Memory impairment effect of Cannabidiol Oil and Prednisolone Treatment: Alteration of Neuro-oxidant markers and Acetylcholinesterase Activity in the Hippocampus of Cadmium-Induced Toxicity in Male Wistar Rats

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Keywords: ABSTRACT

Background: There have been conflicting reports on the effect of Cannabis products on learning and memory. Hence this study investigated CBD oil's and prednisolone treatment's cognitive impact on cadmium-induced toxicity in male Wistar rats. Methods: Forty rats weighing between 150g to 200g were assigned into 8 groups (1-8) of five animals each. Group A control, Group B-H received 1mg/kg body weight prednisolone; 1.5mg/kg Cadmium; 1mg/kg pred+0.2mg/kg CBD-oil; 0.2mg/kg CBD-oil+2mg/kg cadmium; 3mg/kg pred+2mg/kg cadmium; 0.1mg/kg CBD-oil and 0.2mg/kg CBD-oil respectively. The administration was done using gavage for 14 days. A T-maze test apparatus was used to determine the latency of object recognition before and after administration. Results: There was a significant decrease in latency of object recognition in prednisolone, cadmium, and 0.2mg/kg CBD-oil treated groups than control after administration. Acetylcholinesterase activity significantly (P<0.05) increased in the cadmium+0.1mg/kg CBD-oil treated group and decreased in the pred+CBD-oil group compared to the control. Acetylcholinesterase activity significantly (P<0.05) increased in prednisolone, pred+cadmium, and 0.2mg/kg CBD-oil treated groups and decreased in cadmium, pred+CBD-oil, cadmium+CBD-oil, and 0.1mg/kg CBD-oil treated groups compared to control. Catalase activity significantly increased in pred+cadmium, 0.1mg/kg CBD-oil, and 0.2mg/kg CBD-oil treated groups compared to control. SOD activity significantly decreased in the treatment groups than the control. Malondialdehyde significantly increased in cadmium, pred+CBD-oil, cadmium+CBD-oil, 0.1mg/kg CBD-oil, and 0.2mg/kg CBD-oil than control. Glutathione peroxidase significantly decreased in treated groups compared to control. Reduced glutathione significantly decreased across treated groups than the control. Histology of the hippocampus revealed visible pathologic changes in pred+cadmium, 0.1mg/kg CBD-oil, and 0.2mg/kg CBD-oil treated groups with cellular vacuolization, Perivascular leucocyte infiltration, and pycnotic nuclei, indicating slight inflammation and detrimental effects of the treatment in the histoarchitecture of the hippocampus. Conclusions: CBD oil, prednisolone, and cadmium administration at different doses induced biochemical alterations, and exacerbated cognitive and neurobehavioral decline by enhancing oxidative stress, acetylcholinesterase activity, and alteration in the cytoarchitecture of the hippocampus. All articles published in this journal are licensed under the Creative Commons Attribution 4.0 International (CC BY 4.0) license.

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**1. Introduction**
In recent times, the combined use of cannabidiol (CBD) oil and prednisolone in Nigeria for treatment of some disorders such as pain, asthma, anxiety, anorexia, muscle spasms, depression, and insomnia have generated concern about their likely adverse effects, majorly in brain cognitive function (Braun et al., 1989; Sachs et al., 2015). Cannabis use as a social drug was established to be due to its psychoactive effect produced by a tetrahydrocannabinol compound that presents a feeling of highness (Ahrens, et al., 2009). Cognitive defects have been reported in cannabinoid users (Pope et al., 2001; Bolla et al., 2002). A momentary harmful consequence on working memory and short-term has been reported on Cannabis users (Riedel et al., 2005). It was reported that cannabidiol (CBD), an isomer of tetrahydrocannabinol (THC) harmonizes the potency of the psychoactive compound (THC) as well as controls THC metabolism via the inactivation of cytochrome p450 enzyme that metabolizes drugs (Watson et al., 2000). It was reported in mouse models that CBD caused elevated brain THC concentration as its key metabolite. This was suggested to be a result of its reduced clearance rate from the body (Watson et al., 2000).

Cannabinoid receptors (CB1 and CB2) were reported to be among the G protein-coupled receptor family as they were found in both intracellular and extracellular cell membranes (Pertwee, 1997). CB1 receptors are more copious in the brain whereas CB2 receptors are morphologically diverse and are typically present in cells of the immune system commonly in natural killer cells, B-cells and monocytes, polymorphonuclear neutrophil cells, T8 cells, and T4 cells (Pertwee, 1997). Cannabinoid binding to its receptor reduces adenylyl cyclase action and inhibits Ca^{2+} and K^{+} channels (Pertwee, 1997).

Cannabinoids affect motor control, memory, and pain modulation by interacting with naturally produced dopamine for pain transmission (Abadinsky, 2013). It was reported that as cannabinoids enter the brain via blood flow and bind to the CB1 receptor, it leads to alterations in dopamine and norepinephrine concentrations causing euphoria, altered conscious perception, feeling of well-being, stress reduction or relaxation, elevated positive reception of humor, music joviality, improved memory, better awareness of sensation, metacognition and introspection, (Abadinsky, 2013). Cadmium is a general toxic agent. Chin-Chan et al., (2015) reported that cadmium caused neuropathological and neurochemical changes in the central nervous system, resulting in cognitive impairment. Hence, this study investigated the cognitive impact of CBD oil and prednisolone combined treatment on cadmium-induced toxicity in male Wistar rats.

**2. MATERIALS AND METHODS**

**Drugs:** Prednisolone used for this study was purchased from Unicure Pharmaceutical Limited, Lagos, Nigeria. The cadmium chloride was purchased from Sigma-Aldrich Limited Germany with EC number 233-296-7. Cannabidiol (CBD) oil was purchased from TEEMU Premium, California, USA.

**2.1 Laboratory animals**
Forty (40) male Wistar rats weighing 150–200g were used for this study. The animals were housed in the Department of Physiology Animal House, University of Calabar, Nigeria. Standard animal cages (435 x 290 x 150mm) with wood shavings as bedding were used in housing the animals (5 rats per cage). They were given ad libitum access to feed (AEC Agrosystem limited, PortHarcourt, Rivers State, Nigeria) and fresh water, and exposed to 12/12 hr light/dark phase. They were acclimatized for 7 days and kept in line with laid down ethics for animal care approved by the National Committee for Research Ethics in Science and Technology (NENT), 2018. University of Calabar animal ethics committee permitted our research procedure with approval number 040PHY3719.

**2.2 Experimental Design and Administration of Drugs**
The animals were arbitrarily allotted into 8 separate groups (n = 5). At the end of 7 days of acclimatization, CBD oil, prednisolone, and cadmium administration began. The drugs were given through oral means using gavage (dose per rat outlined in Table 1),

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once, daily, to animals in treatment groups (2 to 8), using the doses outlined in Table 1, whereas the control group was given feed and 0.5ml normal saline as a vehicle. CBD oil and prednisolone administration lasted for fourteen (14) days, whereas, cadmium chloride was given for just 2 days, thereafter, the rats were anesthetized with chloroform, blood samples were collected from rat via ocular puncture, and the hippocampus of the control and treated rats were isolated after fixing whole skull with 10% buffered formaldehyde for 60 minutes for tissue homogenization analysis. The samples were stored in an ice pack and immediately utilized for analysis.

Table 1. Study Design and Drugs Administration

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of rats</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Control)</td>
<td>5</td>
<td>Feed + 0.5ml of normal saline as a vehicle throughout the experimental period.</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>1mg/kg bw of prednisolone</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>1.5mg/kg bw of Cadmium</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1mg/kg bw of prednisolone + 0.2mg/kg bw of CBD Oil.</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0.2mg/kg bw of CBD oil + 2mg/kg bw of cadmium</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>3mg/kg bw of prednisolone + 2mg/kg of cadmium</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>0.1mg/kg bw of CBD Oil low dose</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>0.2mg/kg bw of CBD oil high dose</td>
</tr>
</tbody>
</table>

2.3 Behavioural, learning, and memory assessment

The latency of object recognition was done using T-mase apparatus (30 x 10 x 20cm) arms.

Procedure: The object was placed on either the left or right arm gates, opened, the rat was placed in the start arm, and recording was done with a stopwatch. The start gate was opened gently when the rat was facing away from the goal's arms. When all four paws enter one arm, the other arm gate is closed and arm entry is recorded. The arm gate was closed when the rat returned from the arm and the start gate was closed upon re-entry into the starting arm. If the animal is motionless in the goal arm for longer than the 90s, the rat is gently touched with a rolled paper towel to guide it toward the start arm. The procedure was repeated for 3 trials, and timing and recording were done accordingly. Method used by Alipour et al., (2023)

2.4 Hippocampal Antioxidant Assessment

The hippocampus of each rat was isolated after fixing the whole brain in 10% buffered formaldehyde for 60 minutes and homogenized using a Potter-Elvehjem homogenizer. Twenty percent (1/5 w/v) of tissue homogenate was placed in 50 mm Tris–HCl buffer (pH 7.4) with 1.15% potassium chloride and centrifuged at 10,000 rpm at 4°C for 10 minutes. The supernatant was obtained for catalase (CAT) assay with hydrogen peroxide as substrate. Reduced Glutathione (GSH) was assayed at 412 nm using the method of Luchese et al., (2009). Glutathione peroxidase (GPx) was assayed using hydrogen peroxide as substrate (Lucchese et al.,
Superoxide dismutase (SOD) was assayed using the method described by Misra and Fridovich (1972). Malondialdehyde (MDA) was assessed in thiobarbituric acid reacting substances (TBARS) as explained by Meenakshi, et al., (2007); Okhawa, et al., (1979). Afterward, the mixed reaction generated 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution matched to pH 3.5 with sodium hydroxide, and 1.5 ml of 0.8% water solution of thiobarbituric acid was added to 0.2 ml of 10%(w/v) of homogenate. The mixture was moved up to 4.0 ml using distilled water and heated at 95°C for 60 mins. Almost 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) was added and centrifuged ice cooling at 4000rpm. The crude layer was discarded and absorbance was summed at 532 nm and added with results obtained from MDA standards. The concentrations were calculated from absorption values as normal absorption.

2.6 Acetylcholinesterase activity assessment
Acetyl-cholinesterase enzyme activity was obtained using the Ellman (1961) method. Aliquots of supernatant (0.4 mL) of hippocampus homogenate were added to 2.6 mL of phosphate buffer (0.1 M, pH 7.4) followed by 0.1 mL of 5, 5-dithiol-bis (2-nitrobenzoic acid) (DTNB). Then, 0.1 mL of acetylthiocholine iodide was added to the reaction mixture. A spectrophotometer (412 nm) was later employed to read the absorbance (10 min). Absorbance changes at 2-minute intervals were recorded. The activity of AChE was measured following an increase in color produced from thiocholine after reacting with DTNB. The change in absorbance per minute was determined and the rate of AChE activity was calculated and expressed as μmol/min/mg protein Ellman et al., (1961); Wopara et al., (2021).

2.7. Measurement of serum Calcium ion concentration
The serum calcium ion concentration was determined by the o-cresolphthalein complexone method (Baginsky et al., 1973). This method is based on calcium ion reacting with o-cresolphthalein complexone in an alkaline solution to form an intense violet colored complex.

**Procedure:** The test tubes (Becton, Dickinson, and Company, USA) were labeled as test, standard, and blank. Thereafter, 1.0 ml of the reagent was introduced into all the test tubes. Then 0.025ml of the samples were added into appropriate tubes and mixed vigorously. The mixture was allowed for 5 minutes, thereafter, the absorbance was read and recorded at 590nm. The increase in absorbance of the mixture is directly proportional to the calcium ion concentration in the sample. The concentration of serum calcium ion was calculated by dividing the absorbance of the test by the absorbance of the standard, multiple by the concentration of the standard which is 2.5mmol/l.

2.8 Histological studies
The hippocampus of the control and treated rats were isolated after fixing the whole skull with 10% buffered formaldehyde for 48 hours. A microtome (Sakura Finetek, USA) was used to obtain tissue sections (5um in thickness). Thereafter, the cut tissue was floated over a water bath (Labotech International Co., Ltd, Tokyo, Japan) at a temperature of 6°C to remove wrinkles and distortion in the tissue, and picked up on a slide. The sections were stained with hematoxylin and eosin (H & E) stains. The microscopic slides were labeled appropriately. Photomicrographs were taken at ×125 magnifications using a light microscope (Leica DM 750, Switzerland). The cytoarchitectural changes of the hippocampus were examined with blinding to reduce bias. Method used by Mobisson et al., (2023).

2.9 Statistical analysis
All results are presented as mean ± SEM, n=5. One-way analysis of variance (ANOVA) was utilized in comparing the differences within groups, followed by post hoc multiple comparisons. Computer software SPSS version 17.0 and Excel analyzer were used for the analysis. The level of significance was set at p<0.05.
3. RESULTS

3.1. The Mean latency of object recognition during the T-maze test before and after drug administration in control and different experimental groups

In Figure 1 below, the Mean latency of object recognition during the T-maze test in rats administered 0.2mg/kg bw of CBD oil significantly decreased (p<0.01) after administration compared to control and before administration. However, a significant increase was shown in groups administered prednisolone alone and cadmium alone after administration compared to before administration and control.

3.2. Comparison of hippocampal antioxidant markers in control and different experimental groups.

In Figure 2 below, there was a significant increase(p<0.05) in the concentration of catalase present in the group administered CBD oil (0.2ml), compared to the control. However, it also shows an increase in groups administered Prednisolone + cadmium and CBD oil(0.1ml) compared to groups administered Prednisolone alone and Cadmium alone. Figure 3 below showed a significant decrease(p<0.05) in superoxide dismutase in all treated groups compared to control. However, it also showed a significant decrease(p<0.05) in the group administered CBD Oil(0.1ml) compared to groups administered prednisolone alone and cadmium alone. Figure 4 below showed a significant increase(p<0.05) in the concentration of malondialdehyde in all treated groups except groups treated with prednisolone alone and pred+CBD oil alone compared to the control. Figure 5 below showed a significant decrease (p<0.05) in glutathione peroxidase in all treated groups except groups administered pred+ CBD oil alone and cadmium+ CBD oil compared to control. However, a significant decrease (P<0.05) was shown in groups administered pred + CBD oil, cadmium + CBD oil, prednisolone + cadmium, and CBD oil (0.2ml) compared to groups administered prednisolone alone and cadmium alone. A significant increase (P<0.05) was also shown in the group administered Pred+ cadmium and CBD oil(0.1ml) compared to the group administered CBD oil (0.2ml). Figure 6 below showed a significant increase(p<0.05) in the concentration of reduced glutathione in all treated groups compared to the control. However, a significant increase(p<0.05) was shown in groups administered pred+ CBD oil, cadmium+ CBD oil, pred+ cadmium, and CBD oil (0.2ml) compared to groups administered prednisolone alone and cadmium alone. There was a significant increase(p<0.05) in the group administered CBD oil(0.1ml) compared to the group administered Pred+ CBD oil.
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3.3. Comparison of hippocampal acetylcholinesterase (AChE) concentration in the different experimental groups

Figure 7 below showed a significant increase (p<0.05) in the concentration of acetylcholinesterase in the group administered prednisolone alone, and a significant decrease (p<0.05) in the group administered CBD oil (0.1ml) alone compared to the control. However, there was a significant decrease in all treated groups compared to the group administered Prednisolone alone.
3.4. Serum calcium concentration in the different experimental groups

Figure 8 below shows a significant increase (p<0.05) in the concentration of serum calcium level in the group administered cadmium alone compared to the control. However, a significant decrease (p<0.05) was shown in groups administered Pred+ CBD oil and CBD oil (0.1ml) compared to groups administered cadmium alone.

3.5. Histological examination of the Hippocampus after administration of the treatments

Plate 1a below shows a photomicrograph of control (group 1) with four layers of hippocampal Cornu ammonis CA (1-4). CA1 and CA2 contain small pyramidal neurons; CA3 and CA4 contain large pyramidal neurons. CA4 projects into the concavity of the dentate gyrus. The dentate gyrus contains stellate cells.

Plate 1b (group 2) shows a photomicrograph of a prednisolone-treated rat with similar histological features as in the control group, with no visible histopathologic change noticed.

Plate 1c (group 3) shows a photomicrograph of a representative rat from the cadmium treated group appearing as in the control.

Plate 1d (group 4) shows a pred+CBD oil-treated rat with no histopathologic change observed.

Plate 1e (group 5) shows cadmium+CBD oil-treated rat with no histological changes seen.

Plate 1f (group 6) shows pred+cadmium treated rat with cellular vacuolization, indicating slight detrimental effects of the treatment in the histoarchitecture of the hippocampus and perivascular leucocyte infiltration.

Plate 1g (group 7) shows CBD oil (0.1ml) fed rats with various pycnotic nuclei.

Plate 1h (group 8) showed CBD oil (0.2ml) fed rats with perivascular leucocyte infiltration, a sign of slight inflammation. Many Cells Vacuolization.
4. DISCUSSION
Traditional medicine including the use of natural products from plants, is widely practiced around the world, assuming that certain natural products contain enhancing and healing properties that may have an essential role in the human system. Over the past few years, considerable attention has focused on cannabidiol (CBD), a major non-psychotropic constituent of Cannabis (Erin, et al., 2020). Prednisolone, as a cortico-steroid was first introduced into medical practice in the late 1940s, since then, it has been used by almost all medical specialists as an effective treatment for autoimmune and inflammatory conditions (Renner et al., 1986).

This study investigated the effect of cannabidiol oil and prednisolone on cadmium-induced toxicity on memory and neurobehavior with T-maze, endogenous antioxidant system, calcium, and neurotransmitter alteration in rat's brain regions, and hippocampal histologic examination.

4.1. Latency of object recognition
The latency of object recognition was significantly reduced in rats fed with 0.2mg/kg body weight of CBD oil compared with the control. However, the groups administered prednisolone alone and cadmium alone significantly increased compared to the groups administered CBD oil (0.2mg/kg). The significant reduction in latency of object recognition in this group may likely be due to the effects of CBD oil in the hippocampus in declarative, episodic, and recognition memory. This effect was suggested to be mediated by endocannabinoid receptors (CB1) within the brain areas (Kaczocha, 2009). This counters the report made by Osborne et al., (2017), that CBD does not affect cognition in healthy individuals, but can improve cognitive processes including attention, and executive function (Osborne et al. 2017), though, this may likely be due to the differences between humans and animals.

Furthermore, studies have suggested that CBD acutely reduces THC-related learning and memory impairments in well-controlled humans (Englund et al., 2013) and was also replicated in animal studies (Vann et al., 2008). A study conducted by Englund et al., (2013), reported better episodic memory with oral CBD consumption. The stated findings served as backup to the effects of cannabis products (CBD and THC) in learning and memory as shown in Figure 1.

4.2. Serum Calcium ion concentration
The Serum calcium ion concentration significantly increased in the group administered cadmium alone compared to the control. The increase in serum calcium concentration may likely be attributed to cadmium's effect on the parathyroid gland's stimulation (Zbigniew, et al., 2020). It could also be attributed to increasing the concentration of acetylcholinesterase as calcium ion plays an important role in acetylcholine release (Baux and Fossier, 1992). The significant increase in the concentration of serum calcium ions in cadmium-treated rats corresponds with the report made by Zbigniew, et al., (2020), that suggested increased calcium ion levels in cadmium and lead-exposed workers may be useful in lowering cadmium concentration. Furthermore, the degree of hypocalcemia was proportional to the dose of cadmium used. It was suggested that this fall in serum calcium ion level may likely be due to increased renal excretion (Wallin et al., 2013). Furthermore, it was reported that cadmium negatively affects the skeletal system by causing demineralization through direct interaction with bone cells, inhibiting procollagen, C-proteinases, and collagen production (Staessen et al., 1999).

4.3. Hippocampal Acetylcholinesterase concentration
The concentration of hippocampal Acetylcholinesterase significantly increased in the group administered Prednisolone alone and significantly decreased in the group administered CBD Oil (0.1ml) respectively compared to the control. The significant increase in acetylcholinesterase concentration in the prednisolone-treated group may be due to the effect it exerts in increasing the level of myotube acetylcholine receptor expression, initiating an anti-inflammatory effect, by binding to cellular glucocorticoid receptors, prednisolone acts to inhibit inflammatory cells and suppresses expression of inflammatory mediators (Braun et al., 1989). The results showed a significant increase in acetylcholinesterase concentration, as reported by Braun et al., (1989), in Stimulating effects of prednisolone on acetylcholine receptor expression and myogenesis in the primary culture of newborn rat muscle cells. This indicates that prednisolone at concentrations of 10(-5) to 10(-8) mol/l, added to 3-day (day D + 2) tissue cultures of newborn rat
myogenic cells at the time myoblasts are beginning to fuse, increases the level of myotube acetylcholine receptor expression at the 8th day of culture (Braun et al., 1989). This effect is associated with increases in the number and size of formed myotubes, not with a changed affinity of the receptor for its ligand (Murillo-Rodríguez et al., 2018). This was suggested to be probably mediated by one or more extracellular proteins (Murillo-Rodríguez et al., 2018). The significant decrease in acetylcholinesterase in the group administered CBD Oil (0.1ml), may likely be due to the effects of CBD Oil serving as AChE inhibitors and thereby increasing both the level and duration of neurotransmitter action, which play an important role in memory retention and learning processes (Umukoro et al., 2020).

4.4. Hippocampal oxidative stress markers
The catalase concentration significantly increased in the group administered CBD Oil (0.2ml) compared to the control. The increase in the concentration of catalase in CBD oil (0.2ml) treated group, may be due to toxic effects of hydrogen peroxide generated by various reactions and environmental agents such as noise and temperature (Michiels et al., 1994). It was reported that the purpose of catalase in living cells is to protect them from oxidative damage when cells or other molecules in the body come into contact with oxidative compounds (Sun, 1990). The decrease in the concentration of Superoxide dismutase in all treated groups may be a result of the absence or minute traces of harmful superoxide free radicals that could cause tissue damage in all treated groups most especially the group treated with CBD oil (0.1ml) as previously reported by Hayyan et al., (2016) and Wang et al., (2018).

The increase in the concentration of malonaldehyde in the treated groups may likely be an indication of lipid peroxidation as reported by Gaweł et al., (2004); Mobisson et al., (2022). It was reported that an elevated lipid peroxidation can overwhelm the antioxidant defense system and trigger cell apoptosis and pathological processes leading to elevated serum MDA levels that reflect increased free radical production (Arya et al., 2021). The decrease in glutathione concentration may likely be due to little or absence of harmful peroxide free radicals in all treated groups. The biochemical function of glutathione peroxidase is to reduce lipid hydro-peroxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Muthu Kumar et al., 2011). The increase in reduced glutathione concentration may be attributed to the little or absence of cellular damage recorded in this study.

4.5. Histological examination of the hippocampus of treated rats
The changes (cellular vacuolization, Perivascular leucocytes infiltration, pycnotic nucleus, and inflammation) seen in histoarchitecture of the hippocampus in treated rats (plate 1f, plate 1g, and plate 1h specifically) may be attributed to slight detrimental effects of the treatments. Furthermore, these alterations may be linked to increased hippocampal malonaldehyde concentration, indicative of lipid peroxidation. These alterations may be linked to an increase in the latency of object recognition, especially in rats fed with 3mg/kg body weight of prednisolone + 2mg/kg body weight of cadmium (plate 1f). It was reported that prolonged glucocorticoid exposure, particularly cortisol, disrupts explicit memory as it alters the function of the hippocampus (Robert, 2003).

4.6. Conclusion
Our study confirmed that administration of CBD oil, prednisolone, and cadmium-induced biochemical alterations and exacerbates cognitive and neurobehavioral decline by enhancing oxidative stress, and acetylcholinesterase activity, and causing alterations in the cytoarchitecture of the hippocampus.

Author Contributions
Mobisson Samuel Kelechi and Agona Odeh Obembe designed the study and wrote the study protocol. Mobisson Samuel Kelechi and Madu Emmanuel Chibuikem performed laboratory experiments and literature searches. Mobisson Samuel Kelechi drafted the manuscript; Iheanyichukwu Wopara and Onyebuagu Peter Chukwuma worked on data analysis. Iheanyichukwu Wopara performed the statistical analysis. All authors read and approved the final manuscript.

Acknowledgment
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Authors hereby acknowledge the animal ethics committee of the University of Calabar, Nigeria for approving our study protocol.

Conflict of Interest
None declared.

References


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