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Aqueous leaf extract of Phyllanthus amarus protects against oxidative stress and misfiring of dopaminergic neurons in Paraquat-induced Parkinson's disease-like model of adult Wistar rats

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Abstract

Background of the study: Phyllanthus amarus has high nutritional value and is beneficial in managing and treating diverse ailments. This study assessed the role of aqueous leaf extract of Phyllanthus amarus on Paraquat (PQ) induced neurotoxicity in the substantia nigra of Wistar rats.

Materials and methods: The role of aqueous leaves extract of Phyllanthus amarus was assessed using an open field test (OFT) for motor activity, oxidative stress biomarkers [Catalase (CAT), and Superoxide Dismutase (SOD)], histological examination (H and E stained) for cytoarchitectural changes and immunohistochemical studies using tyrosine hydroxylase (TH) as a marker for dopaminergic neurons. Forty-two (42) rats were categorized into six groups (n = 7); group 1: control was administered 0.5 ml/kg distilled water, group 2: received 10 mg/kg PQ + 10 mg/kg L-dopa as reference drug, group 3; received 10 mg/kg PQ, while group 4: received 10 mg/kg PQ + 200 mg/kg P. amarus, group 5: received 10 mg/kg PQ + 300 mg/kg P. amarus, and group 6: received 10 mg/kg PQ + 400 mg/kg P. amarus respectively, for 14 days. All administrations were done orally; a significant difference was set at $p<0.05$.

Results and discussion: The study's open field test (OFT) revealed no motor activity deficit with Paraquat (PQ) exposure. Also, cytoarchitectural distortions were not observed with Paraquat (PQ) only treatment group compared to the control and other groups pretreated with P. amarus and L-dopa. Moreover, the Paraquat (PQ) only treatment group showed oxidative stress by significantly decreasing the antioxidant enzyme (SOD) compared to the control and L-dopa pretreated group. A significant decrease in tyrosine hydroxylase (TH) expressing dopaminergic neurons was also observed in Paraquat (PQ) only treatment. However, P. amarus treatment showed therapeutic properties by significantly increasing tyrosine hydroxylase (TH) expressing dopaminergic neuron levels relative to control.

Conclusion: Aqueous leaf extract of Phyllanthus amarus possesses therapeutic properties against Paraquat (PQ) induced changes in the substantia nigra of Wistar rats.

1.0. Introduction

Neurotoxicity is any reversible or irreversible adverse effect on the nervous system’s structure, function, or chemistry during development or maturity, produced by physical or chemical causes. (Zahra et al., 2020). The main mechanisms for neurotoxicity involve the excessive production of reactive oxygen species leading to oxidative stress, the release of cytokines causing neuroinflammation, and dysregulations of apoptosis leading to neuronal death (Teleanu et al., 2018). Parkinson's disease (PD) is a good example of a neurodegenerative disease that is associated with a group of motor disorders in the elderly population (Vaccari et al., 2019) and has affected millions around the world from 1990 to 2015. The neuropathological features of Parkinson's disease (PD) are the loss of dopaminergic neurons in vulnerable brain regions, especially in the substantia nigra (SN) (Liddell and White, 2018; Rai et al., 2019). Further, the substantia nigra (SN) is a midbrain dopaminergic nucleus critical in modulating motor movement and reward functions as part of the basal ganglia circuitry (Sonne et al., 2019). The

substantia nigra (SN) is a dopaminergic nucleus located in the midbrain. It is critical in modulating motor movement and reward functions as part of the basal nucleus circuitry (Sonne et al., 2019).

In the recent past, the awareness of the serious consequences related to the use of herbicides discovered during the second world war on humans has increased among developing nations in Africa and Asia. A very good example of such herbicide is paraquat. Paraquat (PQ) is considered to be among the main herbicide involved in intentional and accidental poisoning despite its use as a weed control. Examples of illness caused by paraquat PQ causes toxicity in vital regions of the brain, including substantial nigra, which plays a critical role in motor coordination, supporting the idea that exposure to this herbicide may contribute to the pathophysiology of neurodegenerative diseases like PD Paraquat (PQ), as a kind of pesticide in agriculture, is a widely used herbicide that was identified as a neurotoxicant and is linked to increased Parkinson's disease (PD) risk and Parkinson's disease (PD)-like neuropathology (Chia et al., 2020; Yadav et al., 2017). Protein aggregation, mitochondrial dysfunction, altered dopamine levels, and increased oxidative stress are majorly reported mechanisms by which Paraquat (PQ) causes neurological disease conditions (Dick, 2006; Rappold et al., 2011; Zhang et al., 2016).

Several researchers have worked on how medical plants can ameliorate this disease among the aging population. Phyllanthus amarus, a member of the Euphorbiaceae family, is conventionally utilized for gonorrhea, dysentery, kidney ailments, pain, and diabetes. Phyllanthus amarus has been reported to have the following properties: anti-oxidant properties (Deora et al., 2021), anti-inflammatory properties (Deora et al., 2021), anti-diabetic properties (Shetti et al., 2012), and Anti-cancerous properties (Lee et al., 2011). Phyllanthus amarus contains such principal constituents as phyllanthin, hypophyllanthin, corilagin, geraniin, amariin, repandusinic acid, phyllanthusiin D, rutin and quercetin 3-O-glucoside; all of which are reported to potently scavenge free radicals in a range of systems (Londhe et al., 2008). Although the therapeutic activity of Phyllanthus amarus against Lipopolysaccharide-induced neurotoxicity has been reported (Alagan et al., 2019), there needs to be more relevant research evidence to demonstrate a similar therapeutic activity against Paraquat-induced neurotoxicity. This study explores its anti-oxidant and anti-inflammatory properties as it concerns the therapeutic role of the aqueous leaf extract of Phyllanthus amarus against Paraquat-induced neurotoxicity in the substantia nigra of Wistar rats.

2.0. Materials And Methods

This study was performed in line with the principles of the Declaration of Helsinki, as revised in 2013.

2.1. Plant material collection and identification

Fresh leaves of Phyllanthus amarus were collected in bulk in the swampy areas of Federal Housing Estate Farm, Igba, Ondo City, Ondo State. Collected plant material was identified (herbarium voucher label UNIMED/P.B.T.H/013) in the Department of Biology Biotechnology Department, University of Medical Sciences, Ondo City, Ondo State, Nigeria.

2.1.1. Plant material extraction

Aqueous leaf extract of Phyllanthus amarus extract was prepared in the Department of Anatomy, Faculty of Basic Medical Sciences, University of Medical Sciences, Laje Road, Ondo City, Ondo state, Nigeria. The maceration method of extraction, as described by Shetti et al., 2012 was adopted.

2.2. Experimental animals

Healthy forty-two (42) adult Wistar rats (100–150 g) were obtained from the Animal House facility of the University of Medical Sciences Ondo and housed under standard laboratory conditions, light and dark cycles of 12 h provided, and fed with rat chow and water ad libitum. Rats were allowed to acclimatize for fifteen (15) days before the commencement of experimentation.

2.3. Drugs

In this study, paraquat PQ (Paracot® PQ Dichloride) was obtained and used as a neurotoxin. Hubei Xianlong Chemical Industry Co, China, manufactures the product. Levodopa (Sinemet) tablet was obtained and used as a reference drug to evaluate the therapeutic property of P A. Mylan Pharmaceuticals, Inc., Morgantown, USA, manufacture the product. Ketamine (Ketamine Hydrochloride injection USP, 50 mg/ ml) was obtained and used for anesthesia. Swiss Parenterals PVT Ltd, Gujarat, India, manufactures the product.

2.4. Experimental design

Forty-two (42) rats were categorized into six (6) groups (groups 1 to 7) of seven (7) rats each for a (14) days period of treatments via oral route; group 1 served as the control group, treated with distilled water (0.2 ml/kg), group 2 was treated with PQ.35 mg/kg) + Ldopa (10 mg/kg), groups group 3 was treated with PQ 35 mg/kg; 28.4% LD50 oral, while groups 4,5 and 6 were treated with PQ + Phyllanthus amarus (200 mg/kg), PQ + Phyllanthus amarus (300 mg/kg) and Phyllanthus amarus (400 mg/kg) respectively [Table 1]. Rats were euthanized using Ketamine (75 kg/mg i. p) anesthesia on day 16, the rats were decapitated, and their skull dissected to remove the brain. Harvested whole brains were dissected sagittally at the midline into two halves, one homogenized for biochemical analysis and the other fixed in 10% neutral buffered formalin for histological processing immunohistochemical studies.

Table 1
Experimental groups and their respective treatments

Groups	Treatment	Duration (days)
1	Distilled Water (0.5 ml/Kg)	15 days
2	Paraquat (10 mg/kg) + L-dopa (10 mg/kg) as a reference drug	15 days
3	Paraquat only (10 mg/kg)	15 days
4	Paraquat (10 mg/kg) + Aqueous leaves extract of Phyllanthus amarus (200 mg/kg)	15 days
5	Paraquat (10 mg/kg) + Aqueous leaves extract of Phyllanthus amarus (300 mg/kg)	15 days
6	Paraquat (10 mg/kg) + Aqueous leaves extract of Phyllanthus amarus (400 mg/kg)	15 days

2.5. Neurobehavioral study

The neurobehavioral assessment was conducted on experimental day eight and day 15 using an open field test (OFT). All experiment procedures were conducted in a quiet room in the Department of Human Anatomy, Faculty of Basic Medical Sciences, University of Medical Sciences, Ondo City, Ondo State, Nigeria. This test measured the experimental animals' locomotion, exploration, and anxiety (Brown et al., 1999). The open field utilized in this study was a square wooden arena (100cm x 100cm x 38cm) with lines on its floor dividing it into 18cm by 18cm square. The open field apparatus was cleaned with alcohol between each rat to avoid irritability due to odor. The rats were conveyed to the test room in their cages, and each rat was tested individually once for five (5) minutes in an open field apparatus, and behaviors were scored. The behaviors scored included: line crossing, rearing, walling, and center square activity (Frequency of entry into a central square and Duration spent in the center square).

2.6. Biochemical studies

Brains were weighed using a digital weighing scale (Acculab Vicon VIC-511 Precision Balance/Scale, USA, 0.001 g) and mechanically homogenized in 0.1 M phosphate buffer (pH 7.4) (1 g tissue/4 ml) according to already established protocols. Homogenate was analyzed for oxidative stress biomarkers superoxide dismutase, [SOD], and catalase (CAT). Biochemical analysis was conducted at the Department of Human Anatomy, ABU, Zaria. Enzymatic antioxidant activity was estimated by using appropriate enzyme lysate immunosorbent assay kits.

2.7. Histological studies using H & E techniques

The recommended procedure of Alagan et al., 2019 was adopted. Histological paraffin sections were processed and stained with Hematoxylin and Eosin (H and E) stains for demonstration of the cytoarchitecture of SN in the Histology Unit of the Department of Human Anatomy, FUTA, Ondo.

Microscopy (using Digital Microscopic Camera, MA 500 AmScope®, USA) was conducted in the Microscopy Research Laboratory of FUTA, Akure.

2.8. Immunohistochemical Staining with Tyrosine Hydroxylase (TH) Procedures

Brain samples were taken to the Department of Anatomy FUTA, where tissues were processed for immunohistochemical studies. The sections were deparaffinized in xylene and taken to water with descending grades of alcohol. Antigen retrieval was performed. The slides were washed in phosphate-buffered saline (PBS) for about 2 minutes. Endogenous peroxidase blocking was performed using 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 minutes. The slides were washed with phosphate-buffered saline (PBS). The sections were blocked in 2.5% normal animal serum for 20 minutes. The sections were incubated in primary antibody (anti-tyrosine hydroxylase) at 1:7500 for 3 hours at room temperature. The sections were then washed in PBS for 5 minutes. Sections were incubated in ImmPRESS (peroxidase) Polymer Anti-Rabbit IgG Reagent, made in horse for 30 minutes. The sections were washed twice, 5 minutes at a time. The color was developed with a DAB peroxidase (HRP) Substrate kit (Vector®). Sections were then rinsed well in tap water and Counter-stained in hematoxylin, dehydrated using graded alcohols, cleared using xylene, mounted, and coverslipped using Distyrene Plasticizer Xylene (DPX). Slides were subjected to quality control assessment and stored at room temperature before photomicrography. The processed tissues were viewed under a Digital Light microscope, and digital photomicrographs were taken by an attached camera at x400 magnification using OMAX software. Using the cell counter plugin Field, NIH-sponsored ImageJ software was used to digitally analyze photomicrographs (Edobor et al., 2021).

2.9. Data analysis

Data obtained from this study were expressed as mean ± standard error of the mean (SEM). One-way analysis of variance was used to compare the mean difference between groups, followed by Tukey's Posthoc test, two-way analysis of variance, and Bonferroni's multiple comparisons tests. GraphPad Prism version 8.0.2(263) was used for statistical analysis. A significant difference was set at $p < 0.05$.

3.0. Results

3.1. Neurobehavioral study

3.1.1. Open field test

Figure 1:bar graphs comparing days eight and 15 of the Open field test. Graph **A** represents the frequency of line crossing made by the experimental rats in all groups over horizontal and vertical lines and shows no significant difference between day eight and day 15 in the frequency of line crossing across all experimental groups ($p > 0.05$). Graph **B** bar graph shows a comparison between day eight and day 15 of the frequency of rearing made by the experimental rats. There was a significant increase in the

frequency of rearing in the PQ (10mg/kg) + P. amarus (400mg/kg) group on Day 15 compared to PQ (10mg/kg) + P. amarus (400mg/kg) group on day 8 ($p < 0.05$), n = 3. All other comparisons of experimental groups between days 8 and 15 are not significantly different (ns) $p > 0.05$. graph **C** bar graph shows a comparison between day eight and day 15 of the frequency of walling made by the experimental rats. There was a significant decrease in the frequency of walling in the control group on day 15 compared to the control group on day 8 ($p < 0.05$), n = 3. All other comparisons of experimental groups between days 8 and 15 are not significantly different (ns) ($p > 0.05$). Graph **D** bar graph compares day eight and day 15 in the entry frequency into a center square (square center activity) made by the experimental rats. There was no significant difference between day eight and day 15 in the frequency of entry into a center square (square center activity) across all experimental groups ($p > 0.05$). Graph **E** bar graph compares days eight and 15 of the experimental rats' duration in the center square (square center activity). There was no significant difference between day eight and day 15 in the duration spent in the center square (square center activity) across all experimental groups ($p > 0.05$). * depicts a significant difference ($p < 0.05$).

3.2. Biochemical analysis (oxidative stress biomarkers)

3.2.1. catalase and SOD activity tests

Figure 2:bar graphs showing the level of oxidative stress markers among treatment groups. Graph **A** shows no significant difference in the level of catalase across all experimental groups ($p > 0.05$); control [31.00 ± 0.5774], PQ (10mg/kg) + l-dopa [32.43 ± 0.8647], PQ (10mg/kg) [27.00 ± 1.155], PQ (10mg/kg) + P. amarus (200mg/kg) [29.67 ± 0.8819], PQ (10mg/kg) + P. amarus (300mg/kg) [28.00 ± 0.5774], and PQ (10mg/kg) + P. amarus (400mg/kg) [31.67 ± 0.3333]. Graph **B** shows a significant decrease in the levels of superoxide dismutase in PQ (10mg/kg) [26.00 ± 0.000], PQ (10mg/kg) + P. amarus (200mg/kg) [28.00 ± 0.5774], PQ (10mg/kg) + P. amarus (300mg/kg) [26.00 ± 0.000], and PQ (10mg/kg) + P. amarus (400mg/kg) [29.67 ± 0.3333] treated groups compared to control [31.53 ± 0.2906] and PQ (10mg/kg) + l-dopa [32.97 ± 0.1453] groups. *** depict significant decrease in superoxide dismutase level in PQ (10mg/kg), PQ (10mg/kg) + P. amarus (200mg/kg), PQ (10mg/kg) + P. amarus (300mg/kg), and PQ (10mg/kg) + P. amarus (400mg/kg) groups compared to control and PQ (10mg/kg) + l-dopa groups ($p < 0.001$).

3.3. Histoarchitecture of the SN using hematoxylin and Eosin Stain (H and E Stain)

Figure 3: shows the histology of the substantia nigra. In **A** (control), there are large nigral neurons with a large round nucleus, prominent nucleoli, and small glial cells. All other groups; PQ (10mg/kg) + l-dopa (**B**), PQ (10mg/kg) (**C**), PQ (10mg/kg) + P. amarus (200mg/kg) (**D**), PQ (10mg/kg) + P. amarus (300mg/kg) (**E**), and PQ (10mg/kg) + P. amarus (400mg/kg) (**F**) show intact substantia nigra histology architecture as described in the control group.

3.4. Immunohistochemical assessments using tyrosine hydroxylase (TH)

Figure 4 :shows the immunohistochemical localization of tyrosine hydroxylase (TH) immunoexpression in large dopaminergic neurons in control (A), PQ (10mg/kg) + l-dopa (B), PQ (10mg/kg) (C), PQ (10mg/kg) + P. amarus (200mg/kg) (D), PQ (10mg/kg) + P. amarus (300mg/kg) (E) and PQ (10mg/kg) + P. amarus (400mg/kg) (F). Arrows indicate TH expressing dopaminergic neurons. (TH stain, x400). The bar graph depicts the quantification of tyrosine hydroxylase (TH) Immunoreactive Cells in the Substantia Nigra. ** denotes a significant decrease in the number of TH expressing dopaminergic neurons in PQ (10mg/kg) [53.95 ± 3.564] and PQ (10mg/kg) + P. amarus (200mg/kg)[53.95 ± 3.564] treated groups compared to control, PQ (10mg/kg) + l-dopa, PQ (10mg/kg) + P. amarus (300mg/kg) and PQ (10mg/kg) + P. amarus (400mg/kg) groups ($p < 0.001$).

4.0. Discussion

In this study, the role of the aqueous leaves extract of Phyllanthus amarus on paraquat-induced neurotoxicity in the substantia nigra of Wistar rats was studied using neurobehavioral, biochemical, histological, and immunohistochemical assessments.

The open field test is widely used to evaluate motor function in Parkinson's disease animal models (Crist ´ova˜o et al., 2020), and epidemiologic studies link paraquat exposure to Parkinson's disease (PD) development (Brouwer et al., 2017). Line crossing, rearing, walling, frequency, and duration in the center square are usually used as measures of locomotor activity as well as exploration and anxiety in open field tests, with a higher frequency of these behaviors indicating increased locomotion and exploration and low anxiety (Walsh and Cummins, 1976). This study assessed an open field test for deficit or improved motor function. A comparison of open field tests across the study period revealed no motor function deficit with the paraquat-only treatment group. This finding is at variance with reports on the paraquat-induced motor deficit. Following paraquat exposure, Fernandes et al. and Ait-Bali et al. reported significant difficulties, reduced motor activities, and modified motor coordination, attributing these adverse observations to paraquat neurotoxicity. Variance in findings with other reports could be attributed to differences in paraquat dosage administered, duration, and routes of administration adopted in this study. Besides, Rojo et al. demonstrated that paraquat administered at different intranasal doses for four weeks (28 days) did not cause motor deficits in rats which could be similar to the findings of this study. However, L-dopa and P. amarus (400 kg/mg) pretreated group showed improvement in motor coordination across the period of this study, as shown in Fig. 4.7, Fig. 4.8, and Fig. 4.12. This suggests the therapeutic properties of these treatments against paraquat-induced motor deficits in rodents.

Furthermore, antioxidant agents have been reported to improve motor coordination functions in paraquat-induced motor deficits in rodents (Ateş et al., 2019; Mirshekar et al., 2020). An established L-dopa action mechanism is the biological system's down-regulation of reactive oxygen species (ROS)

(Olanow, 2015). P. amarus also contains beneficial compounds with strong antioxidant activities, such as phyllanthin and hypophyllanthin, which are reported to potentially scavenge free radicals in various systems (Londhe et al., 2008). Oxidative stress is the imbalance in intracellular biochemical redox activity, ultimately impairing cellular integrity and function. The central nervous system is especially sensitive to oxidative stress because of the relatively high oxygen utilization, high polyunsaturated fat, and low antioxidant level (Carvalho et al., 2014), and paraquat increases the formation of free radicals and oxidative stress (Ranjbar et al., 2018). Superoxide dismutase (SOD) and catalase (CAT) are critical to the antioxidant system. Superoxide dismutase (SOD) breaks down highly reactive superoxide into less reactive hydrogen peroxide. Hydrogen peroxide is further disintegrated into water molecules and oxygen by catalase (CAT). Exacerbating the level of superoxide and hydrogen peroxide will eventually downplay the activities of superoxide dismutase (SOD) and catalase (CAT), respectively, thereby resulting in oxidative stress (Omotoso et al., 2018).

Widdowson et al. demonstrated that the administration of paraquat at a dose of 5 mg/kg/day for four weeks (28 days) does not cause biochemical changes, which partially reinforces the finding of this study where no significant difference was observed in catalase (CAT) activity in paraquat, only treatment group, compared to the control group as shown in Fig. 4.16. except for superoxide dismutase (SOD) activity, where a significant reduction in superoxide dismutase (SOD) activity was observed in paraquat only treatment group when compared to the control group and was alleviated by L-dopa pretreatment as shown in Fig. 4.17. This agrees with a previous report on L-dopa therapeutic activity against oxidative stress-triggered neurotoxicity (Olanow et al., 2015).

In addition, Edobor et al. reported cytoarchitectural distortions in the substantia nigra of rats following paraquat exposure for four weeks (28 days). This is at variance with this present study in which cytoarchitectural distortions were not observed in the paraquat (PQ) only treatment group compared to the control and other groups pretreated with P. amarus L-dopa as shown in Fig. 4.18, Fig. 4.19, and Fig. 4.20. Variance in findings could be attributed to differences in paraquat dosage administered and the duration adopted in this study. The gold standard marker used to identify a dopaminergic neuron is the presence of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine, catalyzing the conversion of L-tyrosine to L3, 4-dihydroxyphenylalanine (L-DOPA) and broadly expressed in noradrenergic and dopaminergic neurons in the central nervous system (White and Thomas, 2012; Levitt et al., 1965).

Fahim et al. reported a remarkably decreased number of dopaminergic neurons in the substantia nigra exposed to paraquat. They attributed the loss of neurons to paraquat-triggered oxidative stress, which is in agreement with this study, where the immunohistochemical assessments showed a significant decrease in the number of tyrosine hydroxylase (TH) expressing dopaminergic neurons in paraquat only treatment group compared to the control group, P. amarus (300 mg/kg), P. amarus (400 mg/kg), and L-dopa pretreated groups as shown in Fig. 4.24. This suggests that the reduction in the number of dopaminergic neurons in the substantia nigra induced by paraquat (PQ) (10 mg/kg) was alleviated by P. amarus (300 mg/kg), and P. amarus (400 mg/kg), as well as L-dopa. Previous reports have shown that

phyllanthin, a major lignan in Phyllanthus amarus leaves, is a potent neuroprotective agent (Tao et al., 2020; Yuan et al., 2021). In agreement with these reports, the observed therapeutic activity of Phyllanthus amarus leaves extract against paraquat-triggered pathological changes in the substantia nigra of Wistar rats in this study may be attributable to phyllanthin. Thus, P. amarus, especially at 300 mg/kg and 400 mg/kg doses, possesses therapeutic properties comparable to the reference drug, L-dopa.

5.0. Conclusion

In conclusion, the aqueous leaf extract of Phyllanthus amarus possesses therapeutic properties against paraquat-triggered pathological changes in the substantia nigra of Wistar rats. Therapeutic properties could be attributed to bioactive compounds in aqueous leaves extract of Phyllanthus amarus with potent antioxidant activities against reactive oxygen species-associated paraquat-triggered pathologies.

Declarations

Acknowledgment

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Ethical approval

This study was submitted for review, and approval was granted by the Research Ethics Committee of the University of Medical Sciences, Ondo, Nigeria, with approval number NHREC/TR/UNIMED-HREC-Ondo St/22/06/21. The animals received human care following the principle of human care and the use of laboratory animals.

Competing interests

The author discloses no conflict of interest.

Authors contribution:

Felix, Kingsley, and Blessing conceptualized the research work and designed the experiment. Felix, Kingsley, and Blessing performed the experiment. Felix, Kingsley, Blessing, and Raphael analyzed the data and wrote the manuscript. Felix and Kingsley reviewed the manuscript.

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Availability of data set and materials

Data are available on request.

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Figures

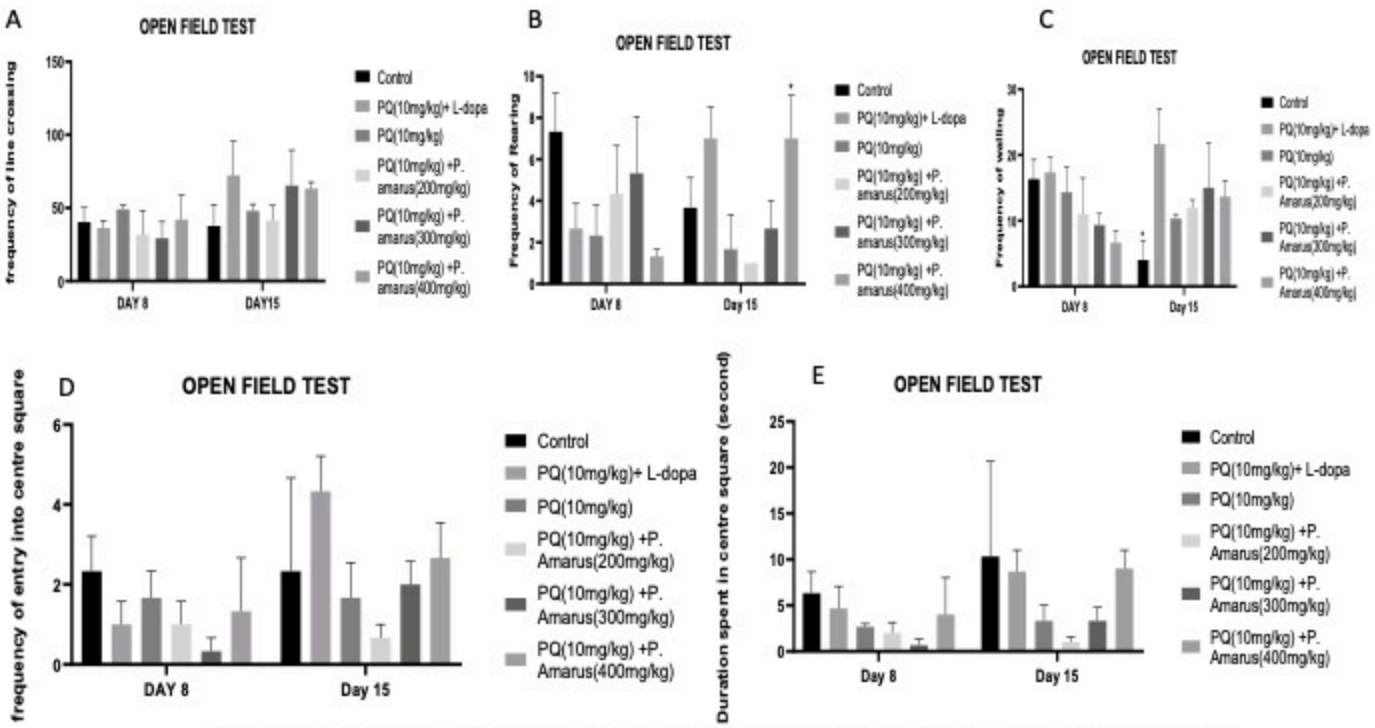


Fig 1: bar graphs showing a comparison between day eight and day 15 in the Open field test

Figure 1

See image above for figure legend.

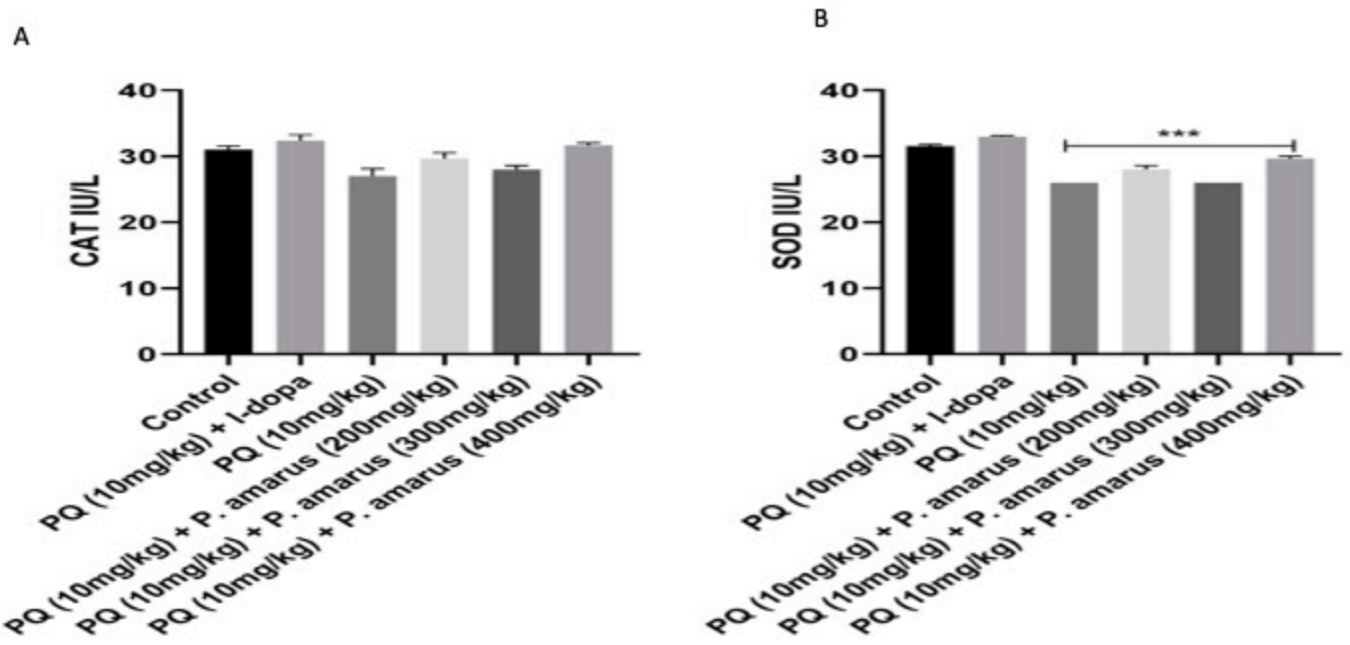


Fig 2: A bar graphs showing oxidative stress markers level

Figure 2

See image above for figure legend.

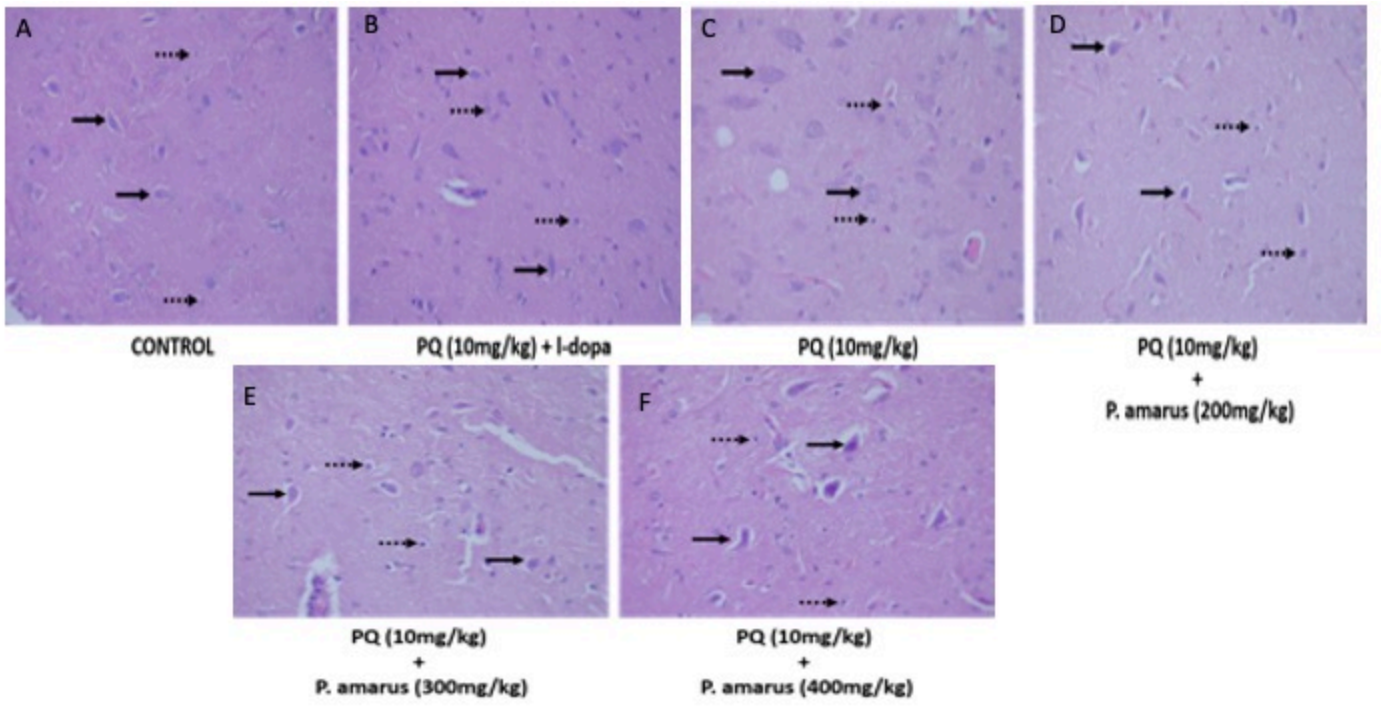


Fig 3: Photomicrographs of the the substantia nigra (H and E , x400

Figure 3

See image above for figure legend.

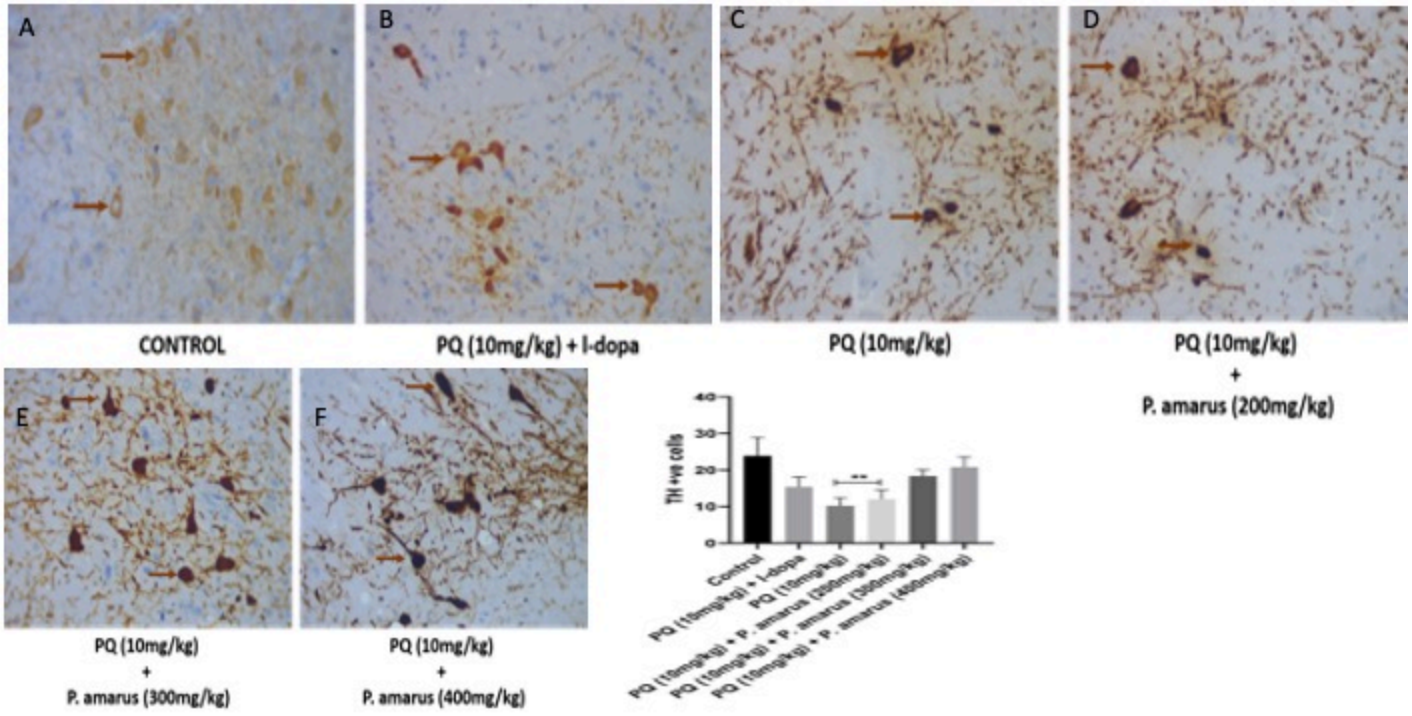


Fig 4: Photomicrographs of the substantia nigra of Wistar rats (TH stain, x400)

Figure 4

See image above for figure legend.