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Effects of Vitamin C on Aluminum Chloride- Induced Neurotoxicity on the Hippocampal Cortex of Adult Wistar Male Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study aimed to evaluate the neuroprotective effects of Vitamin C on aluminum chloride ($AlCl_3$)-induced brain damage, focusing on behavioral, biochemical, and histomorphological outcomes in a rodent model.

Study Design: A total of 42 healthy male rats were randomly assigned to three groups: control, $AlCl_3$ -only, and $AlCl_3$ plus Vitamin C (500 mg/kg and 1000 mg/kg). The $AlCl_3$ groups received daily doses of $AlCl_3$, while the Vitamin C groups received concurrent Vitamin C supplementation.

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Methodology: Body weight, behavioral changes, biochemical markers of oxidative stress (MDA, SOD, GSH), and inflammation (TNF-α, IL-6) were assessed. Histomorphological analysis of the hippocampus was performed to evaluate structural damage. Behavioral assessments included locomotor and exploratory activity tests. Biochemical analyses measured oxidative stress and antioxidant enzyme activities.
Results: $AlCl_3$ administration resulted in significant body weight loss, increased oxidative stress (elevated MDA, and SOD levels), and heightened inflammation (elevated TNF-α and IL-6). Behavioral tests showed increased excitatory activities, and increased anxiety levels as seen in increased rearing and grooming activities. Histomorphological examination revealed significant hippocampal damages as seen in the pale staining, shrunken pyramidal cells. Vitamin C co-administration mitigated these adverse effects, demonstrating reduced body weight loss, lower oxidative stress, decreased inflammation, and improved behavioral performance. Histological analysis indicated less hippocampal damage in the Vitamin C treated groups.
Conclusion: As an antioxidant, Vitamin C effectively attenuates the neurotoxic effects of $AlCl_3$ by reducing oxidative stress and inflammation, and by protecting the hippocampal cellular integrity. These findings suggest that Vitamin C holds potential as a therapeutic agent for mitigating neurotoxicity induced by aluminum compounds.

Keywords: Aluminum chloride neurotoxicity; vitamin C neuroprotection; Oxidative stress in the brain; Inflammation and aluminum exposure; Hippocampal damage and neurodegeneration; Antioxidant effects of Vitamin C.

1. INTRODUCTION

"Aluminum (Al) is a ubiquitous metal that naturally exists in the trivalent state ($Al3^+$). It may associate with elements such as Sulphur, Chlorine, Fluorine, and may also form complexes with organic matter" [1]; (Igbokwe et al., 2019). "Humans get exposed to Al via activities such as recycling of scrap metals, ore processing, mining, and manufacturing processes in which Al is used. Moreover, humans and animals living in places polluted by industrial wastes have higher exposure to Al" [2]. "Various Al containing compounds such as Aluminium chloride, aluminium nitrate, and Aluminium sulphate are found in widely used products such as detergents, drugs, ceramics, cement and paints, and aluminium chloride happens to be the most prevalent in everyday consumer products" (Newairy et al., 2009). "However, weathering of rocks discharges higher levels of Al to the environment when compared with human activities" (Igbokwe et al., 2019). "Previous studies showed that on the average, adults take 3–12 mg of Al perday" [3].

"Even though Al does not play any physiological function in metabolism, it can constitute a toxicant to both humans and animals, if present at high levels in the body after exposure to its natural and unnatural sources" [1]. "Aluminium gains access to the brain through transferrin mediated transport, which subsequently leads to neurotoxicity" [4]. "It adversely affects series of biological reactions in the brain, by modulating synthesis of neurotransmitters such as acetylcholine and glutamate (Kawahara and Kato-Negishi, 2011) activating acetylcholinesterase" (Kaizer et al., 2005); modulating phosphorylation and dephosphorylation, as well as protein degradation (Huat et al., 2019). Other reports suggest the mechanism of neurological toxicities of Al is through the activation of oxidative and inflammatory processes [5];(Maya et al., 2016), increased levels of amyloid beta (Aβ) protein (Praticò 2002), development of oxidative stress due to increased generation of reactive oxygen species and reduced antioxidant defense mechanisms (Esparza et al.,., 2019; Sahebkar et al.,., 2018), degeneration of cholinergic terminals in hippocampus being one of the unique regions in brain where the neurogenesis (Bonfanti et al., 2011) continues even in adult life. It contains two parts: Cornu ammonis (hippocampus proper) and dentate gyrus. Both of these parts are separated by hippocampal sulcus and curve into each other. Below the sulcus lies subiculum. and cortex (Platt et al., 2001), neuronal apoptosis (Niu et al., 2007) and hyperphosphorylated tau protein development [6]. Studies have shown that aluminium chloride ($AlCl_3$) and other aluminium compounds are neurotoxic in both humans and animals, and give rise to neurodegenerative diseases [7] ; (Miu et al., 2004; Rondeau et al., 2009). In the light of this, it is imperative that research continues towards formulation and identification of supplement,

agents or drugs that can ameliorate the depilating effects that results from the exposure to Aluminum Chloride.

1.1 Statement of Problem

There is increasing demand worldwide for drugs that has neuroprotective effects against the toxicity that results from exposure to compounds such as Aluminum Chloride ($AlCl_3$) as well as the adverse effects of drugs. However, there are emerging evidences that supplements such as L-ascorbic acids could play important roles in the management of neurotoxicity [8]. While L-ascorbic acids have shown promise, scientific research are needed to ascertain its potential use as sole agents in the management neurotoxicity from Aluminum Chloride.

Vitamin C (ascorbic acid) is a potent antioxidant and a vital cofactor in several enzymatic reactions, which makes it a promising candidate for counteracting aluminum chloride-induced oxidative damage [9]. Vitamin C's ability to scavenge free radicals, reduce oxidative stress, and modulate inflammation presents a potential solution for mitigating aluminum chloride's neurotoxic effects. This research aims to investigate the efficacy of Vitamin C in protecting against aluminum chloride-induced brain damage by examining its impact on behavioral, biochemical, and histomorphological markers of brain integrity in a rodent model.

1.2 Review of Related Literature

The adverse effects of aluminum chloride ($AlCl_3$) on brain health have been well- documented in scientific literature, reflecting a growing concern over its neurotoxic impact. Numerous studies have explored the mechanisms underlying aluminum chloride-induced neurotoxicity, emphasizing its role in oxidative stress and cognitive impairment. Buraimoh [10] provided significant insights into the detrimental effects of $AlCl_3$, demonstrating that exposure to this compound in Wistar rats led to pronounced oxidative stress and cognitive deficits. This finding aligns with the broader body of research highlighting aluminum chloride's impact on brain function.

In a complementary study, Popovic et al. [11] further underscored the role of oxidative stress in aluminum chloride-induced neurotoxicity. Their research elucidated how aluminum chloride exposure leads to an imbalance in oxidative and antioxidative mechanisms, contributing to neuronal damage. The accumulation of reactive oxygen species (ROS) and subsequent lipid peroxidation are critical aspects of this neurotoxic process, suggesting that oxidative stress is a central pathway through which aluminum chloride exerts its harmful effects on the brain.

In response to these challenges, the neuroprotective potential of Vitamin C (ascorbic acid) has garnered considerable attention. Vitamin C is renowned for its antioxidant properties, which have been extensively studied in the context of neuroprotection. Research by Khassaf et al. [12] highlighted the ability of Vitamin C supplementation to restore antioxidant defenses and mitigate oxidative damage. Their study demonstrated that Vitamin C could counteract oxidative stress induced by various insults, including heavy metals like aluminum chloride. This restorative effect is attributed to Vitamin C's capacity to scavenge free radicals and enhance endogenous antioxidant systems.

The efficacy of Vitamin C extends beyond its antioxidant role. Al-Fahham and Ali [13,14] investigated the impact of Vitamin C on anxiety-related behaviors in animal models, revealing its potential therapeutic benefits in neurodegenerative conditions. Their findings indicated that Vitamin C supplementation could alleviate anxiety symptoms, suggesting that it may offer broader neuroprotective effects beyond just oxidative stress mitigation. This is particularly relevant given the behavioral disturbances often associated with neurotoxic exposures, including those caused by aluminum chloride.

Despite the promising evidence supporting Vitamin C's neuroprotective properties, there remains a significant gap in the literature regarding its comprehensive effects on a range of neurochemical and behavioral outcomes, particularly in the context of aluminum chloride exposure. Existing studies primarily focus on specific aspects of Vitamin C's action, such as its antioxidant capacity or its impact on anxiety. However, a more holistic examination of Vitamin C's effects on various neurochemical markers and behavioral changes is needed to fully understand its potentialtherapeutic benefits.

Moreover, while the neurotoxic effects of aluminum chloride and the protective role of Vitamin C have been individually studied, there is a lack of integrated research exploring their

combined impact. This includes assessing how Vitamin C can modulate oxidative stress, influence neurochemical alterations, and address behavioral impairments induced by aluminum chloride. Addressing these gaps could provide valuable insights into potential therapeutic strategies for mitigating aluminum chloride-induced neurotoxicity and improving overall brain health.

1.3 Scope and Justification

The scope of this research encompasses the examination of Vitamin C's protective effects against aluminum chloride-induced neurotoxicity in rodents. This includes assessing changes in body weight, behavioral parameters, oxidative stress markers, and histomorphological alterations in the hippocampus. The justification for this study lies in the increasing environmental and occupational exposure to aluminum chloride and the consequent need for effective interventions to safeguard brain health. By providing insights into Vitamin C's potential as a neuroprotective agent, this research aims to contribute to the development of strategies for mitigating aluminum chloride's adverse effects and improving public health outcomes.

1.4 Research Aim

This research aimed to evaluate the Effects of Vitamin C (Ascorbic acid) on Aluminium-Chloride- Induced Neurotoxicity on the Hippocampal cortex of Adult male Wistar rats.

1.5 Research Objectives

The specific objectives of the study were to;

I. Assess the effects of ascorbic acid on weekly change in body weight following aluminum Chlorideinduced toxicity.
II. investigate the effects of ascorbic acid on open field behavior and anxiety related behaviors;
III. assess the effects of ascorbic acid on oxidative stress and antioxidant markers (Malondialdehyde, Nitric oxide, Superoxide Dismutase, Glutathione, catalase, Glutamate Dehydrogenase, and Acetycholinesterase.
IV. evaluate the effects of ascorbic acid on inflammatory markers (Tumour Necrotic Factor Alpha and Interleukin-6).
V. evaluate the effects of ascorbic acid on the microanatomy of the hippocampal cortex in aluminum chloride-induced toxicity in Wistar rats.

1.6 Research Question

1. What is the effect of ascorbic acid on weekly changes in body weight following aluminum chloride-induced toxicity in Wistar rats?
2. How does ascorbic acid influence open field behavior and anxiety-related behaviors in aluminum chloride-induced neurotoxicity?
3. What are the effects of ascorbic acid on oxidative stress and antioxidant markers (such as malondialdehyde, nitric oxide, superoxide dismutase, glutathione, catalase, glutamate dehydrogenase, and acetylcholinesterase) in aluminum chloride-induced neurotoxicity?
4. What impact does ascorbic acid have on inflammatory markers (including Tumor Necrotic Factor Alpha and Interleukin-6) in the context of aluminum chloride-induced neurotoxicity?
5. What histological changes occur in the hippocampal cortex of Wistar rats exposed to aluminum chloride, and how does ascorbic acid influence these changes?

2. MATERIALS AND METHODS

2.1 Animals

Forty-two adult male Wistar rats, weighing between 120 g and 150 g, were sourced from Empire Farms in Osogbo, Osun State, Nigeria. The animals were housed in plastic cages within temperature-controlled quarters, maintained between 23°C and 26°C, with a light cycle of 12 hours (lights on at 7:00 a.m.) in the animal house of the Department of Anatomy, Ladoke Akintola University of Technology (LAUTECH), Ogbomoso. The animals were provided ad libitum access to food and water throughout the experimental period. The commercial standard rodent chow used

was obtained from TOP FEEDS Nigeria LTD. All rats were acclimatized for two weeks before the commencement of the study.

2.2 Materials

The materials used in the study included plastic cages, an electronic precision balance (Mettler Toledo), oral cannula, Petri dishes, cotton wool,

universal bottles, dissecting sets, slides, and cover slips. The cages were cleaned regularly to maintain a sterile environment for the animals.

2.3 Chemicals and Reagents

Ascorbic acid (Anhui Sinotech Industrial Co. Ltd) was procured from Sumther Pharmacy, Ogbomoso, and used as an antioxidant agent in this study. Aluminum chloride, assay kits for lipid peroxidation, nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), superoxide dismutase (SOD), interleukin-10 (IL-10), acetylcholinesterase (AChe), malondialdehyde (MDA), catalase (CAT), and glutamate dehydrogenase (GDH) were obtained from Idi-Araba chemicals Ogbomoso.

2.4 Diet

All animals were fed commercially available rodent chow (Top Feed, TOP Feed Ltd, Ibadan, Nigeria), which contained 11% fat and 58% carbohydrates. The diet was consistent for all experimental groups to ensure uniformity in nutrient intake throughout the study.

2.5 Experimental Design and Groupings

Forty-two rats weighing between 150 g and 200 g were randomly assigned to six groups (A–F), with seven animals per group. The experimental period spanned 30 days, during which aluminum chloride and Vitamin C (L-ascorbic acid) were administered orally by gavage for 28 days. The groupings and treatments were as follows:

Group A (Control): Administered normal saline (10 mL/kg).

Group B (Aluminum Chloride Control): Administered aluminum chloride (150 mg/kg).

Group C (L-Ascorbic Acid 500 mg/kg): Administered L-ascorbic acid (500 mg/kg).

Group D (L-Ascorbic Acid 1000 mg/kg): Administered L-ascorbic acid (1000 mg/kg).

Group E (Aluminum Chloride + L-Ascorbic Acid 500 mg/kg): Administered aluminum chloride (150mg/kg) and L-ascorbic acid (500 mg/kg).

Group F (Aluminum Chloride + L-Ascorbic Acid 1000 mg/kg): Administered aluminum chloride (150mg/kg) and L-ascorbic acid (1000 mg/kg).

Body weights of all animals were measured weekly using an electronic Mettler weighing balance (Mettler Toledo Type BD6000, Switzerland). Behavioral tests, including the open field and elevated plus maze, were conducted on days 14 and 28 of the experimental period. Following the final behavioral test, animals were fasted overnight, euthanized by cervical dislocation, and their brains were dissected and processed for histological and biochemical analyses.

2.6 Determination of Body Weight

Body weights were measured weekly using an electronic precision balance. The weight measurements helped monitor the animals' growth and responses to the treatments throughout theexperimental period.

2.7 Behavioral Analysis

Behavioral assessments were conducted using the open field and elevated plus maze tests, as previously documented by Ajibade et al. [15], Seibenhener and Wooten [16], and Onaolapo et al. (2017). Behavior was observed by two independent observers who were blind to the groupings.

2.7.1 Open field test

In the op field test, each rat was placed in the center of a rectangular arena (36 x 36 x 26 cm) made of white-painted wood. The floor was divided into 16 squares by permanent red marker lines. The behavioral parameters recorded during the 5-minute test period included:

Total horizontal locomotion: Number of squares crossed.
Rearing frequency: Number of times the rat stood on its hind legs.
Grooming frequency: Number of body-cleaning actions performed by the rat.

The test assesses anxiety and exploratory behavior, as rats typically prefer the edges of the arena (thigmotaxis). Increased time spent in the center of the arena was interpreted as reduced anxiety.

Fig. 1: The Open Field Setup

(Figure illustrating the open field apparatus used for the behavioral test)

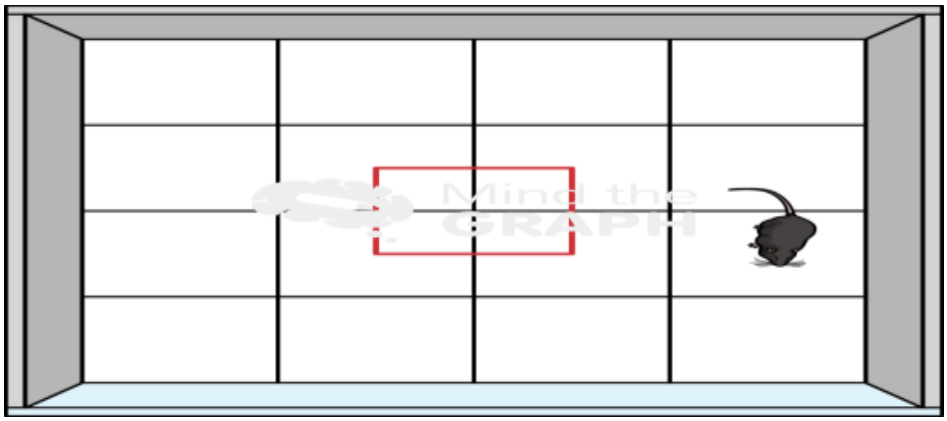

Fig. 1. The Open field (Appendix A)

2.7.2 Elevated plus maze

The elevated plus maze is a plus-shaped apparatus used to assess anxiety-related behavior in rodents. It consists of two open arms (25 x 5 x 0.5 cm) and two closed arms (25 x 5 x 16 cm), elevated above the floor. Rats were placed in the center of the maze, and the time spent in each arm was recorded. Anxiety was measured based on the percentage of time spent in the open armsversus the closed arms.

Fig. 2: The Elevated Plus Maze Setup

(Figure illustrating the elevated plus maze apparatus used for anxiety testing)

2.8 Biochemical Analysis

Following the behavioral tests, animals were euthanized by cervical dislocation, the hippocampus were excised. and homogenized in ice-cold phosphate-buffered saline using a Teflon-glass homogenizer. Supernatants were used for the analysis of all biochemical parameters.

2.8.1 Hippocampal homogenization

The hippocampus was dissected from each brain and homogenized in ice-cold phosphate-buffered saline using a Teflon-glass homogenizer. The homogenate was centrifuged at 5000 rpm at 4°C for 15 minutes, and the supernatant was used for the analysis of lipid peroxidation and antioxidant activity. **(Appendix B)**

2.8.2 Determination of Glutathione (GSH) Activity

Glutathione (GSH) level was assayed based on Ellman's reagent's (DTNB) reaction with free thiol groups. Sample were mixed with 0.4M Tris-HCL buffer (pH 8.9) and 0.01M DTNB following the manufacturer's instructions. GSH activity was determined by absorbance at 412nm, and expressed as nM (nanomoles) of GSH [17].

2.8.3 Determination of lipid peroxidation (Malondialdehyde, MDA). (Appendix B)

Lipid peroxidation was determined using a thiobarbituric acid reactive substances (TBARS) assay to measure malondialdehyde (MDA) levels, which indicate lipid peroxidation. Levels of malondialdehyde (MDA) a marker of lipid peroxidation was assayed from plasma by measuring thiobarbituric reactive species. Reactive substances of the thiobarbituric acid react with thiobarbituric acid producing a red coloured complex which is measured at an absorbance of 532nm [18].

2.8.4 Determination of Superoxide Dismutase (SOD) Activity. (Appendix B)

The principle for determining superoxide dismutase (SOD) activity is based on the ability of SOD to inhibit the auto-oxidation of adrenaline (epinephrine) at an alkaline pH. Superoxide radicals (O_2-) are generated through the auto-oxidation of adrenaline at pH 10.2 [19]. SOD catalyzes the dismutation of O_2- into oxygen (O_2) and hydrogen peroxide (H_2O_2). The degree of inhibition of adrenaline auto-oxidation by SOD is measured spectrophotometrically at 480 nm [19]. A higher inhibition of adrenaline auto-oxidation indicates higher SOD activity. The SOD activity is expressed as the amount of enzyme required to inhibit the auto- oxidation of adrenaline by 50%, which is defined as 1 unit of SOD activity [19]

Fig. 2. The Elevated Plus maze (Appendix A)

2.8.5 Determination of Acetylcholinesterase (AChE) Activity. (Appendix B)

Brain acetylcholinesterase activity was measured by monitoring the increase in light emission produced by the accumulation of choline or by determining the amount of choline generated after a short interval using a colorimetric method. The assay is rapid and sensitive, and uses the natural substrate of the enzyme (Birman, 1985); [20].

2.8.6 Determination of Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6). (Appendix B)

Tumour necrosis factor-α and interleukin IL-6 were measured using enzyme-linked immunosorbent assay (ELISA) techniques with commercially available kits (Enzo Life Sciences Inc. NY, USA) designed to measure the 'total' (bound and unbound) amount of the respective cytokines, and the results were expressed as pg/mL of serum. Method used was as described by Olofinnade et al., [21].

2.9 Histological Procedure. (Appendix C)

The brains were dissected, and the hippocampal regions were excised and fixed in 10% formol-saline for 24 hours. The tissues were dehydrated through graded alcohol concentrations (70%, 80%, 90%, and absolute alcohol), cleared using xylene, and embedded in paraffin wax. Sections were cut using a rotary microtome at 5 µm thickness, and the slides were stained with hematoxylin and eosin (H&E) and cresyl violet for histological analysis.

2.9.1 Dehydration

The tissues were passed through ascending concentrations of alcohol to remove water, beginning with 70% ethanol and progressing to absolute alcohol [22].

2.9.2 Clearing

Tissue sections were cleared using xylene to ensure the removal of alcohol residues before paraffin embedding by the method described by Aziz and Zeman-Pocrnich, [22].

2.9.3 Embedding and Sectioning

After clearing, the tissues were infiltrated with paraffin wax, embedded, and sectioned at 5 µm thickness using a rotary microtome. The sections were stained with hematoxylin and eosin (H&E) for general morphology and cresyl violet for neuronal integrity. [22].

2.9.4 Microscopy (Appendix C)

The slides were examined under a light microscope (Olympus BX51), and photomicrographs were taken using an attached digital camera (Moticam, Motic China Group Co., Ltd).

2.10 Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Differences between groups were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Induced Toxicity in Wistar Rats

The results of this study, as illustrated in Fig. 3, and Fig. 2 reveal the effect of vitamin C on the weekly changes in body weight of Wistar rats exposed to aluminum chloride ($AlCl_3$). There was a significant reduction in body weight across all groups exposed to $AlCl_3$ when compared to the control group ($p < 0.001$). Interestingly, rats that received ascorbic acid treatment (500 mg/kg and 1000 mg/kg) in combination with $AlCl_3$ showed a significant increase in body weight compared to the $AlCl_3$-only group, though the increase was still below the levels observed in the control group. This suggests that ascorbic acid partially mitigates the weight loss caused by $AlCl_3$ toxicity. The dose-dependent effect of ascorbic acid, where the 1000 mg/kg group showed a more pronounced weight gain in week four compared to the 500 mg/kg group, indicates that higher doses of ascorbic acid may offer better protective effects.

$AlCl_3$ exposure is known to induce oxidative stress and neurotoxicity, which likely contributes to reduced body weight. The observed weight loss in $AlCl_3$-treated rats in weeks two and four can be attributed to toxicity-induced anorexia, poor nutrient absorption, and metabolic disturbances. The protective role of ascorbic acid in attenuating weight loss is likely due to its antioxidant properties, which help in scavenging free radicals and reducing oxidative stress. Ascorbic acid's ability to preserve body weight suggests its potential as a protective agent against$AlCl_3$-induced metabolic dysfunctions.

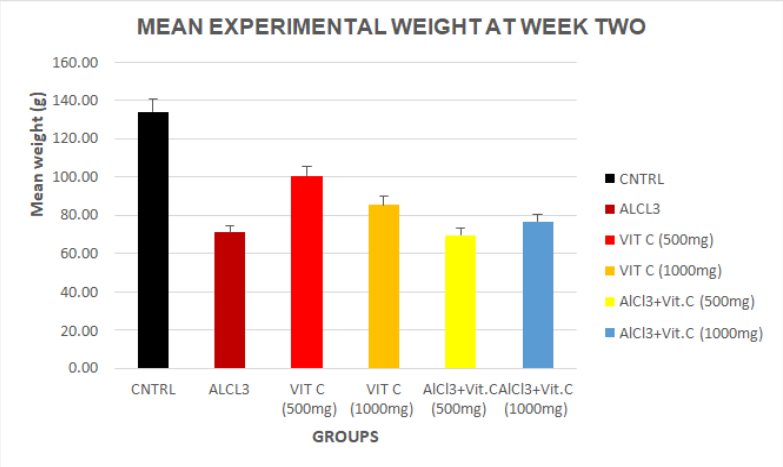

Fig. 3. Effect of Vitamin C on weekly body weight in Aluminium Chloride ($AlCl_3$) treated rats (week two). Each bar represents Mean ± S.E.M. (Appendix A)

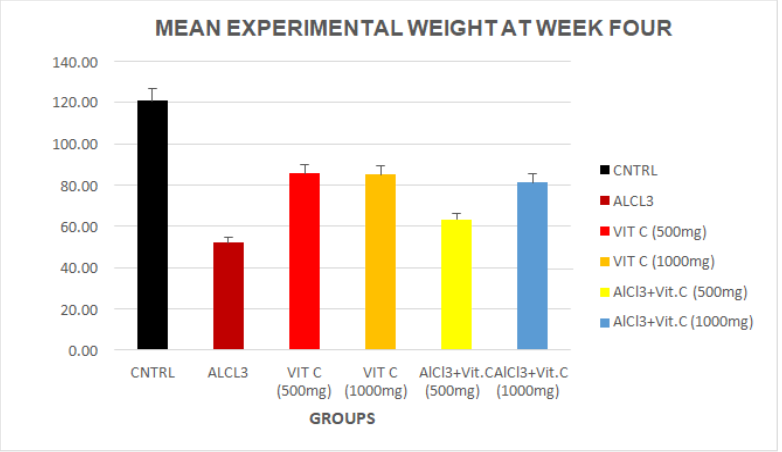

Fig. 4. Effect of Vitamin C on weekly body weight in Aluminium Chloride ($AlCl_3$) treated rats (week four). Each bar represents Mean ± S.E.M. (Appendix A)

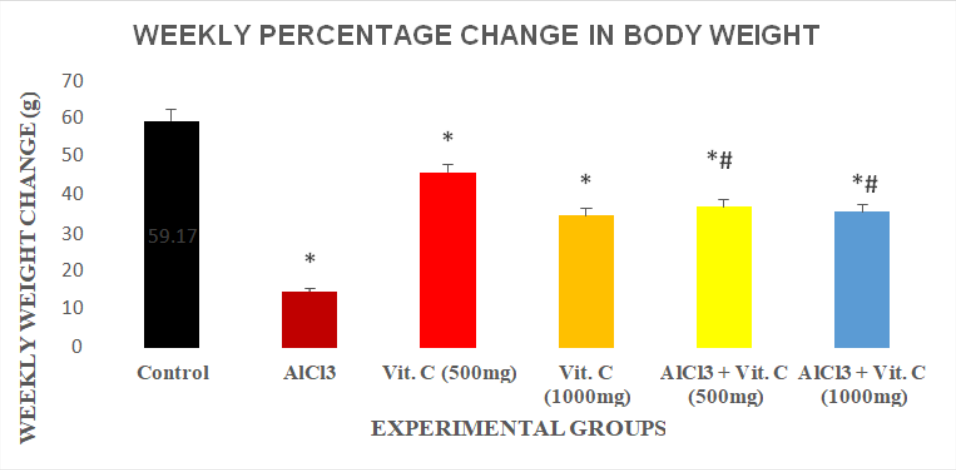

Fig. 5. Effect of Vitamin C on weekly percentage change in body weight in AluminiumChloride ($AlCl_3$) treated rats. Each bar represents Mean ± S.E.M. (Appendix A)

3.2 Neurobehavioral Indices

3.2.1 Grooming Activity

As shown in Fig. 4, grooming behavior exhibited notable changes over the experimental period. In the second week, $AlCl_3$ exposure significantly increased grooming activity in the rats, which is an indicator of anxiety-like behavior. However, rats treated with ascorbic acid (500 mg/kg and 1000 mg/kg) showed a significant reduction in grooming compared to the $AlCl_3$ group, suggesting that ascorbic acid reduces anxiety-related grooming behavior. By the fourth week, grooming activity decreased in all groups, including those treated with $AlCl_3$ and ascorbic acid.

The initial increase in grooming activity following $AlCl_3$ exposure aligns with established findings that neurotoxicity can heighten anxiety-like behaviors in rodents. The reduction in grooming in ascorbic acid-treated groups suggests an anxiolytic effect, likely linked to its ability to counteract oxidative stress and neuroinflammation induced by $AlCl_3$. The overall decline in grooming in the fourth week may reflect the body's adaptation to the experimental conditions or a cumulative effect of ascorbic acid's neuroprotective properties.

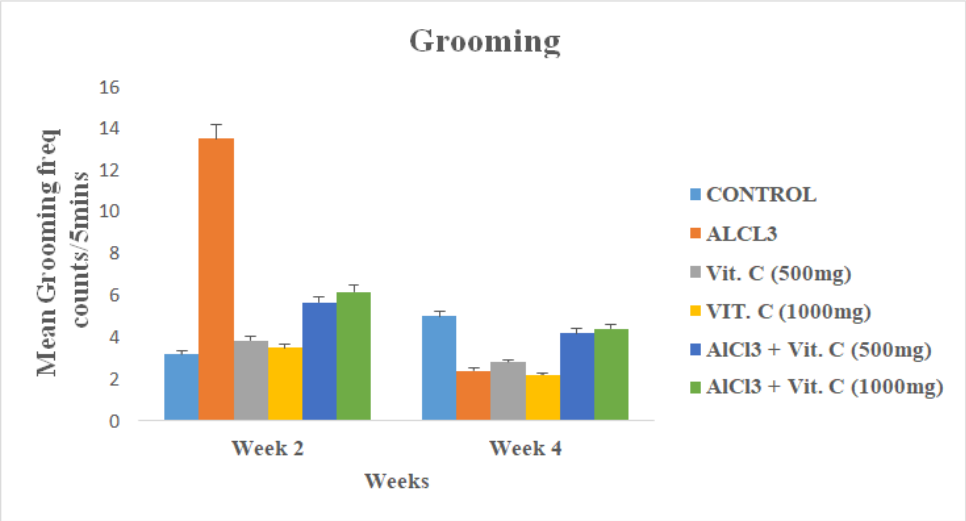

Fig. 6. Effect of Vitamin C on grooming activities in $AlCl_3$ and vitamin C treated rats.Each bar represents Mean ± S.E.M. (Appendix A)

3.2.2 Line Crossing Activity

As seen in Fig. 7, line crossing activity, a measure of horizontal locomotion, increased across all groups in the second week. However, by the fourth week, $AlCl_3$ exposure significantly reduced line crossing activity. In contrast, the ascorbic acid-treated groups (500 mg/kg and 1000 mg/kg) showed improved locomotion compared to the $AlCl_3$-only group, suggesting a reversal of $AlCl_3$-induced motor deficits. $AlCl_3$-induced neurotoxicity is known to impair motor function, as seen in the reduction of line crossing activity. The ability of ascorbic acid to improve locomotion further supports its neuroprotective effect, possibly through modulation of oxidative stress pathways and the restoration of neurotransmitter balance.

3.2.3 Rearing Activity

Fig. 8 illustrates the effect of $AlCl_3$ and ascorbic acid on rearing, a measure of vertical locomotion. Rearing activity decreased significantly in the $AlCl_3$ group during the second week but improved in the Vitamin C-treated groups, particularly at higher doses. By the fourth week, a similar trend was observed, with ascorbic acid reversing the $AlCl_3$-induced suppression of vertical locomotion. Rearing behavior reflects exploratory activity and motor function, both of which are impaired by neurotoxicity. The ability of vitamin c to enhance rearing in $AlCl_3$-treated rats reinforces its potential role in mitigating $AlCl_3$-induced behavioral deficits. Rearing behavior reflects exploratory activity and motor function, both of which are impaired by neurotoxicity. The ability of ascorbic acid to enhance rearing in $AlCl_3$-treated rats reinforces its potential role in mitigating $AlCl_3$-induced behavioral deficits.

3.2.4 Time Spent in Open and Closed Arms (Elevated Plus Maze)

The elevated plus maze test, as shown in Fig. 9 and 10, measured anxiety-related behaviors by analyzing time spent in open and closed arms. In the $AlCl_3$ group, rats spent less time in the open arms, indicating heightened anxiety. However, ascorbic acid treatment, especially at 1000 mg/kg, increased the time spent in the open arms while reducing time in the closed arms, suggesting an anxiolytic effect.

The elevated plus maze is a widely used test for assessing anxiety-like behaviors in rodents. The reduced open arm time in $AlCl_3$-treated rats is indicative of anxiety, which may stem from neuroinflammation and oxidative stress. Ascorbic acid's ability to increase open arm time supports its anxiolytic potential, possibly through the reduction of neuroinflammation and protection of the hippocampus from $AlCl_3$-induced damage.

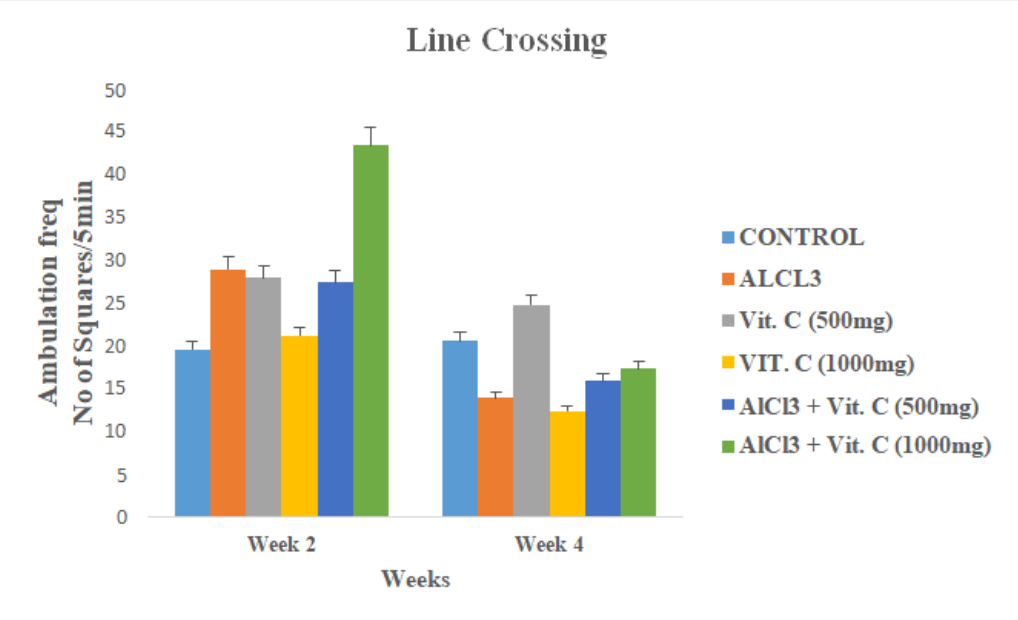

Fig. 7. Effect of Vitamin C on Line crossing activities in AlCl3 and vitamin C treated rats. (Appendix A)

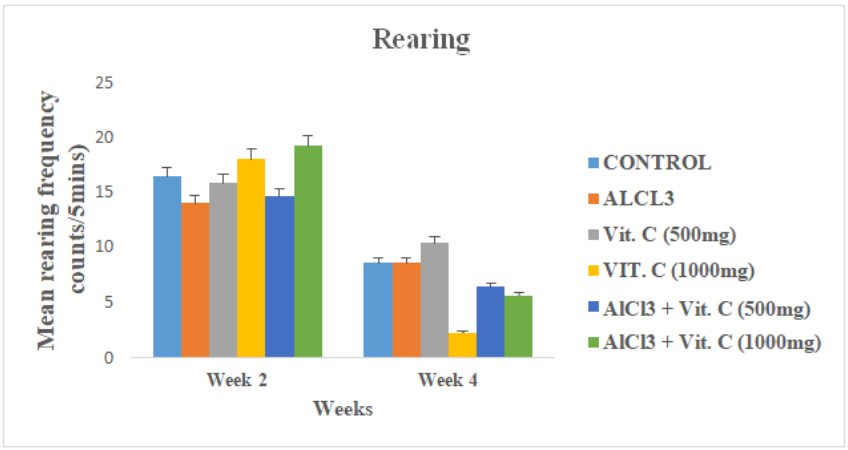

Fig. 8. Effect of Vitamin C on rearing activities in $AlCl_3$ and vitamin C treated rats. (Appendix A)

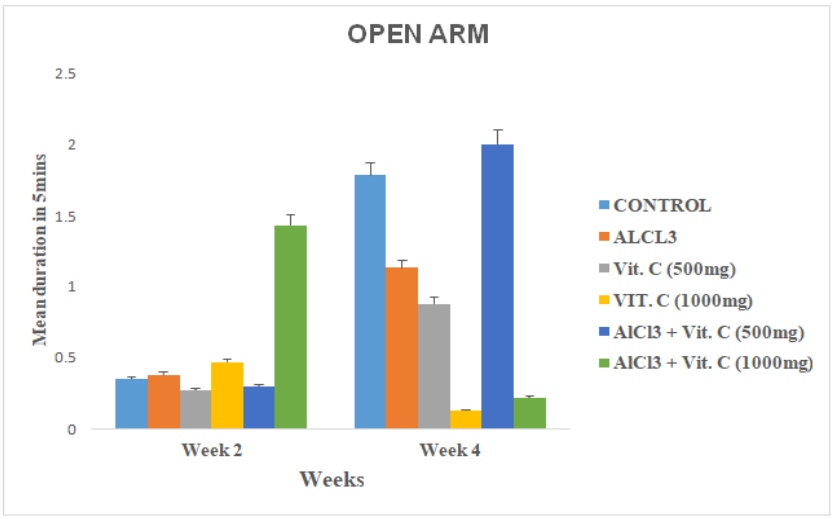

Fig. 9. Effect of vitamin C on Open arm time and Closed arm time in $AlCl_3$ treated rats.Each bar represents Mean ± S.E.M. (Appendix A)

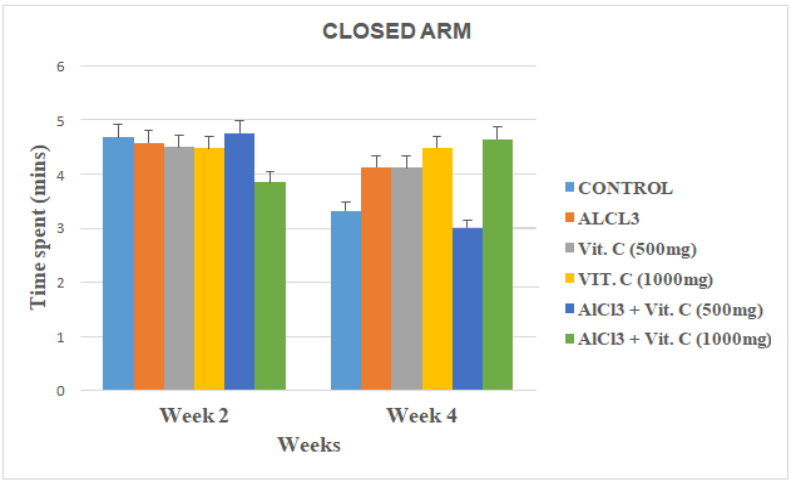

Fig. 10. Effect of vitamin C on time spent in the closed arm time in $AlCl_3$ treated at theend of. (Appendix A) the experimental period week (4) in $AlCl_3$ treated rats. Each bar represents Mean ±S.E.M

3.3 Oxidative Stress and Antioxidant Markers

3.3.1 Malondialdehyde (MDA) and Nitric Oxide (NO) Levels

As shown in Table 1, MDA and NO levels were significantly elevated in $AlCl_3$-treated rats, indicating increased oxidative stress. Treatment with ascorbic acid (500 mg/kg and 1000 mg/kg) significantly reduced both MDA and NO levels, restoring them to near-control levels.

3.3.2 Superoxide Dismutase (SOD), Catalase (CAT), Glutathione (GSH), and Acetylcholinesterase (AChE)

AlCl3 exposure significantly reduced the activity of antioxidant enzymes (SOD, CAT, GSH) and increased acetylcholinesterase (AChE) levels. However, ascorbic acid treatment restored antioxidant enzyme levels and reduced AChE activity, as shown in Table 1.

The suppression of antioxidant enzymes in the $AlCl_3$ group underscores the oxidative imbalance caused by neurotoxicity. Ascorbic acid's ability to restore these enzymes suggests its role in maintaining redox homeostasis. Additionally, the reduction in AChE activity points to ascorbic acid's neuroprotective effects, possibly through the prevention of cholinergic dysfunctionassociated with $AlCl_3$ toxicity.

3.4 Inflammatory Markers: Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6)

Table 2 shows that AlCl3 exposure significantly increased pro-inflammatory cytokines TNF-α and IL-6, while ascorbic acid treatment significantly reduced these inflammatory markers. The elevation of TNF-α and IL-6 in $AlCl_3$-treated rats is indicative of neuroinflammation, a hallmark of $AlCl_3$-induced toxicity. Ascorbic acid's ability to reduce these cytokines highlights its anti-inflammatory potential, likely contributing to the observed neuroprotective effects. This reduction in inflammation may be critical in preventing neuronal damage and behavioral impairments.

3.5 Histological Changes in the Hippocampal Cortex

Histological analysis revealed that $AlCl_3$ caused substantial damage to the hippocampal cortex, including neuronal loss and structural degeneration. Vitamin C treatment, particularly at higher doses, preserved hippocampal integrity and prevented AlCl3-induced neuronal damage.

The hippocampus is highly susceptible to oxidative stress and inflammation, both of which are induced by $AlCl_3$. Ascorbic acid's protective effect on hippocampal morphology is likely due to its antioxidant and anti-inflammatory actions, preventing the cascade of neuronal damage associatedwith $AlCl_3$ neurotoxicity.

4. DISCUSSION

This section provides a comprehensive interpretation of the data obtained in relation to the research questions, examining the effects of Vitamin C on brain behavior, biochemical markers, histomorphological integrity, oxidant–antioxidant status, and inflammation in healthy rats administered with aluminum chloride ($AlCl_3$). The data interpretation follows each of the research questions and aligns with the stated discussion guidelines.

The results from the current study show that administration of $AlCl_3$ leads to a significant decrease in body weight, as confirmed in Fig. 10. This finding is consistent with Buraimoh [23], who reported that exposure to $AlCl_3$ induces oxidative stress, which can negatively affect metabolic processes and body weight. In contrast, the groups co-administered Vitamin C (500 mg/kg and 1000 mg/kg) showed a reversal of the weight loss, suggesting that Vitamin C's antioxidant properties mitigate the harmful effects of oxidative stress. The reversal of weight loss in the Vitamin C groups can be attributed to the vitamin's ability to reduce free radical damage, thereby promoting cellular recovery and metabolic stabilization.

In terms of behavioral changes, the study revealed excitatory effects on locomotor and exploratory activities in the $AlCl_3$ -administered groups, particularly at the fourth week. This observation suggests that prolonged exposure to $AlCl_3$ may induce hyperactivity or anxiety-like behavior. However, in the groups administered Vitamin C, a reduction in these excitatory effects was observed, with behavior returning closer to that of the control group. This aligns with earlier findings by Al-Fahham and Ali [13,14], who demonstrated the anxiolytic effects of Vitamin C supplementation. These results suggest that Vitamin C can attenuate the neurobehavioral changes induced by $AlCl_3$, particularly anxiety-like behavior, through its neuroprotective role in mitigating oxidative stress and restoring neuronal function.

Table 1. Showing The Effect of Vitamin C on Levels of Malondialdehyde (MdA), SuperoxideDismutase(SOD), Catalase (CAT), Glutamate Dehydrogenase (GDH), Glutathione (GSH), Acetylcholinesterase (AcHe) And Nitric Oxide(NO) Activity in AlCl3-Treated Rats

Groups	MDA (nmol/g)	SOD (U/mg/protein)	CAT (µM/mg)	GDH (µmol/minL)	GSH (pg/mg/protein)	ACHe (nmol/mg)	NO (ng/mg)
Control	21.16 ± 0.24	35.05±1.06	16.98 ±0.43	4.48 ± 0.42	1.12 ± 0.02	0.03 ±0.00	9.93±0.09
AlCl3	36.07 ± 1.26*	9.45±0.30*	7.34±0.93*	1.61 ±0.00*	0.44 ± 0.03*	0.09 ±0.01	19.02±0.76*
Vit. C(500mg)	16.66 ± 0.59*	43.49±2.06*#	32.17±1.00*	5.15± 0.11*	1.11 ± 0.37*	0.03 ±0.00	7.61±0.28*
Vit. C(1000mg)	16.29 ± 1.28*	48.48 ±1.50*#	39.03±0.93*#	6.49 ±0.62*#	1.38 ± 0.03*#	0.021 ±0.00*#	6.48±0. 17*#
AlCl3 + Vit. C (500mg	30.07± 1.60*#	17.29 ±1.22*#	12.96±0.23*#	2.94±0.13*#	0.61±0.30*	0.04 ±0.00	14.92±0.32*#
AlCl3 +Vit. C (1000mg)	23.87± 0.26&	23.23 ±0.72*&	17.23±1.06*&	3.45±0.62*&	0.71±0.31*&	0.44 ±0.00	13.71 ±0.35*&

Table 2. Shows The Effect of Vitamin C on Levels of Inflammatory Cytokines in Alcl3-Treated Rats

Groups	Interleukin 6 (pg/ml)	TNF-α(pg/ml)
Control	97.86±8.50*	27.79 ± 0.89*
AlCl3	264.26±3.80*	51.56 ± 2.86*
Vit. C (500mg)	54.01 ± 2.59*	36.89 ± 0.85*
Vit. C (1000mg)	60.16 ± 13.10*#	29.76 ± 2.53*#
AlCl3 + Vit. C (500mg)	249.75 ± 1.18*#	39.38 ± 0.60*#
AlCl3 +Vit. C (1000mg)	234.42 ± 1.46	35.32±1.66

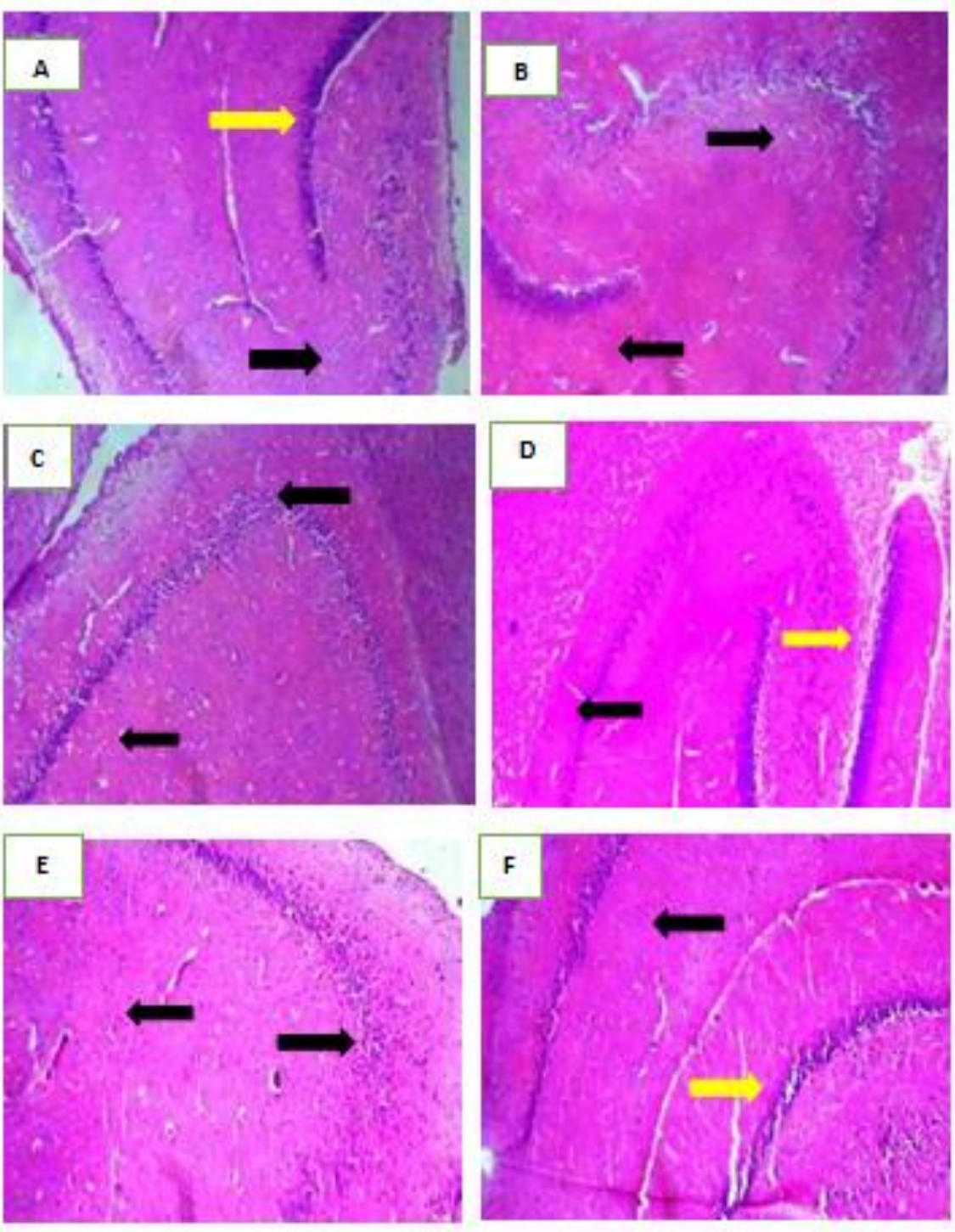

Plate 1. Photomicrograph of Hippocampus stained sections by hematoxylin and eosin (H &E) slides revealed distinct layers of the hippocampus. Cornu Ammonis including CA1, and CA2, (black arrow) dentate gyrus (yellow arrow), with presence ofnumerous granule cells, pyramidal cells and neuroglia. (Mag X100) (Appendix D)

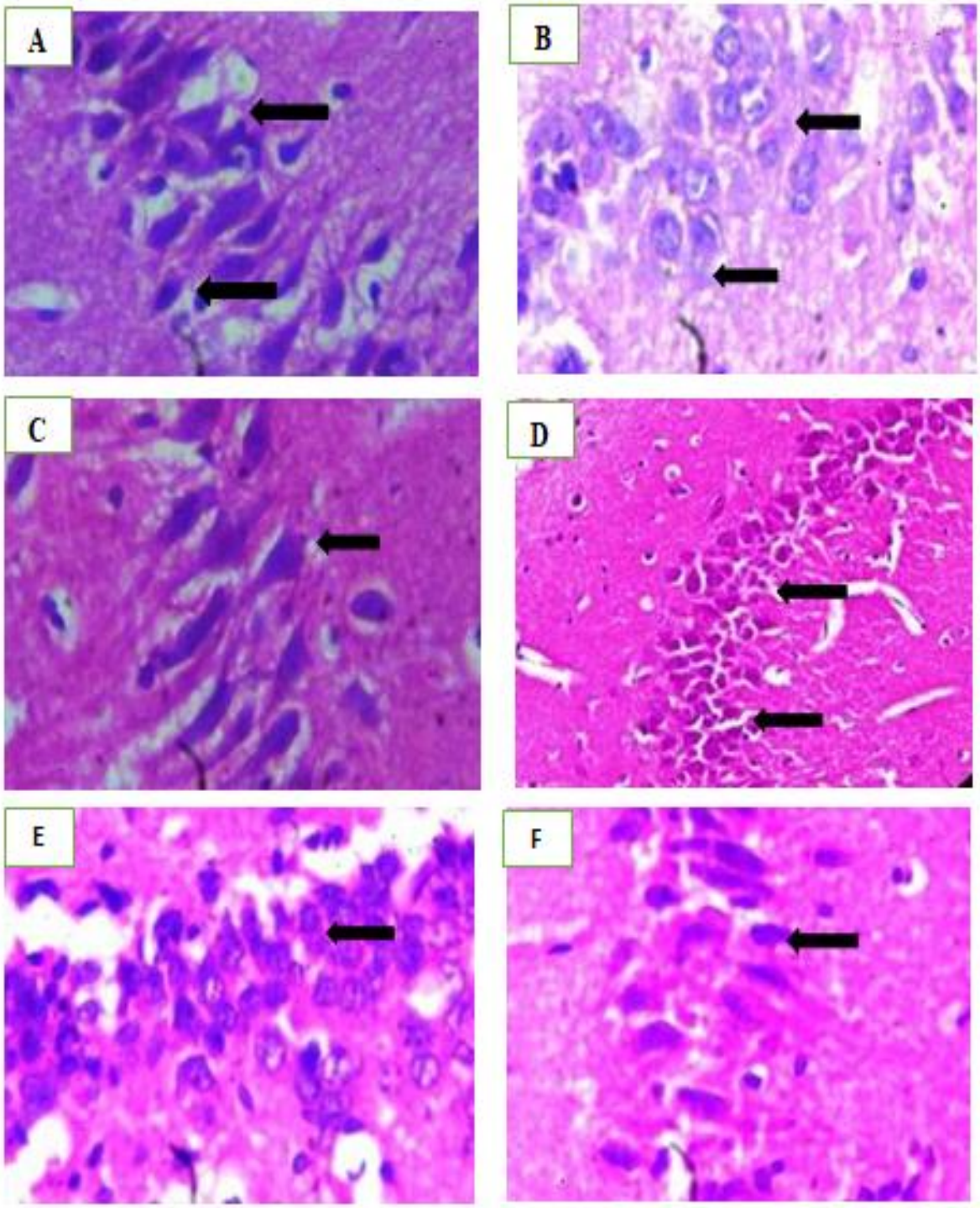

Plate 2. Photomicrograph of Hippocampus stained sections by hematoxylin and eosin (H &E) stained slides revealed distinct layers of the hippocampus with presenceof numerous pyramidal cells (black arrows) and neuroglia in rats administered aluminium chloride, and vit.c. Multipolar shaped pyramidal cells with large, rounded, vesicular nucleus is seen scattered throughout the neuropil; granular neurons with large open-faced nuclei, prominent nucleoli and scantcytoplasm are also observed. (Mag X400) (Appendix D)

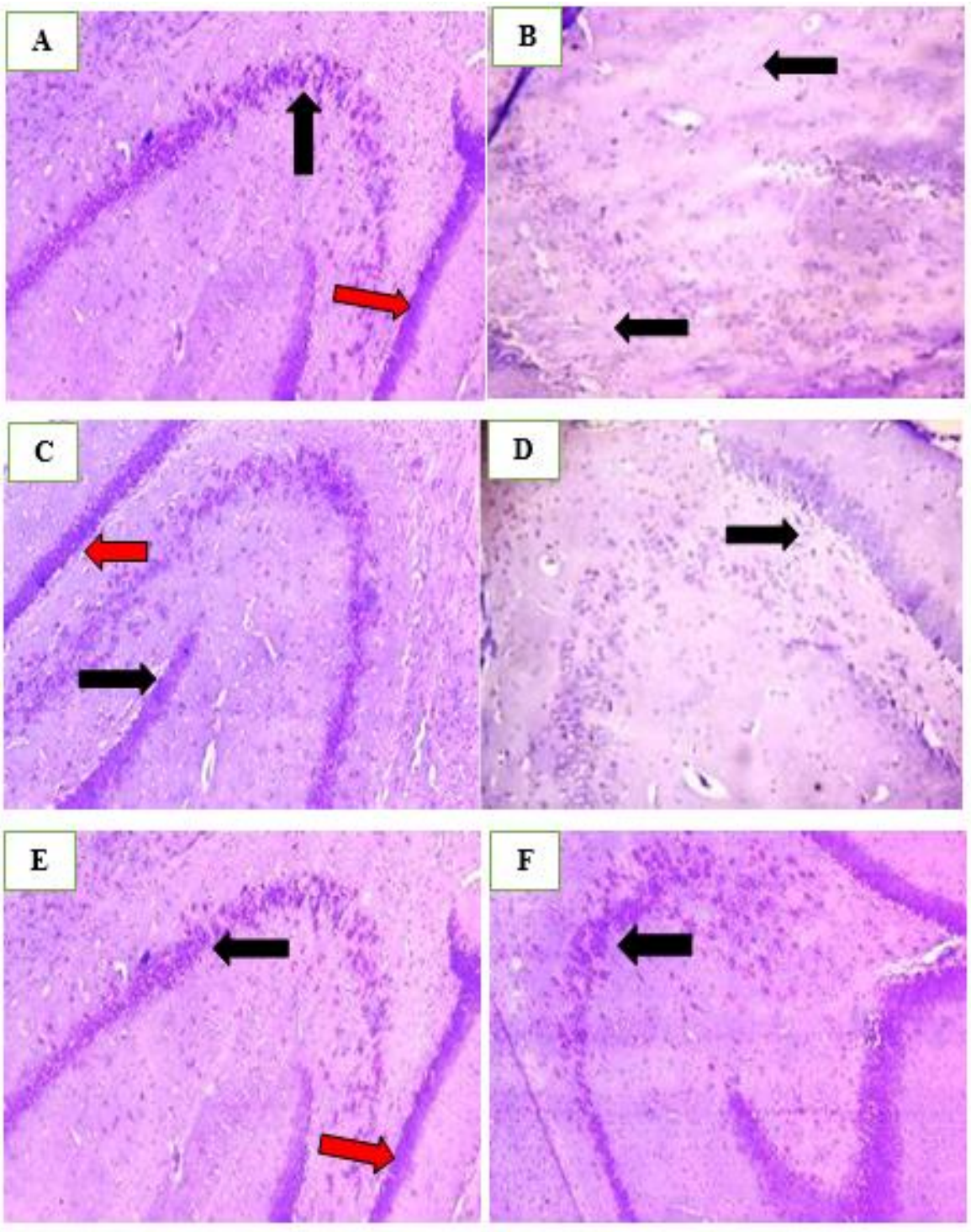

Plate 3. Photomicrograph of Hippocampus stained sections by cresyl fast violet. Thepink-staining background which is the neuropil is better appreciated in the cresyl fast violet-stained slides. the layers of the Hippocampus; the cornu ammoris (CA1 & CA2;black arrows), the dentate gyrus (Red arrows). (Mag X100) (Appendix D)

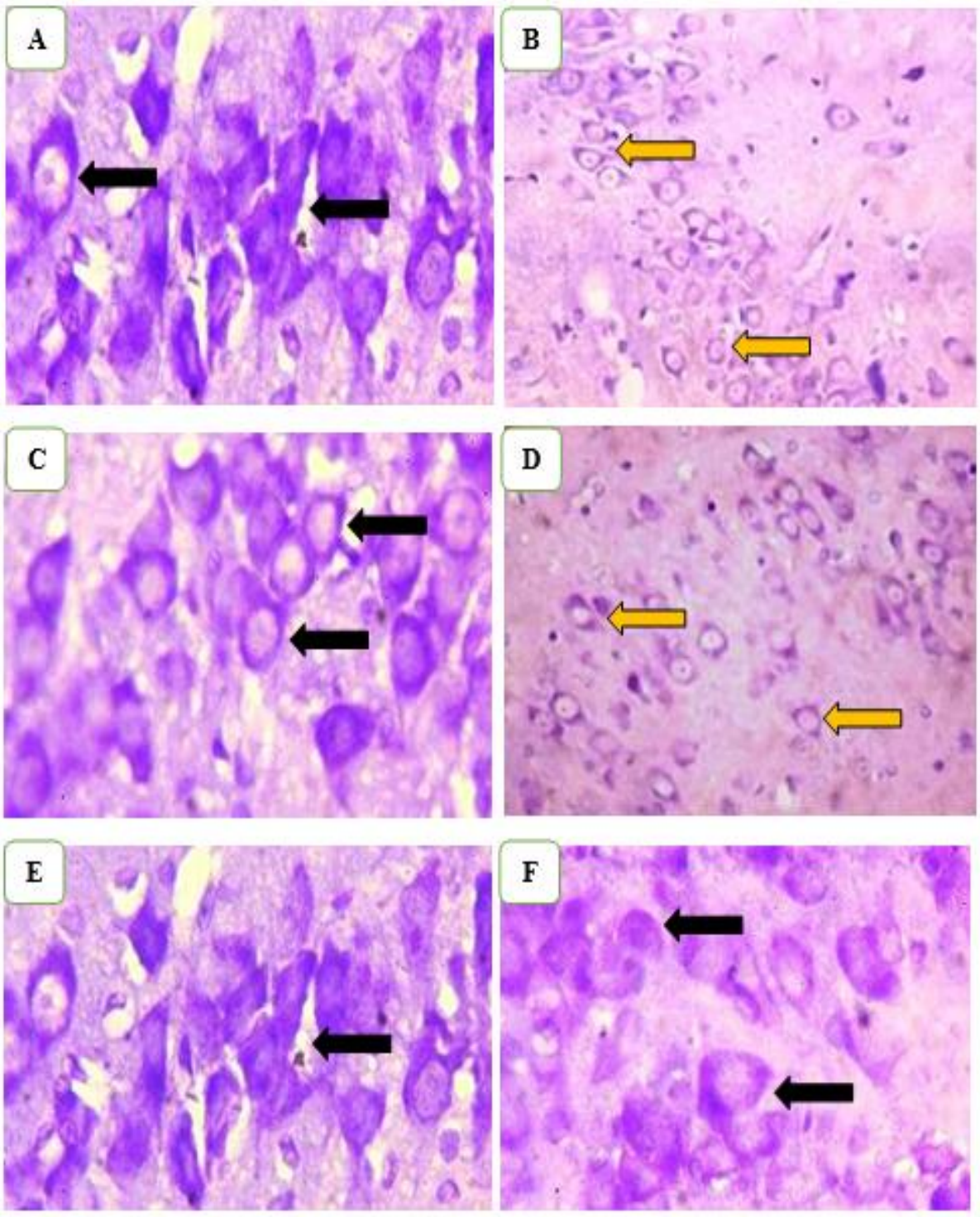

Plate 4. Photomicrograph of Hippocampus stained sections by cresyl fast violet. Thepink-staining background which is the neuropil is better appreciated in the cresyl fast violet stained slides (black arrows). In the group administered AlCl3 numerous degenerating pyramidal and granule cells are observed, degenerating neurons are pale-staining with shrunken pale staining nuclei, these features are in keeping with neuronal injury (yellow arrow). In groups treated with Vitamin C (4.4 e-f) was observeda protection against $AlCl_3$-induced neuronal injury. (Mag X400). (Appendix D)

The study showed a significant increase in pro-inflammatory cytokines, TNF-α, and IL-6, in the $AlCl_3$ -administered groups. This is consistent with the known pro-inflammatory effects of $AlCl_3$, which can induce oxidative stress and stimulate the release of cytokines that contribute to systemic inflammation [24]. TNF-α, in particular, plays a crucial role in mediating inflammatory responses, and its elevated levels in $AlCl_3$ -treated groups suggest the potential for tissue damage and inflammation in the brain.

However, co-administration of Vitamin C significantly reduced the levels of TNF-α and IL-6 in the treatment groups, indicating its anti-inflammatory effects. Vitamin C's ability to inhibit the production of these cytokines aligns with previous studies showing its role in modulating immune responses and reducing inflammation [25]. The reduction in TNF-α and IL-6 levels suggests that Vitamin C can suppress inflammatory processes, likely through its antioxidant action, which reduces oxidative stress and limits the activation of pro-inflammatory pathways. This highlights Vitamin C's potential therapeutic role in alleviating $AlCl_3$ -induced inflammation, particularly in brain tissues.

$AlCl_3$ exposure significantly increased oxidative stress in rats, as indicated by elevated levels of MDA, a key marker of lipid peroxidation. This finding is in agreement with previous studies, such as Popovic et al. [11], which demonstrated that exposure to metals like $AlCl_3$ can enhance free radical generation and increase MDA levels. The elevated MDA levels reflect damage to cell membranes and other structures due to excessive oxidative stress, which can lead to cellular dysfunction.

Conversely, administration of Vitamin C at both 500 mg/kg and 1000 mg/kg doses reduced MDA levels in the treatment groups, indicating its capacity to neutralize free radicals and limit lipid peroxidation. This suggests that Vitamin C can effectively mitigate oxidative damage, primarily through its role as an antioxidant that scavenges reactive oxygen species (ROS).

The activity of the antioxidant enzymes SOD and GSH was significantly reduced in the $AlCl_3$ groups, highlighting the overwhelming oxidative stress induced by the metal. These enzymes play critical roles in defending against oxidative damage, and their depletion indicates a compromised antioxidant defense system in $AlCl_3$ -treated animals. However, in groups treated with Vitamin C, both SOD and GSH levels were restored, consistent with findings by Khassaf et al. [12] and Vodošek et al. [26]. Vitamin C's ability to regenerate GSH and support the activity of SOD suggests a potent mechanism by which it restores redox balance and enhances the body's ability to counteract oxidative stress.

The study examined the effects of $AlCl_3$ on the histomorphological integrity of the brain, particularly the hippocampus, a region crucial for memory and learning. The results showed significant damage to the hippocampal architecture in $AlCl_3$ -administered rats, consistent with reports of neurotoxic effects of aluminum compounds on brain tissues [27]. However, Vitamin C treatment reversed these histomorphological changes, with the hippocampal structure in Vitamin C-treated rats appearing closer to normal, as observed in the control group. This suggests that Vitamin C can protect against $AlCl_3$ -induced neurotoxicity, potentially through its antioxidant and anti-inflammatory properties, which reduce oxidative damage and inflammation in brain tissues.

In terms of neurochemical markers, the study showed an increase in Acetylcholinesterase (AChE) activity in the $AlCl_3$ -treated groups, indicating enhanced degradation of acetylcholine, a neurotransmitter involved in cognitive functions. This aligns with findings by Al-Otaibi et al. [28], who reported that $AlCl_3$ exposure can impair cholinergic transmission by increasing AChE activity. Interestingly, Vitamin C co-administration also increased AChE activity, particularly at the 1000 mg/kg dose, suggesting a complex interaction between Vitamin C and $AlCl_3$. This observation could indicate that while Vitamin C protects against oxidative stress, its effects on cholinergic transmission may be dose-dependent and require further investigation [29-31].

Additionally, the study observed increased nitric oxide (NO) levels in both $AlCl_3$ and Vitamin C groups, which contrasts with the expected deleterious effects of $AlCl_3$. This finding suggests that, under certain conditions, $AlCl_3$ may have beneficial effects related to NO signaling, such as promoting blood vessel dilation and improving cerebral blood flow. Vitamin C's effects on NO levels further support its potential benefits in maintaining vascular function, though the mechanisms require further exploration.

5. CONCLUSION

The discussion highlights the protective effects of Vitamin C against $AlCl_3$ -induced changes in body weight, behavior, inflammation, oxidative stress, and brain integrity. Vitamin C's role in mitigating oxidative damage, reducing inflammation, and preserving histomorphological structure suggests its potential as a therapeutic supplement for preventing or reversing $AlCl_3$ -induced neurotoxicity. The findings provide new insights into the interaction between $AlCl_3$ and Vitamin C, particularly in their effects on neurochemical markers, and underscore the importance of further studies to elucidate the optimal dosage and mechanisms involved.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

I hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

CONSENT

It's not applicable.

ETHICAL APPROVAL

The experimental protocol was approved by the Ethical Research Committee of the Faculty of Basic Medical Sciences, LAUTECH (approval number ERCFBMSLAUTECH:019/01/2024). All procedures adhered to institutional guidelines for animal care and use, and experimental procedures followed internationally recognized standards for animal welfare.

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COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript. If any such competing interests existed, they have been disclosed as required by journal policy.

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Appendix A: Figures and Setup Illustrations

Figure 1: The Open Field Setup
Figure 2: The Elevated Plus Maze Setup
Figure 3 Graph showing mean weight in week two Figure 4 Graph showing mean weight in week four.
Figure 5 Graph showing percentage change in body weight Figure 6 Graph showing mean Grooming (anxiety) activity
Figure 7 Graph showing mean line crossing (locomotive) activity Figure 8 Graph showing mean rearing (anxiety) activity
Figure 9 Graph showing mean open arm (anxiety) activity Figure 10 Graph showing closed arm activity

Appendix B: Biochemical Analysis Procedures

B.1 Hippocampal Homogenization
B.2 Determination of Glutathione (GSH) Activity
B.3 Determination of Lipid Peroxidation (MDA Levels)
B.4 Determination of Superoxide Dismutase (SOD) Activity
B.5 Determination of Acetylcholinesterase (AChE) Activity
B.6 Determination of Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6)

Appendix C: Histological Procedures

C.1 Tissue Processing for Histology
C.2 Microscopy

Appendix D: Histological Analysis and Photomicrograph Descriptions

Plate 1: A photomicrograph of the hippocampus showing sections stained with hematoxylin and eosin (H&E). (Mag X100)

Plate 2: A photomicrograph of the hippocampus stained with H&E, showing distinct hippocampal layers in rats administered with a $AlCl_3$, and Vit C. (Mag X400)

Plate 3: A photomicrograph of the hippocampus stained with cresyl fast violet. (Mag X100)

Plate 4: A photomicrograph of the hippocampus stained with cresyl fast violet. (Mag X400)

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