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Epigallocatechin -3- gallate mitigates diazinon neurotoxicity via suppression of pro-inflammatory genes and upregulation of antioxidant pathways

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Abstract

Diazinon is a commonly used organophosphate (OP) insecticide especially in developing countries for the control of insect pests, however, exposure to its toxic impact especially in humans and other non-target species remains an important public health concern. The study aimed to investigate the effect of epigallocatechin -3- gallate (EGCG), abundant in green tea plants on neurobehavioural, biochemical, and pathological changes in the brain of male Wistar rats following exposure to diazinon toxicity. Sixty adult male Wistar rats were acclimatized for seven days and subsequently randomly assigned into six treatment groups as follows: Group I: Control group (0.2 mL distilled water); Group II: Diazinon at 3 mg/kg (1% LD50); Group III: Diazinon (3 mg/kg) + EGCG (50 mg/kg, ~ 2% of LD50); Group IV: Diazinon (3 mg/kg) + EGCG (100 mg/kg, ~ 5% of LD50); Group V: EGCG (50 mg/kg) and Group VI: EGCG (100 mg/kg). All treatments were administered orally once daily for 14 days. Neurobehavioural studies, biomarkers of oxidative stress, histology, immunohistochemistry, and quantitative polymerase chain reaction (RT qPCR) were performed. Diazinon alone impaired recognition memory, increased oxidative stress markers and altered antioxidant defense in the brain. It upregulated TNF-α and IL-6 genes and repressed GPx 4 gene expressions. It was also associated with increased GFAP, Tau, and α-SN immunoreactivity. Microscopic examination revealed loss of Purkinje and hippocampal cells in brain. Co-treatment with EGCG however improved cognition, lowered oxidative stress markers, improved antioxidant status and suppressed TNF-α and IL-6. In conclusion, findings from this study demonstrated that EGCG offered protection against diazinon-induced neurotoxicity. Hence, natural sources of epigallocatechin -3- gallate such as fruits and vegetables could offer immense benefits by protecting against oxidative stress and inflammation in neurodegenerative disease conditions.

Clinical trial number Not applicable.

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Introduction

Pesticide exposure remains a persistent public health concern especially among the subpopulations that by virtue of their occupation and/or routine activities are constantly in the vicinity where these compounds are either produced or applied [1–2]. Even in residential urban settings, the presence of pesticides or their metabolites on food stuffs and water meant for domestic purposes pose a source of exposure risk [3–5].

Diazinon is a commonly used organophosphate pesticide (OP) for the control of agricultural and household pests including insect vectors of human diseases [6]. It works as a contact insecticide and kills target pests by inhibiting acetylcholinesterase enzyme activity producing the so called "cholinergic crisis". However, this mechanism is non-specific and affects both target pests and non-target species, including humans [7]. In the last few decades, however, diazinon as with many OP have been documented to produce oxidative stress (OS) and promote inflammation in tissues via the actions of reactive oxygen species [8–9]. This is of relevance because OS and chronic inflammation have been linked to the development of many chronic neurodegenerative diseases including cognitive disorders (such as Alzheimer's disease, dementia) and motor disorders (such as Parkinson-like diseases) either as a causal factor or somewhat involved in the progression of these diseases [10–11]. Diazinon-induced neurotoxicity occurs in part via the induction of oxidative stress (OS), interference with antioxidant defense and promotion of neuroinflammation [12–13]. Previous studies have shown that diazinon exposure in Wistar rats resulted in progressive and/or persistent neurobehavioral deficits, impairment in cognitive functions and memory, as well as neurochemical alterations [13–14]. Hence, exposure to OP such as diazinon could have long-term global impact on human mental health such as cognition, learning, and memory. Therefore, unnecessary exposure to OP should be avoided and preventive measure in mitigation OP should be in place.

The cholinergic system includes neurons located in the basal forebrain and long axons that reach the cerebral cortex and the hippocampus. This system modulates cognitive function. Cognitive impairment is associated with progressive damage to cholinergic fibres, which leads us to the cholinergic hypothesis for Alzheimer's disease (AD) as previously reported [15–16]. Alterations or dysfunction in noradrenergic and cholinergic systems have been shown to occur in each of the major neurodegenerative diseases of ageing, including Alzheimer's disease, Parkinson's disease, Lewy body dementia, frontotemporal dementia, and progressive supranuclear palsy [17–18]. Therefore, natural products including plat-derived products and synthetic agents have been reported to offer significant improvement in the cholinergic pathway thereby enhancing cognitive impairment [19–23].

Antioxidants are structurally and functionally diverse compounds that employ a variety of mechanisms to prevent the accumulation of ROS (and other prooxidants species) and therefore the development of oxidative stress in the body [24–27]. Investigations into their use to ameliorate or reverse the toxic effects of chemical and environmental pollutant exposures are actively ongoing. Much effort in this regard is focused on the identification and isolation of bioactive compounds from plants with health promoting potentials. Several antioxidants have been obtained from plants and investigated in a variety of neurological diseases [19–21; 26–27]. Epigallocatechin -3-gallate (EGCG) is a polyphenolic flavanol (or catechin) richly present in green tea plant (*Camellia sinensis*) [28–29]. A number of studies have shown that EGCG is endowed with antioxidant, anti-inflammatory, and immunomodulatory properties [30–32]. The take out from most of these studies is that EGCG employs complex mechanisms in exerting its health promoting attributes. There is however, a gap as to its efficacy in pesticide-exposed animals. This is crucial because different toxicants may employ unique mechanism(s) in promoting their cytotoxicity.

This study was therefore aimed at investigating the ameliorative effect of EGCG on neurobehavioural, biochemical, and histopathological changes induced by diazinon in the brain of exposed male Wistar rats. In clinical setting, supplementation and adequate intake of EGCG, a major polyphenol in green tea, holds promise in supporting cognition, memory, and learning due to its neuroprotective and neuro-enhancing properties. While preclinical evidence is promising, more clinical trials are needed to confirm its efficacy and optimal usage in humans.

Materials and methods

Experimental animals, housing and management

This research was conducted at the Experimental laboratory of the Department of Veterinary Physiology and Biochemistry, Faculty of Veterinary Medicine, University of Ibadan, Oyo State, Nigeria.

A total of sixty (60) adult male Wistar rats weighing between 180 and 200 g were used for the study. The rats were sourced from a pure breed rat's colony in the Experimental Animal House of the Faculty of Veterinary

Medicine, University of Ibadan. They were housed in spacious cages (5 rats/per cage) to minimize stress and enhance normal species-specific behaviour. The cages were kept in a well-ventilated room under natural lighting conditions of 12 h light and 12 h dark daily throughout the experimental period. They were fed commercially formulated broiler finisher feed produced by Top Feeds®. Feed and clean water was provided *ad libitum*. Dry wood shavings were used as litter material and this was regularly changed as required upon visual inspection. All experimental protocol implemented was in conformity with the guidelines of the Animal Research Review Panel [33] for the care and use of laboratory animals.

Acquisition and reconstitution of test compounds

Commercial grade diazinon marketed as Knock Out® 12% EC (100 ml, Agrochemical Nigeria Limited) was reconstituted in water to yield a 1.8 mg/ml working solution that was used for the study. Commercial grade EGCG 98% (HPLC) (5 g, LC44509PU2, AK Scientific®, USA) was used for this study. It was reconstituted in dimethyl sulfoxide (DMSO) into a 100 mg/mL working solution and stored at 4°C throughout the experimental period.

Experimental design

A completely randomized design was used for the study. After seven (7) days of acclimatization, the rats were weighed using a digital weighing scale to obtain their initial weight. They were then randomly assigned to six (6) independent treatment groups. Each group had ten (10) rats. All treatments were administered by oral gavage once daily for 14 days. The various treatment groups were as follows (see Table 1):

Group I: Control group (0.2 mL distilled water).

Group II: Diazinon (3 mg/kg representing 1% LD_{50}).

Group III: Diazinon (3 mg/kg) + EGCG (50 mg/kg which represents 2% LD_{50}).

Group IV: Diazinon (3 mg/kg) + EGCG (100 mg/kg which represents 5% LD_{50}).

Group V: EGCG (50 mg/kg).

Group VI: EGCG (100 mg/kg).

Table 1 Experimental design

Groups ($n = 10$)	Compound(s) administered (per os)	Duration (Experimental days)
I	Distilled water (0.2 mL)	1–14
II	Diazinon (3 mg/kg)	1–14
III	Diazinon (3 mg/kg) + EGCG (50 mg/kg)	1–14
IV	Diazinon (3 mg/kg) + EGCG (100 mg/kg)	1–14
V	EGCG (50 mg/kg)	1–14
VI	EGCG (100 mg/kg)	1–14

The dosage of diazinon was based on the work of Ajibade et al. [34], while that of EGCG was selected from the findings of Isbrucker et al. [35]. Any sign of adverse reaction was recorded daily. On days 10 to 12 of the experiment, neurobehavioural evaluations were performed on the rats using appropriate test procedures. All rats were sacrificed on day 15 of the experiment. Five (5) rats were randomly selected from each group, sacrificed by quick cervical dislocation and brains harvested for biochemical and PCR assay. Harvested brains were stored at -20°C until analysed. The remaining 5 rats were euthanised with ketamine (100 mg/kg, i.p.); they were then perfused intracardially with normal saline first and then 10% phosphate buffered formalin (PBF). Brains were harvested and post-fixed in PBF for 5 days and thereafter subjected to histological procedures for histopathological and immunohistochemical analysis [36]. All experimental protocol was implemented in conformity with the guidelines of the Animal Research Review Panel for the care and use of laboratory animals. Ethical approval for the study and consent for the use of animals was obtained from University of Ibadan, Animal Care and Use Research Committee (UI-ACUREC) with approval number UI-ACUREC/059–0324/28.

Neurobehavioural evaluation

The following tests were conducted to assess neurobehavioural function: open field test, elevated plus maze (EPM) test, novel object recognition test, and hanging wire test. Tests were performed on days 10–12 (Table 2).

Open field test (OFT)

Anxiety and general motor function were assessed using an OFT initially developed by Hall [37] and modified by several researchers through the years. The open field apparatus was a 100 cm-by-100 cm-by-80 cm box. The floor was divided into 16 small squares with a centre square. The assessment time for each rat was 5 min after which the apparatus was thoroughly cleaned with methylated spirit to remove any previous animal's odour which may confound test results. The variables recorded include number of squares crossed, frequency of entry into centre square, frequency of rearing and grooming, and number of faecal bolus(es) passed out. Tests were carried out on experimental day 12.

Elevated plus maze (EPM) test

This test is used to evaluate anxiety-like behaviour in rat and other rodents. It is based on their reluctance to enter 'unprotected' spaces balanced out by their innate desire for exploration in a new environment. The EPM apparatus has two oppositely placed open and closed arms with a small centre area in between where the rat is dropped. The time spent in these 3 compartments (open, closed

Table 2 Protocol schedule

	Experimental Days														
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15
Exposure to test compounds															
Novel object recognition tests															
OFT, EPM & hanging wire tests															
Sacrifice, brain harvesting and isolation															

and centre) within a 5 min period was recorded [38]. This test was performed on experimental day 12.

Hanging wire test

This test as described [39] is used to evaluate muscle strength in rats. Each rat was hung with its fore paw on a wire support, at a height of about 60 cm and the time until it drops to the floor is recorded [40]. Each rat will be allowed three trials with a resting period between consecutive attempts of 5 min. The average duration for each rat was then recorded. This test was performed on experimental day 12.

Novel object recognition test (NORT)

This test is used to evaluate cognition in rats as it relates to different aspects of learning and memory [41]. A general description for the test is as follows: in an open field box, the rats are first familiarized with two identical objects (habituation phase). Twenty-four hours later (test phase) one of the objects is replaced with a new object and the rats are allowed to explore both objects for 5 min and the time spent exploring each object was recorded. NORT was performed on experimental days 10–12; rats were trained on days 10 and 11 and tests carried out on day 12.

Tissue biochemical assay

Tissue homogenization

The brain samples were homogenized in buffer (0.1 M Phosphate Buffer, pH 7.4) at a dilution ratio of 1:8 using a Teflon® homogenizer. The resulting homogenate was then centrifuged using a cold centrifuge at 10,000 rpm for 10 min at 4°C. The resulting supernatant was collected into plain sample bottles and stored at -20°C until use.

Determination of biomarkers of oxidative stress and antioxidant status

Lipid peroxidation was evaluated by measuring the formation of Thiobarbituric Acid Reactive Substances (TBARS) according to previously described methods [42]. Hydrogen Peroxide concentration was determined as described [43], reduced glutathione content was measured [44] and glutathione Peroxidase activity was determined [45]. The activity of SOD was determined as previously described [46] and with a slight modification [47]. Glutathione S-transferase was determined as described [48]. The concentration of nitric oxide (NO) was determined using Griess reagent [49].

Determination of acetylcholinesterase (AChE) activity

The activity of AChE in brain samples was determined as previously described [50].

Gene expression analysis

Real time quantitative polymerase chain reaction (qPCR) assay to quantify mRNA levels of glutathione peroxidase (GPx), interleukin 6 (IL-6), and TNF-α in the brain heart and kidneys was conducted [51].

Procedure

Total RNA extraction, RNA quality control, and cDNA synthesis

Total RNA was extracted using a modified Cetyltrimethyl ammonium bromide (CTAB) extraction protocol. The extracted RNA was then treated with NEB DNase 1 (M0303) to totally eliminate extracted DNA.

Gene quantification

Gene quantification was performed using Luna® Universal qPCR Master Mix (New England Biolabs, Massachusetts, USA) protocol (M3003). Actin was used as internal control.

Primer design

Gene	Forward primer seq	Reverse primer sequence
GPX4	CCGATATGCTGAGTGTGGTTTA	GGCTGCAAACTCCTTGATTTC
TNF-Alpha	TCTTCAAGGGACAAGGCTGC	CTTGATGGCAGAGAGGAGGC
IL6	GCAAGAGACTTCCAGCCA	CTGGTCTGTTGTGGGTGG
actin	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG

Immunohistochemistry

Immunohistochemical staining to visualize and quantify tissue expression level for the following proteins: glial fibrillary associated protein (GFAP), alpha synuclein and tau protein was as described by Oyagbemi et al. [24]. Slight modification using 2-step plus Poly-HRP Anti Mouse/Rabbit IgG Detection System with DAB solution (Catalog number: E-IR-R217 from Elabscience Biotechnology®, China) was employed.

Statistical analysis

All values obtained were expressed as mean ± standard error. One-way analysis of variance (ANOVA) was used to test for significance at $p < 0.05$ and Tukey HSD post-hoc test was used for pair-wise comparison [52]. All analysis was performed using "GraphPad Prism 8" statistical software.

Results

Effect of treatment on neurobehavioural assessments and cognitive functions

Open field test/maze There was a significant ($p < 0.05$) decrease in the number of lines crossed in rats that received only diazinon compared to the control (Fig. 1A). Rearing frequency and frequency of entry into centre square was also significantly ($p < 0.05$) reduced in the diazinon alone group compared to their control counterpart. When compared to the diazinon only group, co-treatment with EGCG significantly increased ($p < 0.05$) the rearing frequency but not the number of lines crossed or the frequency of entry into the centre square (Fig. 1B-C). Grooming frequency was not significantly different ($p > 0.05$) in rats that received diazinon alone relative to the control (Fig. 1D).

Elevated plus maze (EPM) test The time spent in either the closed or open arm of the EPM apparatus was significantly affected by the treatments (Fig. 2). Rats that received diazinon alone spent significantly less time ($p < 0.01$) in the

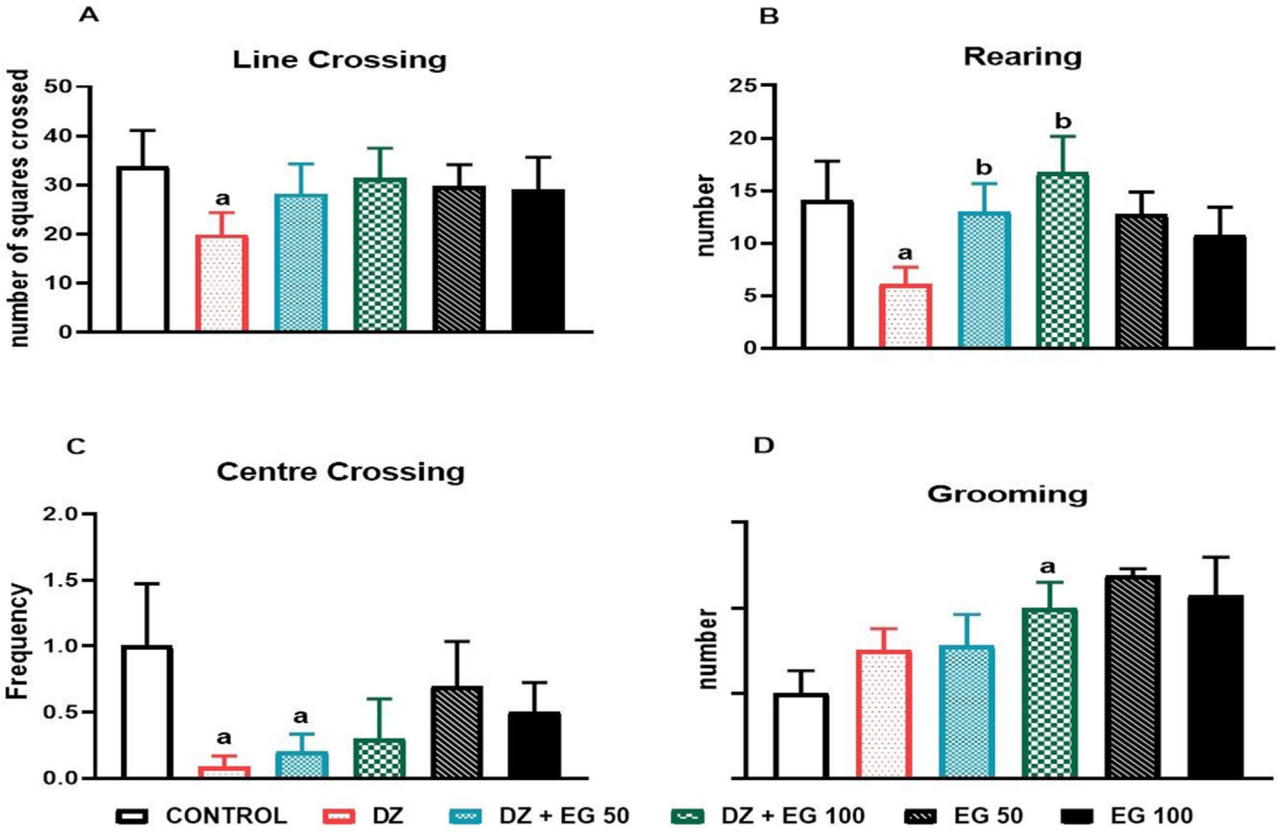

Fig. 1 Open Field Maze (OFM) results of rats treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Values are means ± SEM ($n = 10$). Alphabet "a" indicates significant difference when compared to the control at $P < 0.05$ while "b" indicates significant difference when compared to diazinon only (DZN) group at $P < 0.05$. Mean ± SEM ($n = 10$)

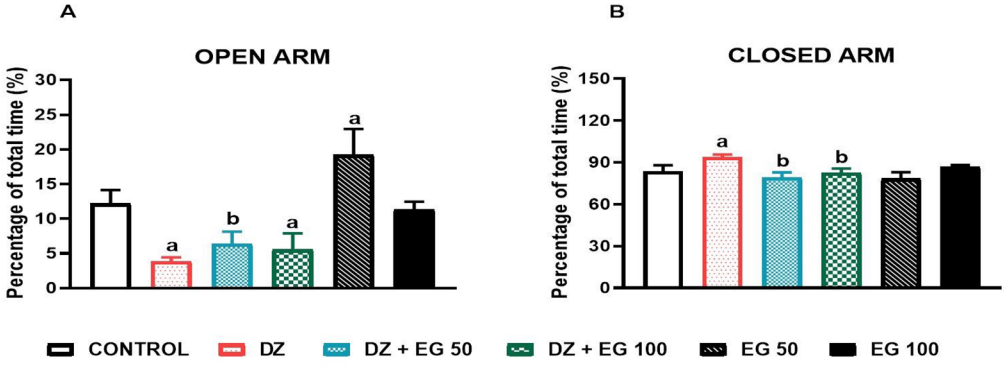

Fig. 2 Elevated plus maze results of rats treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Values are means ± SEM ($n = 10$). Alphabet "a" indicates significant difference when compared to the control at $P < 0.05$ while "b" indicates significant difference when compared to diazinon only (DZN) group at $P < 0.05$. Mean ± SEM ($n = 10$)

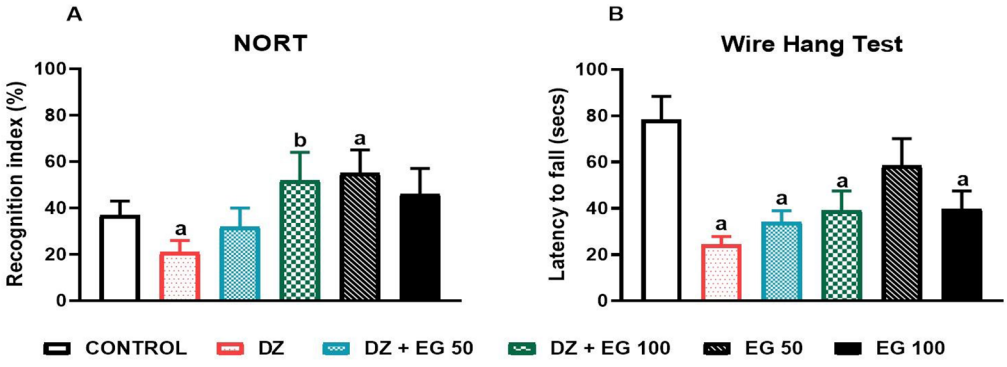

Fig. 3 Wire hang test and novel object recognition test (NORT) results of rats treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Values are means ± SEM ($n = 10$). Alphabet "a" indicates significant difference when compared to the control at $P < 0.05$ while "b" indicates significant difference when compared to diazinon only (DZN) group at $P < 0.05$. Mean ± SEM ($n = 10$)

open arm (Fig. 2A) and significantly ($p < 0.05$) more time in the closed arm of the apparatus compared to those in the control group (Fig. 2B). However, co-treatment with EGCG at 50 mg/kg significantly increased ($p > 0.05$) the time spent in the open arm, while the time spent in the closed arm was significantly decreased ($p > 0.05$) following co-treatment with EGCG at both dosage levels, as shown in Fig. 2A&B. Interestingly, EGCG alone at 50 mg/kg significantly improved ($p > 0.05$) the time spent in the open arena of the plus maze compared to the control.

Novel object recognition The recognition index for novel object was decreased significantly ($p > 0.05$) in rats exposed to diazinon alone relative to the untreated control, while a corresponding significant improvement ($p < 0.05$) in the recognition index was observed following co-treatment with EGCG at 100 mg/kg (Fig. 3A). Interestingly, recognition index in rats was significantly increased ($p > 0.05$) by EGCG alone at 50 mg/kg compared to the control.

Hanging wire test The latency until fall decreased ($p < 0.05$) significantly in the rats treated with diazinon alone compared to control. Co-administration of EGCG did not significantly increase ($p > 0.05$) the hanging latency when compared to the diazinon alone group (Fig. 3B). Latency to fall was also significantly decreased ($p > 0.05$) by EGCG alone at 100 mg/kg compared to the control.

Effect of treatment on tissue biochemical parameters

Treatment with diazinon alone significantly increased MDA contents in the cerebrum ($p < 0.01$) and cerebellum ($p < 0.01$) of rats compared to control group. A corresponding decrease in MDA levels ($p < 0.05$) was however observed following co-treatment with EGCG at 50 mg/kg and 100 mg/kg respectively compared to the diazinon alone group, as shown in Fig. 4A-B. Treatment with diazinon alone significantly increased hydrogen peroxide (H_2O_2) generation in the cerebrum ($p < 0.05$) and cerebellum ($p < 0.05$) of rats compared to the untreated controls while H_2O_2 concentration in these tissues was significant ($p < 0.05$) lowered by the co-treatment of rats with EGCG at both 50 mg/kg and 100 mg/kg respectively compared to the diazinon alone group, as shown in Fig. 4C-D. GSH levels were found to decrease ($p < 0.05$) significantly in the cerebrum and cerebellum of diazinon alone exposed rats compared to control, while co-treatment with EGCG

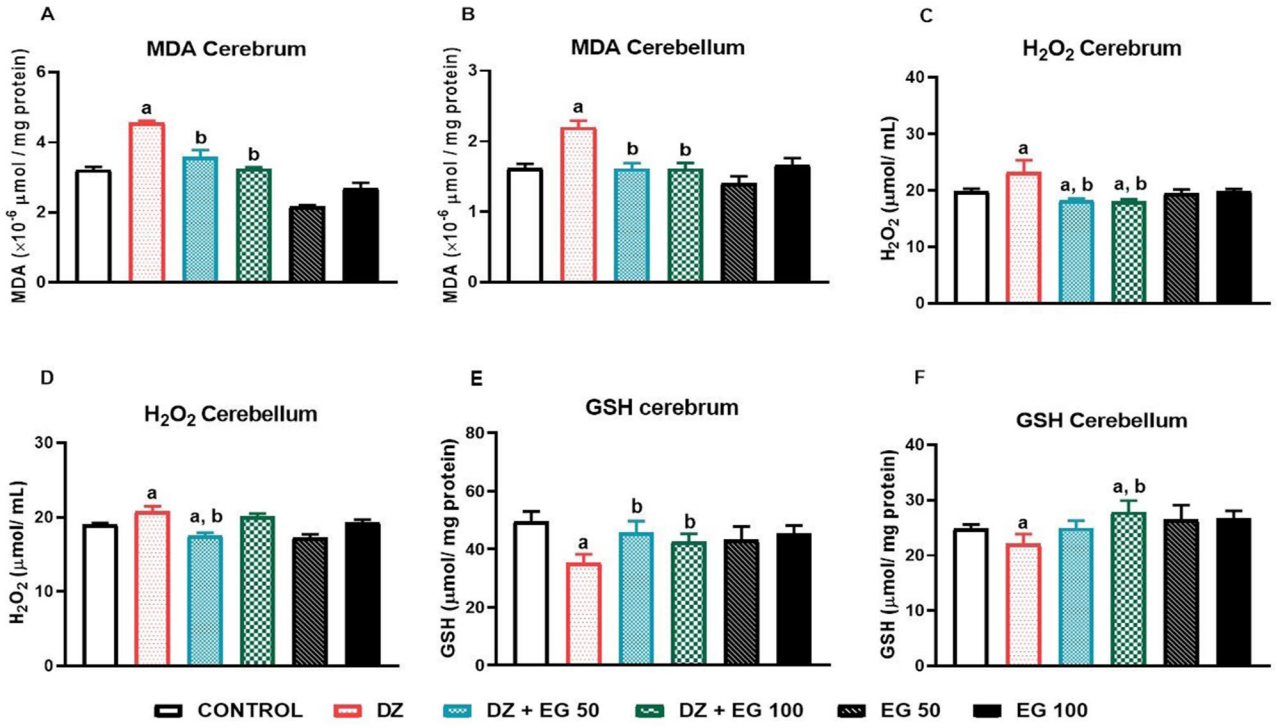

Fig. 4 MDA concentration (µmol/ mg protein) of rats treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Values are means ± SEM ($n = 10$). Alphabet "a" indicates significant difference when compared to the control at $P < 0.05$ while "b" indicates significant difference when compared to diazinon only (DZN) group at $P < 0.05$. Mean ± SEM ($n = 10$)

at 50 mg/kg and 100 mg/kg significantly increased GSH ($p < 0.05$) in the cerebrum and cerebellum respectively compared to the diazinon alone group, as shown in Fig. 4E-F.

The activity of GPx reduced significantly ($p < 0.05$) in the cerebrum and cerebellum of rats treated with diazinon alone compared to the untreated controls. However, in combination with EGCG at 50 mg/kg and/or 100 mg/kg, GPx activity improved significantly in the cerebrum and cerebellum at $p < 0.05$ (Fig. 5A-B). Treatment with diazinon alone significantly increased ($p < 0.05$) SOD activity in the cerebrum and cerebellum of rats relative to those in the control group. Interestingly, relative to the diazinon alone group, co-treatment with EGCG did not significantly reduce ($p > 0.05$) SOD activity in these brain regions to levels observed in the untreated controls (Fig. 5C-D). In both brain regions (cerebrum and cerebellum) GST enzyme activity did not show any significantly change in rats treated with diazinon alone compared to those in the untreated control group (Fig. 5E-F).

Effect on brain nitric oxide (NO) content and acetylcholinesterase (AChE) activity

Nitric oxide (NO) level was increased significantly ($P < 0.05$) in the cerebrum and cerebellum of rats treated with diazinon alone compared to the control group. Conversely, co-treatment with EGCG at 50 mg/kg and 100 mg/kg significantly lowered NO levels ($P < 0.05$) in these brain regions when compared to the diazinon alone group, as shown in Fig. 6A-B.

Cerebral and cerebellar AChE activity of was significantly increased in rats treated with diazinon alone ($P < 0.05$) compared to the untreated control. However, in both brain regions, co-treatment with EGCG at 100 mg/kg significantly lowered AChE activity ($P < 0.05$) relative to the diazinon alone group. Interestingly, EGCG alone inhibited the activity of AChE in the cerebrum (at both 50 mg/kg and 100 mg/kg) and cerebellum (at 100 mg/kg only) compared to the control, as shown in Fig. 6C-D.

Effect of treatment on gene expression for glutathione peroxidase 4 (GPX 4), Interleukin 6 (IL 6) and tumour necrosis factor alpha (TNF-α)

Exposure of rats to diazinon alone significantly suppressed ($p < 0.05$) GPx 4 enzyme gene expression and upregulated ($p < 0.05$) those of TNF-α and IL 6 relative to the untreated control group. Interestingly, compared to the diazinon alone group, co-treatment with EGCG significantly suppressed TNF-α and IL 6 expression ($p < 0.05$) in a dose dependent manner while GPx 4 expression was significantly upregulated at $p < 0.05$, as shown in Fig. 7A-C.

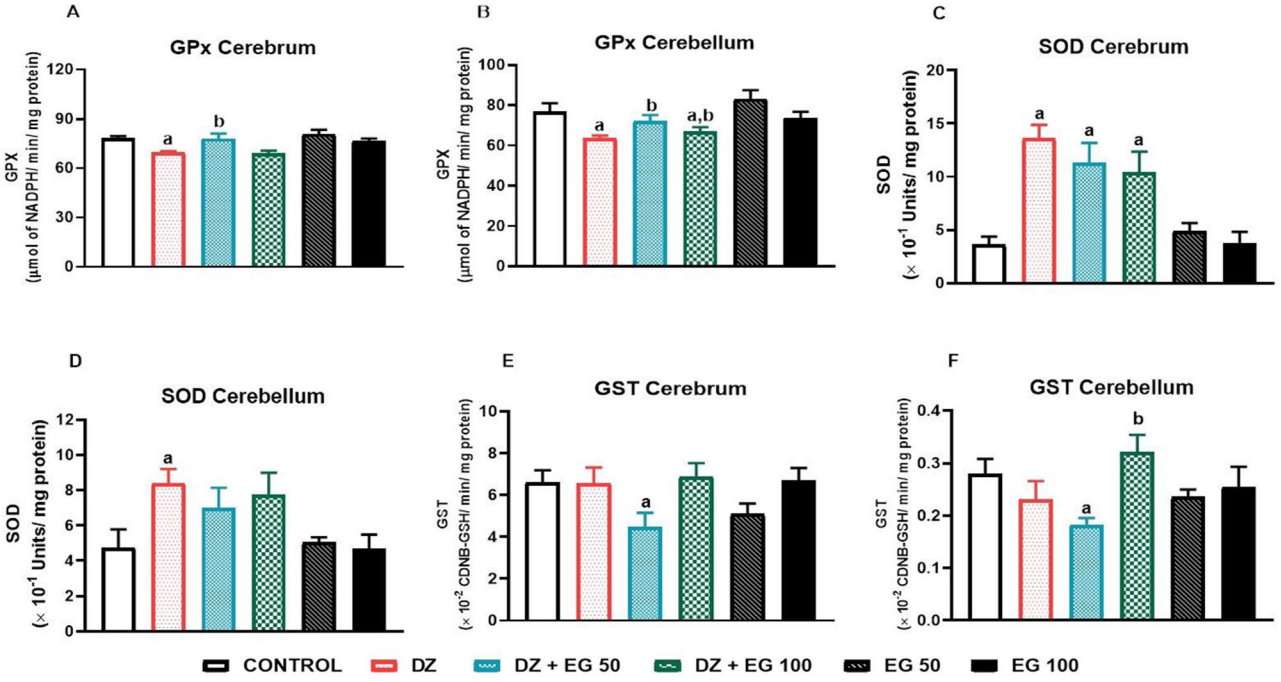

Fig. 5 GPX activity of rats treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Values are means ± SEM ($n = 10$). Alphabet "a" indicates significant difference when compared to the control at $P < 0.05$ while "b" indicates significant difference when compared to diazinon only (DZN) group at $P < 0.05$. Mean ± SEM ($n = 10$)

Effect of treatment on neurons, alpha synuclein, glial fibrillary associated protein and Tau protein expression

Cresyl-stained sections of the rats' cerebellum revealed Purkinje cells loss, and reduction in dendritic arborisation in DZN-treated rats. This neurodegeneration was rescued with EGCG administration (Fig. 8). The CA2 (Fig. 9) and CA3 (Fig. 10) hippocampal regions of rat brain treated with diazinon (DZN) alone showed relatively decreased cell number compared to the EGCG co-treated groups.

The cerebral cortical sections of the rats treated with diazinon revealed more immunoreactivity of α-synuclein protein compared to the control. This intensity was however moderated with fewer localizations of deeply stained sections following co-treatment with EGCG (Fig. 11).

Astrocytic (GFAP) immunostaining revealed an increase in astrocytic number and a more pronounced staining intensity especially in the cerebellar regions in rats exposed to diazinon alone, compared to the untreated controls. Co-treatment with EGCG also resulted in a less astrocytic activation relative to the diazinon alone group (Fig. 12).

There were more localized and deeply staining areas showing immunoreaction with tau proteins in rats exposed to diazinon alone compared to the control group. This intensity was however more moderated and diffused in rats co-treated with EGCG compared to the diazinon alone group (Fig. 13).

Discussion

Diazinon is a commonly used organophosphate (OP) insecticide in developing countries and exposure to its toxic impact especially in humans and other non-target species remains an important public health concern. It is well documented that oxidative stress coupled with significant alteration in overall endogenous antioxidant defence contributes to the toxicity caused by OPs. This study was set out to investigate the role of EGCG in ameliorating the toxic effects of diazinon in the brain of male Wistar rats.

The open field maze (OFM) test is a conventionally used technique in investigating the influence of various pharmacological and non-pharmacological compounds on neurobehavioural performance using small rodent models such as rats and mice [53–54]. This is based on parameters of the test that evaluate locomotor, emotionality and/or anxiety-related behaviour in these species [53]. Our study revealed that exposure of rats to diazinon alone significantly reduced ambulatory (locomotor) activity compared to the untreated control group. This may be indicative of increased anxiogenic tendencies in these rats. It can be argued that the significant dissimilarity in ambulation which is indicative of inactivity may confound the treatment effect, further spatial analysis of the open field data may be used to discriminate anxiety-related behaviour from ambulation. First, rats in the diazinon alone group generally explored the open field

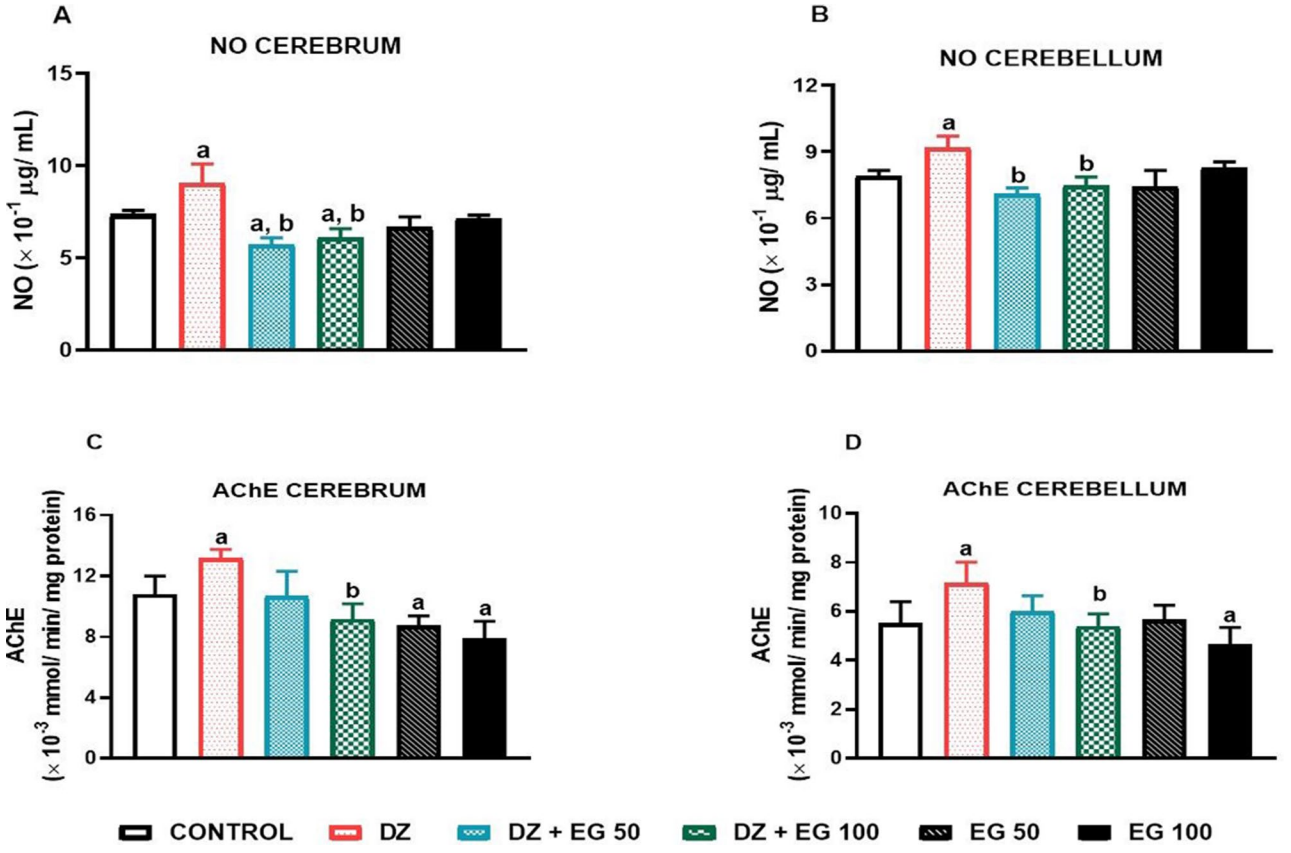

Fig. 6 Nitric oxide (NO) content and acetylcholinesterase (AChE) of rats treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Values are means ± SEM ($n = 10$). Alphabet "a" indicates significant difference when compared to the control at $P < 0.05$ while "b" indicates significant difference when compared to diazinon only (DZN) group at $P < 0.05$

maze to a significantly lesser degree as shown by the frequency of centre crossings in the OFM box compared to those in the control group. Furthermore, they demonstrated significantly increased thigmotaxic tendencies (movement along walls) evaluated by the significantly decreased frequency of entry and crossing of the centre square. Increased thigmotaxis correlates positively with increased levels of anxiety in rodents [54]. Second, rearing behaviour is considered an exploratory behaviour in rats and mice and is also used as a measure of anxiety in these species [55]. The significantly decreased rearing frequency in diazinon-exposed rats relative to those in the control group may also suggest increased levels of anxiety-related behaviour and/or emotionality in these animals [56]. Although, OFM results may be difficult to interpret, studies have indicated that rearing behaviour and ambulatory activity are reliable indices for emotionality in rats in a novel setting [57–58]. Third, OFM test results were similar to those obtained from the Elevated Plus Maze (EPM) test. This test is also used score emotionality in rats and is based on the balance between their avoidance of elevated and unprotected (open) spaces and their innate tendency to explore new areas [56]. The fact that diazinon alone-exposed rats stayed for longer duration in the closed areas and shorter duration in the open areas of the apparatus may also be indicative of increased display of emotionality and reduced exploratory tendencies. The number of fecal boli expelled by diazinon intoxicated rats (results not shown) was also significantly higher than those in the control group. Increased defecation was shown by [37] to correlate with increased levels of anxiety in rats. However, the validity of this measure as an index of anxiety-like behaviour has been mixed and still remains unclear. Taken together therefore, the decreased exploration of the entire open field space by diazinon alone treated rats may be attributed to increases inactivity, increased thigmotaxic behaviour or increased emotionality and/or anxiety-linked behaviour induced by diazinon. Increased thigmotaxic behaviour and reduced exploration of the open field maze should however be regarded as a preliminary test which should be supported with more specific anxiety-related tests. Dopaminergic receptors are known to be involved in the regulation of emotionality in rats. Enhanced anxiogenic behaviour in novelty may therefore be related to impaired dopaminergic activity in higher cortical brain centres.

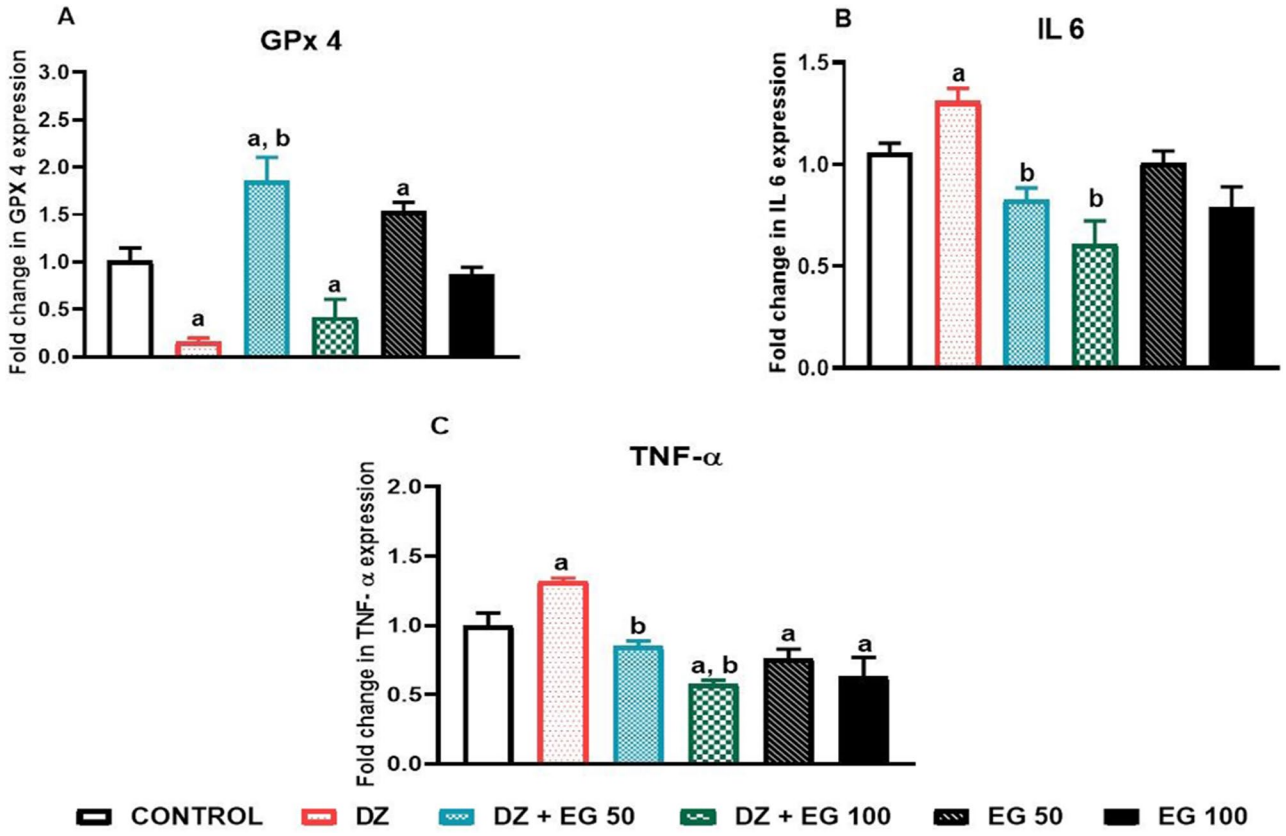

Fig. 7 GPx 4 gene expression level of rats treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Values are means ± SEM ($n = 10$). Alphabet "a" indicates significant difference when compared to the control at $P < 0.05$ while "b" indicates significant difference when compared to diazinon only (DZN) group at $P < 0.05$

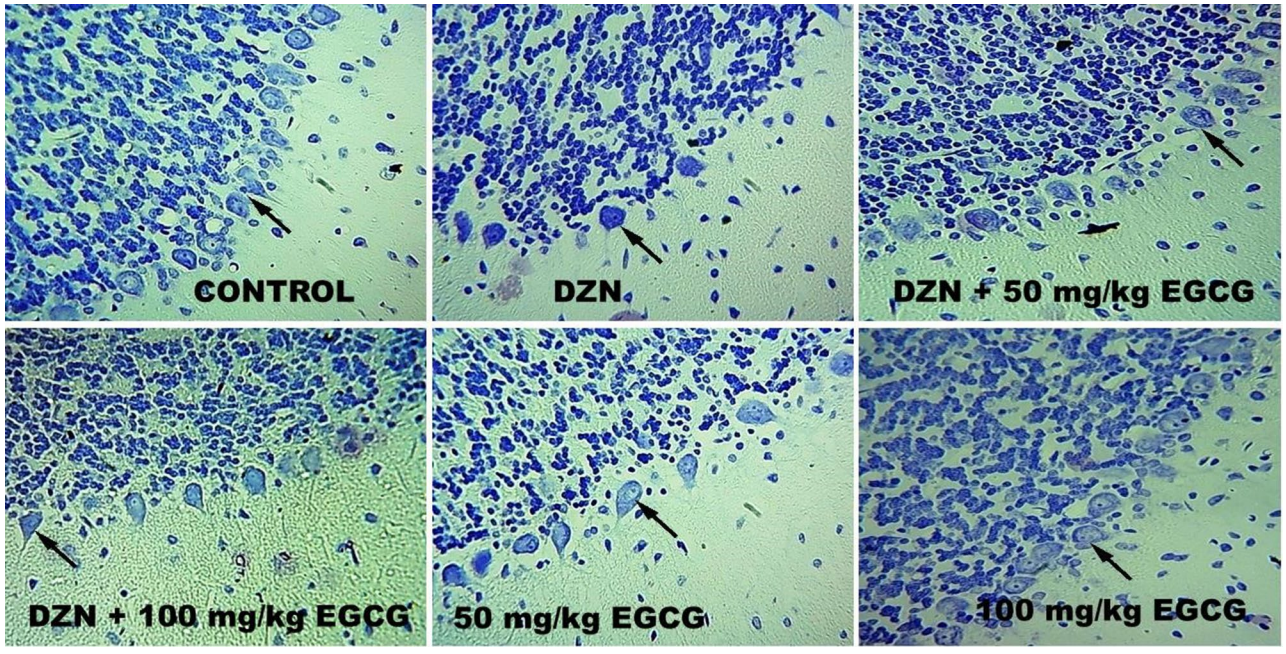

Fig. 8 Cresyl violet stain, cerebellum of rat brain treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Sections were stained with cresyl violet stain with bold arrows showing Purkinje cell death and loss of dendritic arborization in rats treated with DZN; this was rescued with EGCG administration. Scale bar– 50 µm

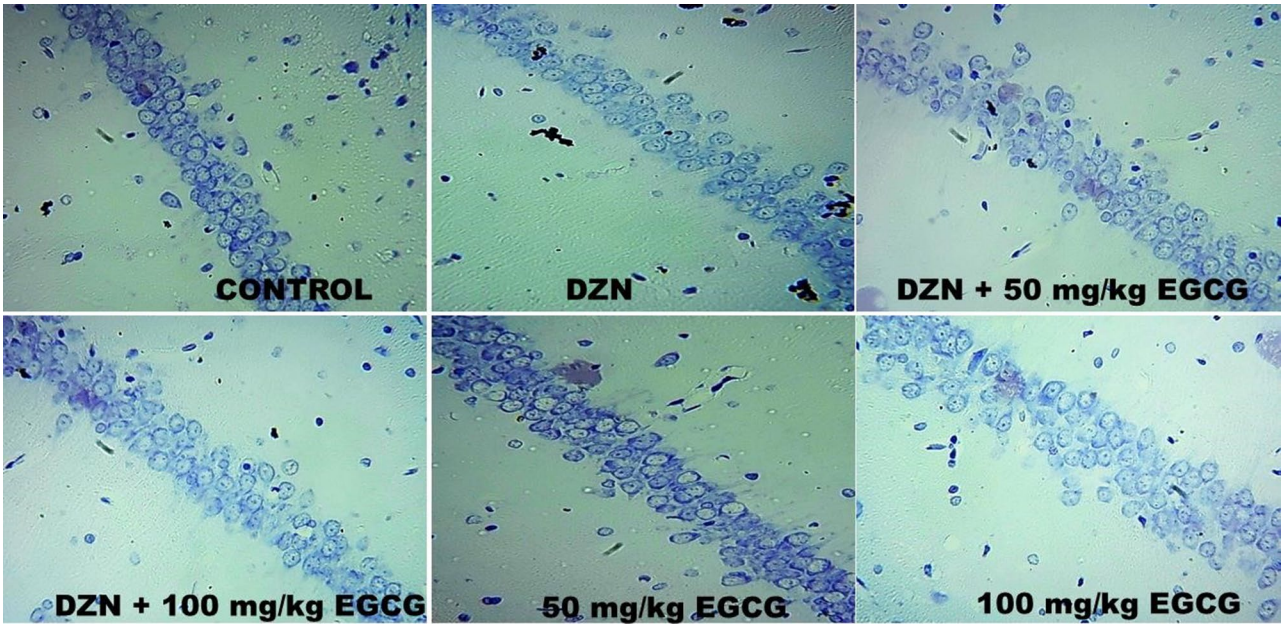

Fig. 9 Cresyl violet stain, hippocampal CA2 region of rat brain treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Note the neuronal cell loss observed with DZN administration, which was rescued with treatment with EGCG. Scale bar– 50 µm

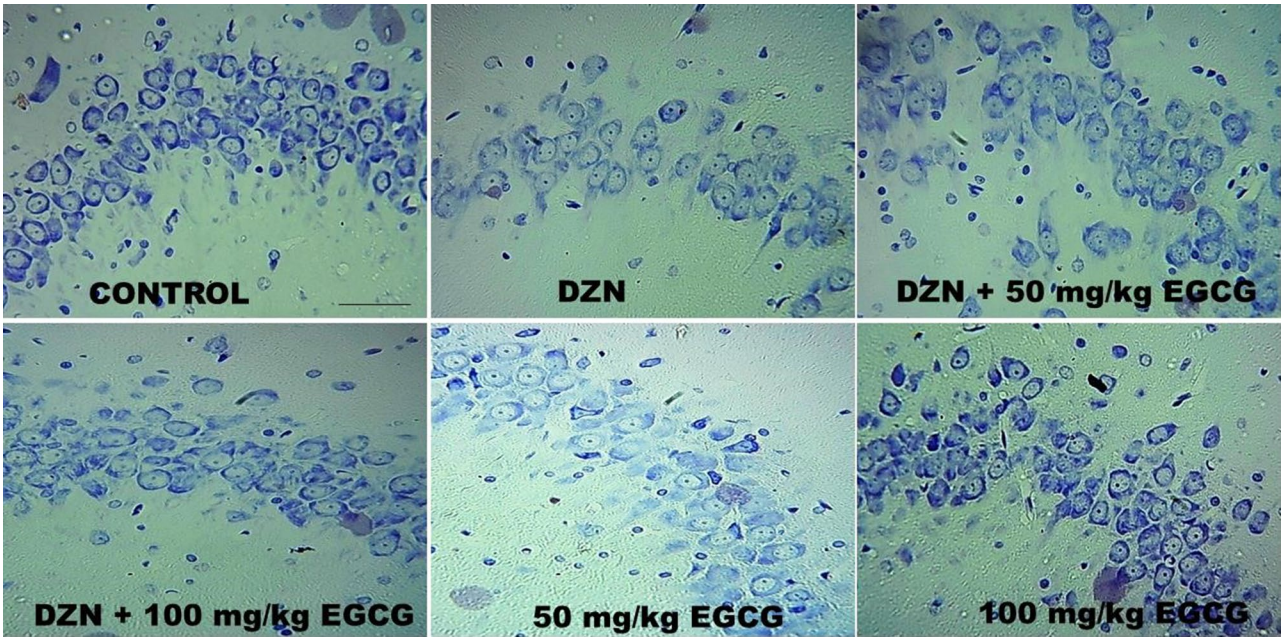

Fig. 10 Cresyl violet stain, hippocampal CA3 region of rat brain treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Note the severe neurodegeneration observed with DZN administration and the mitigation due DZN administration. Scale bar– 50 µm

This dysfunction in dopaminergic neuron activity may be induced via interaction with D1 and D2 receptors or following increased loss of dopaminergic neurons in specific regions of the brain [14, 59–60]. Whether diazinon or its breakdown metabolic products directly interact with dopaminergic receptors is unclear. However, studies show that organophosphate pesticide intoxication in rats is associated with increased generation of reactive oxygen species (ROS), inflammation and increased expression of proapoptotic markers in rat brain [61–63]. These results improved in the co-treated rats and those exposed to EGCG alone at 50 mg/kg suggesting a role of EGCG in decreasing emotionality and improving exploratory behaviour in rats. Recognition memory in diazinon alone-exposed rats was severely impaired. This was however improved following co-treatment with 100 mg/ kg of

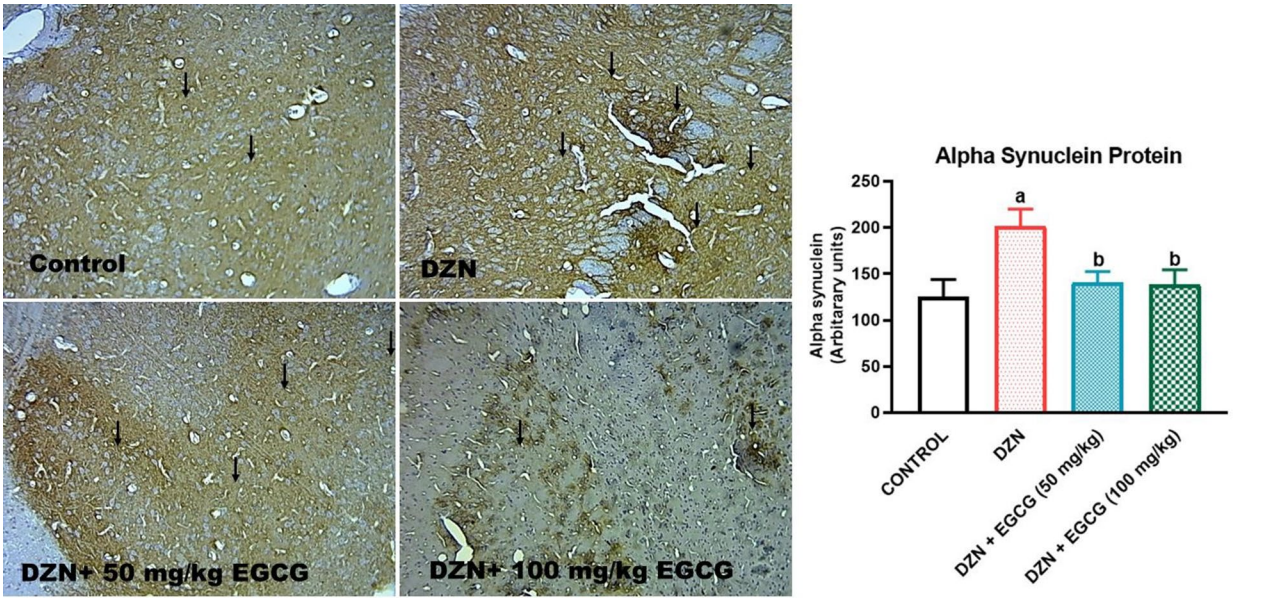

Fig. 11 Immunohistochemical photomicrograph of α-synuclein protein expression in rat hippocampus (CA1) treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Slides were stained with high-definition Haematoxylin with bold arrows showing α- synuclein protein. Scale bar– 200 µm

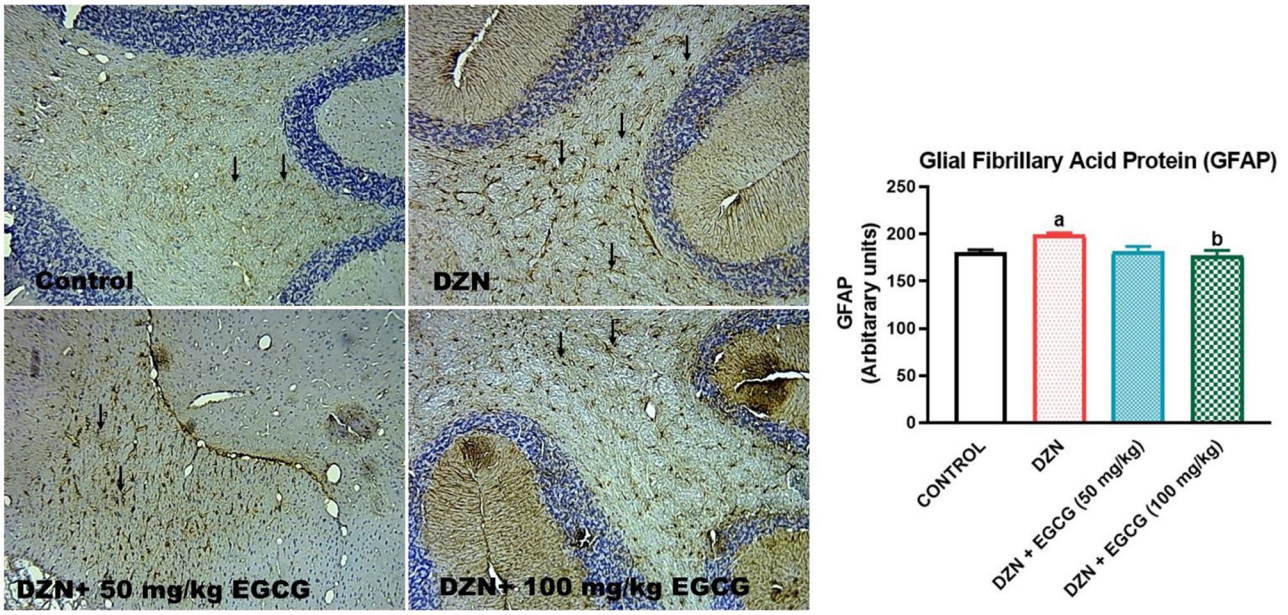

Fig. 12 Immunohistochemical photomicrograph of glial fibrillary acidic protein (GFAP) expression in cerebellum treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Slides were stained with high-definition Haematoxylin with bold arrows showing GFAP. Scale bar– 200 µm

EGCG and EGCG alone at 50 mg/kg. Neurobehavioural deficit following organophosphate pesticide intoxication have been reported in other studies (16, 64–65). Chlorpyrifos, a commonly used OP was reported by [64–65] to induce oxidative stress; enhance anxiety in rats and impair neuro-cognitive function. Delavar et al. [16] had previously demonstrated that diazinon exposure in male Wistar rats impaired spatial memory. Hawkey et al. [14] reported that developmental exposure of rats to diazinon resulted in neurochemical alteration with progressive neurobehavioural deficit which persisted into adulthood. Diazinon exposure in rats was also reported to impaired learning and memory [66]. The improved cognitive function in rats co-treated with EGCG or EGCG alone may be related to its ability to improve emotionality, enhance antioxidant defence against oxidative damage and/or modulate neurochemical homeostasis in brain regions involved with cognitive functions [14, 64–66]. In our

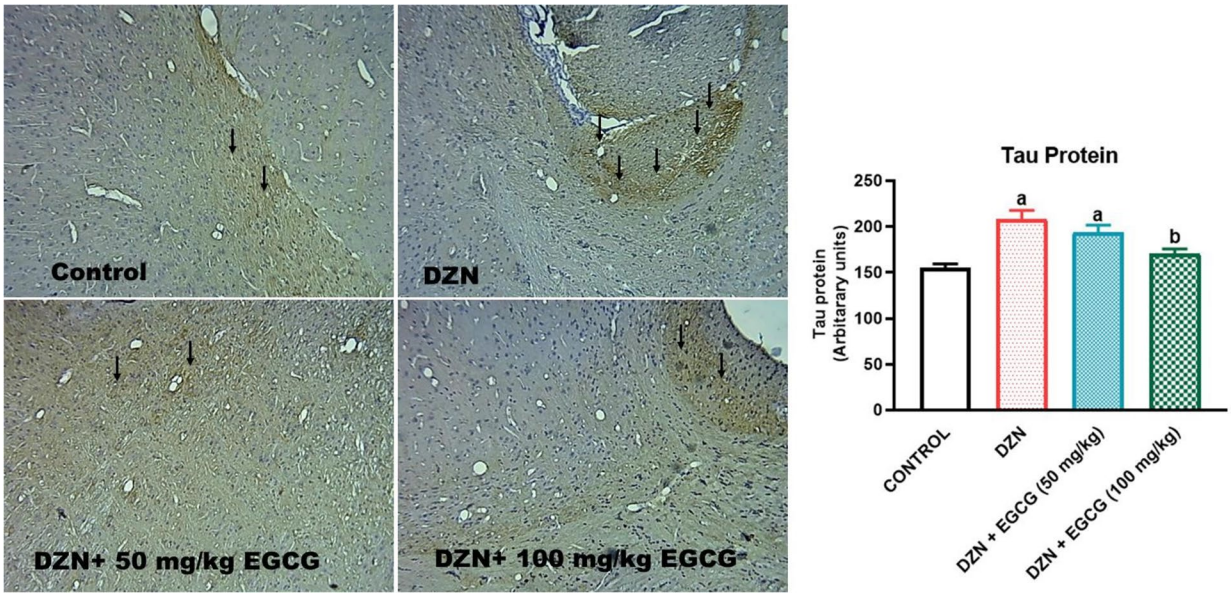

Fig. 13 Immunohistochemical photomicrograph of tau protein expression in rat brain treated with diazinon (DZN) and/or epigallocatechin -3- gallate (EGCG) for 14 days. Slides were stained with high-definition Haematoxylin, with bold arrows showing tau proteins. Scale bar– 500 µm

study, the inhibition of AChE by EGCG may have contributed to enhanced levels of the neurotransmitter, acetylcholine, known to facilitate neuronal communication. The link between activity of cholinergic neurons and recognition memory is still being explored [66]. The inability of EGCG either alone or in combination with diazinon to improve muscular strength to the level observed in the control group may indicate impairment in neuromuscular integrity. Both diazinon and EGCG have been documented to interfere with AChE activity [66].

Diazinon alone increased oxidative stress (OS) makers and altered antioxidant defense in the brain. A conventionally used OS markers namely: MDA, a stable molecular product of lipid oxidation (or peroxidation); and hydrogen peroxide (H_2O_2), a ROS were both elevated following treatment with diazinon alone when compared to the untreated control group. This increase is suggestive of increased ROS generation in the brain. The oxidative metabolism of xenobiotics (including diazinon) by enzymes of the cytochrome P450 monooxygenase system and other oxidoreductases (such as NADPH oxidases or NOXs) have been reported to be accompanied by increased generation of reactive oxygen and nitrogen species in the cell [67–68]. Overproduction of these highly reactive electrophilic species may overwhelm the body's antioxidant defence capacity resulting in a net shift in redox homeostasis in the towards a prooxidative state. More importantly however is that reactive radical species may begin to attack susceptible cellular biomolecules including lipids, proteins and nucleic acid required for cellular stability and survival [69]. Among the most susceptible lipid fractions are the polyunsaturated fatty acids which undergo increases peroxidative damage yielding a variety of stable oxidation by-products which can be quantified spectrophotometrically [70]. Neuronal cells are richly endowed with PUFAs as such may be selectively susceptible to lipoperoxidative damage by ROS following impairment in antioxidant defense. Rats in the diazinon alone group also showed significantly increased SOD activity, relatively normal GST activity and at least in one brain region, depletion of GSH and/or decreased GPx activity. Studies that show OPs mediate their neurotoxic actions in part by inducing OS and impairing antioxidant defenses have been reported [71]. The depletion of GPx and its cofactor GSH may indicate increased utilization for peroxide decomposition into less toxic forms that is more easily eliminated [72]. SODs primarily convert superoxides to H_2O_2 and may be considered as a first line of defence against increased superoxide radical production [73]. The observed increase in their activity may therefore be suggestive of some kind of ROS-mediated enzyme induction. GSTs are an important family of phase two detoxifying enzyme and play a cytoprotective role in cells under chemical stress via enhanced adaptive response mechanisms [74]. SODs and GSTs are components of the adaptive response system that enables the body cope with stress induced by environmental pollutants [75–77]. Increase in SOD activity in Wistar rats exposed to permethrin, a pyrethroid pesticide compound has been earlier reported by [78]. Non-chemical stress in wistar rats have also been reported to induce SOD activity [78]. The significant increase in the activities of SOD, GPx, and GST in group intoxicated with diazinon could be attributed with adaptive response which has been

extensively reported in literature elsewhere [79–80]. The lowering of OS and improvement in antioxidant defence in rats co-treated with EGCG is therefore suggestive its antioxidant role.

Nitric oxide (NO) level in rats exposed to diazinon alone was significantly elevated in the cerebrum and cerebellum, the brain region generally involved in cognition, memory and learning, muscle activity and other motor functions. This may indicate increased levels of oxidative stress in these brain areas. Nitric oxide is an important physiological molecule that facilitates neuronal communication and function in the brain [81]. However, conditions that induce uncontrolled production of this free radical may result in the promotion of its neurotoxic effect via the promotion of oxidative stress, apoptosis and neuronal damage [82–83]. In the presence of the superoxide radical, NO is oxidized to peroxynitrite anion ($ONOO^-$), a potent apoptotic molecule [82]. The lowering of NO levels in these brain regions following co-treatment of rats with EGCG is suggestive of its ability to offer neuroprotection by reducing oxidative stress and improve NO metabolism.

Our study also revealed that repeated exposure of rats to diazinon alone enhanced that activity of AChE in the cerebrum and cerebellum. This increased enzyme activity may be due to increased rate of spontaneous reactivation of phosphorylated (inhibited) enzyme [84]. Generally, organophosphate (OP) compounds are known cholinesterase inhibitors; however, the toxicity of individual OP compounds also depends on the exposure doses and stability of the inhibited (phosphorylated) enzyme. The ability of EGCG both in combination with diazinon or alone to decrease the activity of AChE may suggest its role in improving brain acetylcholine levels and the activity of cholinergic neurons [27, 66].

Intoxication with diazinon alone suppressed expression of glutathione peroxidase 4 (GPx 4), a selenoprotein antioxidant molecule involved in the neutralization of peroxides [85–86]. Furthermore, diazinon alone exposure in rats increased expression of IL-6, a pro-inflammatory cytokine and potent mediator of inflammation [87]. TNF-α expression was also increased in diazinon alone exposed rats. TNF-α is considered as a multi-functional cytokine that is involved in cellular immunity [88–89]. At low levels this protein plays a beneficial role in activating host defense response. However, overproduction may induce systemic inflammatory response and wide spread tissue damage [89]. Taken together, diazinon exposure impairs brain antioxidant defense and contributes to the development of a state of neuroinflammation. Co-administration of EGCG enhanced GPx 4 expression and repressed IL 6 and TNF-α expression which is suggesting its role in the activation of oxidative stress-related genes (such as GPx 4), anti-inflammatory and immunomodulatory effects.

Intracellular proteins facilitate important cellular processes and provide structural support within the cell. Their visualization, analysis, and quantification in the cell under specific conditions may provide invaluable insight into their function and more crucially, any underlying physiological and/or pathologic process occurring within the cell. In this study, results from immunohistochemical staining for specific proteins in the brain revealed the following:

The increased deposition of alpha synuclein (α-SN) in the brain of diazinon alone treated rats over those observed in the control group and their subsequent reduction following co-treatment with EGCG may be indicative of increased expression of α-SN in the brain. α-SN is a soluble unfolded motor protein that may be thought to be associated with the cytoskeletal network of the cell and facilitates and/or regulate vesicular transport within the cell, exocytosis (especially of neurotransmitters) and endocytosis [90]. Increased insoluble aggregate of α-SN are associated with many neurodegenerative conditions such as Parkinson's disease and other dementia-like diseases or synucleinopathies [91–93]. Neurodegeneration involves progressive loss of neurons in the brain [94]. α-synuclein, a protein critically involved in Parkinson disease. We hypothesized that exposure to organophosphates such as diazinon could precipitate Parkinson-like disease as indicated with loss of muscular strength [93, 95].

Conditions such as changes in α-SN gene function due to mutation or altered gene expression, accumulation of cytotoxic substance or even aging those results in excessive, misformed or misfolding and aggregation of α-SN may result in impaired cytoskeletal function, loss of neurotransmitter release, and neuronal death [90–92, 95]. Modulation of increased α-SN immunodeposition by EGCG co-treated rats may be suggestive of its neuroprotective effect.

Immune reactivity to glial fibrillary acidic protein (GFAP) in diazinon alone treated rats was higher than in untreated controls while co-treatment with EGCG resulted in lowering of GFAP synthesis. GFAP is a protein involved with the cytoskeletal support system in the cell and the regulation of specific neurotransmitter necessary for proper brain function [96]. It is a relevant astrocytic marker in the brain. High levels of this protein in brain may be indicative of increased astrocyte activation [96]. Astrocytes are important neuroglial or supporting cells with multifunctional role in the CNS as well as participation in immune responses [97]. In the presence of injury and/or brain damage, astrocytes have been reported to increase the expression of GFAP [98]. Amelioration of GFAP reactivity in the astrocytes of co-treated rats with

EGCG may indicate this antioxidant's role in immunomodulation and neuroprotection.

The deposition and/or expression of tau protein were higher in diazinon alone intoxicated rats compared to the untreated controls. This may be indicative of neurotoxicity. Tau is an "intrinsically disordered" (or "natively unfolded") protein that plays a role in the stabilization of tubulin sub units in neurons [99]. This microtubulary system is necessary for the cell division and translocation of substances within the neuron [100–101]. Studies show that to be active, tau requires phosphorylation by specific kinases [102]. However, mutational modification of tau genes, oxidative stress, neuronal injury and/or neuroinflammation may activate kinases resulting in the hyperphosphorylation of tau [102–104]. Hyperphosphorylated tau is susceptible to misfolding, oligomerization, and aggregation [103]. Aggregation of tau into neurofibrillary structures results in loss of function and is resistant to proteosomal processing and degradation [102–103]. The loss of microtubule integrity results in their catastrophic collapse, neuronal death, and progressive neurodegeneration. Increased deposition of tau has been associated with learning and memory deficit and impaired cognitive function [103–104]. Fewer depositions of tau proteins following co-treatment with EGCG may be indicative of its neuroprotective effect.

Microscopic examination of the brain of rats treated with diazinon alone, revealed decrease in Purkinje and hippocampal cells. This may be suggestive of increased neuronal loss in these brain regions. Increased levels of proapoptotic markers, neuronal damage and neuroinflammation have been reported following exposure of rats to OPs [62–63]. The increased neuronal cell count in these brain regions of rats co-treated with EGCG may suggest a neuroprotective role of this compound via the inhibition of neuronal apoptotic loss.

Conclusion

This study has demonstrated that repeated low dose exposure of rats to diazinon is associated with toxicity in the brain by inducing OS, impairing antioxidant defence, suppressing GPx 4 gene expression, promoting inflammatory cytokines release and altering the levels of relevant tissue proteins involved in facilitating various cellular processes in these tissues. At the same time, EGCG, a flavanol and abundant in green tea ameliorated and, in some instances, completely reversed the toxic effect of diazinon in these tissues by employing complex mechanisms including lowering oxidative stress, improving antioxidant defence, upregulating GPx 4 expressions, and decreasing inflammation. The cytoprotective effects of EGCG at 50 mg/ kg was comparable to those produced at 100 mg/ kg in nearly all instances which is indicative of the potency (or efficacy) and safety of EGCG as an antioxidant.

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Author contributions
Conceptualization– Ademola Oyagbemi, Omobowale, Ajibade; Animal experiments– Onukak, Obasa, Adeogun; Histological and biochemical analyses– Onukak, Igado, Obasa, Femi-Akinlosotu, Ademola Oyagbemi, Ajibade, Omobowale, Folarin, Esan, Taiwo Oyagbemi, Oguntibeju, Yusuf, Yakubu, Ola-Davies, Olopade; Data analysis and interpretation– Ademola Oyagbemi, Obasa, Igado, Femi-Akinlosotu, Omobowale; Initial manuscript draft– Onukak, Ademola Oyagbemi, Igado; manuscript editing, review and final version– Onukak, Igado, Obasa, Femi-Akinlosotu, Ademola Oyagbemi, Ajibade, Omobowale, Folarin, Esan, Taiwo Oyagbemi, Oguntibeju, Yusuf, Yakubu, Ola-Davies, Olopade; Project supervision– Omobowale, Ademola Oyagbemi, Ajibade.

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Data availability
Data is provided within the manuscript or supplementary information files and is available on reasonable request from corresponding author.

Declarations

Ethical approval and consent to participate
Ethical approval for the study was obtained from the University of Ibadan, Animal Care and Use Research Committee (UI-ACUREC) with approval number UI-ACUREC/059–0324/28.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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