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Effects of *Daucus carota* Ethanolic Leaf Extract in Cadmium-Induced Toxicity on the Oxidative Stress Markers in the Hippocampus and Prefrontal Cortex of Adult Wistar Rats

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Abstract:

- ***Background:***

Cadmium (Cd) is a heavy metal with very high toxicity, whose prolonged contact is linked to neurodegenerative disorders due to its ability to induce oxidative stress, neuro-inflammation, and apoptosis in critical brain regions such as the hippocampus and prefrontal cortex.

- ***Objectives:***

This study evaluated the effects of *Daucus carota* ethanolic leaf extract in cadmium-induced toxicity on the oxidative stress markers in the hippocampus and prefrontal cortex of adult wistar rats.

- ***Methods:***

Thirty adults male Wistar rats (weighing 150–180 g) were randomly assigned into five groups (6 per group). Group 1 (normal control) received water, Group 2 (Cd-only) was administered cadmium chloride (5 mg/kg) to induce neurotoxicity. Group 3 received only CLE (400 mg/kg). Groups 4 and 5 were received cadmium chloride and CLE at doses of 200 mg/kg and 400 mg/kg, respectively. All treatments were administered orally for 28 days. At the end of the experiment, brain tissues were harvested for biochemical analysis of oxidative stress markers (MDA, ROS and 4-HNE) and anti-oxidant enzyme (SOD, CAT, GSH) activities. Data were analyzed using GraphPad Prism version 8 and presented as Mean ± SEM. Statistical comparisons were made using one-way ANOVA followed by Tukey's post hoc test, with significance set at $p < 0.05$.

- ***Results:***

Cadmium exposure significantly increased oxidative stress, and triggered neuro-inflammation, as evidenced by elevated MDA, ROS and 4-HNE levels and reduced antioxidant enzyme activity (SOD, CAT, GSH). However, CLE treatment ameliorated these changes in a dose-dependent manner. The Cd + CLE (200 mg/kg) and Cd + CLE (400 mg/kg) groups exhibited significant improvements compared to the Cd-only group, showing reduced oxidative damage. The highest dose (400 mg/kg) demonstrated the most pronounced neuroprotective effects, with biochemical parameters approaching those of the control group.

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➢ ***Conclusion:***

This study provides compelling evidence that *Daucus carota* ethanolic leaf extract exhibits potent neuroprotective properties against cadmium-induced neurotoxicity. The observed anti-oxidative effects suggest that CLE could serve as a promising natural intervention for mitigating heavy metal-induced cognitive and neuronal impairments.

Keywords: *Cadmium, Extract, Oxidative Stress, Toxicity, Hippocampus.*

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I. INTRODUCTION

The brain, a highly complex and vital organ, is responsible for cognitive functions, memory, emotions, and overall neural coordination.[1] It is highly sensitive to various internal and external insults, including environmental toxins, oxidative stress, and neurodegenerative conditions.[2] Among the brain regions, the hippocampus and prefrontal cortex (PFC) play crucial roles in learning, memory processing, and executive functions.[3,4] Any disruption to these areas can result in cognitive impairment, mood disorders, and neurodegenerative diseases.[5-8] Increasing evidence suggests that environmental pollutants, including heavy metals, can severely impact brain health, leading to neurotoxicity and long-term neurological dysfunctions.[9-12]

Cadmium toxicity poses a serious environmental and public health concern due to its widespread industrial use and persistence in the environment.[13-15] Chronic exposure to cadmium through contaminated food, water, air, and cigarette smoke leads to bioaccumulation in various organs, including the brain, where it exerts profound neurotoxic effects.[16,17] The hippocampus and prefrontal cortex which are critical for learning, memory, and executive functions, are predominantly susceptible to cadmium-related damage.[4] Researches had demonstrated that cadmium exposure disrupts neuronal integrity,[18] induces oxidative stress,[19] triggers neuro-inflammation,[20] and promotes apoptosis,[21] ultimately contributing to cognitive decline,[6] behavioural deficits, and an increased risk of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease.[22,23]

Despite the known dangers of cadmium neurotoxicity, effective therapeutic interventions remain limited.[24] Conventional treatments often focus on chelation therapy, which can be ineffective in reversing neuronal damage or restoring cognitive function.[25] This highlights the urgent need for alternative neuroprotective strategies that can counteract the toxic effects of cadmium at a cellular and molecular level.[23,26] Plant-based antioxidants, such as those found in carrot leaf extract (CLE), have shown promising potential in mitigating oxidative stress and inflammation,[17,27] yet their role in neuroprotection remains largely unexplored. Investigating the neuroprotective effects of CLE against cadmium-induced toxicity could provide a natural, cost-effective therapeutic approach to preserving brain health and preventing heavy metal-induced cognitive impairments. This study seeks to bridge this knowledge gap by evaluating the protective effects of CLE on the hippocampus and PFC, offering new insights into its potential as a neuroprotective agent.

II. MATERIALS AND METHODS

➢ *Materials*

Carrot leaves, Cadmium chloride, 35 adult Wistar rats, Reagents: Normal saline, 10% formalin, Alcohol, Xylene, Animal feed (growers), Netted cages, dissecting board and kit, Hand gloves, Laboratory coat, Sample bottle, Weighing balance, Syringe and needle, Beakers, Glass slides and coverslips.

➢ *Plant Collection and Identification*

Fresh carrot leaves were obtained from a community in the study area. It was identified and authenticated by a Botanist in the Department of Science and Biotechnology, University of Nigeria, Nsukka, with herbarium number 1017b.

➢ *Cadmium Chloride*

Cadmium chloride was purchased from Zayo-Sigma Chemicals Ltd, Jos, Northern part of Nigeria, with molecular weight 201.32g/mol, batch number 117 09, product number 80683 and pack size 250g, which was used as the toxin for this experiment, the LD50 of cadmium is 88mg/kg. It was prepared using 1g of cadmium which was dissolved in 50ml of distilled water. The constituted solution was shaken for proper dissolution and then preserved in a refrigerator for use.

➢ *Extract Preparation*

Carrot leaves were collected and allowed to dry under shade for two weeks to prevent the direct effects of sunlight on the active constituents of the leaves, after which they were grounded into powdery form in a milling machine. The powder was sieved to obtain uniform particle size that was used in the extraction process by the maceration method. The leaves were dissolved in water at a ratio of 1:7, using 450 ml of water. The mixture was stirred every 6 hours over 48 hours. After this duration, it was sieved to extract the liquid content and subsequently strained again using litmus paper. The supernatant was dried at 40°C in a water bath. About 2.5g, 5g and 7.5g of the supernatant were stirred in 30mls, 50mls and 70mls of water to obtain the various doses of the treatment, respectively.[28]

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➢ *Animal Procurement*

Thirty-five (35) Adult Wistar rats weighing 100-120g were purchased from the Animal house of the study institution. The animals were housed in well-ventilated wired cages and allowed to acclimatize for two weeks in the animal house. They were maintained under standard photoperiodic conditions of 12 hours of light/dark cycle at a temperature of 27^0C -30^0C and relative humidity of 50 ± 50C. The animals were fed with rat pellets (Top Feed Ltd, Nigeria) and allowed unrestricted drinking water access.

➢ *Experimental Design*

The experimental study involved five (5) groups (Groups A-E) of arbitrarily divided thirty-five (35) adult Wistar rats, separately involving seven (7) randomized rats, tagged and housed in separate cages. For the preparation of stock solution, 20g of CLE was dissolved in 100 ml of distilled water, from which subsequent concentrations for administration were derived. According to Ijomone *et al*. (2020), 20 mg of Cdcl2 does not cause morbidity.[29] Furthermore, according to Ahmad *et al*. (2023), 800 mg/kg of CLE does not cause morbidity.[30]

- Group A (Control): Received normal rat feed and water only for 28 days.
- Group B received 8-mg/kg body weight (bwt) of $CdCl_2$ for 14 days.
- Group C was administered 400-mg/kg bwt of CLE for 14 days.
- Group D received 8-mg/kg body weight (bwt) of $CdCl_2$ in saline and 200-mg/kg bwt of CLE for 28 days
- Group Ewas administered 8-mg/kg bwt of $CdCl_2$ in saline and 400-mg/kg bwt of CLE for 28 days.

➢ *Animal Sacrifice and Sample Collection*

After 24 hours of fasting at the end of 28 days, we slaughtered the animals using cervical dislodgment. Blood samples for biochemical studies were obtained from the apex of the heart while the skull was excised, the prefrontal cortex and hippocampus were harvested and fixed in 10% formalin for histological studies.

➢ *Biochemical Studies*

The samples were obtained, homogenised and spinned at 1000rmp in 10mins. The resulting serum was used for the assessment of the Glutathione-S-Transferase (GST), Malondialdehyde (MDA), Catalase (CAT) and Superoxide dismutase (SOD).

➢ *Determination of Oxidative Stress Markers and Lipid Peroxidation*

- *Malondialdehyde:*

The MDA level in brain tissue of rats was estimated using a adapted technique by Lykkesfeldt (2001).[31] Briefly, trichloroacetic acid (0.5ml of 0%) was mixed with 0.4 ml of the tissue serum and 1.6 ml of TrisKCl was added to it. This was followed by addition of thiobarbituric acid (0.5ml), and the mixture secured for 45 minutes under optimal condition before being read at 532 nm wavelength in nmol/ml tissue.

- *Superoxide Dismutase (SOD) Activity:*

This was assessed by modified methods, following the procedures according to Sun & Zigma (1978),[32] and based on the enzyme's capacity to inhibit the auto-oxidation of epinephrine, determined by the increase in absorbance at 480 nm. The reaction mixture included 2.95 ml of 0.05 M sodium carbonate buffer at pH 10.2, 0.02 ml of the homogenate, and 0.03 ml of epinephrine in 0.005 N HCl to initiate the reaction. Enzyme activity was calculated by measuring the change in absorbance at 480 nm over a 5-minute duration.

- *Catalase Determination:*

This was estimated using a adapted spectrophotometric technique designated by Aebi (1984).[33] It was based on the putrefaction of H_2O_2.

- *Glutathione (GSH) Level:*

This was done by titrating 0.1 mmol/L of 5,5'-Dithibios (2-nitrobenzoic acid) in a 0.1 mol/L disodium phosphate buffer solution with a pH of 8. At 412 nm, the reduced product of thionitrobenzene's production was quantified spectrophotometrically (Güntherberg & Rost, 1966).[34] The GSH concentration was given as mol/g of moist tissue.[35]

- *Glutathione S-Transferase Estimation:*

The activities of this enzyme were estimated by observing the thioether link between GST and 1-chloro-2,4-dinitrobenzene (CDNB) spectrophotometrically at a wavelength of 340nm for 5 minutes.

- *Detection of Reactive Oxygen Species (ROS):*

ROS levels were assessed using a fluorescence spectrophotometer with dichlorodihydrofluorescein diacetate (DCFH-DA) as the fluorescent probe. The probe was hydrolyzed intracellularly to dichlorodihydrofluorescein (DCFH) and subsequently oxidized by ROS to form dichlorofluorescein (DCF). The fluorescence intensity of DCF was measured at an excitation/emission wavelength of 488/525 nm, and the recorded fluorescence values correlated with the ROS levels in the samples.

- *Detection of 4-Hydroxy-2-Nonenal (4-HNE):*

4-HNE levels were quantified using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (MyBioSource company). Samples were added to wells pre-coated with anti-4-HNE antibodies and incubated to allow antigen-antibody binding. A secondary enzyme-linked antibody was introduced, followed by a chromogenic substrate. The resulting colour change was measured using a microplate reader, and the absorbance values were compared against a standard curve to determine the concentration of 4-HNE in the samples.

- *Detection of Acetylcholine (ACh):*

Acetylcholine levels were measured using ELISA kits (MyBioSource company). Samples were added to microplate wells pre-coated with anti-acetylcholine antibodies and incubated to allow specific binding. A horseradish peroxidase (HRP)-conjugated secondary antibody was then introduced,

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followed by the addition of a chromogenic substrate. The enzymatic reaction produced a colour change, and the absorbance was measured using a microplate reader. The concentration of acetylcholine in the samples was determined by comparing the absorbance values to a standard calibration curve.

- *Tau protein:*

Serum level of tau protein was measured using a commercial ELISA kit (MyBioSource company) with sensitivity of 2.0 pg/ml and a detection range of 15.6 to 500 pg/ml.

➢ *Data Analysis*

The experimental data obtained was analyzed using Graph Pad prism version 9.0.1.5 and results were presented in a tabular form as Mean ± standard error of mean (SEM). The statistical differences in the means were established with ANOVA. Multiple comparisons of Turkey's Post Hoc Test were adopted to check the significance level at $p \leq 0.05$.

III. RESULTS

➢ *Effects of Daucus carota Ethanolic Leaf Extract on Cadmium Toxicity in 4-HNE, ACH and Tau Protein Concentrations*

As shown in Table 1, 4-HNE levels were higher in group B compared to group A. Group C had significantly lower 4-HNE levels compared to group B ($P<0.05$), while group D was significantly higher than group C ($P<0.05$). Group E showed a decrease compared to group B, but this was not statistically significant. Furthermore, ACH levels were significantly higher in group B compared to group A ($P<0.05$). Groups C and D had significantly lower ACH levels compared to group B ($P<0.05$). Group E also showed a significant reduction in ACH compared to group B ($P<0.05$).

Tau Protein levels were significantly lower in group B compared to group A ($P<0.05$). Groups C and D showed an increase in Tau Protein compared to group B, but these differences were not statistically significant. Group A remained statistically and significantly higher than group E ($P<0.05$).

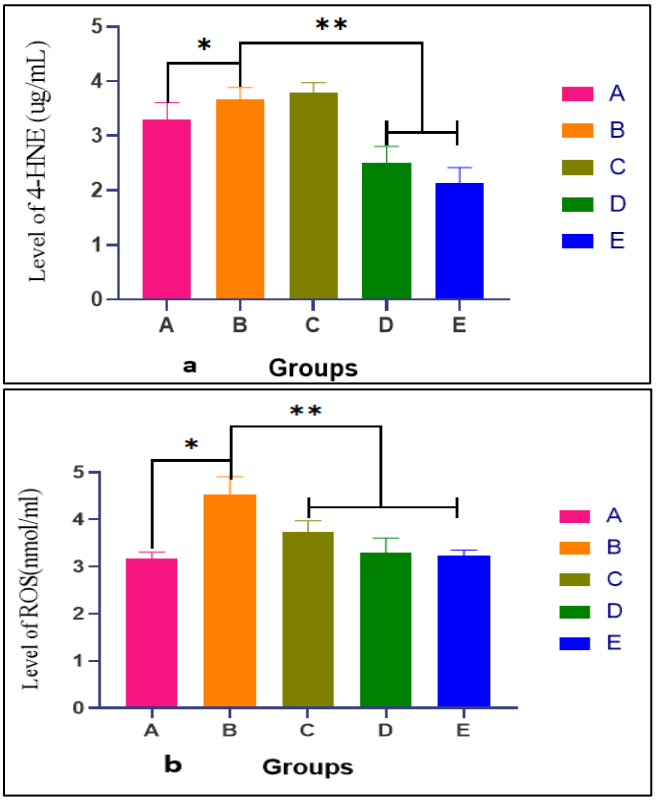
Table 1 Effects of *Daucus carota* Ethanolic Leaf Extract on Cadmium Toxicity in 4-HNE, ACH and Tau Protein Concentrations.

Groups	4-HNE	ACH	Tau Protein
A	3.30±0.31	16.67±0.47	12.47±0.41
B	3.67±0.22	26.73±1.13[a]	8.10±0.30[a]
C	2.13±0.29[b]	16.50±0.46[b]	9.77±0.96
D	3.80±0.17[c]	16.97±0.90[b]	10.47±0.81
E	2.50±0.31	12.53±2.81[b]	8.30±0.47[a]

a = Significant Difference when Compared to A; b = Significant Difference when Compared to B; c = Significant Difference when Compared to C; d = Significant Difference when Compared to D; e = Significant Difference when Compared to E.
KEYS: 4-HNE = 4-Hydroxy-Nonenal; ACH = Acetylcholine

➢ *Effects of Cadmium and Carrot Leaves Extract on Oxidative Markers*

The oxidative markers measured during this experiment are presented in Figures 1a-c. In Figure 1a, the levels of 4-hydroxylnonela (4-HNE) significantly increased in group B compared to the control group A ($p<0.05$). In contrast, the level of 4-HNE was decreased considerably in the treated groups compared to group B ($p<0.05$). Figure 1b showed that the level of reactive oxygen species (ROS) was increased significantly in group B compared to group A ($p<0.05$), and decreased significantly in all the treated groups (C, D, and E). Also, the malondialdehyde (MDA) level shown in Figure 1c increased significantly in group B compared to group A ($p<0.05$). At the same time, the MDA level decreased substantially among extract-treated groups matched to group B at $p<0.05$.



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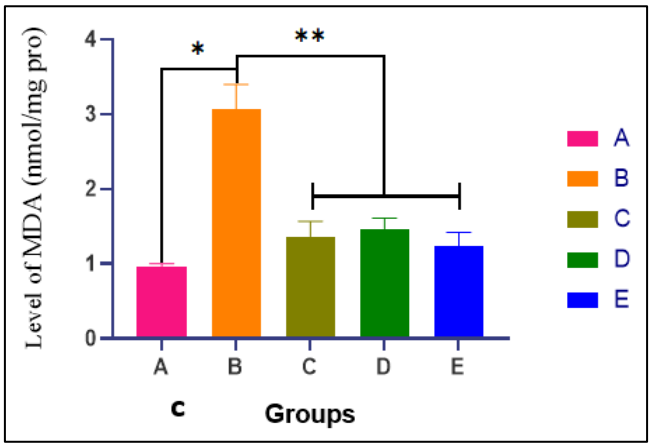


Fig 1 Effects of Cadmium and Carrot Leaves Extract on the Levels of Oxidative Markers During the Experiment. (a) 4-Hydroxylnonela (4-HNE) Levels; (b)Reactive Oxygen Species (ROS) Level; and (c) Malondialdehyde (MDA) levels. *Significant Increase at p<0.05; and **Significant Decrease at p<0.05

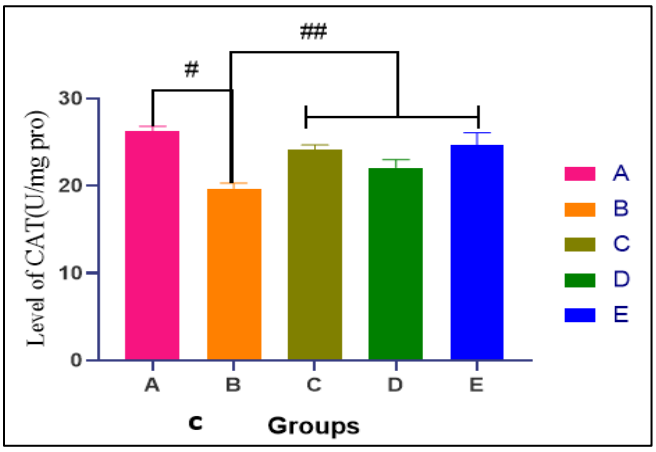
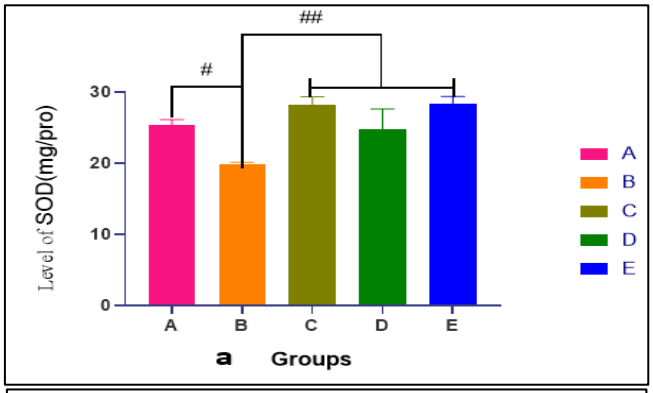
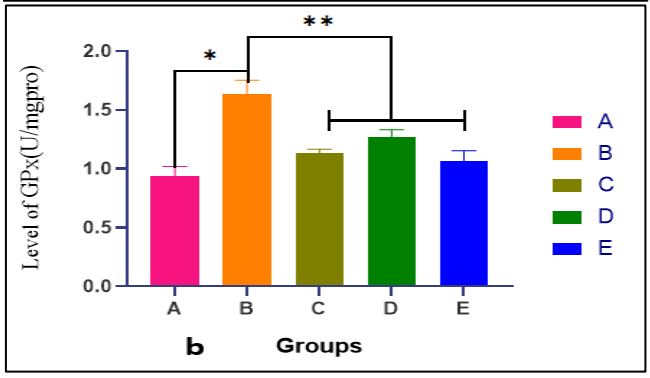


Fig 2 Effects of Cadmium and Carrot Leaves Extract on the Levels of Antioxidant Markers During the Experiment. (a) Superoxide Dismutase (SOD) Levels; (b) Glutathione Peroxidase (GPx) Level; and (c) Catalase (CAT) levels. #Significant Decrease at p<0.05; and ##Significant Increase at p<0.05

➢ *Effects of Cadmium and Carrot Leaves Extract on Antioxidant Levels*

The antioxidant levels determined in this study are shown in Figures 2a-c. Figure 2a showed that the superoxide dismutase (SOD) was significantly decreased in groups B compared to group A (p<0.05). In contrast, the SOD level was increased considerably in the extract groups compared to group B (p<0.05). The result in Figure 2b showed that the catalase (CAT) activity level was reduced significantly only in group E, compared to group B (p<0.05). The glutathione peroxidase (GPx) level as shown in Figure 2c was decreased significantly in group B compared to group A (p<0.05). At the same time, the GPx level was increased substantially in groups C and D compared to group B (p<0.05).





IV. DISCUSSION

➢ *Effects of CLE and Cd on Biochemical Analysis*

The biochemical analysis in this study assessed oxidative stress markers and antioxidant enzyme activity in the hippocampus and prefrontal cortex of cadmium-exposed rats and the potential curative effects of CLE. The results indicate that cadmium exposure led to significant oxidative stress, evidenced by increased levels of ROS, MDA and 4-HNE (Fig 1), and decreased levels of antioxidant enzymes (SOD, CAT, and GPX) (Fig 2). However, treatment with CLE demonstrated varying degrees of amelioration against these cadmium-induced alterations, with some parameters significantly improved in CLE-treated groups.

The results in Fig 2a and 2c showed that SOD and CAT activity were significantly lower in group B (cadmium-only) compared to the control group (Group A), indicating severe oxidative stress. This finding is consistent with previous research showing that cadmium exposure reduces antioxidant enzyme activity, thereby promoting the accumulation of ROS and exacerbating neuronal damage.[36] The SOD plays a crucial role in converting superoxide radicals into hydrogen peroxide, which is further detoxified by CAT to prevent oxidative damage.[37]

The reduction in these enzymes suggests that cadmium exposure overwhelms the brain's antioxidant defense mechanisms, leading to increased oxidative stress and neuronal injury.[38] Importantly, treatment with *Daucus carota* extract significantly improved SOD and CAT activity in groups D and E, indicating its curative antioxidant effects. This aligns with findings that *Daucus carota* contains bioactive compounds such as flavonoids, terpenes, and polyphenols, which enhance the activity of antioxidant enzymes and reduce oxidative stress.[39] The ability of *Daucus carota* to restore SOD and CAT activity suggests that its bioactive components help counteract cadmium-induced free radical production, thereby preserving neuronal function.

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Glutathione peroxidase (GPX) activity as shown in Fig 2b was significantly higher in group B compared to the control group, indicating a cellular adaptive response to cadmium toxicity. This is consistent with previous findings that suggest elevated GPX activity in cadmium-exposed cells is a compensatory mechanism to neutralize increased lipid peroxidation.[40] However, groups D and E, which received CLE treatment, showed significantly lower GPX activity compared to group B, suggesting that CLE supplementation reduced oxidative stress, thereby normalizing GPX levels.

Similar findings have been reported where antioxidant-rich plant extracts restored GPX activity by reducing oxidative stress load in cadmium-exposed animals .[39] Cadmium exposure is also known to deplete intracellular GSH levels by binding to thiol (-SH) groups, thereby impairing the brain's redox balance.[41] The ability of *Daucus carota* to enhance glutathione-related antioxidant defense mechanisms has been previously documented, with its polyphenolic compounds shown to upregulate GSH synthesis .[42]

Lipid peroxidation is a key indicator of oxidative stress and is commonly assessed by measuring malondialdehyde (MDA) and 4-hydroxy-nonenal (4-HNE) levels. In this study, 4-HNE and MDA levels were significantly higher in group B (Fig 1a and c) confirming that cadmium exposure induces lipid peroxidation and neuronal membrane damage. These findings align with previous reports that cadmium increases MDA levels due to its ability to generate free radicals, which attack polyunsaturated fatty acids in cell membranes.[43] Studies have shown that lipid peroxidation is one of the primary mechanisms through which cadmium exerts neurotoxicity, leading to cognitive deficits and neuronal apoptosis.[44]

The CLE treatment (Groups D and E) significantly reduced MDA and 4-HNE levels, suggesting that its antioxidant properties counteract lipid peroxidation. These results support previous findings that *Daucus carota* essential oils and polyphenols can reduce oxidative damage by scavenging free radicals and enhancing antioxidant enzyme activity.[45] The presence of sesquiterpenes such as β-caryophyllene in *Daucus carota* has been linked to the inhibition of lipid peroxidation.[46]

ROS levels were significantly elevated in group B, confirming that cadmium exposure promotes oxidative stress by generating excessive free radicals. This aligns with studies showing that cadmium increases ROS production, leading to neuronal dysfunction and cognitive impairments.[36] The CLE treatment significantly reduced ROS levels, particularly in groups C and E, indicating its effectiveness in scavenging free radicals. These findings are in agreement with previous research demonstrating that *Daucus carota* extracts possess potent radical-scavenging activity due to their high phenolic and flavonoid content.[39]

As shown in table 1, Cadmium exposure significantly increased acetylcholine (ACH) levels in group B, indicating disrupted cholinergic neurotransmission. Studies suggest that cadmium interferes with acetylcholine metabolism by inhibiting acetylcholinesterase activity, leading to excessive ACH accumulation and neuronal hyperactivity.[47] This dysregulation has been implicated in cognitive impairments and neurodegenerative conditions.[48] Treatment with CLE significantly reduced ACH levels in groups C, D, and E, suggesting that it may help restore cholinergic balance and improve cognitive function.

Tau protein levels, which are crucial for neuronal stability, were significantly lower in group B, indicating cadmium-induced neurotoxicity. This aligns with research suggesting that cadmium disrupts cytoskeletal integrity by impairing tau protein phosphorylation.[49] While CLE treatment in groups D and E increased tau protein levels, these changes were not statistically significant, suggesting that further investigation is needed to understand the precise role of *Daucus carota* in tau protein regulation.

V. CONCLUSION

This study evaluated the neuro-ameliorating effects of *Daucus carota* ethanolic leaf extract against cadmium-induced toxicity in adult Wistar rats, assessing its impact on oxidative stress markers and neurotransmitters in the hippocampus and prefrontal cortex. The findings confirmed that cadmium exposure led to significant increased oxidative stress, and severe neuronal degeneration.

However, CLE treatment, in a dose dependent mannerr, demonstrated protective effects by enhancing antioxidant enzyme activity (SOD, CAT, GPX), and reducing oxidative stress markers (MDA, ROS, 4-HNE). While this study highlights the potential of CLE as a natural therapeutic agent against cadmium-induced neurotoxicity, further research is needed to explore its precise mechanisms of action, optimal dosing strategies, and long-term effects in both experimental and clinical settings.

➢ *Ethical Approval*

Ethical approval was sought and got from the Research Ethics Committee of the Faculty of Basic Medical Sciences Ebonyi State University, Abakaliki, Ebonyi State, with number EBSU/REC/2024/7299. This research also complied with the Helsinki declaration of 2013 as it concerns animal studies.

- *Conflict of Interests:*

The authors have no potential conflict of interests to declare.

- *Data Availability Statement:*

All data about this research is available on reasonable request to the corresponding author on ogashstanly90@yahoo.com.

- *Author Contributions:*

The authors contributed equally to all aspects of this research.

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