVA, respectively. * significant different compared to the control at $p < 0.05$.

rC1L-1β

The mean rC1L-1β of Wistar rats in the control, E1, E2, and E3 groups were 1.09 ± 0.06, 1.26 ± 0.08, 0.91 ± 0.05, and 1.33 ± 0.15, respectively. The mean rC1L-1β of E1 ($p = 0.640$) and E3 ($p = 0.362$) showed no significant difference when compared to the control group. There was a lower mean rC1L-1β in the E2 ($p = 0.558$), which was not significantly different when compared to the control group (Fig. 4).

rCAChE

The mean rCAChE of the male Wistar rats in the control, E1, E2, and E3 groups were 1.05 ± 0.05, 0.80 ± 0.04, 0.60 ± 0.03, and 0.98 ± 0.06, respectively. The mean rCAChE of groups E1 ($p = 0.023$) and E2 ($p = 0.001$) were significantly lower when compared to the control group. The mean rCAChE of group E3 ($p = 0.773$) was higher but not significantly different when compared to the control group (Fig. 5).

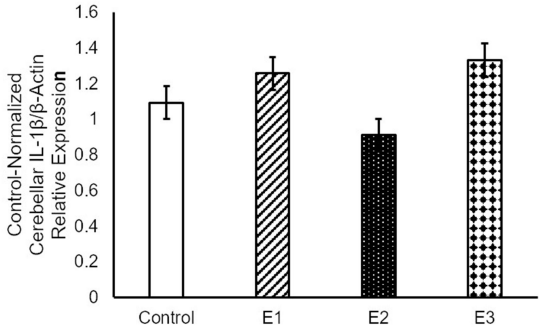

Fig. 4: The mean relative gene expression of cerebellar rC1L-1β. The control group received only feed and water. E1, E2, and E3 groups received 100 mg/kg, 200 mg/kg, and 400 mg/kg body weight of ELEVA, respectively. No significant difference was observed at $p < 0.05$

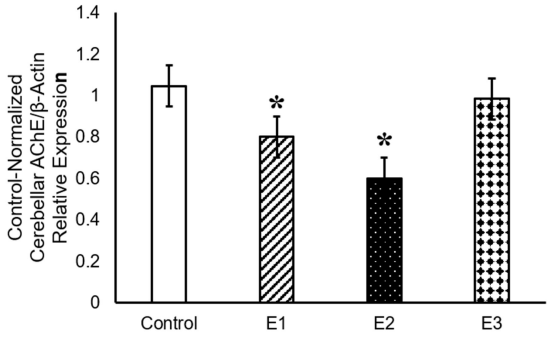

Fig. 5: The mean relative gene expression of cerebellar acetylcholinesterase (rCAChE). The control group received only feed and water. E1, E2, and E3 groups received 100 mg/kg, 200 mg/kg, and 400 mg/kg of ELEVA, respectively. * significant different compared to the control at $p < 0.05$.

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DISCUSSION

Medicinal plants and their products have gained worldwide recognition as effective components in treatment plans for various diseases, often with fewer side effects. Scientifically verified effects of VA on key cellular activities, vital enzymes, or proteins in the CNS, especially the cerebellum, have remained scarce. Narrowing this knowledge gap will significantly assist in their proper use to address ongoing therapeutic needs. This study evaluated the expressions of rCCAT, rCGPx-1, rCTNF-α, rCIL-1β, and rCAChE following the administration of ELEVA in male Wistar rats.

A uniform downregulation in the expression of cerebellar catalase was observed following the administration of ELEVA, reaching significant levels in the three test groups compared to the control group. This decline in expression implies reduced translation for the synthesis of catalase in the Purkinje cells and in neurons of the molecular and the granular layer, where expression of cerebellar catalase occurs (Schad *et al.*, 2003). The observed decline in rCCAT suggests that hydrogen peroxide will accumulate in the cerebellar cortex, subsequently increasing the risk of oxidative stress, as the function of catalase, which is responsible for eliminating hydrogen peroxide, will be disrupted (Rasheed, 2024). As an antioxidant enzyme, its reduction due to a decline in the rCCAT may lead to mi-

croglia activation or neuronal damage in the cerebellar cortex (Alrafiah, 2021) and also may disrupt synaptic plasticity (Ahn *et al.*, 2016).

GPx supports protection against ROS and reactive nitrogen-induced cell damage (Oliveira-Silva *et al.*, 2019), and GPx-1 contributes to these general functions through its ability to decrease hydrogen peroxide and lipid hydroperoxides (Handy and Loscalzo, 2022). This study specifically focused on the expression of GPx-1, which is one of the five selenium-containing glutathione peroxidase types (GPx-1 to GPx-4 and GPx-6) (Brigelius-Flohé and Flohé, 2020), and showed significant upregulation of rCGPx-1 at 400 mg/kg of ELEVA. This may suggest increased synthesis of GPx-1, supporting the classification of VA as a medicinal plant with antioxidant properties (Builders *et al.*, 2011) due to its ability to facilitate the expression of GPx-1 in the cerebellum, as shown in this study. This finding suggests ELEVA may play a vital role at this concentration in offering protection to neural cells from oxidative stress by decomposing hydrogen peroxide to water, hence limiting its toxic effects (Cueto-Ureña *et al.*, 2023). Furthermore, in their review centred on GPx-1 in disease and health, Handy and Loscalzo (2022) stated that the expression of GPx-1 at the physiological levels is as important as excessive upregulation of GPx-1, as the latter is also highly beneficial in ROS-induced cellular damage. This study, therefore, supports the reported antioxidant properties of VA as stated by Builders *et al.* (2011). However, such properties may result from the observed inverse relationship between rCCAT and rCGPx-1 or solely from the increased activity of GPx-1 due to the upregulation in rCGPx-1 observed in this study.

Of the two evaluated pro-inflammatory cytokines in this study, ELEVA stimulated a significant increase in rCTNF-α. These elevated levels of rCTNF-α may contribute to communication between glial cells, resulting in the sustenance of the physiological levels of glutamate in the synaptic cleft, within the cerebral cortex, thereby inducing neuronal plasticity (Shim et al., 2018). Furthermore, the upregulation of rCTNF-α levels may lead to an increase in ROS (Hariharan and Dharmaraj, 2020), which will subsequently elicit neuronal death and increased blood-brain barrier permeability (Caldito, 2023). This study also suggests that ELEVA may lack anti-inflammatory potential in the cerebellar cortex due to the significant increase in rCTNF-α, which is contrary to the anti-inflammatory properties shared by other plants of the same genus, such as *Vernonia amygdalin*a (Georgewill and Georgewill, 2010) and *Vernonia cinerea* L. (Theja and Nirmala, 2024). The finding from this study suggests that the administration of VA may pose a risk of neurotoxicity.

Conversely, the observed elevation of rCIL-1β due to ELEVA administration indicates that the extract may not alter the physiological roles of IL-1β, which, according to Hewett *et al.* (2012), include neuronal protection, tissue remodelling, and repair. This is because rCIL-1β remained within the physiological range, as no significant changes were observed in the expression levels of cerebellar IL-1β upon administration of various concentrations of ELEVA compared to the control group.

The observed downregulation in rCAChE due to ELEVA administration suggests that ELEVA possesses the ability to suppress the activity of AChE. Other plants with similar AChE inhibitory effects include *Acinos arvensis*, *Lavandula angustifolia* (Vladimir-Knežević *et al.*, 2014) and *Vernonia amygdalina* (Oladele *et al.*, 2020). This implies that ELEVA may increase the level of acetylcholine, thereby influencing the physiology of the synaptic cleft and cholinergic system in the cerebellum.

Conclusion

The ELEVA exhibited concentration-dependent effects that reduced rCCAT and rCAChE, but increased rCGPx-1 and rCTNF-α. Therefore, ELEVA may decrease AChE activity, offering potential therapeutic benefits while posing a risk of raised inflammatory responses. Additionally, the antioxidant effects of ELEVA may rely on elevated rCGPx-1, as it reduced rCCAT. However, there is a need for further study to understand the observed dynamics between antioxidant enzymes, pro-inflammatory cytokines and AChE following the administration of ELEVA.

DECLARATION

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This work was self-funded.

Conflict of Interest

None declared.

Ethical Approval

This study followed the stipulated guideline provided in the Guide for the Care and Use of Experimental Animals Committee (National Research Council, 2011), which was approved by the Faculty of Basic Medical Sciences Research and Ethics Committee of Ebonyi State University, Abakaliki, Ebonyi State, Nigeria, with the assigned ethical approval number of EBSU/FBMS/REC/2019/08/009.

Consent to Participate and Publish Data

Not applicable.

Authors' Contribution

All aspects of this study were contributed by the author.

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