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Protocatechuic acid ameliorates neurobehavioral deficits via suppression of oxidative damage, inflammation, caspase-3 and acetylcholinesterase activities in diabetic rats

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ABSTRACT

Clinical and experimental data have demonstrated that diabetes is associated with neurological complications. Protocatechuic acid (PCA) is a phenolic phytochemical widely reported to possess antidiabetic property. However, there is no scientific information on the influence of PCA on diabetes-induced neurotoxicity. The present study aimed at investigating the neuroprotective mechanism of PCA in streptozotozin (STZ)-induced type 1 diabetic rats orally treated with PCA (50 mg/kg body weight) or glibenclamide (5 mg/kg body weight) for 45 consecutive days. Locomotor behavior was analyzed using video-tracking software during the 8-min trial in a novel environment whereas the pancreas, cerebrum and cerebellum of the rats were processed for biochemical analyses. Results showed that treatment of diabetic rats with PCA at 50 mg/kg significantly ($p < 0.05$) improved the locomotor and motor activities including the average speed, total time mobile, distance travelled, body rotation, turn angle, forelimb grip and grooming when compared with untreated diabetic rats. Moreover, the prevention of diabetes-mediated increase in acetylcholinesterase activity, biomarkers of inflammatory and oxidative stress as well as caspase 3 activity by PCA treatment was accompanied by improved pancreatic, cerebral and cerebellar architectures. Collectively, the neuroprotective mechanisms of PCA is related to its antioxidant, anti-inflammatory and anti-apoptotic activities.

1. Introduction

Diabetes is a non-communicable metabolic disorder which is characterized by hyperglycemia due to defective insulin secretion, resistance to insulin action or both (Samad et al., 2009; Amutha and Mohan, 2016). Epidemiological data by the International Diabetes Federation showed that about 387 million people are suffering from diabetes while the number was predicted to increase to 590 million by 2035 (Guariguata et al., 2014). Diabetes mellitus is one of the five leading risk factors for death globally (Ritchie and Roser, 2018). Besides, clinical and experimental data have demonstrated a positive correlation between diabetes and neurological complications in both peripheral and central nervous system (CNS) (Manschot et al., 2008; Erbaş et al., 2016; Bădescu et al., 2016).

It is well known that diabetes-related alterations of the CNS increase the risk of neurobehavioral disorders such as cognition, depression, anxiety and locomotor disturbances (Kodl and Seaquist, 2008; Patel and Udayabanu, 2017). Several studies indicated that the prevalence of anxiety disorder is about 60% among diabetic individuals when compared to non-diabetic population (Maia et al., 2014; Castellano-Guerrero et al., 2018; Purewal and Fisher, 2018). Anxiety comorbid with diabetes reportedly worsen quality of life, glycemic control and increased morbidity and mortality rates (Huang et al., 2011; Maia et al., 2014). The adverse effects of diabetes on the CNS has been associated with a series of neurochemical, neurophysiological and structural abnormalities (Vukojević et al., 2014; Jawale et al., 2016). Specifically, clinical epidemiological studies evidenced that epilepsy (Dafoulas et al., 2017), psychomotor retardation (Li et al., 2017; Nunley et al., 2017), ischaemic and haemorrhagic stroke (Ståhl et al., 2017) are common CNS complications associated with type 1 diabetes. Moreover, it is well known that type 1 diabetes adversely impact both somatic and autonomic nerves resulting in motor dysfunction (Ziegler et al., 1988; Andersen, 2012) which is associated with locomotor impairments namely altered gait and balance, increased risk of falling and body sway (Petrofsky et al., 2005; Orlando et al., 2017).

Oxidative stress has been established to play a fundamental role in brain and neuronal damage in both clinical and experimental diabetes (Martín-Gallán et al., 2007; Hoffman et al., 2011). The neurotoxic

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effects of hyperglycemia are mediated through an increased reactive oxygen species (ROS) production during glucose autooxidation (Van Dam and Bravenboer, 1997). Elevated ROS production results in lipid peroxidation, oxidation of proteins and DNA damage which consequently contribute to neuronal death (Kahya et al., 2017). Moreover, neuroinflamation and alterations in glutamatergic neurotransmission have also been associated with neurotoxic effects of hyperglycemia (Li et al., 2014; Zhou et al., 2017). The effectiveness of the currently available anti-diabetes drugs during prolonged therapy has been associated with adverse side effects. The assessment of the chemoprotective role of natural compounds in this pathology is now a high priority in biomedical research. Hence, potent, but non-toxic, anti-diabetic phytochemicals are the main scientific interest in order to reduce the economic and clinical burden of diabetes (Dragan et al., 2015; Rehman and Akash, 2016; Solayman et al., 2016).

Protocatechuic acid (PCA) otherwise known as 3, 4-dihydroxybenzoic acid is a phenolic compound widely present in many edible fruits, vegetables, wines and plant-derived beverages. Besides, diets rich in PCA reportedly elicited several beneficial health effects in humans (Masella et al., 2012). PCA was reported to elicit anti-hyperglycemic effect by decreasing gluconeogenic enzymes activities while increasing glycolytic enzyme glucokinase activity in the liver of STZ-induced diabetic rats (Harini and Pugalendi, 2010a). It ameliorated oxidative stress, hyperlipidaemia and restored vascular responses in STZ-induced diabetic rats (Harini and Pugalendi, 2010b; Semaming et al., 2016). Recently, PCA reportedly elicited antioxidative burst activity on polymorphonuclear neutrophils and macrophages as well as anti-proliferative activity on T-cells (Erukainure et al., 2017). PCA has also been reported to suppress diabetic cardiomyopathy in rats (Bhattacharjee et al., 2017). Indeed, PCA has been suggested to be a good chemotherapeutic agent for oxidative stress-induced neurodegenerative diseases including Parkinson's disease (Zhang et al., 2015; Krzysztoforska et al., 2017). Hitherto, there is no scientific information on the influence of PCA on diabetes-induced neurotoxicity in rats.

Streptozotocin (STZ)-induced type 1 diabetic rat is a useful animal model to determine the underlying cause of CNS complications. The model is known to exhibit pathological features that resemble diabetes in humans. Indeed, diabetogenic agent STZ induces specific necrosis of pancreatic beta cells leading to insulin dependent diabetes (Lenzen, 2008). STZ is known to cause elevation in the intracellular ROS, proinflammatory mediators and redox imbalance in experimental animals (Yonguc et al., 2015; Tian et al., 2016). The present study aimed at investigating the role of PCA in diabetic-induced neurotoxicity by assessing some locomotor activities and exploratory profiles in experimental rats using a standard behavioral protocol (Antunes et al., 2011; Quines et al., 2014; Zimcikova et al., 2017; Adedara et al., 2017) and a video-tracking software. Moreover, in order to further understand the neuroprotective mechanisms of PCA in STZ-induced type 1 diabetic rats, evaluation of oxidative stress and inflammatory indices in conjunction with acetylcholinesterase (AChE), antioxidant enzymes and caspase-3 activities were analyzed in the pancreas, cerebrum and cerebellum of the treated rats for the first time.

2. Materials and methods

2.1. Chemicals

Streptozotocin (STZ), protocatechuic acid (PCA, ≥97%), 5′, 5′-dithiobis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), 1-chloro-2,4-dinitrobenzene (CDNB), hydrogen peroxide epinephrine, glutathione (GSH) and trichloroacetic acid (TCA) were procured from Sigma Chemical Co. (St Louis, MO, USA). All other reagents were of analytical grade and were purchased from the British Drug Houses (Poole, Dorset, UK).

2.2. Animal model

Sixty male rats of Wistar strain (6 weeks old, 140 ± 8 g) obtained from the Faculty of Veterinary Medicine, University of Ibadan, Ibadan were used for this study. The animals (10 rats per cage) were housed in plastic cages (50 × 36 × 28 cm) placed in a well-ventilated vivarium, provided rat chow and water *ad libitum* and subjected to natural photoperiod of 12 h light/dark. The rats were acclimatized for one week before the commencement of the experiment. Sufficient quantity of wood shavings which cover the whole floor was provided as bedding. Animal care and experimental protocols were performed in accordance with the approved guidelines set by the University of Ibadan Ethical Committee, which is in agreement with the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Science (NAS) and published by the National Institute of Health.

2.3. Induction of experimental diabetes in rats

The induction of experimental diabetes was achieved with a single intraperitoneal administration of STZ (60 mg/kg) in a freshly prepared citrate buffer (0.1 M, pH 4.5) to sixty rats following an overnight fast. Ten normoglycemic control rats were injected with citrate buffer alone. The induction of diabetes was verified 72 h after STZ injection by measuring the blood glucose level in a drop of blood from the tail vein of the rats using test strips impregnated with glucose oxidase (Accu-Check Active™, Roche Diagnostics GmbH, Mannheim, Germany). The fifty rats with fasting blood glucose levels higher than 250 mg/dL were considered as diabetic and selected for the study. The 7-week old rats were injected with STZ in the present study in order to mimic the real-life youth-onset Type 1 diabetes (Costacou et al., 2018; Campbell et al., 2018; Reynolds et al., 2018).

2.4. Experimental protocol

The study consisted of five groups of ten rats each. The schematic diagram of the study paradigm showing the experimental groups, 45 days of treatment period with PCA or glibenclamide as well as the neurobehavioral and the biochemical analysis performed in this study are represented in Fig. 1. Stock solution of PCA and glibenclamide (100 mg/mL) was prepared fresh every other day using normal saline as a vehicle. The doses of PCA and glibenclamide used in the present study were chosen based on the results from the pilot study in our laboratory and previously published data (Boudjelal et al., 2015; Semaming et al., 2016; Adedara et al., 2018).

2.5. Behavioral measurements in a novel environment

Twenty-four hours after the last treatment, the behavioral tests were performed between 10:00 a.m. and 4:00 p.m. in a novel apparatus (wooden box of 56 cm width x 56 cm length x 20 cm height) according to established protocol (Adedara et al., 2017). Briefly, the rats from each group were randomly selected, placed in the center of the apparatus and allowed to freely explore the arena. The behavior of the rats was captured throughout the 8-min trial using a webcam (DNE webcam, Porto Alegre, Brazil) positioned directly above the apparatus and connected to a laptop computer. Subsequently, the video movies of the rats were analyzed and the behavioral parameters automatically computed at a rate of 30 frames per second using suitable video-tracking software (ANY-maze, Stoelting Co, USA). The apparatus was carefully cleansed with cotton wool soaked in 70% ethanol at the end of each test.

2.5.1. Neurobehavioral and locomotor activity assessment

Locomotor, motor and exploratory activities were evaluated in the novel environment with the aim of reflecting habituation to novelty stress. The total distance travelled, average speed, total time mobile, body rotation, grooming time and absolute turn angle were used to measure locomotor and motor patterns. The exploratory activities of the rats in the novel environment were evaluated using representative

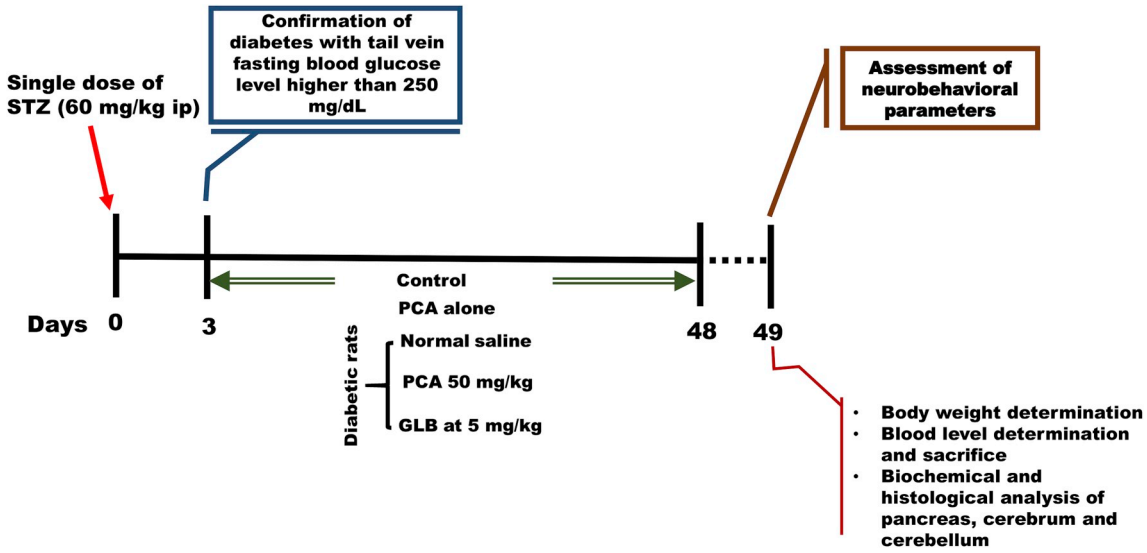

Fig. 1. Schematic diagram of the protocol showing the experimental groups, treatment period with PCA or glibenclamide as well as the neurobehavioral and the biochemical endpoints assessed.

track plots automatically produced by the video-tracking software (ANY-maze, Stoelting Co, USA). Fear/anxiety-related behavior was determined by counting fecal pellets (Quines et al., 2014; Zimcikova et al., 2017; Adedara et al., 2017). Briefly, the rats from each group were randomly selected, placed in the center of the novel apparatus and allowed to roam freely without interference for 8 min. Subsequently, the total number of fecal pellets in the area were counted and recorded. The apparatus was cleaned using cotton wool and 70% alcohol at the end of each trial.

2.6. Forelimb grip test

The rats were suspended with their forepaws on a horizontal, 4-mm-diameter metal rope, stretched horizontally 40 cm over a foam pad. The time taken for the animals to fall off the rope was video captured and recorded (Darbra et al., 2003; van Wijk et al., 2008; Folarin et al., 2016).

2.7. Biochemical estimations

2.7.1. Neurotoxicity and oxidative stress biomarkers

Following behavioral assessment, the final body weights of the rats were taken before they were sacrificed by cervical dislocation. The pancreas was carefully excised and the cranium opened to get the cerebrum and cerebellum of the rats. Subsequently, the pancreas, cerebrum and cerebellum were homogenized in eight volumes of 50 mM Tris–HCl buffer (pH 7.4) containing 1.15% potassium chloride. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4 °C and the resulting supernatant used for biochemical estimations. The protein concentration of the supernatant was assessed according to the method described by Bradford (1976). Acetylcholinesterase (AChE) activity was assessed according to the method described by Ellman et al. (1961). Superoxide dismutase (SOD) activity was assayed according to the method described by Misra and Fridovich (1972), catalase (CAT) activity according to Clairborne (1995), glutathione peroxidase (GPx) activity according to Rotruck et al. (1973) whereas glutathione-S-transferase (GST) activity was assayed according to Habig et al. (1974). Moreover, glutathione (GSH) level was assayed according to Jollow et al. (1974), hydrogen peroxide generation according to Wolff (1994) whereas lipid peroxidation (LPO) was assayed according to Farombi et al. (2000) with slight modification. Activities of SOD and CAT were measured using 752S UV-VIS Spectrophotometer (Ningbo, China) whereas all other assays were performed with the aid of a SpectraMax plate reader (Molecular Devices, CA, USA).

2.7.2. Assessment of pro-inflammatory biomarkers and caspase-3 activity in pancreas, cerebrum and cerebellum

Nitric oxide (NO) level was assayed according to Green et al. (1982). Myeloperoxidase (MPO) activity was assayed according to Granell et al. (2003). Assessment of the tumour necrosis factor alpha (TNF-α) level was determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer recommendations (ABCAM PLC, UK). Caspase-3 activity was evaluated using commercially available Rat CASP3 (Caspase 3) ELISA Kit (Elabscience Biotechnology Company, Beijing, China) in accordance with the procedure described in the assay manual. The optical density was measured at a wavelength of 450 nm ± 2 nm with the aid of SpectraMax plate reader (Molecular Devices, CA, USA).

2.8. Light microscopic examination

Samples of pancreas, cerebrum and cerebellum were fixed in 10% neutral-buffered formalin and processed for histology according to standard protocol (Bancroft and Gamble, 2008). Briefly, the fixed tissues were dehydrated using increasing concentrations of alcohol, cleared by xylene and embedded in paraffin wax. The tissues were subsequently cut into 4–5 µm sections with the aid of a microtome, fixed on the slides and stained with hematoxylin and eosin (H & E). All the slides were coded prior to viewing under a light microscope (Olympus CH; Olympus, Tokyo, Japan) and capturing of the photomicrographs using a Sony DSC-W 30 Cyber-shot (Sony, Tokyo, Japan) by pathologists.

2.9. Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) to compare the experimental groups followed by Bonferroni's post-hoc test using GRAPHPAD PRISM 5 software (Version 4; GraphPad Software, La Jolla, California, USA). Values of $p < 0.05$ were considered significant.

3. Results

3.1. Influence of PCA on mortality, blood glucose level and body weight of STZ-induced diabetic rats

Table 1 shows the details of STZ administration. At the end of the treatments, 20% of the untreated diabetic rats were found dead

Table 1
Effects of PCA on body weight and survival rate of STZ-induced diabetic rats.

	Control	PCA alone	Diabetic alone	Diabetic + PCA	Diabetic + GLB
Initial wt. (g)	147.5 ± 2.11	146.2 ± 1.85	148.1 ± 2.53	149.4 ± 1.82	148.1 ± 2.21
Final wt. (g)	233.4 ± 3.26	243.8 ± 2.74	174.9 ± 1.82[a]	202.4 ± 4.62[b]	218.42 ± 3.54[b]
Mortality	0/10	0/10	2/10	0/10	0/10
Survival rate	100%	100%	80%	100%	100%

Wt, Weight; PCA, 50 mg/kg protocatechuic acid; GLB, 5 mg/kg glibenclamide. The values in parentheses are percentage of rats that survived during the treatment. Values represents mean ± SD of rats per group. n = 8 for diabetic alone group whereas n = 10 for remaining groups. a: $p < 0.05$ against control. b: $p < 0.05$ against diabetic alone.

whereas all the rats in control, PCA alone and diabetic rats treated with PCA or glibenclamide groups survived. Moreover, there was a significant ($p < 0.05$) decrease in the final body weight of untreated diabetic rats whereas groups treated with either PCA or glibenclamide exhibited significant increase in the final body weight when compared with untreated diabetic rats.

Fig. 2 depicts the blood glucose level (Fig. 2A and B) and percentage change in body weight (Fig. 2C) of the control and experimental rats after 45 days of treatment. The untreated diabetic rats were hyperglycemic evidenced by a significant increase in the blood glucose level when compared with the control rats. The increase in the blood glucose level of untreated diabetic rats was 324% above the control rats. However, oral administration of PCA at 50 mg/kg and glibenclamide to the diabetic rats resulted in 52.4% ($p < 0.01$) and 48.8% ($p < 0.01$) blood glucose lowering effects following 45 days of treatment respectively, which were significant in comparison with the untreated diabetic rats. Moreover, the body weight gain of untreated treated diabetic rats decreased significantly ($p < 0.05$) when compared with the control rats. However, oral administration of PCA at 50 mg/kg and reference drug, glibenclamide at 5 mg/kg to the diabetic rats significantly increased the body weight gain when compared with untreated diabetic rats. There were no treatment-related effects on the blood glucose level and the body weight gain and in rats administered with PCA alone when compared with control rats.

3.2. PCA improved locomotor activities, forelimb grip, time of grooming and incidence of fecal pellets in diabetic rats

Table 2 shows the results of endpoint analyses of the general locomotor activities, forelimb grip, time of grooming and incidence of fecal pellets in diabetic rats during the 8-min trial in the novel environment. Untreated diabetic rats demonstrated significant ($p < 0.05$) decrease in the total distance travelled, average speed, total time mobile, absolute turn angle and total body rotation in comparison with control. However, diabetic rats administered PCA at 50 mg/kg or glibenclamide showed significant improvement in the motor and locomotor activities evidenced by marked increase in the average speed, total distance travelled, total time mobile, total body rotation and absolute turn angle when compared with untreated diabetic rats. Moreover, untreated diabetic rats exhibited a significant lesser time to hang on the rope with two arms (i.e. forelimb grip) whereas they demonstrated marked increase in time of grooming and incidence of fecal pellets than the control. Rats administered PCA alone showed no treatment-related effects on the forelimb grip, time of grooming and incidence of fecal pellets in comparison with the control. However, administration of PCA to diabetic rats significantly decreased the time of grooming and incidence of fecal pellets but increased the forelimb grip in the treated rats when compared with untreated diabetic rats.

3.3. PCA enhanced the exploratory profile in diabetic rats

Fig. 3 depicts the representative track plots of the walking traces of rats in control and experimental rats within the novel apparatus. The control rats exhibited a normal behavioral profile by walking around the novel apparatus. There were no treatment-related effects on the track plot density of rats administered with PCA at alone when compared with control rats. However, there were marked decrease in the density of track plots of untreated diabetic rats, thus confirming the reduction in locomotor activity. Administration of PCA at 50 mg/kg or glibenclamide significantly ameliorated diabetes-mediated reduction in the track plot density.

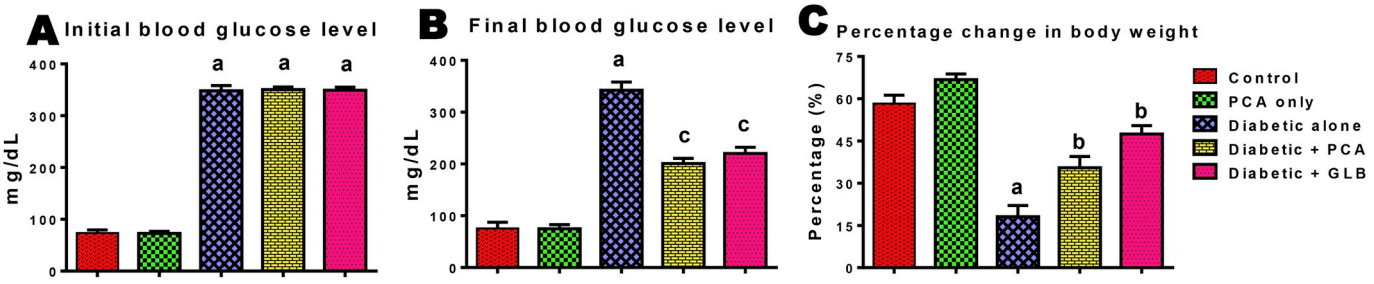

Fig. 2. Influence of PCA on the blood glucose level and body weight gain in STZ-induced diabetic rats. PCA, 50 mg/kg protocatechuic acid; GLB, 5 mg/kg glibenclamide. Each bar represents mean ± SD of rats per group. n = 8 for diabetic alone group whereas n = 10 for remaining groups. a: $p < 0.05$ against control. b: $p < 0.05$ against diabetic alone. c: $p < 0.01$ against diabetic alone. d: $p < 0.001$ against diabetic alone.

Table 2
Influence of PCA on locomotion activities, forelimb grip, grooming time and incidence of fecal pellets in STZ-induced diabetic rats.

	Control	PCA alone	Diabetic alone	Diabetic + PCA	Diabetic + GLB
Total distance travelled	39.12 ± 3.44	38.86 ± 2.25	18.55 ± 2.03[a]	35.41 ± 2.11[b]	36.52 ± 1.87[b]
Average speed	0.08 ± 0.01	0.09 ± 0.01	0.05 ± 0.01[a]	0.07 ± 0.01[b]	0.08 ± 0.01[b]
Total time mobile	470.2 ± 12.9	467.3 ± 10.4	318.9 ± 15.1[a]	459.9 ± 14.4[b]	414.4 ± 16.2[b]
Absolute turn angle	55.9 ± 3.74	56.5 ± 6.12	34.2 ± 2.75[a]	49.7 ± 4.64[b]	45.6 ± 3.89[b]
Total body rotation	21.8 ± 1.39	22.85 ± 2.11	9.45 ± 1.83[a]	18.75 ± 1.65[b]	16.08 ± 1.87[b]
Forelimb grip	40.67 ± 2.86	42.33 ± 4.17	16.03 ± 1.43[a]	31.72 ± 3.08[b]	33.52 ± 3.52[b]
Grooming time	71.51 ± 4.11	75.08 ± 2.94	142.4 ± 6.25[a]	85.16 ± 3.87[b]	94.27 ± 5.34[b]
Fecal pellets	0.75 ± 0.15	0.68 ± 0.17	2.68 ± 0.16[a]	1.24 ± 0.13[b]	1.17 ± 0.18[b]

PCA, 50 mg/kg protocatechuic acid; GLB, 5 mg/kg glibenclamide. Total distance travelled (Metres); Average speed (Metres/second); Total time mobile (Seconds); Absolute turn angle (Degree); Total body rotation (Degree); Forelimb grip (Seconds); Grooming time (Seconds) and Fecal pellets (Frequency). Values represents mean ± SD of rats per group. n = 8 for diabetic alone group whereas n = 10 for remaining groups. a: Values differ significantly from control ($p < 0.05$). b: Values differ significantly from diabetic alone.

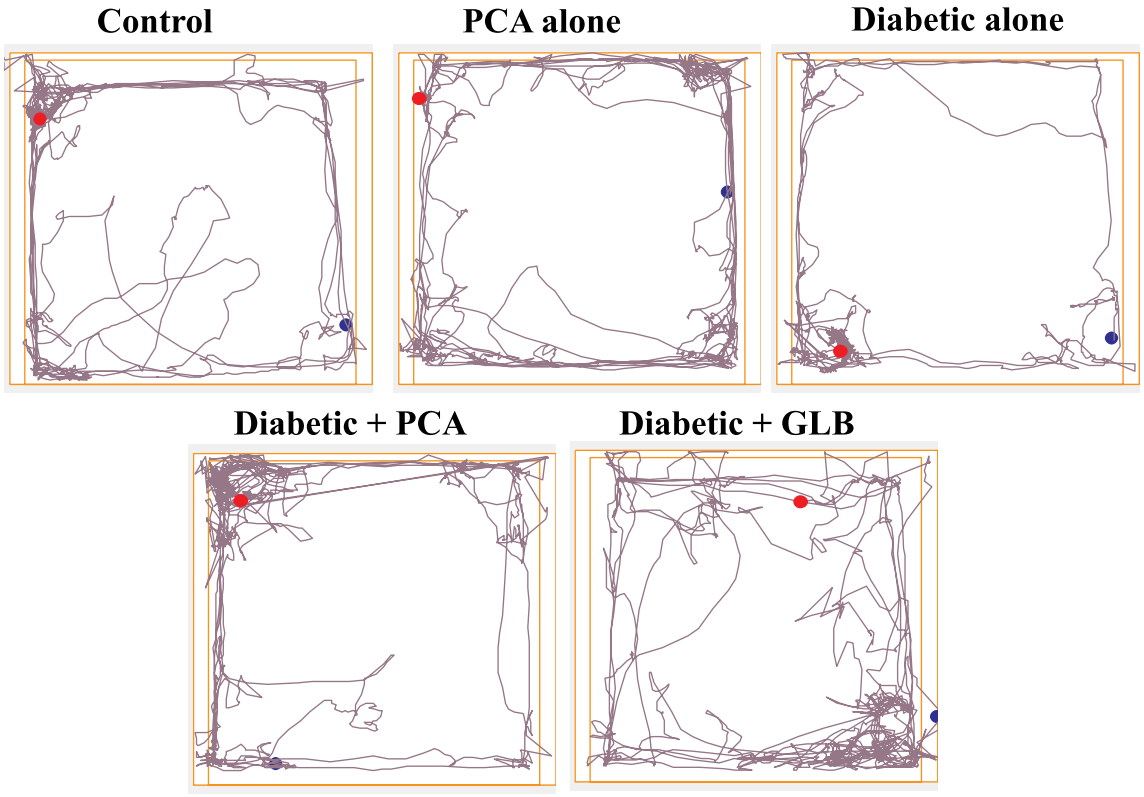

Fig. 3. The representative track plots show the path travelled by experimental rats in the novel apparatus during the 8-min trial of 8 rats in diabetic alone group and 10 rats each for remaining groups. PCA, 50 mg/kg protocatechuic acid; GLB, 5 mg/kg glibenclamide. The data were analyzed using video-tracking software (ANY-maze, Stoelting CO, USA).

3.4. Influence of PCA on acetylcholinesterase activity and glutathione level in diabetic rats

Fig. 4 depicts the modulatory effects of PCA treatment on the AChE activity and GSH level in diabetic rats. Untreated diabetic rats demonstrated a significant ($p < 0.05$) increase in AChE activity in the cerebrum and cerebellum of the treated rats. However, administration of PCA at 50 mg/kg to diabetic rats significantly ($p < 0.01$) decreased the AChE activity when compared with untreated rats (Figs. 4A and 6B). Moreover, there was a significant decrease in cerebral GSH level whereas pancreatic and cerebellar GSH were not significantly decreased in untreated diabetic rats when compared with control. However, administration of PCA at 50 mg/kg or glibenclamide to diabetic rats significantly increased the cerebral GSH level when compared with untreated rats (Fig. 4C, D and 4E).

3.5. PCA improved antioxidant enzymes activities in pancreas, cerebrum and cerebellum of diabetic rats

Table 3 shows the antioxidant activities of SOD, CAT, GPx and GST

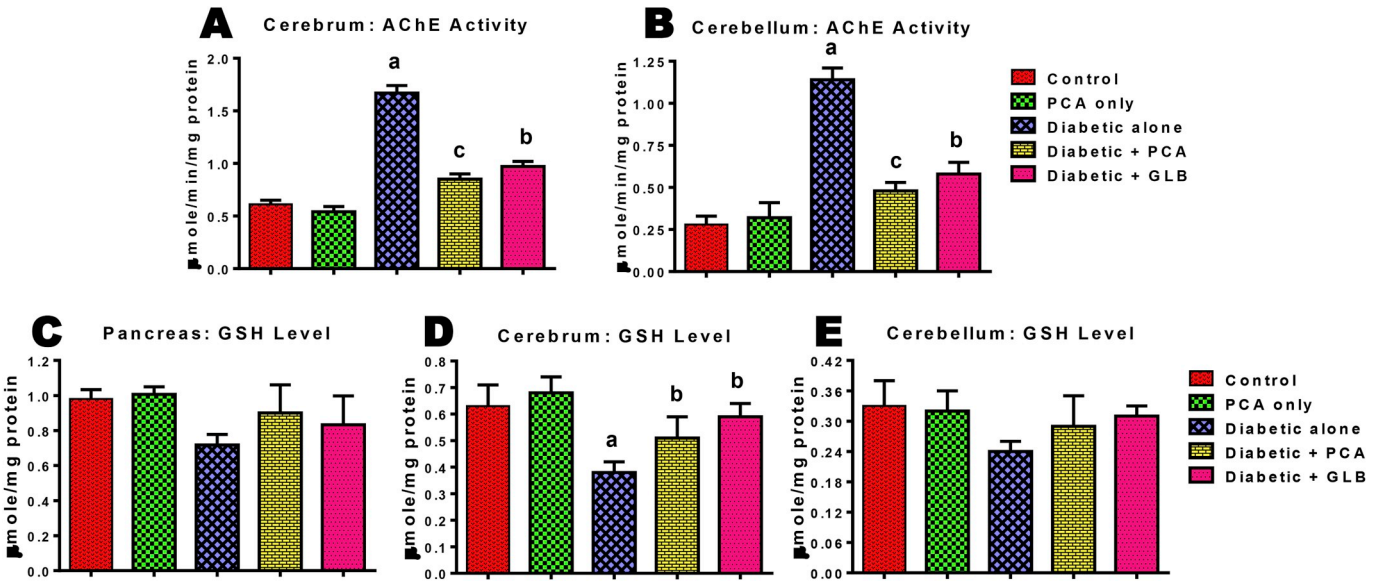

Fig. 4. Influence of PCA on AChE activity and GSH level in the pancreas, cerebrum and cerebellum of STZ-induced diabetic rats. PCA, 50 mg/kg protocatechuic acid; GLB, 5 mg/kg glibenclamide. Each bar represents mean ± SD of rats per group. n = 8 for diabetic alone group whereas n = 10 for remaining groups. a: $p < 0.05$ against control. b: $p < 0.05$ against diabetic alone. c: $p < 0.01$ against diabetic alone. d: $p < 0.001$ against diabetic alone.

in the pancreas, cerebrum and cerebellum of control and experimental rats. Rats administered PCA alone showed no treatment-related effects on the SOD, CAT, GPx and GST activities when compared with the control. There was a significant decrease in the cerebral and cerebellar SOD activity whereas pancreatic SOD activity was unaffected in the untreated diabetic rats. Moreover, CAT activity was significantly decreased in pancreas, cerebrum and cerebellum of untreated diabetic rats when compared to the control. Administration of PCA at 50 mg/kg or glibenclamide significantly increased the activities of SOD and CAT in the treated rats when compared with untreated diabetic rats. Untreated diabetic rats showed a significant decrease in pancreatic GPx and GST activities whereas they were not significantly affected in the cerebrum and cerebellum when compared with the control. However, administration of PCA at 50 mg/kg or glibenclamide significantly increased the pancreatic GPx and GST activities in the treated rats when compared with untreated diabetic rats.

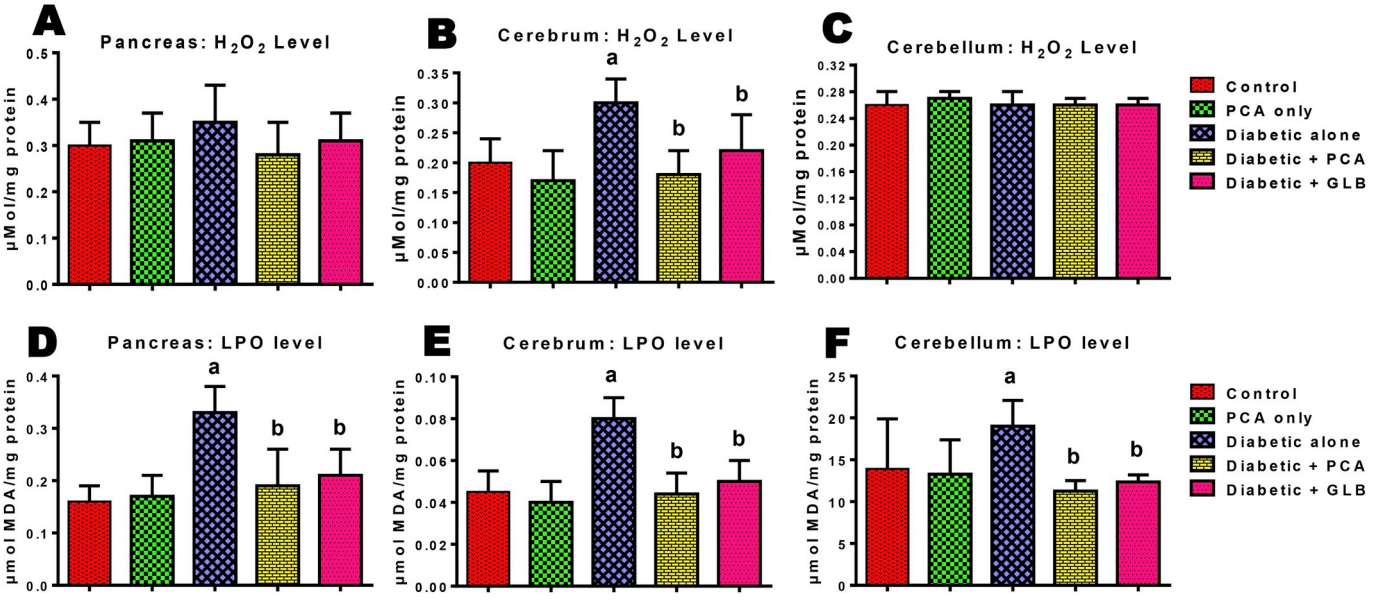

Fig. 5. Influence of PCA on H_2O_2 and LPO levels in the pancreas, cerebrum and cerebellum of STZ-induced diabetic rats. PCA, 50 mg/kg protocatechuic acid; GLB, 5 mg/kg glibenclamide. Each bar represents mean ± SD of rats per group. n = 8 for diabetic alone group whereas n = 10 for remaining groups. a: $p < 0.05$ against control. b: $p < 0.05$ against diabetic alone.

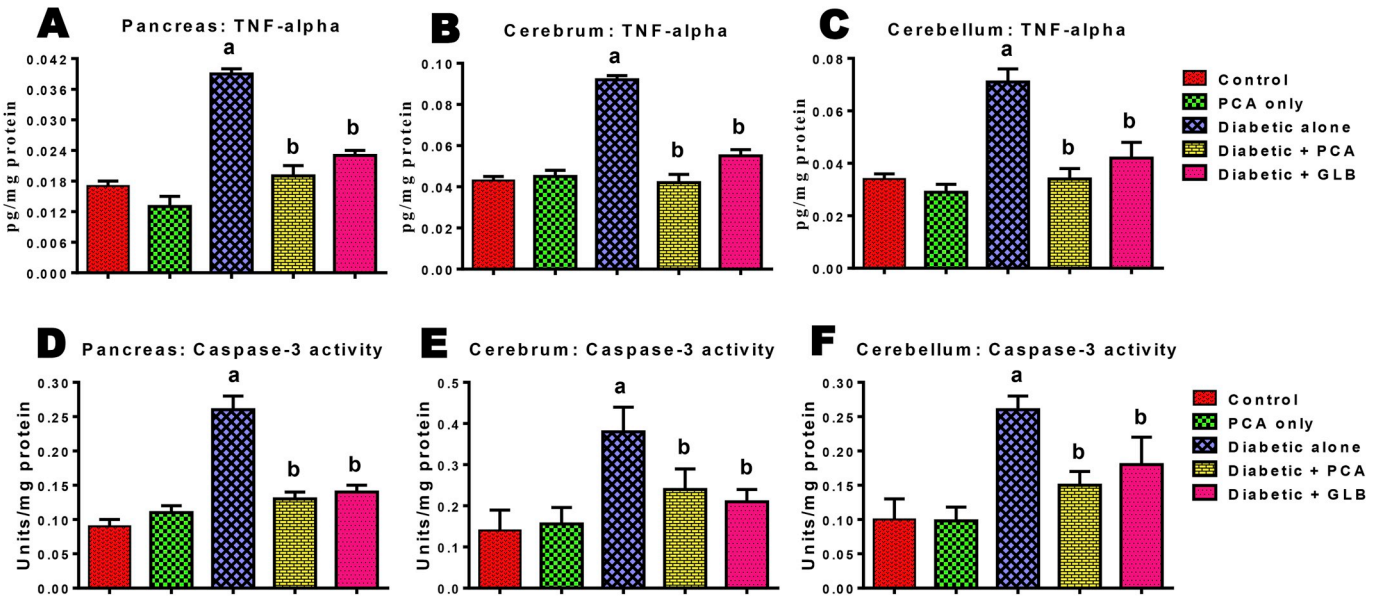

Fig. 6. Influence of PCA on TNF-α level and caspase-3 activity in the pancreas, cerebrum and cerebellum of STZ-induced diabetic rats. PCA, 50 mg/kg protocatechuic acid; GLB, 5 mg/kg glibenclamide. Each bar represents mean ± SD of rats per group. n = 8 for diabetic alone group whereas n = 10 for remaining groups. a: $p < 0.05$ against control. b: $p < 0.05$ against diabetic alone.

Table 3
Influence of PCA on antioxidant enzymes activities in the pancreas, cerebrum and cerebellum of STZ-induced diabetic rats.

		Control	PCA alone	Diabetic alone	Diabetic + PCA	Diabetic + GLB
SOD	Pancreas	1.52 ± 0.05	1.54 ± 0.01	1.44 ± 0.04	1.44 ± 0.03	1.48 ± 0.03
	Cerebrum	1.20 ± 0.06	1.28 ± 0.07	0.68 ± 0.08[a]	1.16 ± 0.17[b]	0.98 ± 0.06[b]
	Cerebellum	1.15 ± 0.08	1.25 ± 0.06	0.63 ± 0.06[a]	0.93 ± 0.04[b]	0.81 ± 0.03[b]
CAT	Pancreas	1.86 ± 0.26	1.79 ± 0.39	0.89 ± 0.08[a]	1.76 ± 0.26[b]	1.38 ± 0.11[b]
	Cerebrum	0.51 ± 0.08	0.55 ± 0.04	0.19 ± 0.06[a]	0.48 ± 0.07[b]	0.39 ± 0.08[b]
	Cerebellum	0.34 ± 0.07	0.36 ± 0.08	0.13 ± 0.05[a]	0.31 ± 0.07[b]	0.22 ± 0.05[b]
GPx	Pancreas	9.58 ± 0.75	8.93 ± 0.86	5.27 ± 0.55[a]	8.06 ± 0.55[b]	7.86 ± 0.76[b]
	Cerebrum	6.39 ± 1.03	6.83 ± 0.58	5.49 ± 0.93	6.39 ± 0.24	6.25 ± 0.81
	Cerebellum	4.81 ± 0.52	4.79 ± 0.72	4.07 ± 0.50	4.41 ± 0.46	4.38 ± 0.43
GST	Pancreas	2.85 ± 0.45	2.78 ± 0.35	1.41 ± 0.48[a]	2.56 ± 0.36[b]	1.91 ± 0.45[b]
	Cerebrum	7.44 ± 0.93	7.58 ± 0.82	6.57 ± 0.85	7.12 ± 0.98	7.27 ± 0.78
	Cerebellum	2.04 ± 0.16	2.08 ± 0.18	1.76 ± 0.15	1.96 ± 0.25	1.89 ± 0.32

PCA, 50 mg/kg protocatechuic acid; GLB, 5 mg/kg glibenclamide. SOD activity (nmoles epinephrine oxidized/min/mg protein); CAT activity (μmole H_2O_2 consumed/min/mg protein); GPx activity (nmole of residual GSH/mg protein), GST activity (μmole CDNB–GSH complex formed/minute/mg protein). Values represents mean ± SD of rats per group. n = 8 for diabetic alone group whereas n = 10 for remaining groups. a: Values differ significantly from control ($p < 0.05$). b: Values differ significantly from diabetic alone.

3.6. PCA abrogated oxidative stress biomarkers in pancreas, cerebrum and cerebellum of diabetic rats

Fig. 5 depicts the levels of oxidative stress biomarkers evaluated in the pancreas, cerebrum and cerebellum of control and experimental rats. There were no treatment-related effects between the oxidative stress parameters namely the levels of H_2O_2 generation and MDA (an index of lipid peroxidation), in the control and rats treated with PCA alone. However, untreated diabetic rats demonstrated significant increases in H_2O_2 generation and LPO levels in the pancreas, cerebrum and cerebellum when compared with the control group. Administration of PCA at 50 mg/kg or glibenclamide significantly ($p < 0.05$) decreased the H_2O_2 and LPO levels when compared with untreated diabetic rats (Fig. 5A–F).

3.7. PCA suppressed pro-inflammatory biomarkers and caspase-3 activity in pancreas, cerebrum and cerebellum of diabetic rats

Moreover, the influence of PCA on the pro-inflammatory biomarkers and caspase-3 activity in pancreas, cerebrum and cerebellum of the control and experimental rats was assessed. Rats administered PCA alone showed no treatment-related effects on these inflammatory biomarkers and caspase-3 activity when compared with the control. Untreated diabetic rats showed a significant elevation in the

Table 4
Influence of PCA on NO level and MPO activity in the pancreas, cerebrum and cerebellum of STZ-induced diabetic rats.

		Control	PCA alone	Diabetic alone	Diabetic + PCA	Diabetic + GLB
NO	Pancreas	0.82 ± 0.06	0.87 ± 0.07	1.47 ± 0.03[a]	0.92 ± 0.08[b]	1.18 ± 0.07[b]
	Cerebrum	0.21 ± 0.04	0.23 ± 0.04	0.52 ± 0.03[a]	0.24 ± 0.05[b]	0.22 ± 0.04[b]
	Cerebellum	0.25 ± 0.01	0.27 ± 0.03	0.35 ± 0.01[a]	0.25 ± 0.02[b]	0.26 ± 0.03[b]
MPO	Pancreas	0.38 ± 0.33	0.43 ± 0.05	0.96 ± 0.05[a]	0.72 ± 0.04[b]	0.68 ± 0.05[b]
	Cerebrum	0.27 ± 0.07	0.25 ± 0.04	0.76 ± 0.07[a]	0.43 ± 0.03[b]	0.38 ± 0.04[b]
	Cerebellum	0.32 ± 0.05	0.38 ± 0.06	0.82 ± 0.12[a]	0.48 ± 0.14[b]	0.53 ± 0.17[b]

PCA, 50 mg/kg protocatechuic acid; GLB, 5 mg/kg glibenclamide. NO level (Units/mg protein); MPO activity (Units/mg protein). Values represents mean ± SD of rats per group. n = 8 for diabetic alone group whereas n = 10 for remaining groups. a: Values differ significantly from control ($p < 0.05$). b: Values differ significantly from diabetic alone.

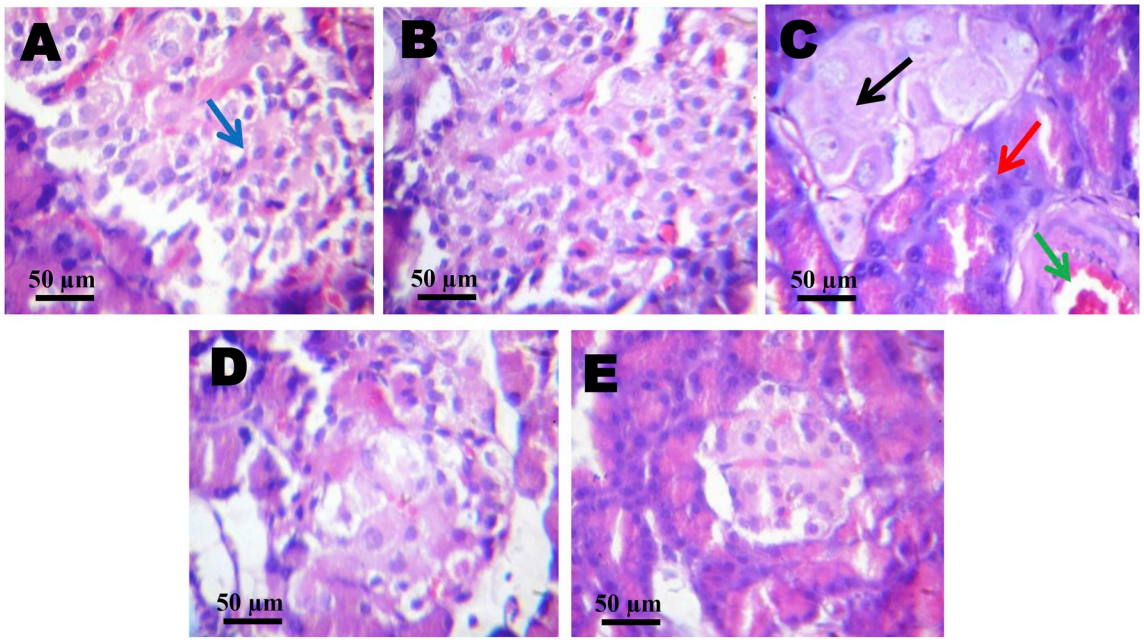

Fig. 7. Representative histopathological sections of the pancreas. Control (**A**) and PCA alone rats (**B**) showing normal exocrine acini with deep-staining zymogen granules, the intralobular and interlobular ducts are essentially normal with pancreatic secretion and the islets appear normal in varying sizes (blue arrow). Diabetic rats (**C**) showing focal area of extensive haemorrhagic lesion (green arrow), infiltration of the acini by inflammatory cells (red arrow) and necrosis of the islet (black arrow). Pancreatic sections of PCA-treated rats (**D**) showing normal architecture with normal exocrine acini and islets. Pancreatic sections of glibenclamide (GLB)-treated rats (**E**) showing normal exocrine acini, the intralobular and interlobular ducts with normal but few islets. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

inflammatory biomarkers namely MPO activity, NO (Table 4) and TNF-α levels as well as caspase-3 activity (Figs. 6A–F) in pancreas, cerebrum and cerebellum when compared with the control. However, administration of PCA at 50 mg/kg or glibenclamide significantly suppressed NO and TNF-α levels as well as MPO and caspase-3 activities in the pancreas, cerebrum and cerebellum when compared with untreated diabetic rats.

3.8. PCA abrogated histological alterations in pancreas, cerebrum and cerebellum of diabetic rats

Figs. 7–9 depict the histological alterations seen with the light microscope in the pancreatic, cerebral and cerebellar sections from the control and experimental rats. The pancreas, cerebrum and cerebellum of rats from control and PCA alone groups appeared structurally and functionally normal. However, obvious pathological lesions were observed in the pancreas, cerebellum and cerebrum sections from untreated diabetic rats. The pancreas of untreated diabetic rats (Fig. 7) showed focal area of extensive haemorrhagic lesion (green arrow), infiltration of the acini by inflammatory cells (black arrow) and necrosis of the islet (yellow notch), the cerebrum (Fig. 8) showed disseminated congestion (black arrow) and haemorrhagic lesion (blue arrow) whereas the cerebellum (Fig. 9) showed marked neuronal degeneration and focal area of central chromatolysis of the Purkinje cells (yellow

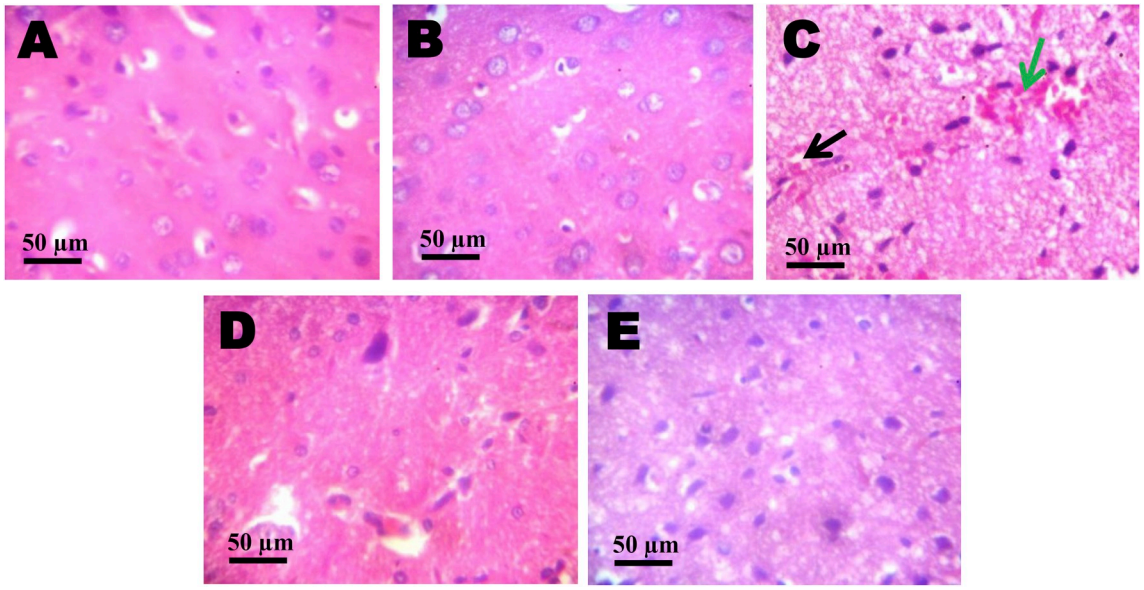

Fig. 8. Representative histopathological sections of the cerebrum. Control (**A**) and PCA alone rats (**B**) showing normal cerebral cortex. Cerebrum of untreated diabetic rats (**C**) showing disseminated congestion (black arrow) and mild haemorrhagic lesion (green arrow) whereas cerebral sections of PCA-treated rats (**D**) and glibenclamide (GLB)-treated rats (**E**) showing normal architecture with Purkinje cells somewhat similar to control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

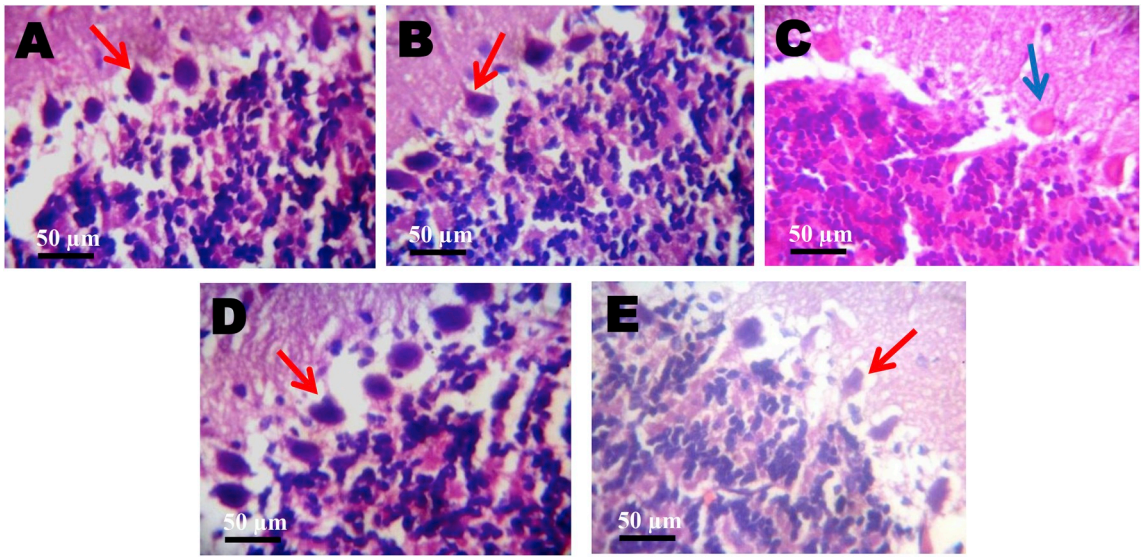

Fig. 9. Representative histopathological sections of the cerebellum. Control (**A**) and PCA alone rats (**B**) showing normal cerebellum with large and deeply basophilic Purkinje cells (red arrows). Cerebellum of untreated diabetic rats (**C**) showing marked neuronal degeneration and focal area of central chromatolysis of the Purkinje cells (blue arrow) whereas cerebellar sections of PCA-treated rats (**D**) and glibenclamide (GLB)-treated rats (**E**) showing normal architecture with Purkinje cells somewhat similar to control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

arrow). However, the pancreatic, cerebral and cerebellar sections from diabetic rats treated with PCA at 50 mg/kg or glibenclamide showed normal architecture somewhat similar to control (Figs. 7–9).

4. Discussion

The neurotoxicity induced by hyperglycemia is a serious medical concern globally. As a result, the prevention or amelioration of neurotoxicity is a vital clinical interest in order to improve the well-being of diabetic patients. The present study demonstrated that a natural phytochemical, PCA, has notable beneficial effects in preventing neurobehavioural and biochemical deficits in diabetic rats. In the present investigation, untreated diabetic rats exhibited significant increase in the blood glucose level with concomitant marked decrease in the final body weight and survival rate. Several investigators as reviewed by Deeds et al. (2011) have demonstrated significant weight loss and mortality of mice due to STZ toxicity and complications of hyperglycemia if left untreated over time. The severe sarcopenia and muscle wasting associated with STZ administration could impair the growth rate and consequently, the marked decrease in the body weight of the untreated diabetic rats in this study. The observed decrease in the body weight gain in the present study is in agreement with earlier findings (Boudjelal et al., 2015; de Morais et al., 2016; Bequer et al., 2016; Sekiou et al., 2018). However, the significant increase in the body weight gain and the restoration of the blood glucose level to near normal following treatment with PCA or glibenclamide revealed an

improvement in the metabolic condition and prevention of tissue damage due to hyperglycemia in the rats.

The untreated diabetic rats in this study exhibited marked locomotor deficits as evidenced by decrease in total distance moved, speed and total mobility time. Moreover, the decrease in the absolute turn angle, body rotation and forelimb grip which are crucial motor coordination parameters during bodily movements (Riemann and Lephart, 2002; Adedara et al., 2016) indicates an impairment in the coordination between nervous and muscular junctions. However, the increase in these parameters following treatment with PCA or glibenclamide clearly demonstrated their abilities to prevent diabetes-induced locomotor and motor deficits in rats. Rodent self-grooming (that is, cleaning of outer body surface) is a well acceptable index of disturbed motor functions and survival behavioral patterns in models of brain disorders (Tartaglione et al., 2016). Thus, the ability of PCA to prevent diabetes-induced increase in grooming time in the present study further indicates the protective effect of PCA against motor dysfunction in the treated rats.

Experimental animal models demonstrate complex behavioral alterations during exposure to stressors which can induce fear or anxiety-like phenotype. The increased in the incidence of fecal pellets suggests an anxiogenic-like behavior in untreated diabetic rats. However, treatment with PCA or glibenclamide effectively reduced the incidence of fecal pellets thus revealing the inhibitory effect of PCA on anxiety-like behavior in the treated rats. Moreover, one of the major way by which rodents acquire information about their spatial environment is exploration (Gorny et al., 2002). The reduction in the exploratory activity of untreated diabetic rats was confirmed by the decrease in density of track plots. The improvement in the exploratory activity as verified by the increase in the densities of track plots in diabetic rats treated with PCA or glibenclamide in this study indicates the protective effects of these compounds against disorganization of exploratory and spatial behavior in the treated rats.

Moreover, AChE is well documented to hydrolyze acetylcholine, an essential neurotransmitter in the regulation of motor function and locomotion (Day et al., 1991). The increase in the AChE activity in the untreated diabetic rats in the present study could lead to shortage of acetylcholine, at the synaptic cleft due to excessive breakdown by AChE thereby leading to impairment in the normal neurotransmission in the rats. The observed increase in the AChE activity in the present study corroborates earlier findings (Kuhad et al., 2008; Maciel et al., 2016). The reduction in AChE activity by PCA treatment consequently increased acetylcholine level in synapse, improve cholinergic neurotransmission and finally enhanced locomotor functions. Evaluation of antioxidants including SOD, CAT, GPx and GST activities and MDA level, the end-products of lipid peroxidation (LPO) are well-known to provide evidence about oxidative damage (Valko et al., 2016). The reduction in the activities of these antioxidant enzymes result in the accumulation of ROS including H_2O_2 which eventually leads to LPO (Adedara et al., 2015). Neuronal membranes which are rich in peroxidizable fatty acids undergo peroxidation to give MDA and 4-hydroxynonenol (Shichiri, 2014). The present study indicated that treatment of diabetic rats with PCA or glibenclamide significantly prevented LPO evinced by decreased MDA level in treated rats. Thus, PCA not only suppressed the induction of LPO but also enhanced antioxidant defense mechanism.

Several possible mechanisms identified in the pancreatic β-cell dysfunction in diabetes include the toxic effects of elevated glucose level, ROS generation, amyloid deposition and altered levels of inflammatory mediators (Newsholme et al., 2016). The present study showed that untreated diabetic rats exhibited increased MPO activity with concomitant elevation in the levels of NO and TNF-α in the pancreas, cerebrum and cerebellum. This observation indicates involvement of aggravated inflammatory response in diabetes-induced neurotoxicity. Moreover, the untreated diabetic rats in the present study demonstrated significant increase in caspase-3 activity in pancreas, cerebrum and cerebellum. Caspase-3 belongs to a family of aspartate-specific cysteine proteases and is a well-known downstream key apoptotic initiator (D'Amelio et al., 2010). The increase in caspase-3 activity indicates induction of apoptotic cell death in the untreated diabetic rats. However, treatment of diabetic rats with PCA markedly suppressed the increase in NO and TNF-α levels as well as MPO and caspase-3 activities, thus indicating the anti-inflammatory and anti-apoptotic properties of PCA.

Histological alterations identified in the pancreas, cerebrum and cerebellum of untreated diabetic rats in the present study were characterized by focal area of extensive haemorrhagic lesion, infiltration of the acini by inflammatory cells and necrosis of the pancreatic islet,

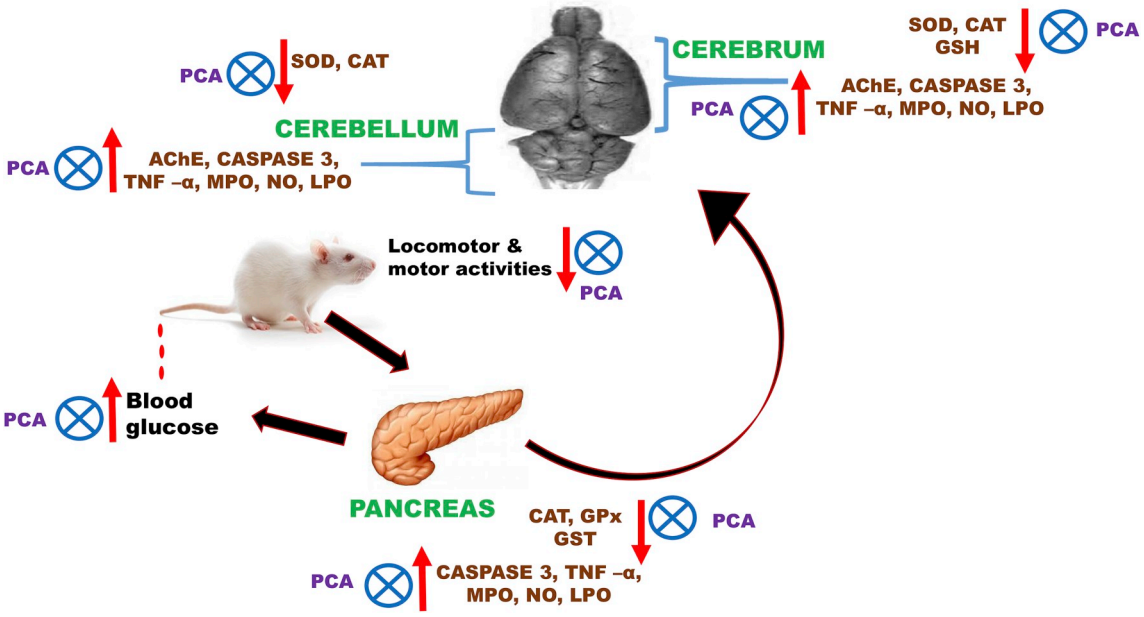

Fig. 10. Proposed pathway delimiting the protective mechanisms of PCA in the pancreas, cerebrum and cerebellum of STZ-induced diabetic rats. PCA: protochatechuic acid; STZ: streptozotocin; SOD: superoxide dismutase activity; CAT: catalase; GST, glutathione S-transferase; GPx: glutathione peroxidase; GSH: reduced glutathione; LPO: lipid peroxidation; TNF-α: tumour necrosis factor alpha; NO: nitric oxide; MPO: myeloperoxidase. AChE: acetylcholinesterase.

disseminated congestion and haemorrhagic lesion of the cerebrum while marked neuronal degeneration and focal area of central chromatolysis of the Purkinje cells observed in the cerebellum of the rats. The observed histopathological lesions may be associated with the induction of inflammation, oxidative damage and caspase-3 activity in the pancreas and brain of the treated rats. However, PCA treatment effectively ameliorated these lesions and preserved the structures of pancreas, cerebrum and cerebellum somewhat similar to the control, thus corroborating the biochemical data on its neuroprotective role in diabetes associated neurotoxicity in rats.

In conclusion, the chemoprotection of diabetes-induced neurobehavioural and biochemical deficits by PCA in the present study is attributed to improvement in endogenous antioxidant status, abrogation of lipid peroxidation and suppression of inflammation, acetylcholinesterase and caspase-3 activities. The proposed pathway delimiting the protective mechanisms of PCA in the pancreas, cerebrum and cerebellum of STZ-induced type-1 diabetic rats is presented in Fig. 10. It is worthy of note that preliminary studies from our laboratory indicated that there were no dose-dependent effects observed between 25 and 50 mg/kg doses of PCA. In the present study, the chemoprotective effects of PCA at 50 mg/kg dose is comparable to the reference drug glibenclamide. Thus, PCA may be a promising pharmacological agent in the treatment of diabetes and its associated neurotoxicity. However, an assessment of the involvement of nuclear factor E2-related factor-2 (Nrf2), nuclear factor-kappa B (NF-κB), phosphatidylinositol-3-kinase/kinase B (PI3K/Akt) or other anti-apoptotic pathways are warranted to further account for the plausible neuroprotective mechanism of PCA in STZ-induced diabetic rats.

Conflicts of interest

The authors have no conflicts of interest to declare.

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