APOPTOTIC CHANGES USING BCL-2 ON ALUMINIUM CHLORIDE INDUCED NEUROTOXICITY TREATED WITH ETHANOLIC EXTRACT OF *CARPOLOBIA LUTEA* AT DIFFERENT DOSES

ABSTRACT

Background: *Carpolobia lutea*is a small tree native to West and Central tropical Africa and it is one of the medicinal plants that play a major role in the health care system of developing countries. It belongs to the plant family polygalaceae. Aluminium is a well-established neurotoxicant involved in the etiology of neurodegenerative diseases.

Aim of the Study: This study was aimed at investigating apoptotic changes using Bcl-2 on aluminium chloride induced hippocampal and striatal damages treated with ethanolic extract of *C. lutea* at different doses.

Materials and Methods: Thirty sixwistar rats weighing 180-200g were used for this study. The animals were randomized into six groups of six rats each. Group A rats received only animal feed and water. Groups B,C,D,E and F were given 100mg/kg bw of aluminium chloride intraperitoneally five times a week for three weeks to induce neurotoxicity.In order to reverse the neurotoxicity, Group C was treated with 10mg/kg bw of donepezil as standard drug for the hippocampus, also Group D was treated with 20mg/kg bw of Levodopa/5mg/kg bw of Carbidopa as standard drug for striatum while group E and F were treated with 200mg/kg and 400mg/kg of *Carpolobia lutea* respectively for 14 days. Histopathological study was done on the hippocampus and striatum of the rat brain and thereafter hematoxylin and eosin staining were

carried out. Evaluation of apoptosis in the hippocampus and striatum were estimated by an immunohistochemical study using Bcl-2 protein expressions.

Results: Our result revealed that 200mg/kg bwof *C. lutea* showed a reduced expression of Bcl-2 immunoreactivity than the standard drug (10mg/kg donepezil). Therefore, it is assume that 200mg/kg *C. lutea* may protect the brain from apoptosis in the hippocampus thereby preventing neurodegenerative diseases.The higher dose (400mg/kg bw*C.lutea*) showed sign of neurotoxicity by having increase Bcl-2 immunoreactivity. In the striatum, we could find out that lower dose of *C. lutea* (200mg/kg bw) may distort the initiation of apoptosis in the striatum by having a reduced Bcl-2 immunoreactive expression but it is not more potent compared to 20mg/kg levodopa/5mg/kg Carbidopa (standard drug). The higher dose (400mg/kg bw of *C. lutea*) may be neurotoxic to the striatum.

Conclusion: In conclusion, the lower dose of *C. lutea* has neuroprotective activity that could have a promising role in ameliorating age-related neurodegeneration or prevent apoptosis in the hippocampus compared to the standard drug (donepezil). In the striatum, the standard drug (20mg/kg Levodopa/5mg/kg Carbidopa) may still protect the brain better against apoptosis than the lower dose of the plant extract.

Keywords: *B-Cell lymphoma-2 (BCL-2); Hippocampal and striatal damage; Wistar male rats; Ethanolic extracts; Carpolobia lutea.*

1. INTRODUCTION

1.1 Background of the Study

Medicinal plants play a major role in the health care system of developing countries such as Nigeria. Despite their high usage, most of these plants lack information on, active chemical constituent, quality, clinical studies, safety and efficacy of which *Carpolobia lutea* is one of them (Odigie et al., 2003).

Carpolobia lutea, commonly called cattle stick or poor man's candle belongs to the plant family polygalaceae (Akhondzadehand Abbas, 2006). It is a small tree native to West and Central tropical Africa. It is common in Rainforest and Guinea savannah of Sierra Leone and Cameroon (Forest et al., 2007). This shrub reaches up to 5m in height and occurs as a dense overgrowth or an evergreen shrub or small tree (Forest et al., 2007). The common names, which the plant is known include cattle stick (English), AbekpokIbuhu (Eket), Ikpafum, Ndiyan, Nyayanga (Ibibio), Agba or Angalagala (Igbo) and Egbo oshunshun (Yoruba) (Essien et al., 2011).

Herbalists in Nigerian tribes use the essence of the root as an aphrodisiac and the treatment of genitourinary infections, gingivitis, and waist pains (Ajibesin et al.,2008; Walker et al., 2004). The tree extract could pave the way for new drugs to tackle patient symptoms but without the unwanted side effects associated with some current treatments (Ajiwhen and Bisong, 2013;Idowo et al., 2005).

Neurodegenerative diseases represent a huge health burden globally, placing pressure on health services and having a negative impact on the lives of patients and their families (Ballard et al., 2011).

Researchers and drug companies are racing to discover new treatments for these disorders and have begun looking to plant extracts as a potential source of novel drugs (Syad and Devi, 2014). In patients with Alzheimer's disease and other diseases such as Parkinson's disease and myasthenia gravis, the activity of the neurotransmitter acetylcholine, is increased, leading to problems with memory and attention (Syad and Devi, 2014).

Current drugs — called acetylcholinesterase inhibitors — reduce the normal breakdown of acetylcholine. Extensive research is underway to find new versions of these drugs but with additional beneficial properties (Parihar and Hemnani, 2004).

According to a study published in Pharmacognosy Review, It is an accepted and commonly utilized herbal booster of libido (Yakubu and Jimoh, 2014; Manfo et al., 2011). It is used to cure male infertility and to boosts libido thereby augmenting male sexual functions or it is used to induce penile erection, and enhance male virility (Tundis et al., 2016; Muanya and Odukoya, 2008). The chewing stick prepared from the stem and root of *Carpolobia lutea* is patronized because it boosts male sexual performance (Mesulam et al., 2002).

The leaf essential oil contains a variety of terpenoids, while polyphenols and triterpenoid saponins have been isolated from the root and leaf extracts respectively (Lockridge, 2015;Nwidu et al., 2011). Other ethnomedicinal uses include curing of stomach ailments, rheumatism, fever, pains, insanity, dermal infection, venereal diseases; to promote childbirth; and as a taeniafuge and vermifuge (Manoharan et al., 2007; Lockridge, 2015).

It has also been reported to possess other anti-inflammatory, anti-arthritic, antimicrobial, antimalarial, and analgesic properties (Bero et al., 2009;Ettebong and Nwafor, 2009). This could be particularly important in Alzheimer's disease as there is more evidence emerging that Alzheimer's patients have inflammation in the brain (Duysen et al., 2007).

Aluminium is a well-established neurotoxicant involved in the etiology of neurodegenerative diseases (Sun et al., 2009). It is an abundant metal on earth with easy access to the human body through agrochemicals,water, food additives, utensils, deodorants and drugs. In the brain, aluminum predominantly accumulates in the hippocampus and frontal cortex, regions known to be particularly susceptible in Alzheimer's disease (Flaten, 2001; Campbell, 2002). It induces misfolding of cytoskeleton proteins which leads to the formation of amyloid beta plaques and tau neurofibrillary tangles (El-Sebae et al., 1993) in the brain. Aluminium supplementation causes neurodegeneration and apoptotic neuronal loss along with cognitive dysfunction, as it is a potent cholinotoxin. Normally, cholinergic activity is necessary for the acquisition and retrieval of learning and memory skills (Domingo, 2006). Hence patients with AD normally demonstrate impaired performance in various cognitive tasks. Various animal studies have also shown that prolonged exposure to aluminium can cause neurochemical, neurobehavioral and neuropathological changes in the brain, which impair the learning ability of the rats (Kaur and Gill, 2006; Prema et al., 2017).

1.2 Aim of the Study: This study was aimed at investigating apoptotic changes using Bcl-2 on aluminium chloride induced hippocampal and striatal damages treated with ethanolic extract of *C. lutea* at different doses.

2. MATERIALS AND METHOD

2.1 Extraction Procedures

The leaves of *Carpolobia lutea* were removed from their stalks. The leaves were oven dried for 72 hours and pulverized. Dried sample was ground into powder mechanically using manual grinder.

Thereafter, 400g of the macerated plant powder was soaked in 1000ml of absolute alcohol and kept in a container for 48 hours. The mixture was shaken vigorously at intervals for another 2 hours, to allow complete extraction.

The resulting mixture was rapidly filtered through whatmann filter paper to obtain a homogenous filtrate.

This filtrate was concentrated in a vacuum at low temperature (37-40^{0}C). The extract was later reconstituted in distilled water at a concentration of 1g/ml before administration. The extract was refrigerated until use.

2.2 Standard Drug

Two standard drugs knownfor their therapeutic management of neurotoxicity, namely Levodopa/Carbidopa and Donepezil were used to ascertain and compare with the potency of ethanolic extract of C*arpolobia lutea* against aluminium chloride induced neurotoxicity. A tablet of these drugs each were dissolved in 20ml of distilled water and mixed thoroughly.

2.3 Recruitment of the Study Population

Thirty sixWistar rats weighing between 180-200g were used for this study. The animals were obtained from the animal house of the Department of Biological Sciences of Rivers State University. They were housed in standard cages and left to acclimatize for 14 days under natural conditions in the animal house of the Department of Human Anatomy of Rivers State University before the commencement of the experiment. The animals were fed with vitakraft complete nutrition rat feed and water ad libitum.

2.4 Study Design

The animals were randomized into six groups (A ,B ,C, D, E & F) of six rats each. Rats in group A received only animal feed and water. All rats in groups B, C, D, E & Fwere injected with aluminium chloride ($AlCl_3$) at 100mg/kg bw intraperitoneally five times a week for three weeks (Aljarari, 2023).

The ethanolic extract of *Carpolobia lutea* and donepezil were administered orally by gavage.

Group B received 100mg/kg bw of aluminium chloride (Aljarari, 2023).

Group C received 10mg/kg bw of donepezil (Shin et al., 2018).

Group D received 20mg/kg bw of Levodopa/5mg/kg bw of Carbidopa (Benedicta et al., 2020).

Group E received 200mg/kg per body weight of ethanolic extract of *Carpolobia lutea*.

Group F received 400mg/kg per body weight of ethanolic extract of *Carpolobia lutea*.

The administration of *Carpolobialutea* and donepezil lasted for 14 days. On the 12^{th},13^{th} and 14^{th} day, the rats were subjected to neuro-behavioural tests after which they were sacrificed by cervical dislocation on the 15^{th} day.

2.5 Surgical procedures

At the end of behavioural studies, animals were euthanized by cervical dislocation. Blood was quickly obtained by cardiac puncture and processed for antioxidant studies. Also, their brains were rapidly excised. Whole brain tissues of two animals per group were fixed in 10% neutral buffer formalin. All tissues were processed for rapid routine paraffin wax embedding for histopathological and immunohistochemical studies.

2.6 Histopathological Studies

After sacrifice, the brains of two rats from each group were fixed whole in 10% formal saline. After 48hours, hippocampus and striatum were excised from each rat brain for histological assessment using H&E.

The fixed tissues were dehydrated to remove water which is not miscible with paraffin (the embedding medium). The tissues were placed in ascending grades of alcohol (70%, 80%, 90%, and absolute). This was done to prevent water from rushing out of the tissue, which may distort and damage the cell structure. The time for each grade of alcohol was one hour and in absolute (100%) it was changed twice, one hour each time. After dehydration **t**he tissues were cleared in 2 changes of xylene for 30 mins each to remove alcohol which removed water from tissue. This was done because paraffin used for impregnation and embedding is not miscible with alcohol.

The tissues were then passed through four changes of molten paraffin wax at constant temperatures of 46-68ºC in an oven of paraffin bath. This was to prepare the tissue for the embedding medium and to confirm firmness to it. This will facilitate the process of sectioning. Metal blocks were taken and filed with paraffin wax and tissues were placed in it immediately with forceps, the face to be cut facing downward. When the paraffin cools, a thin scum of solid paraffin is formed on the bottom of the block which was now immersed in water to solidify and then removed for sectioning.

The solid paraffin blocks were then taken to the rotary microtome where excess paraffin wax was first trimmed off and the tissues cut at 5µm. Paraffin sections were taken to a water bath set at 45^0C to straighten it. The side of the glass slide to receive the section was made sticky by rubbing with egg albumin. The paraffin sections were allowed to float in the water bath to straighten out the wrinkles. Water was drained off and the slide was put in an incubator for the sections to be completely fixed on the slide and allow to dry.

2.7 Staining: Hematoxylin and eosin staining technique

The procedure for H&E staining as described by Robert et al., (2014), Bancroft and Stevens, (2005) was adopted. The sections were dewaxed in xylene, two changes of xylene for 2 minutes each and afterwards rehydrated in descending grades of alcohol, 100%, 95%, 90%, 70%, 50% ethanol for 2 minutes each.

Tissues sections were then rinsed in distilled water and stained in haematoxylin for 10-15minutes. Afterwards sections were rinsed in distilled water for 2-3 minutes and examined to confirm sufficient degree of staining. Excess stain was removed or differentiated in 1% HCL acid alcohol for a second or two as the acid breaks the mordant dye linkage.

The sections are again rinsed in distilled water for 2-3 minutes to regain the bluecolour. Sections were then stained in 1% aqueous eosin for about 3-5 minutes. Surplus stain was washed off in distilled water.

Stained sections were then mounted in distrene plasticizer xylene (DPX) using clean glass cover slide and placed under the microscope for examination. Photomicrographs were taken using the photographic light microscope (National optical).

2.8 Immunohistochemical Studies

Immunohistochemical studies for Bcl-2 was carried out using Novocastra™Novolink™ polymer detection system and appropriate primary monoclonal antibodies.

2.8.1 Immunohistochemistry Using ImmPRESS™ HRP Polymer System

The non-biotin, enzymatic, one-step detection kit, ImmPRESS™ Polymerized Reporter Enzyme Staining System (Vector® Labs, USA), provides very high sensitivity staining with very minimal background inteference in immunohistochemical applications. The ImmPRESS™ Reagent uses an innovative, exclusive approach to conjugate horseradish peroxidase (HRP) micropolymers to affinity-purified, extensively cross-adsorbed secondary antibodies.

2.8.2 Reagents supplied (#MP-7401; Vector® Labs, USA)**:**

- ImmPRESS™ (Peroxidase) Polymer Anti-Rabbit IgG Reagent (made in horse, ready-to-use)
- 2.5% Normal Animal (Horse) Serum for blocking (ready- to-use)

2.8.3 ImmPRESS™Immunohistochemical Detection:

The ImmPRESS™ Reagent is ready-to-use. It requires no mixing or tittering of the ImmPRESS™ reagent to obtain optimal immunohistochemical staining. The staining procedure is performed at room temperature. For optimal performance, the ImmPRESS™ Reagent is equilibrated to room temperature before use. Phosphate buffered saline (PBS) was used as wash buffer.

2.9 Staining Protocol:

The protocol was performed as previously described (Erukainure et al., 2019; Ijomone et al., 2018).

- Paraffin embedded sections where deparaffinized with xylene, and rehydrated through descending grades of ethanol (100%, 95%, 70 % ethanol) and taken to water.
- Heat-mediated antigen retrieval was performed using a citrate-based antigen unmasking solution, pH 6.0 (Vector®, Burlingame, CA, USA; #H3300) in a steamer for 30 mins.
- Wash sections in PBS for 2 mins
- Endogenous peroxidase blocking in 0.3 % hydrogen peroxide solution in PBS for 10 mins.
- Wash sections in PBS for 2 mins
- Sections were incubated in animal free blocker for 20 mins for protein blocking.
- Sections were then incubated at room temperature for 2 hours in primary rat antibodies: GFAP (ThermoFisher, USA; #16825-1-AP), Sections were incubated in ImmPRESS™ HRP Anti-Rabbit IgG (Peroxidase) Polymer Reagent, made in horse for 30 mins
- Wash in PBS for 5 mins x 2
- Colour is developed with DAB Peroxidase (HRP) Substrate Kit (Vector® Labs, USA)

- Sections were rinsed well in tap water
- Sections were counter-stained in hematoxylin
- Sections dehydrated through ascending grades of ethanol (70%, 95%, 100%), cleared in Xylene and mounted with Permount (Fischer Scientific, USA).

Sections without primary antibodies were similarly processed to control for immunohistochemistry procedures. No specific immunoreactivity was detected in control sections.

2.10 Photomicrography and Image Analysis

Stained sections were viewed under a Leica DM750 Digital Light microscope and digital photomicrographs of slides were taken by an attached Leica ICC50 camera. Image Analysis and Processing for Java (Image J), a public domain software sponsored by the National Institute of Health (USA), was used to analyze and quantify photomicrographs. Image J cell counter tool was used to identify and quantify number of intact and degenerating neurons in H&E stained sections. Also the Image J cell counter tool was used to note the number of Bcl-2 positive cells.

2.11 Statistical Analysis

All other data obtained were analysed using one way ANOVA followed by student newman-keuls (SNK) for post test.

Results Of Histological Studies

Haematoxylin And Oesin (H & E) Staining Results

The histological studies were carried out using the Haematoxytm and Oesin (H & E) method. Two parts of the brain were studied, the hippocampus (CA1, CA3and DG Striatum. The histology of the hippocampus, dentate gyrus and their components are presented in figures 1, 2and 3 while those of the straitum are presented in figure 4.

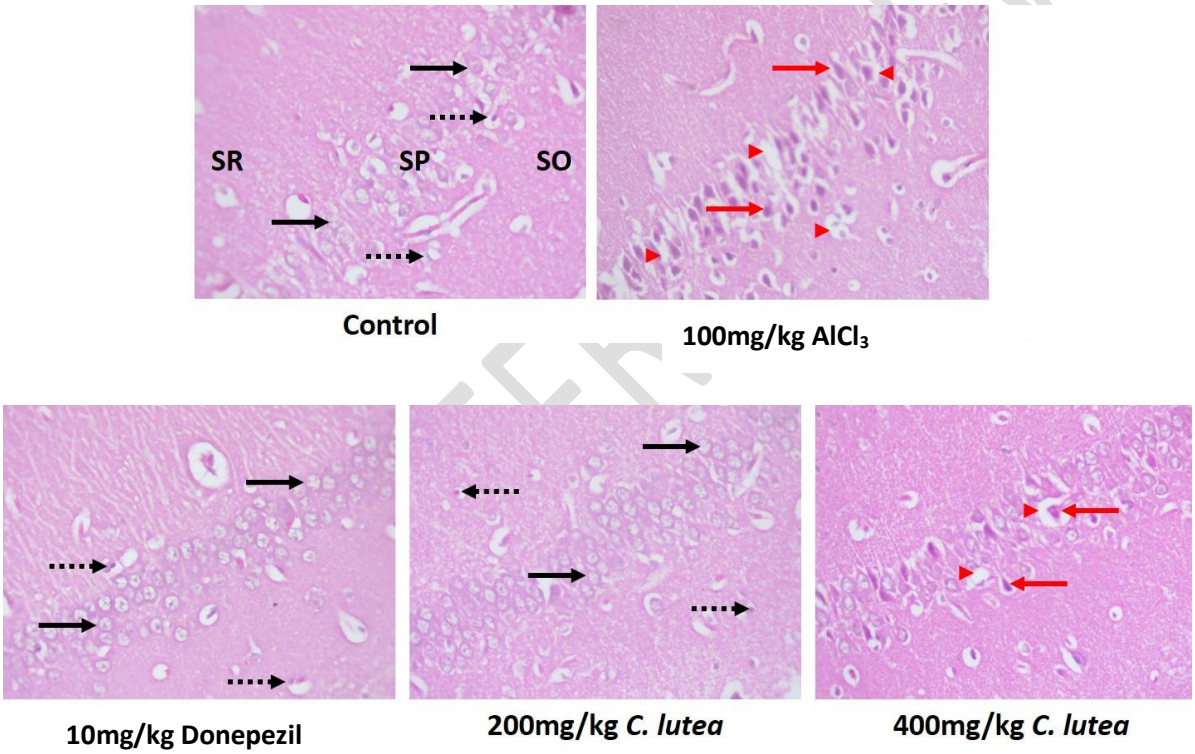
The control group, show typical histological features of the hippocampalCA1 and CA3. There are three distinct layers: stratum radiatum, pyramidalis and oriens. The pyramidal layers are composed of large pyramidal neurons with large round nucleus and prominent nucleoli with glial cells interspaced within the neurons as can be seen in figures 1 and 2.

The negative control group and the treated groups were observed for features that characterize degenerating process in neurons, as can be seen in light microscopystudy of H & E stained sections (Carless et al, 2007, German, 2011). These features include;

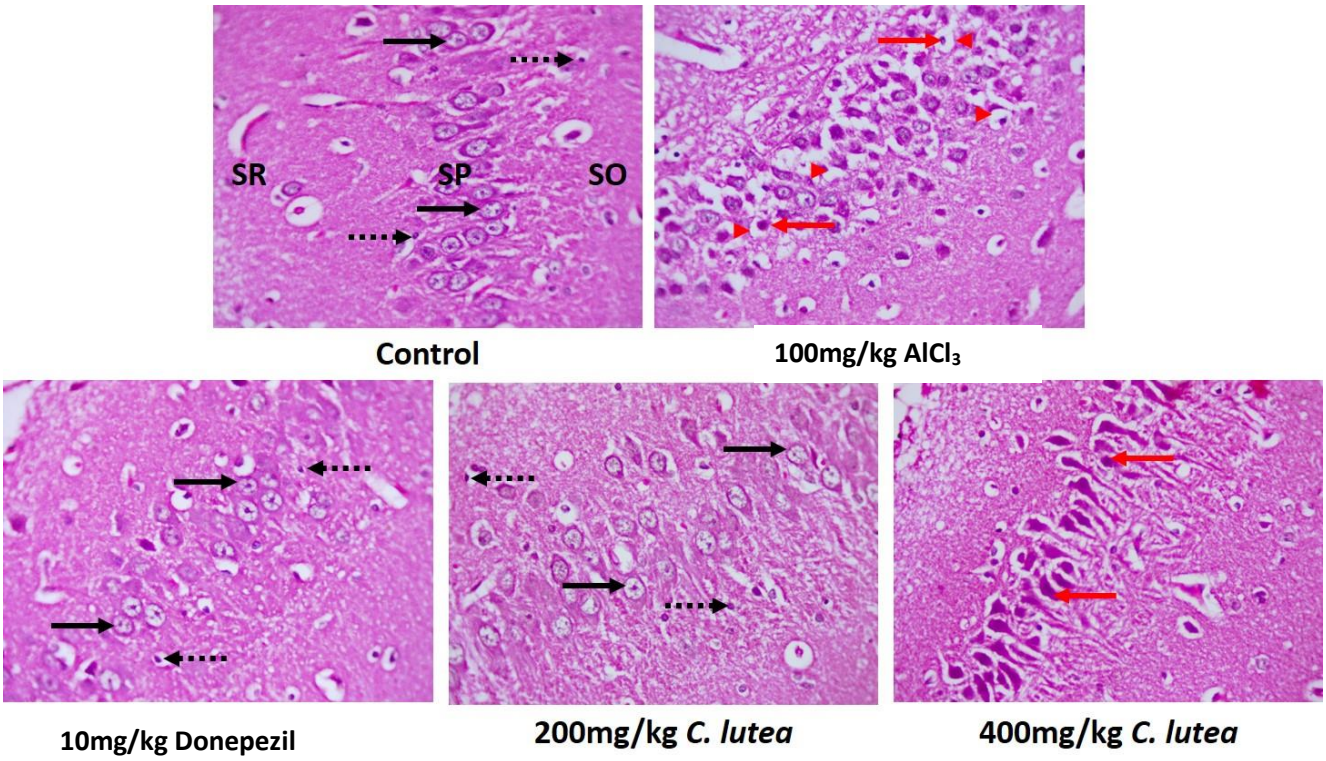
1. Prominent eosinophilic cytoplasm with or without shrunken nuclei
2. Pyknotic nuclei and
3. Neuron swelling and/ or vacuolation within the cytoplasm

The 100mg /kg of $AlCl_3$ group showed degenerative features characterized by pyknotic cells with deeply stained nuclei. There were obvious perinuclear spaces and vacuoles

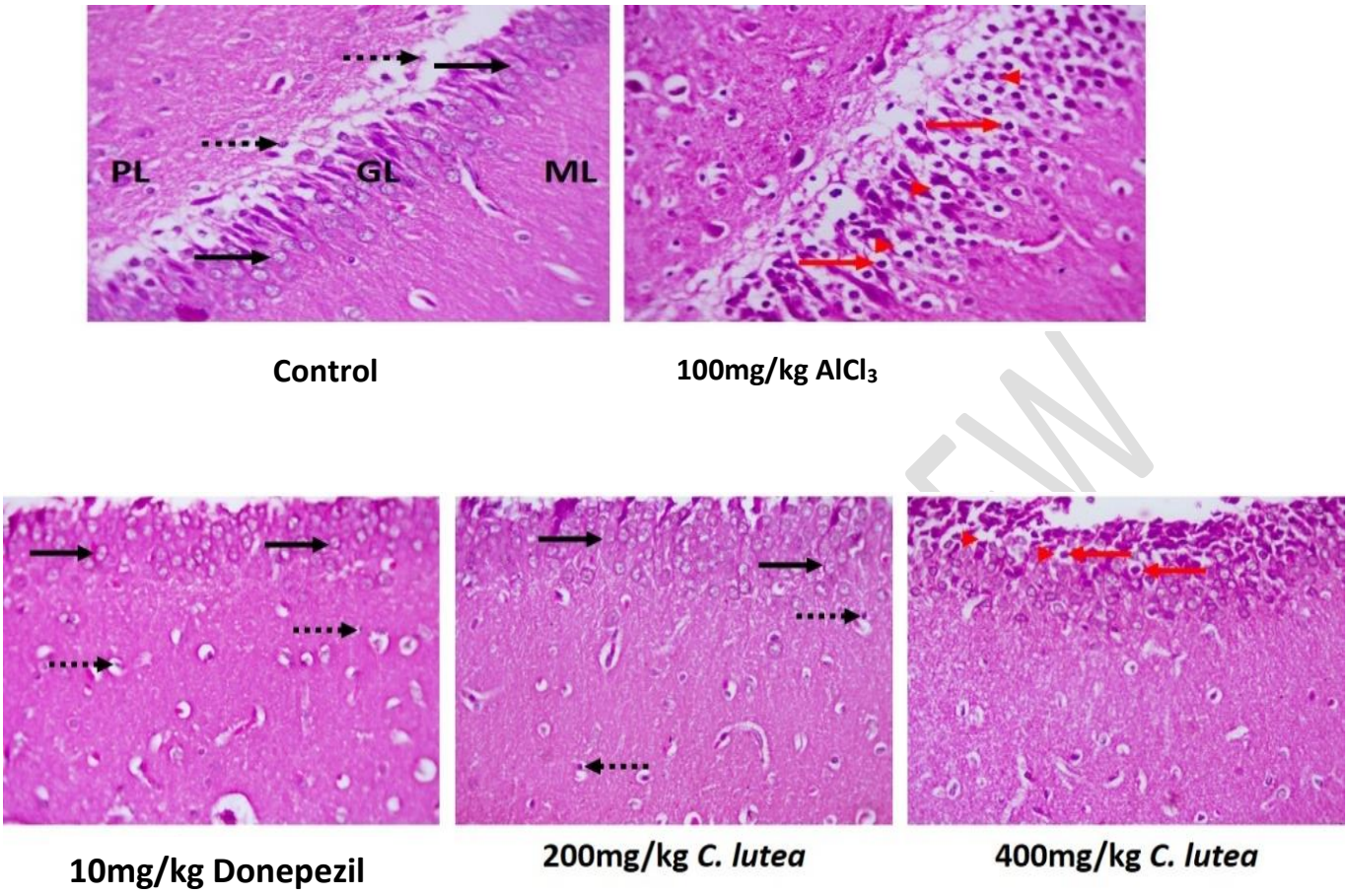
formed from pyknotic neurons and lost neurons respectively as can be seen in Group of figures 1 and 2. The aforementioned degenerative features were also present, albeit mildly in the 400mg/kg of *Capolobia lutea* group when compared to control as seen in figures 1 and 2. Conversely, the 10mg/kg of donepezil and 200mg/kg of *C. lutea* showed no tissue alteration when compared to control.



Group of Figures 1: Histological changes in the hippocampal CA3 of experimental groups. H&E 400x magnification. SR – stratum radiatum; SP – stratum pyramidalis; SO – stratum oriens;black arrows – intact pyramidal neurons; broken/dottedarrows – glial cells; red arrows – pyknotic neurons; red arrow heads – perinuclear spaces.



Group of Figures 2: Histological changes in the hippocampal CA3 of experimental groups. H&E 400x magnification. SR – stratum radiatum; SP – stratum pyramidalis; SO – stratum oriens;black arrows – intact pyramidal neurons; dotted/broken arrows – glial cells; red arrows – pyknotic neurons; red arrow heads – perinuclear spaces.



Group of Figures 3: Histological changes in the hippocampal DG of experimental groups. H&E x400 magnification. PL – polymorphic layer; GL -granular layer; ML – molecular layer;black arrows – intact granular neurons; dotted/broken arrows – glial cells; red arrows – pyknotic neurons; red arrow heads – perinuclear spaces.

Dentate Gyrus as seen in figure 3, the control group exhibits typical histologicalcharacteristics; polymorphic, granular and molecular layers. The granular layer is made up of many neurons with small spherical nuclei and glial cells scattered among them.

The 100mg/kg of $AlCl_3$ group exhibit degenerative characteristic such as pyknotic cells with darkly coloured nuclei. Perinuclear gaps and vacuoles are clearly visible, generated by pyknotic

neurons and lost neurons, respectively. The aforementioned degenerative characteristics are present, however weakly , in the 400mg/kg C. lutea group just as it is in CA1 and CA3.

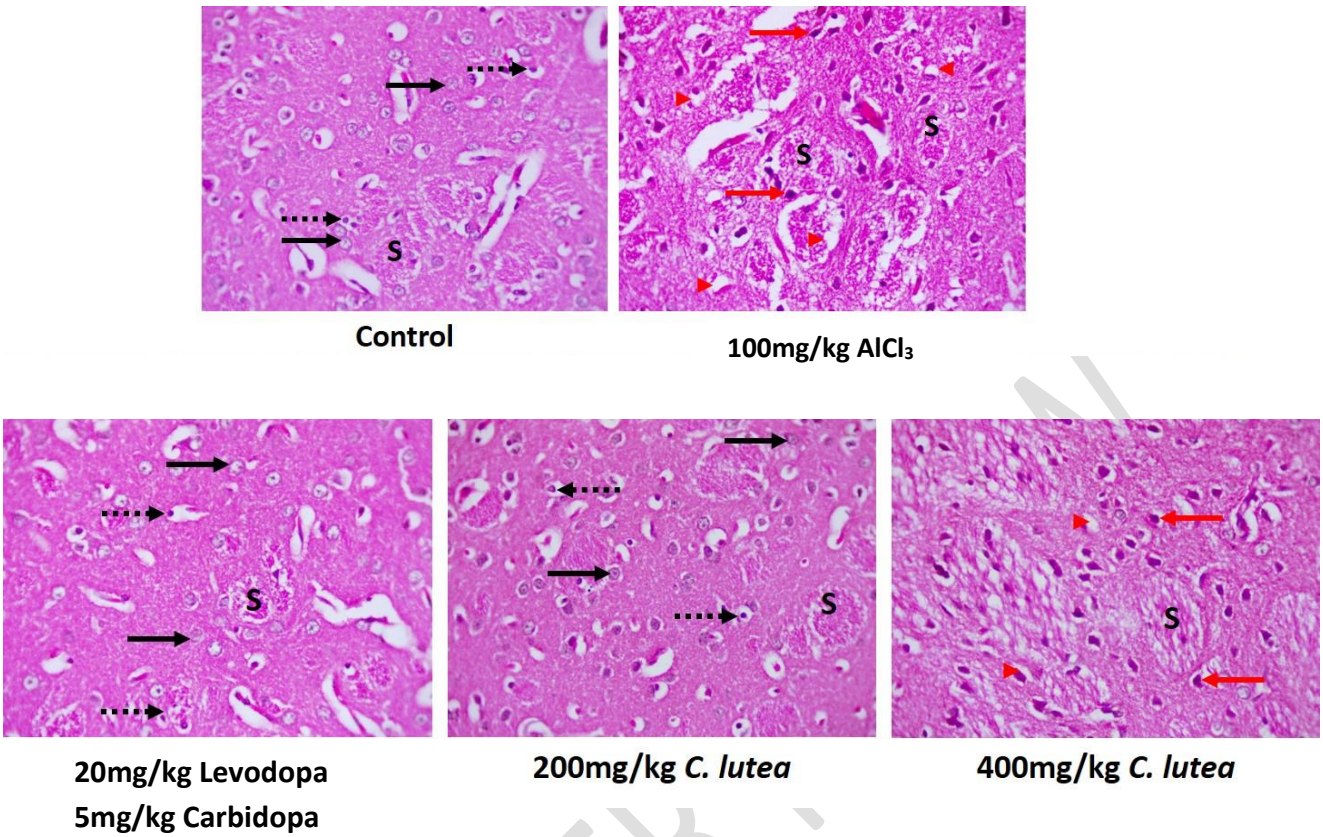
When compared to the control group, the 10mg/kg of Donepezil and 200mg/kg C. lutea groups exhibit no tissue modification as can be seen in figure 3.

The striatum as presented in fig. 4 has typical histological features in the control group. These are separate layers striations generated by nerve bundles and consisting of multiple neurons intermingled with several glial cells.

However infig.4, the 100mg/kg $AlCl_3$ group exhibits degenerated characteristics such as pyknotic cells with darkly discoloured nuclei. There are apparent perinuclear gaps and vacuoles generated by pyknotic neurons and missing neurons, respectively. The aforementioned degenerative characterisitics are also evident, however,minimally in the 400mg/kg *Carpolobia lutea* group.

Also in fig.4 when compared to the control, the 20mg/kg levodopa/5mg/kg bw Carbidopa and 200mg/kg C lutea group exhibited no evident tissue modification.

We can therefore infer that the lower dose of ethanolic extract of the leaf of *Carpolobia lutea* gave better protection on the cytoarchitectureof the hippocampus and striatum.

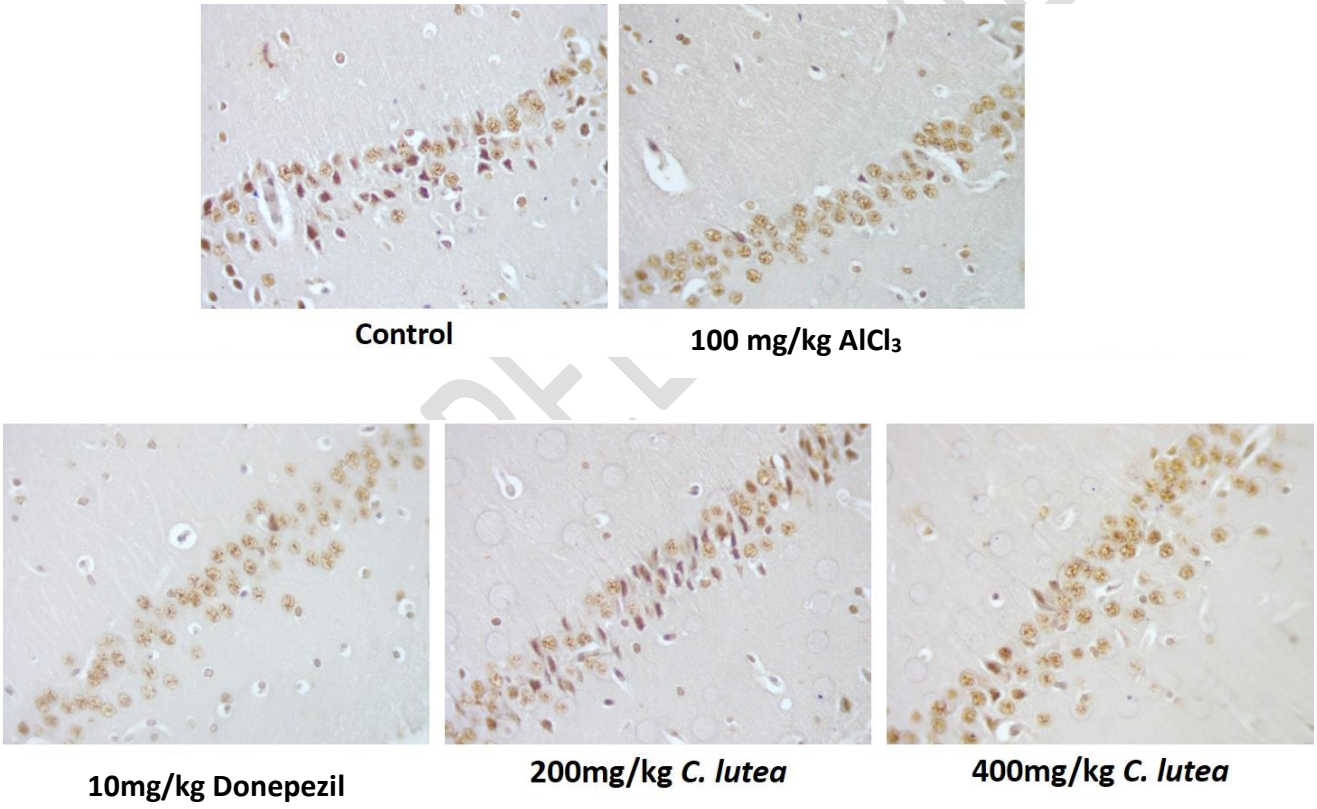


Group of Figures 4: Histological changes in the striatum of experimental groups. H&E x400 magnification.S – Striations;black arrows – intact neurons; broken arrows – glial cells; red arrows – pyknotic neurons; red arrow heads – perinuclear spaces

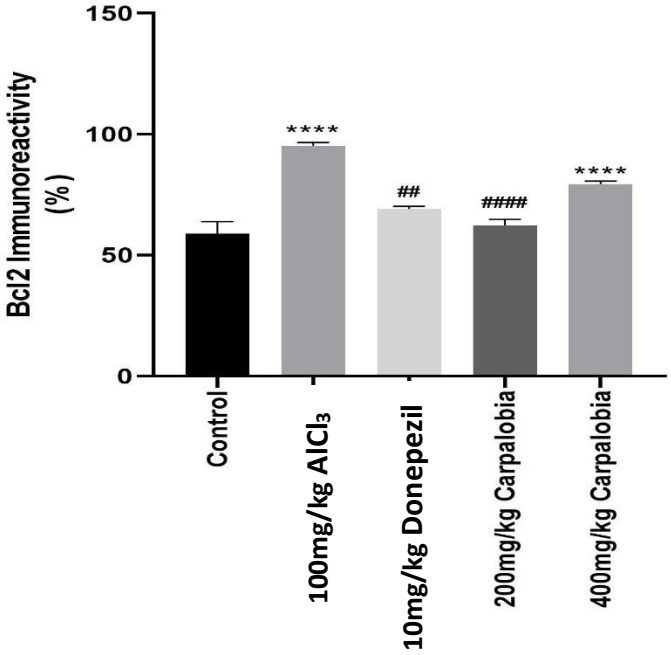
BCL2 – CA1

Immunohistochemical analyses with one-way ANOVA demonstrated significant changes in theimmunoreactivity of Bcl2[F (4, 40) = 30.41, p< 0.0001] in the hippocampal CA1 region following $AlCl_3$ exposure in rats. Post-hoc analysis with Tukey's test indicated that there was significantly more Bcl2 immunoreactivity in the hippocampal CA1 of rats exposed to 100mg/kg $AlCl_3$(p<0.0001)and 400mg/kg *C. lutea*(p< 0.0001)compared to the control. Conversely, in comparison to 100mg/kg $AlCl_3$-exposed rats, there was significantly reduced levels of Bcl2

immunoreactivity in the hippocampal CA1 of 10mg/kg Donepezil- (p<0.01)and 200mg/kg *C. lutea*-treated (p< 0.0001)rats. Furthermore, there was an observable but not significant reduction of Bcl2 immunoreactivity in the hippocampal CA1 of 200mg/kg *C. lutea*-treated group compared to 10mg/kg Donepezil group. Similarly, Bcl2 immunoreactivity was reduced, though not significantly, in 400mg/kg *C. lutea*-treated group compared to 100mg/kg $AlCl_3$-exposed rats (Figure 5).



Group of Figures 5: Immunohistochemical demonstration of BCL2 in the hippocampal CA1 of rats exposed to experimental drugs. 400x magnification.Brown-stained cells are BCL2 expressing cells.

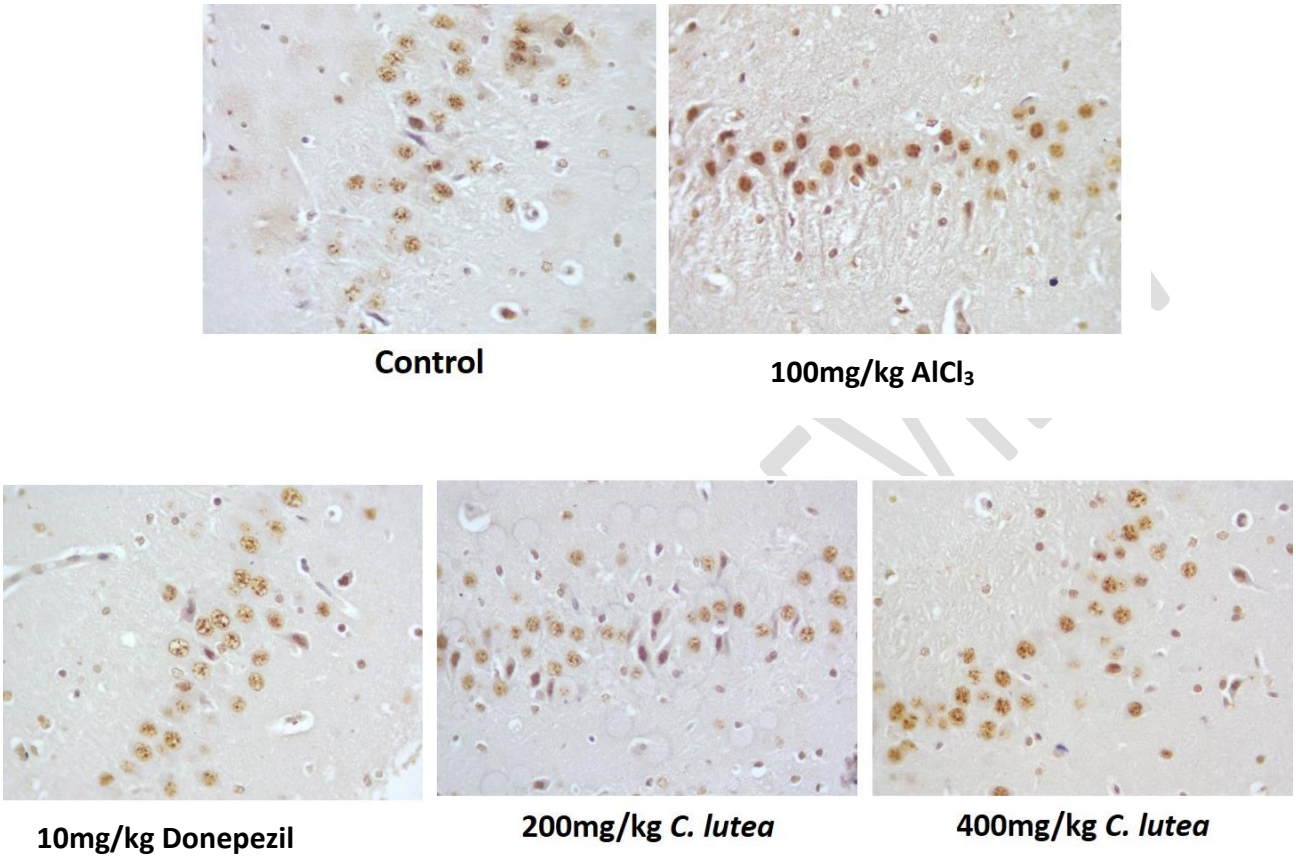


Group of Figures 6: Bar graphs depict the levels of Bcl2 immunoreactivity in experimental rats. Each column represents mean ± S.E.M. Data were analysed using one-way ANOVA followed by Tukey's post-test. ****p < .0001 versus Control; $^{\#\#\#\#}$p < .0001, $^{\#\#}$p < .01 versus 100mg/kg $AlCl_3$.

BCL2 – CA3

Immunohistochemical analysis with one-way ANOVA demonstrated significant changes in theimmunoreactivity of Bcl2 [F (4, 40) = 23.66, p< 0.0001] in the hippocampal CA3 region following $AlCl_3$ exposure in rats. Post-hoc analysis with Tukey's test indicated that there was significantly more Bcl2 immunoreactivity in the hippocampal CA3 of rats exposed to 100mg/kg $AlCl_3$ (p< 0.0001) and 400mg/kg *C. lutea*(p< 0.01) compared to the control. Conversely, in comparison to 100mg/kg $AlCl_3$-exposed rats, there was significantly reduced levels of Bcl2 immunoreactivity in the hippocampal CA3 of 10mg/kg Donepezil- (p< 0.001)and 200mg/kg *C. lutea*-treated (p< 0.0001)rats. Furthermore, there was an observable, albeitinsignificant reduction of Bcl2 immunoreactivity in the hippocampal CA3 of 200mg/kg *C. lutea*-treated group compared to 10mg/kg Donepezil–treated group. Similarly, Bcl2 immunoreactivity was reduced,

though not significantly, in 400mg/kg *C. lutea*-treated group compared to 100mg/kg $AlCl_3$-exposed rats (Figure 7).



Group of Figures 7: Immunohistochemical demonstration of BCL2 in the hippocampal CA3 of rats exposed to experimental drugs. 400x magnification.Brown-stained cells are BCL2-expressing cells.

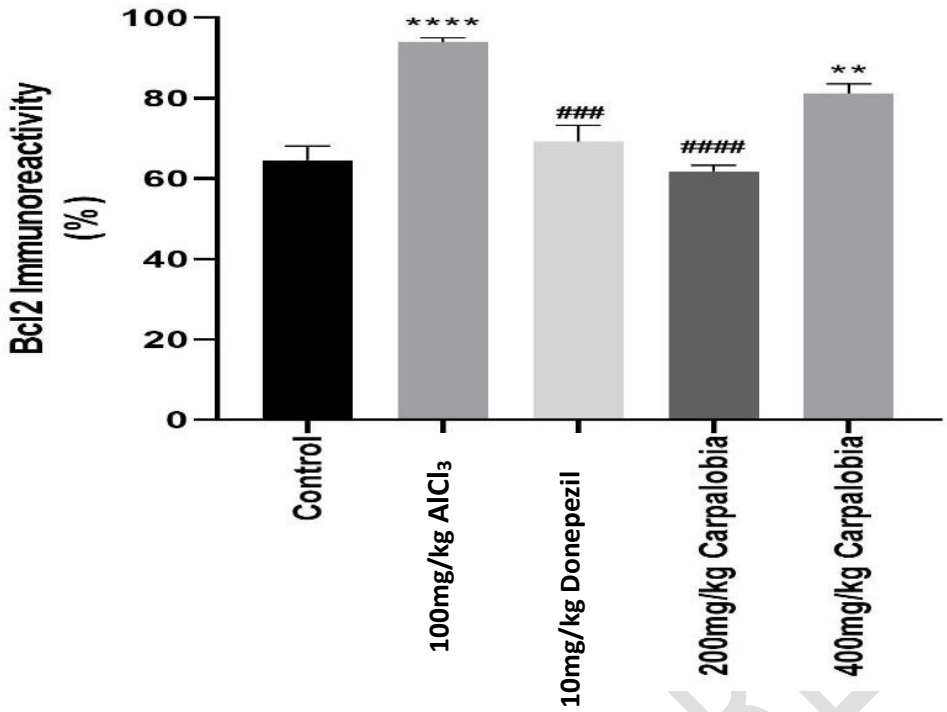
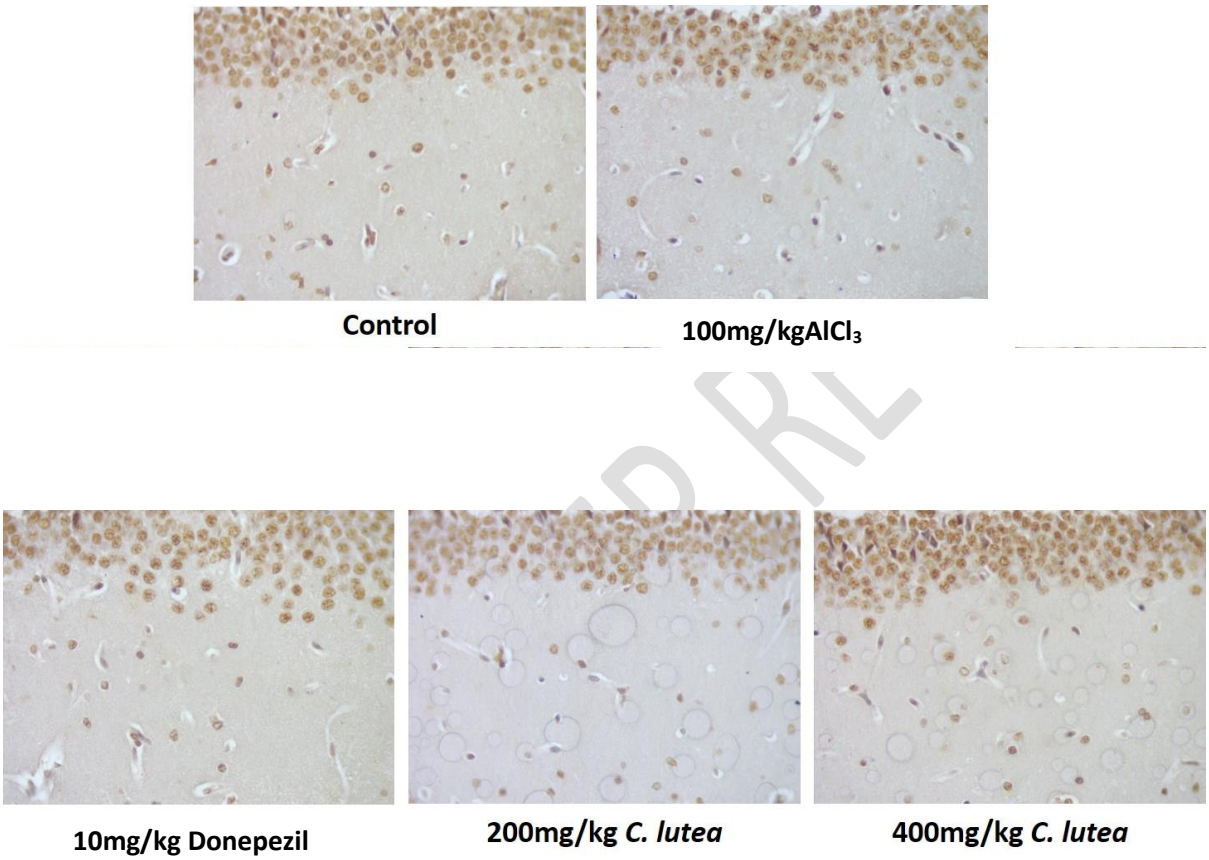


Figure 8: Bar graphs depict the levels of BCL2 immunoreactivity in experimental rats. Each column represents mean ± S.E.M. Data were analysed using one-way ANOVA followed by Tukey's post-test. ****p < .0001, **p < .01 versus Control; ####p < .0001, ###p < .001 versus 100mg/kg $AlCl_3$.

BCL2 – DG

Immunohistochemical analysis with one-way ANOVA demonstrated significant changes in theimmunoreactivityof BCL2 [F (4, 40) = 88.37, p< 0.0001] in the hippocampal DG region following $AlCl_3$ exposure in rats. Post-hoc analysis with Tukey's test indicated that there was significantly more BCL2 immunoreactivity in the hippocampal DG of rats exposed to 100mg/kg $AlCl_3$ (p< 0.0001) and 400mg/kg *C. lutea*(p< 0.001) compared to control. Conversely, in comparison to 100mg/kg $AlCl_3$-exposed rats, there was significantly reduced levels of BCL2 immunoreactivity in the hippocampal DG of 10mg/kg Donoepezil- (p<0.001)and 200mg/kg *C. lutea*-treated (p< 0.0001)rats. Furthermore, there was an observable, albeit insignificant

reduction of BCL2 immunoreactivity in the hippocampal DG of 200mg/kg *C. lutea*-treated group compared to 10mg/kg Donepezil. Similarly, BCL2 immunoreactivity was reduced, though not significantly, in 400mg/kg *C. lutea*-treated group compared to 100mg/kg $AlCl_3$-exposed rats (Figure 9).



Group of Figures 9: Immunohistochemical demonstration of BCL2 in the hippocampal DG of rats exposed to experimental drugs. 400x magnification.Brown-stainedcells are Bcl2-expressing cells.

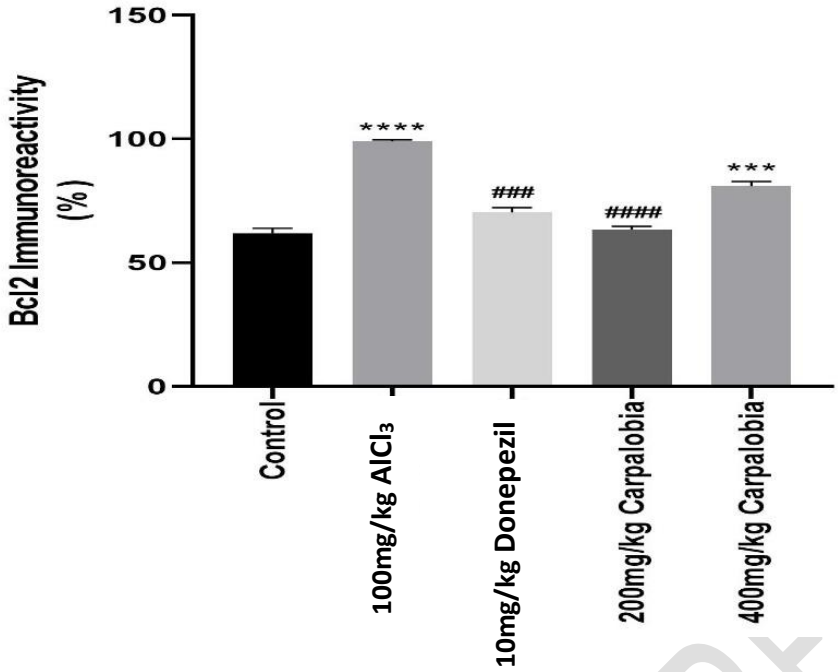
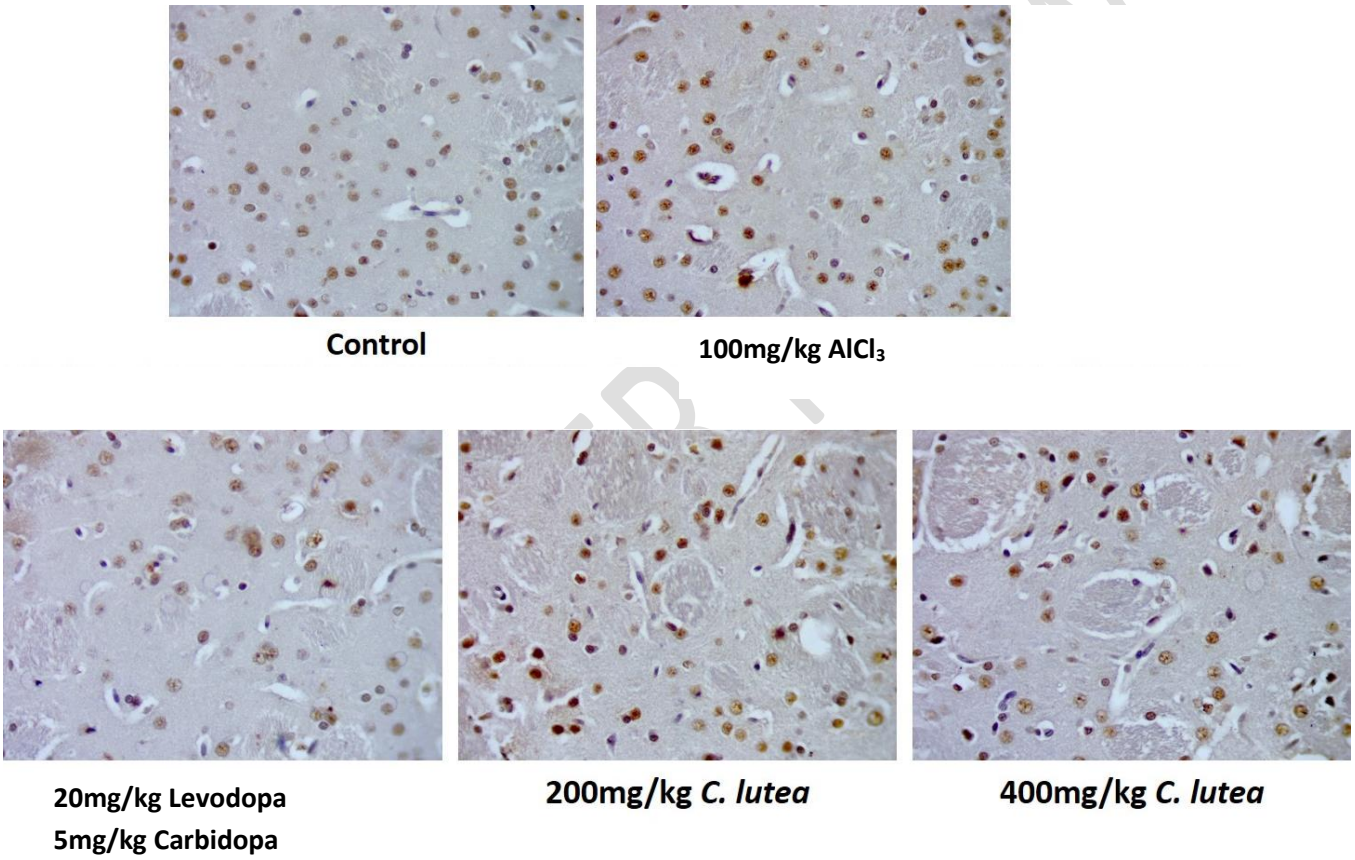


Figure 10: Bar graphs depict the levels of BCL2 immunoreactivity in the hippocampal DG of experimental rats. Each column represents mean ± S.E.M. Data were analysed using one-way ANOVA followed by Tukey's post-test. ****p < .0001, ***p < .001 versus Control; $^{\#\#\#\#}$p < .0001, $^{\#\#\#}$p < .001 versus 100mg/kg $AlCl_3$.

BCL2 – Striatum

Immunohistochemical analysis with one-way ANOVA demonstrated significant changes in the immunoreactivity of BCL2 [F (4, 55) = 108.8, p< 0.0001] in the striatum following $AlCl_3$ exposure in rats. Post-hoc analysis with Tukey's test indicated that there was more BCL2 immunoreactivity in the striatum of rats exposed to 100mg/kg $AlCl_3$ significantly (p<0.0001)and 400mg/kg *C. lutea*significantly (p< 0.001) compared to control. Conversely, in comparison to 100mg/kg $AlCl_3$-exposed rats, there was significantly reduced levels of Bcl2 immunoreactivity in the striatum of 20mg/kg Levodopa/5mg/kg Carbidopa-group (p< 0.0001)and 200mg/kg *C. lutea*-treated (p< 0.001)rats. Furthermore, there was an observable, albeit insignificant reduction of BCL2 immunoreactivity in the striatum of 200mg/kg *C. lutea*-treated group compared to

20mg/kg Levodopa/5mg/kg Carbidopa-treated group. Similarly, BCL2 immunoreactivity was reduced, though not significantly, in 400mg/kg *C. lutea*-treated group compared to 100mg/kg $AlCl_3$-exposed rats(Figure 11).



Group of Figures 11: Immunohistochemical demonstration of BCL2 in the striatum of rats exposed to experimental drugs. 400x magnification.Brown-stained cells are BCL2-expressing cells.

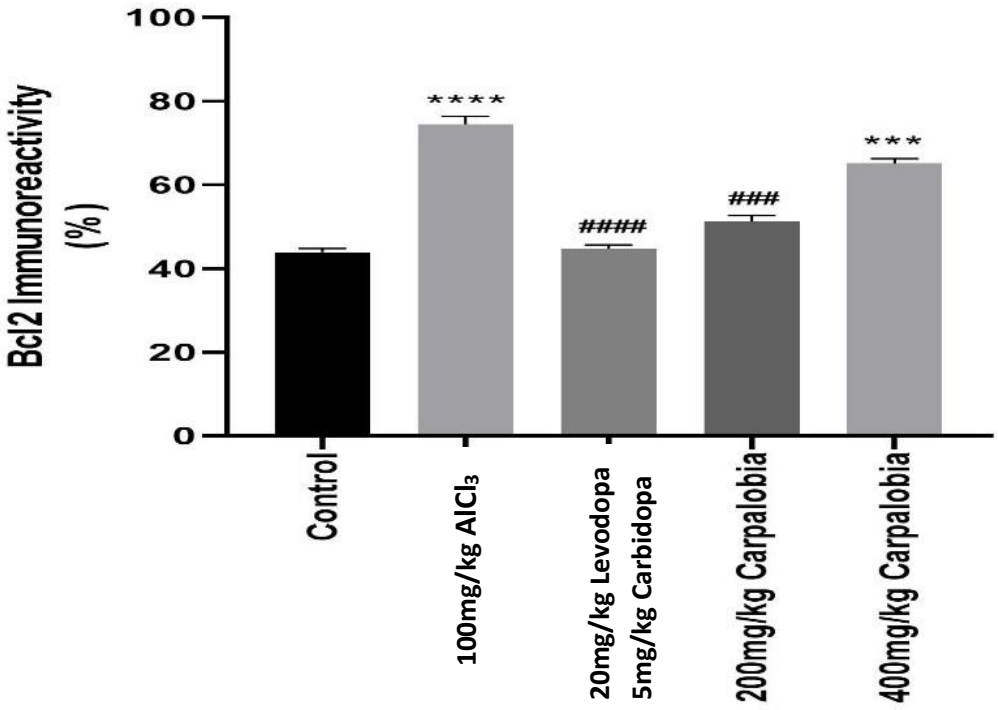


Figure 12: Bar graphs depict the levels of BCL2 immunoreactivity in the striatum of experimental rats. Each column represents mean ± S.E.M. Data were analysed using one-way ANOVA followed by Tukey's post-test. ****p < .0001, ***p < .001 versus Control; $^{\#\#\#\#}$p < .0001, $^{\#\#\#}$p < .001 versus 100mg/kg $AlCl_3$.

Discussion

The present study demonstrated significant changes in the immunoreactivity of BCL2 in the CA1, CA3 and dentate gyrus regions of the hippocampus following $AlCl_3$ exposure in rats compared to the control. This suggests that increased reaction of BCL2 to aluminium chloride may induce apoptosis(Niu et al., 2005). Apoptosis is a controlled form of cell death involving cascades of degradative events (German, 1998). A cell that undergoes apoptosis dies neatly, without damaging it'sneighbours. The cell shrinks and condense. The cytoskeleton collapses, the nuclear envelope disassembles, and the nuclear DNA breaks up into fragrant (Jia et al., 2014).

Compared to 100mg/kg $AlCl_3$ exposed rats, there was significant reduction in the level of BCL2 immunoreactivity in the hippocampal CA1, CA3 and dentate gyrus of the 10mg/kg Donepezil and 200mg/kg *C. lutea* group. Furthermore an observable, though not significant, reduction in BCL2 immunoreactivity in the hippocampal CA1, CA3 and dentate gyrus of the 200mg/kg *C.lutea* treated group when compared to 10mg/kg donepezil.

On the otherhand, increased dosage of 400mg/kg *C. lutea* increased BCL2 immunoreactivity. Similarly, BCL2 immunoreactivity was reduced though not significant in the 400mg/kg *C. lutea* treated group compared to 100mg/kg $AlCl_3$ exposed rats. It could be suggested that higher dose of *C.lutea* might initiate apoptotic pathway while lower dose might suppress apoptosis.

It is noteworthy that neurodegenerative diseases such as Alzheimer's disease are characterized by excessive apoptosis of neurons. For example, Alzheimer's disease is caused by an accumulation of b-amyloids at lesion sites where b-amyloids induce abnormal apoptosis of neurons (Guo and Liang, 2001).

Previous studies on neutoxicity effect on aluminium revealed that aluminium was capable of inducing apoptsis in neuronal (SH-SY5Y), glial and retinal epithelial cells (Toimela and Tahti, 2004). Another study demonstrated that proxidant effect of $AlCl_3$ was associated with significant increase in cerebral levels of caspase -3 and bax,whereas antiapoptotic BCL2 was found to be down regulated by aluminium treatment (Mesole et al., 2020). Jin et al., (2009) asserted that aluminium could influence the activities of learning and memory. A recent study by Reihant et al., (2019) demonstrated that AL^{3+} inhibited profliferation of neural progenitor cells during neural differentiation and induced apoptosis along with modulation of cell cycle.

From the present study, our data revealed that 200mg/kg bwof *C. lutea*showed a reduced expression of Bcl-2 immunoreactivity than the standard drug (10mg/kg donepezil). Therefore, it is assume that 200mg/kg *C. lutea* may protect the brain from apoptosis in the hippocampus thereby preventing neurodegenerative diseases. It is also important to note that donepezil which is the standard drug used in this research does not have better neuroprotective effect than lower dose of *Carpolobia lutea*.

From the present study, in the striatum there was significant changes in the immunoreactivity of BCL2 following $AlCl_3$ exposure in rats compared to the control. This could be the neurotoxicity nature of $AlCl_3$ which may lead to inducement of caspases which has been identified as a key mediator of apoptosis in neuronal cells (Liu et al.,2020). Caspase also act as a regulatory molecule in neurogenesis and synaptic activity(Laabbar et al., 2019).

Conversely, in comparison to 100mg/kg $AlCl_3$ exposed rats, there was significantly reduced levels of BCL2 immunoreactivity in the striatum of 20mg/kg levodopa/5mg/kg Carbidopa and 200mg/kg *C. lutea*. although there was an observable insignificant reduction of BCL2

immunoreactivity in the striatum of 20mg/kg levodopa/5mg/kg Carbidopa compared to 200mg/kg *C. lutea*.

Apoptosis is the main mechanism of neuronal loss on parkinson's disease, as evidenced by the identification of DNA fragmentation and apoptotic chromatin changes in dopaminergic neurons of parkinson's disease patience in postmotem studies (Dudek et al., 2018).

It could be inferred that 200mg/kg C.lutea has the potential of distorting or interrupting the aggregation of caspases-3 which is a precursor to apoptosis in the striatum. Although when comparison was made between 200mg/kg *C. lutea* and 20mg/kg levopoda/5mg/kg Carbidopa, it could be said that levodopa still protect the brain better than 200mg/kg *C. lutea* but not in a significant manner.

Also, 400mg/kg *C. lutea* showed increase BCL2 immunoreactivity when compared to control and has similar but slight reduction in immunoreactivity when compared to 100mg/kg $AlCl_3$. It could be inferred that higher dose of *C. lutea* may be neurotoxic and may not play any significant role in protecting the neurons in the striatum.

Previous studies indicated that mitochondrial dysfunction causes cytochrome cy+ leakage that binds Apaf-1 with subsequent formation of apoptosome and procaspase 9 activation underlying aluminium induced apoptotic neuronal death (savory et al., 2003). Aluminium exposure can be a risk factor for neurodegenerating diseases and may negatively impact the striatum and dopaminergic neurons (Liu et al., 2020). Also the role of Fas/Fasl apoptotic pathway has been comfirmed by the observation of aluminium induced caspases (Qin et al., 2020).

From the data in this study, It could be deduced that lower dose of *C. lutea* may distort the initiation of apoptosis in the striatum but it is not more potent compared to levodopa (standard drug). The higher dose may be neurotoxic to the striatum.

Conclusion

In conclusion, the lower dose of *C. lutea* has neuroprotective activity that could have a promising role in ameliorating age-related neurodegeneration or prevent apoptosis in the hippocampus compared to the standard drug (donepezil). In the striatum, the standard drug (20mg/kg Levodopa/5mg/kg Carbidopa) may still protect the brain better against apoptosis than the lower dose of the plant extract.

Declaimer

Author(s) hereby declare that NOgenerative AItechnologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators has been used during the writing or editing of this manuscript

5. REFERENCES

1. Aljarari RM. Neuroprotective effects of a combination of Boswellia papyrifera and Syzygiumaromaticum on AlCl3 induced Alzheimer's disease in male albino rat. Braz J Biol. 2023 Oct 13;83:e272466. doi: 10.1590/1519-6984.272466. PMID: 37851769

2. Ajibesin KK, Ekpo BA, Bala DN, Essien EE, Adesanya SA. Ethnobotanical survey of Akwa Ibom State of Nigeria. *J Ethnopharmacol.* 2008;115:387–408. [PubMed] [Google Scholar]

3. Ajiwhen IO, Bisong SA. Effect of ethanolic extract of *Carpolobia lutea* G. Don (Polygalaceae) root on learning and memory in CD1 mice. Niger J Physiol Sci. 2013; 28:141–145.[PubMed] [Google Scholar]

4. Akhondzadeh S, Abbasi SH. Herbal medicine in the treatment of Alzheimer's disease. Am J Alzheimers Dis Other Demen, 2006;21:113–118.

5. Ballard C, Corbett A, Brayne C, Aarstand D, Jones E. Alzheimer's disease. Lancet.2001; 377:1019-1031.(Pubmed) (Google scholar).

6. Bero J, Ganfon H, Jonville MC, Frédérich M, Gbaguidi F, DeMol P. *In vitro* antiplasmodial activity of plants used in Benin in traditional medicine to treat malaria. *J Ethnopharmacol.* 2009;122:439–44

7. Benedicta OD, Seth KA, Grace LA, Ismaila A, Nana AG, Henry N. Chitosan Coated Hydroxypropylmethyl Cellulose Microparticles of Levodopa(and Carbidopa): In vitro and Rat Model Kinetic Characteristics. Current Therapeutic Research. 2020; 93:11-39

8. Campbell A.(2002)."The potential role of aluminium in Alzheimer's disease". Nephrology Dialysis Transplantation. 2002; 17(2):17-20.

9. Carliss RD, Radovsky A, Chengelis, CP, O'Neill TP, Shuey DL. Oral administration of dextromethorphan does not produce neuronal vacuolation in the rat brain. NeuroToxicol. 2007; 28:813–818.

10. Domingo JL. (2006). Reproductive and developmental toxicity of aluminum: a review. Neurotoxicology.2006;17(4), 515–521. [Google Scholar]

11. Duysen EG, Li B, Darvesh S, Lockridge O. Sensitivity of Butyrylcholinesterase knockout mice to (-)- huperzine A and donepezil suggest humans with butyrylcholinesterase deficiency may not tolerate Alzheimer's disease drugs and indicates butyrylcholinesterase function.Toxicology. 2007; 233:60-69.

12. Dudek AF, Bolte HKC, SchwemmleM.Partial Inactivation of the Chromatin Remodelers SMARCA2 and SMARCA4 in Virus-Infected Cells by Caspase-Mediated Cleavage. J Virol2018;92:10.1128/jvi.00343-18.

13. El-Sebae,Y.D., Xu,W. U., Zhou, M. V.(2012). "Aluminum induces neurodegeneration and its toxicity arises from increased iron accumulation and reactive oxygen species (ROS) production," Neurobiology of Aging. 2012; 3(1):199-205.

14. Ettebong E, Nwafor P. Report: *In vitro* antimicrobial activities of extracts of Carpolobia lutea root. *Pak J Pharm Sci.* 2009;22:335–8. [PubMed] [Google Scholar]

15. Essien EE, Ajibesin KK, Ekpo BA, Bala DN, Adesanya SA. Ethnobotanical survey of Southern States of Nigeria.J Ethnopharmacol. 2011. 115: 387-408.

16. Flatten TP. Aluminium as a risk factor in Alzheimer's disease, with emphasis on drinking water. Brain Science. 2001; 55(2):187–196.

17. Forest F, Chase MW, Persson C, Crane PR, Hawkins JA. The role of biotic and abiotic factors in evolution of ant dispersal in the milkwort family (polygalaceae) *Evolution.* 2007;61:1675–94. [PubMed] [Google Scholar]

18. Guo GW, Liang YX. Aluminum-induced apoptosis in cultured astrocytes and its effect on calcium homeostasis. *Brain Res*. 2001;888 (2), 221–226. [PubMed] [Google Scholar]

19. Ijomone OM, Olaibi OK, Obi AU, Alese MO. Immunohistochemistry staining protocols; Glial fibrillary acidic protein. NeuroToxicol. 2018;5(1):10-16.

20. Idowo PA, Jones OM, Herbert AO. Phytochemical and antimicrobial screening of three nigerian medicinal plants used to treat infectious diseases traditionally. *J Pharm Biores.* 2005;2:116–9. [Google Scholar

21. Jia X, Zhang Q, Niu Q,. MAPK signaling pathways involved in aluminum-induced apoptosis and necroptosis in SH-SY5Y cells. *J. Hygiene Res.* 2014;43 (6), 917–922. [PubMed] [Google Scholar]

22. Jin C, Liu Q, Wang J, Cai Y. [Effect of aluminium on neural behavior and the expression of Bcl-2 and Fas in hippocampus of weaning rats]. Wei Sheng Yan Jiu. 2009 Jan;38(1):1-3. Chinese. PMID: 19267062.

23. Kaur A, Joshi K, Minz RW, Gill KD. Neurofilament phosphorylation and disruption: a possible mechanism of chronic aluminium toxicity in Wistar rats.Toxicology. 2006; 219(3):1–10.[PubMed] [Google Scholar]..

24. Laabbar W, Elgot A, Elhiba O, Gamrani H, 2019. Curcumin prevents the midbrain dopaminergic innervations and locomotor performance deficiencies resulting from chronic aluminum exposure in rat. *J. Chem. Neuroanat* 100, 101654. [PubMed] [Google Scholar]

25. Liu H, Zhang W, Fang Y, Yang H, Tian L, Li K, Lai W, Bian L, Lin B, Liu X, Xi Z. Neurotoxicity of aluminum oxide nanoparticles and their mechanistic role in dopaminergic neuron injury involving p53-related pathways. J Hazard Mater. 2020 Jun 15;392:122312. doi: 10.1016/j.jhazmat.2020.122312. .PMID: 32105957.

26. Lockridge O. Review of human butyrylcholinesterase structure, function, genetic variants, history of use in the clinic, and potential therapeutic uses. Pharmacol Ther. 2015; 148:34–46. [PubMed] [Google Scholar]

27. Manoharan I, Boopathy R, Darvesh S, Lockridge O. A medical healthreport on individuals with silent butyrylcholinesterase in the Vysya of India. Clin Chim Acta. 2007; 378:128–135. [PubMed] [Google Scholar]

28. Mirza A, King A, Troakes C, Exley C. Aluminium in brain tissue in familial Alzheimer's disease. J. Trace Elem. Med. Biol. 2017; 40:30–36.

29. Mesole SB, Alfred OO, Yusuf UA, Lukubi L, Ndhlovu D. Apoptotic inducement of neuronal cells by aluminium chloride and the neuroprotective effect of eugenol in wistar rats. *Oxid. Med. Cell Longev* 2020. [PMC free article] [PubMed] [Google Scholar]

30. Mesrzzulamn MM, Guillozet A, Shaw P, Levey A, Duysen EG, Lockridge O. Acetylcholinesterase knockouts establish central cholinergic pathway and can use butyrylcholinesterase to hydrolyzeacetylcholine. Neuroscience. 2002; 110:627–639.

31. Muanya CA, Odukoya OA. Lipid peroxidation as index of activity in aphrodisiac herbs. *J Plant Science.* 2008;3:92–8.

32. Manfo FP, Nantia EA, Tchana AN, Monsees TK, Moundipa PF. Evaluation of the effect of Carpolobia alba (Polygalaceae) aqueous extract on male reproduction function in rats. *J Appl Anim Res.* 2011;39:80–4. [Google Scholar]

33. Nwidu LL, Nwafor PA, da Silva VC, Rodrigues CM, dos Santos LC, Vilegas W. Anti-nociceptive effects of Carpolobia lutea G. Don (Polygalaceae) leaf fractions in animal models. *Inflammopharmacology.* 2011;19:215–25. [PubMed] [Google Scholar

34. Niu Q, Wang LP, Chen YL, Zhang HM. [Relationship between apoptosis of rat hippocampus cells induced by aluminum and the copy of the bcl-2 as well as bax mRNA]. Wei Sheng Yan Jiu. 2005 Nov;34(6):671-3. Chinese. PMID: 16535832.

35. Odigie IP, Ettarh RR, Adigun SA. Chronic administration of extracts of *Carpoloia lutea* attenuates hypertension and reverses cardiac hypertrophy in 2K-1 C hypertensive rats. Journal of Ethnopharmacology. 2003; 86(12):181–185.

36. Omeiza NA, Bakre A, Ben-Azu B, Sowunmi AA, Abdulrahim HA, Chimezie J, Lawal SO, Adebayo OG, Alagbonsi AI, Akinola O, Abolaji AO, Aderibigbe AO. Mechanisms underpinning Carpolobia lutea G. Don ethanol extract's neurorestorative and antipsychotic-like activities in an NMDA receptor antagonist model of schizophrenia. J Ethnopharmacol. 2023 Jan 30;301:115767. doi: 10.1016/j.jep.2022.115767.

37. Parihar MS, Hemnani T. Alzheimer's disease pathogenesis and therapeutic interventions. J Clin Neurosci. 2004;11:456–467.

38. Prema A, Justin TA, Manivasagam T, Mohamed EM, Guillemin GJ. Fenugreek seed powder attenuated aluminum chloride induced tau pathology, oxidative stress, and inflammation in a rat model of Alzheimer's disease. J. Alzheimers,2017; 60(1):209–220.

39. Qin X, Li L, Nie X, Niu Q,. Effects of chronic aluminum lactate exposure on neuronal apoptosis and hippocampal synaptic plasticity in rats. *Biol. Trace Elem. Res.* 2020;197 (2), 571–579. [PubMed] [Google Scholar]

40. Reichert KP, Schetinger MRC, Pillat MM, Bottari NB, Palma TV, Gutierres JM, Morsch VM. Aluminum affects neural phenotype determination of embryonic neural progenitor cells. *Arch. Toxicol.* 2019;93 (9), 2515–2524. [PubMed] [Google Scholar]

41. Sun PI, Keen CL, Han B, Golub MS. Aluminum accumulation and neurotoxicity in Swiss-Webster mice after long-term dietary exposure to aluminum and citrate. Journal of Neuroscience. 2009;42:1296 1300.

42. Savory J, Herman MM, Ghribi O. Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain. J. Inorg. Biochem 2003; 97(1),151– 154. [PubMed] [Google Scholar].

43. Syad AN, Devi KP (2014). Botanics: a potential source of new therapies for Alzheimer's disease. Botanics: Targets Therapy. 2014; 4:11–26.

44. Tundis R, Bonesi M, Menichini F, Loizzo MR. Recent knowledge on medicinal plants as source of cholinesterase inhibitors for the treatment of dementia. Mini Rev Med Chem.2016;16:605–618.

45. Shin CY, Kim HS, Cha KH, Won DH, Lee JY, Jang SW, Sohn UD. The Effects of Donepezil, an Acetylcholinesterase Inhibitor, on Impaired Learning and Memory in Rodents. Biomol Ther (Seoul). 2018 May 1;26(3):274-281. doi: 10.4062/biomolther.2017.189. PMID: 29463072; PMCID: PMC5933894

46. Toimela T, Tähti H. Mitochondrial viability and apoptosis induced by aluminum, mercuric mercury and methylmercury in cell lines of neural origin. *Arch. Toxicol.* 2004;78 (10), 565–574. [PubMed] [Google Scholar

47. Yakubu MT, Jimoh RO. *Carpolobia lutea* roots restores sexual arousal and performancein paroxetine-induced sexually impaired male rats. *Rev Intl J Androl.* 2014;12:90-9 [Google Scholar]

48. Walker AR, Silans R. Paris, France: Paul Lechevalier; 2004. Les PlantesUtiles du Gabon; pp. 19–132. [Google Scholar]