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ANTIPARKISONIAN ACTIVITIES OF THE ACTIVE COMPONENTS OF *VERNONIA AMYGDALINA* IN WISTAR RATS

Nwozor C. M.[1*], Ughachukwu P. O.[2], Okonkwo P. O.[2] and Ilo C. E.[3]

[1]Department of Physiology, Faculty of Basic Medical Sciences, Chukwuemeka Odumegwu Ojukwu University, Uli Campus, Anambra State, Nigeria.
[2]Department of Pharmacology and Therapeutics, College of Medicine, Chukwuemeka Odumegwu Ojukwu University, Awka Campus, Nigeria.
[3]Department of Pharmacology and Therapeutics, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, Anambra State, Nigeria.

***Corresponding Author: Dr. Nwozor C. M.**
Department of Physiology, Faculty of Basic Medical Sciences, Chukwuemeka Odumegwu Ojukwu University, Uli Campus, Anambra State, Nigeria.

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ABSTRACT
V amygdalina (VA) is used by traditional medical practitioners as a digestive tonic, appetizer, and for the management of wounds. Parkinson's disease (PD) is a neurodegenerative condition characterized by resting tremor, cogwheel rigidity, bradykinesia, and postural reflex changes due to lack of dopamine in the nigrostriatal area of the brain. This study was undertaken to investigate the antiparkinsonian activities of the active components of *V. amygdalina* in Wistar rats. 54 apparently healthy male Wistar rats weighing between 120-180 g were randomly distributed into nine groups of 6 rats per group. PD was induced in the experimental rats using paraquat (10 mg/kg intra-peritoneally weekly for three weeks. Neurobehavioral tests were assessed using hanging wire/arm grip test, pole test, and elevated-plus maze (EPM). Immunohistochemistry for tyrosine hydroxylase (TH) and α-synuclein was done using brain tissue. There was a decrease in the duration of the time spent on the pole test in the treatment groups compared with the control ($p<0.05$).*V. amygdalina* showed significant neuroprotective effect on tyrosine hydroxylase and α-synuclein levels. It equally had significant antioxidant properties. Methanol extract of *V. amygdalina* contains some important bioactive components. All the three fractions of this extract: ethyl acetate, n-hexane, and methanol fractions demonstrated significant neuroprotective effects. *V. amygdalina* may be useful in the management of PD.

KEYWORDS: *Vernonia amygdalina*, Parkinson's disease, Wistar rats, paraquat, neuroprotective effects.

INTRODUCTION
Parkinson's disease is a neurodegenerative disorder that results in a significant deficiency of dopamine in the nigro-striatal pathway. Parkinson's disease (PD) is the second neurodegenerative disease after Alzheimer's disease. It affects 1% of the population with the average age for the appearance of the disease being between 55-60 years of age while 10-15% of the patients are diagnosed before the age of 50.[1]

There is no known cause of PD, however, environmental factors and /or genetic mutations have been theorized to be implicated.[2] Knowledge about its pathogenesis has increased in recent years. Studies into this disease have been done with various types of animal models which have been well documented.[3] For example, reserpine syndrome has been successfully created in an animal model with the administration of reserpine. The features observed were rigidity and slowness of movement.[4] These features are now commonly associated with PD. Further experimental research revealed that L-DOPA was able to alleviate many of the features associated with reserpine administration. This strengthened the hypothesis that dopamine depletion was at the root of PD pathogenesis. It has been supported by the findings of many researchers in the field of neuroscience.[5]

V. amygdalina has various names depending on the environment. The cooked leaves are a staple vegetable in soups in different parts of Nigeria. The various names include etidot (Ibibio), Onugbu (Igbo), and ewuro (Yoruba).It is called bitter leaf in English. The medicinal properties of *V. amygdalina* have been widely reported. These range from its antibacterial,[6] antiplasmodial and antimalarial,[7] amoebicidal[8] properties to wound management.[9] Apart from its analgesic[7] action there is no report on its possible action in the central nervous system. This research was undertaken to evaluate the effects of methanol leaf extract of *vernonia amygdalina* on paraquat-induced Parkinson's disease in Wistar rat.

MATERIALS and METHODS

Paraquat (Paraefore[R]) (Nanjing Redsun Biochemistry Co Ltd, China), levodopa (Sinemet[R]) (Levodopa/carbidopa-MSD); Human α-synuclein (SNCA) ELISA KIT (CAT. NO: EKHU-1285, Melsin Medical Co Ltd, China); Rat Tyrosine Hydroxylase (TH) ELISA KIT (CAT. NO: EKRAT-0756, Melsin Medical Co. Ltd, China);[10] Immunohistochemical reagents (#MP-7401, Vector® Labs, USA)- ImmPRESS™ (peroxidase) polymer Anti-Rabbit IgG Reagent.

$$\text{Percentage yield} = \frac{\text{Final weight of extract}}{\text{Weight of dry leaves}} \times 100$$

Plant Collection and Taxonomy

The leaves of *vernonia amygdalina* were purchased from afor-egbu market at Uli, Ihiala local government area, Anambra State. The leaves were authenticated by a botanist (Dr Chukwujekwu G. Ukpaka), Department of Biological Sciences, Chukwuemeka Odumegwu Ojukwu University (COOU), Uli campus.

Preparation of Crude Extracts

The fresh leaves were shade-dried and powdered with an electric blender. The powdered sample of *V. amygdalina* weighing 20 g were extracted with methanol solvent (200 mL) by using Soxhlet extractor for 72 h. At the end of the extraction process, the methanol solvent was evaporated by using rotary evaporator (Yamato Rotary Evaporator, model-RE801) under reduced pressure to obtain methanol crude extract. The formula for calculating the yield of the crude extract is as follows:

This crude extract of methanol was suspended in water. It was fractionated successively with different organic solvents such as n-hexane, ethyl acetate to obtain hexane, ethyl acetate and residual methanol fractions respectively. Whatman No. 41 was used to filter the crude extracts. This helped to remove particles. The particle free crude extract was evaporated by using rotary evaporator under reduced pressure. This resulted in dry crude extracts. The combined extracts were concentrated and dried by using rotary evaporator under reduced pressure.[11]

Fractionation Process

When several solvents such as ethyl acetate, n-hexane, and methanol are required for fractionation, they should be added according to the order of increasing polarity.[12] Fractionation was done using column chromatography. The column was made up of a long glass tube (5-50 mm in diameter, 5 cm-1 m long) with a tap and glass wool filter at the bottom. Silica gel, alumina, cellulose or Sephadex could be used as stationary phase, whereas the mobile phase is liquid. The process began by packing 30 g of Silica gel (70/35) into a transparent glass column (80 cm long, 5 cm diameter) without introducing air bubbles. Subsequently the extract to be partitioned was added from the top. Least polar solvent (n-hexane) was first added as a mobile phase and allowed to stand for 1 h in a closed column. The bottom of the column was opened and various fractions of n-hexane collected at an interval. In addition to that, other solvents such as ethyl acetate and methanol were added. Fractions of these solvents were collected individually at different time intervals and finally characterized.[12,13]

Determination of Acute Toxicity (LD50) Of the Three Fractions of *V. Amygdalina*

Acute toxicity of the three fractions of *V. amygdalina* was done using the method of Lorke.[14] Three groups of three rats each were orally administered with *V. amygdalina* at doses of 10 mg/kg, 100 mg/kg, and 1000 mg/kg body weight. The animals were observed for 24 hours. If there was no death in phase I the next phase was phase II. In phase II three groups of one rat each were given the following doses of *V. amygdalina* orally: 1600 mg/kg, 2900 mg/kg, and 5000 mg/kg body weight. They were observed for 24 hours and the number of death was recorded. The same procedure was carried out on each of the three fractions of *V. amygdalina* namely: ethyl acetate, hexane, and methanol. LD50 value was determined using the following formula:

$$LD50 = \sqrt{D0 \times D100}$$

Where D0 = Maximum dose that did not cause death.
D100 = Minimum dose that caused death.

Gas Chromatography Mass Spectroscopy (GC-MS)

The GC-MS analysis of bioactive compounds from the different extracts of the leaves was done using Agilent Technologies GC systems with GC-7890A/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column (30 m in length ×250 µm in diameter × 0.25 µm in thickness of film). Spectroscopic detection by GC-MS involved an electron ionization system which utilized high energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with flow rate of 1 mL/min. The initial temperature was set at 50-150 ^{o}C with increasing rate of 3 ^{o}C/min and holding time of about 10 min. Finally, the temperature was increased to 300 ^{o}C at 10 ^{o}C/min. One microliter of the prepared 1% of the extracts diluted with respective solvents was injected in a splitless mode. Relative quantity of the chemical compounds present in each of the extracts of *V. amygdalina* was expressed as percentage based on peak area produced in the chromatogram.[15]

Identification of Chemical Constituents

Bioactive compounds extracted from different extracts of *V. amygdalina* were identified based on GC retention time on HP-SMS column and matching of the spectra with computer software data of standards (Replib and Mainlab data of GC-MS system).

Experimental Animals

Apparently healthy rats (about 54) from Francis farms Ltd, Nnewi were used. Average weight of each rat was between 140 g to 200 g. The rats were randomly

distributed into nine groups of 6 rats per group: Group A (control), Group B (PD only), Group C (PD plus methanol fraction of VA 100 mg/kg), Group D (PD plus methanol fraction of VA 200 mg/kg), Group E (PD plus L-DOPA, once daily p.o. 200 mg/kg), Group F (PD plus ethyl acetate fraction 100 mg/kg), Group G (PD plus ethylacetate fraction 200 mg/kg), Group H (PD plus n-hexane fraction 100 mg/kg), Group I (PD plus n-hexane fraction 200 mg/kg). Animals were housed at 22 ± 1^{0}C (12-hour light-dark cycle).They were acclimatized for one week before commencement of experiment. Feed and water were made available to the rats *ad libitum.* All experiments were carried out according to high ethical standards.

Study design

Group A (control) received water and feed *ad libitum*. Groups B, C, D, E, F, G, H, and I animals received intraperitoneal injection of paraquat (10mg/kg) weekly for three weeks according to previously published guidelines [16].Groups C and D received 100 mg/kg and 200 mg/kg respectively of methanol fraction of leaf extract of *vernonia amygdalina* by naso-gastric tube daily for 20 days starting 1 hr. after first paraquat injection. Groups F and G rats received ethyl acetate fraction 100 mg/kg and 200 mg/kg respectively, while group H and I rats received 100 mg/kg and 200 mg/kg n-hexane fraction of VA respectively. On the 6^{th} day after the last dose of paraquat the animals were sacrificed and their blood sampled. The serum samples were used to check for the following biomarkers: α-Synuclein, tyrosine hydroxylase, total anti-oxidant capacity (TAC), malondialdehyde (MDA), and Glutathione peroxidase (GPx), superoxide dismutase (SOD). The whole brains of the rats were removed and then fixed in 10 % formal saline for immunohistochemical assay.

Neurobehavioral Tests

Several tests were carried out to evaluate the neurobehavioral activities of the experimental rats. Such tests were: elevated plus-maze, hanging wire (arm grip test), and pole test. These involved test of motor balance and coordination abilities. The elevated plus-maze was used to assess the level of anxiety in the rats. Behavioral tests commenced after five days of paraquat injections.

- Hanging wire (arm grip test) was used to test for the passive motor ability of each rat. The duration for a rat to stay on a horizontal wire was used to assess its motor coordination ability. Hanging wire is a simple method to assess muscle strength and coordination. The apparatus consisted of a horizontal wire across a board containing soft beddings. When placed on the wire the rat would struggle to maintain its balance. It would hold tenaciously to the wire until it got exhausted and fell down. Maximum time was five minutes (300 seconds). A rat with brain lesion, neurological impairment, or neurodegenerative disease (e.g. Parkinson's disease) would spend less time on the wire. Animal weight, balance, and behavior could influence the result of the test.

- Pole test. This test was carried out as described by Matsura et al.,[17] Pole test basically is used to investigate movement disorder caused by dopamine loss in the brain. A rat is placed on a pole (height 50 cm; width 1 cm). The time taken for the rat to climb down with four paws touching the ground was recorded. Total time was five minutes or 300 seconds.

- Elevated Plus- Maze (EPM). Elevated plus-maze measures anxiety. Most putative anxiogenic and anxiolytic drugs are screened using this apparatus. It consists of two elevated closed-arm and two open arm looking like a cross. There is a neutral center square. The EPM is validated for rats and mice.[18] A rat was gently placed in the center square facing the open arm. Each trial lasted for five minutes. The rats were allowed to explore the EPM freely. The EPM was kept clean and fecal droppings removed before a rat was placed in the center square. All experiments were carried out by the same person to reduce error. Stop watch (android phone) was used to time the session. Total time spent in closed and open arms were recorded and analyzed.

Immunohistochemistry

Tyrosine hydroxylase (TH) and α-synuclein immunohistochemistry using ImmPRESS^{TM} HRP Polymer system (Vector® Labs, USA) was done. Paraffin embedded sections where deparaffinized with xylene, and rehydrated through descending grades of ethanol (100%, 95%, 70% ethanol) and taken to water.

- Heat-mediated antigen retrieval was performed using a citrate-based antigen unmasking solution, pH 6.0 (Vector®, Burlingame, CA, USA; #H3300) in a steamer for 30 min. Sections were washed in phosphate buffered saline (PBS) for 2 min. Endogenous peroxidase blocking in 0.3% hydrogen peroxide solution in PBS for 10 min. Sections were washed in PBS for 2 min. Sections were incubated in 2.55 normal animal serum for 20 min for protein blocking. Sections were then incubated for 2 hours at room temperature in primary antibodies; rabbit monoclonal alpha-synuclein (Cell Signalling, USA) at 1: 200, and mouse monoclonal tyrosine hydroxylase (Santa Cruz Biotechnology, USA) at 1: 400.Sections were washed in PBS for 5 min.

- Sections were incubated in ImmPRESS HRP Anti-Rabbit IgG (Peroxidase) Polymer Reagent, made in horse for 30 min. Sections were washed in PBS for 5 min ×2. Color was developed with DAB Peroxidase (HRP) Substrate Kit (Vector® Labs, USA).Sections were rinsed well in tap water, counter-stained in hematoxylin, and dehydrated through ascending grades of ethanol (70%, 95%, and 100%), cleared in

Xylene and mount with Permount (Fischer Scientific, USA).Sections without primary antibodies were similarly processed to control for immunohistochemistry procedures. No specific immunoreactivity was detected in control sections.

Photomicrography of the sections were observed under a digital brightfield microscope (OMAX 40-2000X 3MP Digital Compound Microscope, USA) and photomicrographs were taken 100x or 400x magnification.[19]

Statistical Analysis

Data obtained were expressed as Mean ± SEM. Data were analyzed statistically using SPSS version 21. One-way ANOVA was done followed by post-hoc Bonferroni. For the tyrosine hydroxylase post-hoc Turkey was done. A p-value < 0.05 was considered significant.

RESULTS

Results of LD50: Ethyl Acetate Fraction

LD50 = $\sqrt{D0 \times D100}$. D0= Maximum dose at which no mortality occurred.
D0 = 100 mg/kg
D100 = Minimum dose at which mortality occurred.
D100 = 1000 mg/kg. LD50 = $\sqrt{100 \times 1000}$
316.23 mg/kg

Methanol Fraction: No death was recorded with the maximum dose of 5000 mg/kg.
LD50, therefore, was > 5000 mg/kg.

Hexane Fraction: No death was recorded with the maximum dose of 5000 mg/kg.
LD50 was > 5000 mg/kg

Results of **Gas Chromatography-Mass Spectrometry (GC-MS)**

The GC-MS result revealed the presence of detected compounds in each of the fractions of *V. amygdalina*. The result showed their retention time, concentration (area %), and CAS (Chemical Abstracts Service) numbers. The GC-MS chromatograms showed several peaks. Some bioactive compounds were studied and their molecular structure, molecular weight, chemical formula, and pharmacological activities are shown in table I below. This means that the methanol leaf extract of *V. amygdalina* contains some important bioactive compounds.

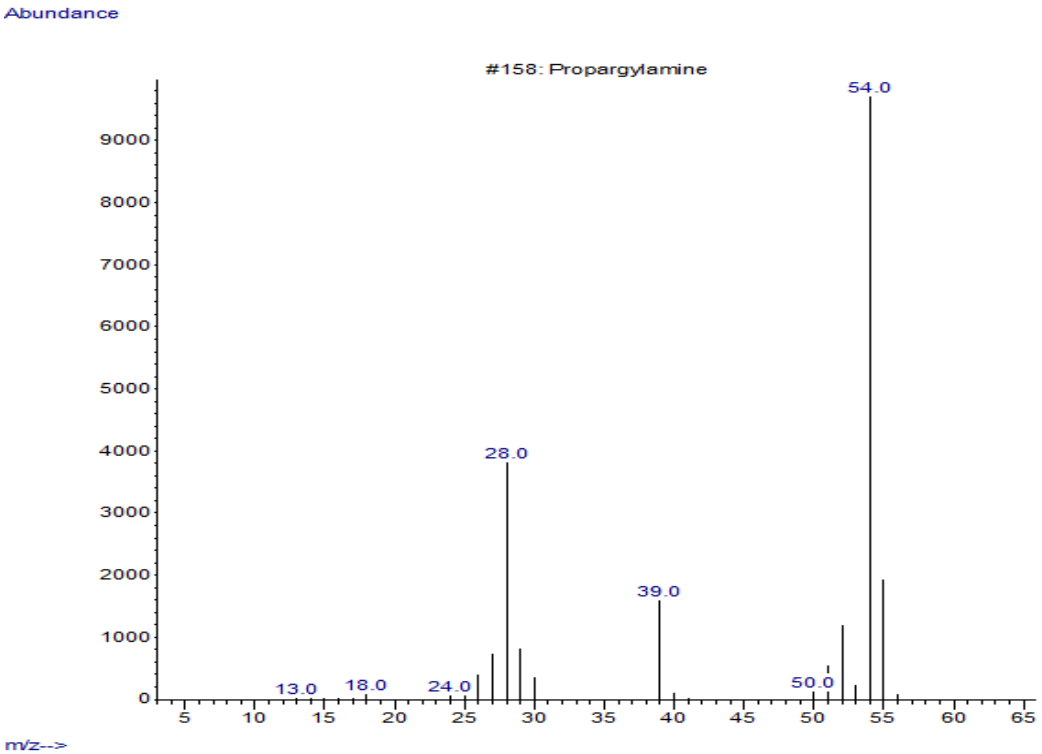

Fig. 1: Chromatogram of propargylamine, a bioactive compound from *V. amygdalina.*

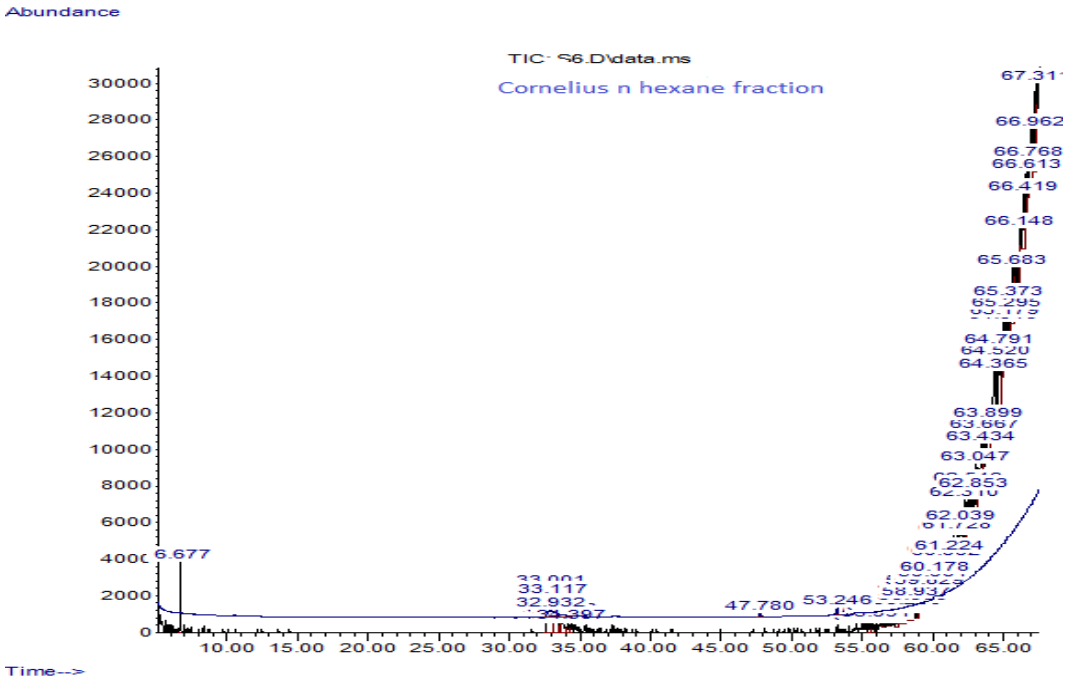

Fig. 2: Chromatogram of n-hexane fraction of *V. amygdalina.*

Some Bioactive Compounds Detected

1) Thiirane: anticancer and antimicrobial.[20]
2) Propargylamine: antiparkinsonian.[21,22]
3) Oxazole: antioxidant.[23]
4) Mephenesin: antiparkinsonian and antispasticity.[24]
5) Triazole: anticancer.[25]
6) Azetidine: antimicrobial and anticancer.[26]
7) 1,5-pentanediol: antiviral.[27]

Physical Observation

After the administration of paraquat, I observed some changes in the experimental animals. There were tremors, hunching, bradykinesia, and decreased locomotor activity.

Neurobehavioral Studies

Elevated Plus Maze (EPM)

In the open arm there was a decrease in the time spent in the open arm in the treatment groups compared with the control. However, this decrease was not statistically significant. In the closed arm some groups (groups B, E, F, G) showed an increase in duration of time while others (groups C, H, I) showed a decrease in duration of time. Group D remained unchanged. There was no statistically significant change in the treatment groups compared with the control.

Pole Test

There was a decrease in the duration of the time spent on the pole test in the treatment groups compared with the control. The decrease was statistically significant (p=0.029) in group G compared with control. The result of post hoc Bonferroni multiple comparison showed that group B vs G was statistically significant (p=0.036). Therefore the result showed that the pole test at 200 mg/kg ethyl acetate fraction of VA had a very significant effect on paraquat-induced movement disorder when compared with the control.

Hanging Wire Test

The results of the hanging wire test showed that there were changes in the duration of time between the control and the treatment groups. However these changes were not statistically significant.

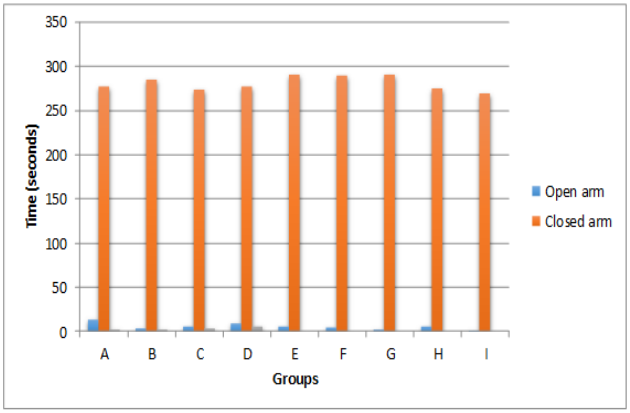

Fig. 3: Elevated plus maze.

Key: Group A= control, Group B= PQ (PD rats), Group C= PQ+100 mg/kg methanol fraction of VA, Group D= PQ+200 mg/kg methanol fraction of VA, Group E= PQ+ 200 mg/kg levodopa, Group F= PQ+ 100 mg/kg ethyl acetate fraction of VA, Group G= PQ+ 200 mg/kg ethyl acetate fraction of VA, Group H= PQ+ 100 mg/kg n-hexane fraction of VA, Group I= PQ+ 200 mg/kg n-hexane fraction of VA. PQ= Paraquat, VA= vernonia amygdalina, PD= Parkinson's disease.

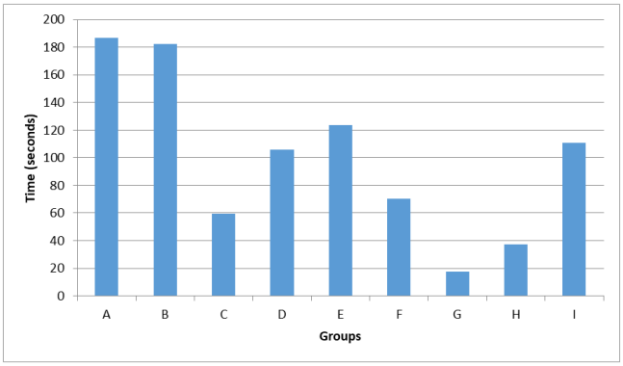

Fig. 4: Pole test.

Key: Group A= control, Group B= PQ (PD rats), Group C= PQ+100 mg/kg methanol fraction of VA, Group D= PQ+200 mg/kg methanol fraction of VA, Group E= PQ+ 200 mg/kg levodopa, Group F= PQ+ 100 mg/kg ethyl acetate fraction of VA, Group G= PQ+ 200 mg/kg ethyl acetate fraction of VA, Group H= PQ+ 100 mg/kg n-hexane fraction of VA, Group I= PQ+ 200 mg/kg n-hexane fraction of VA. PQ= Paraquat, VA= vernonia amygdalina, PD= Parkinson's disease.

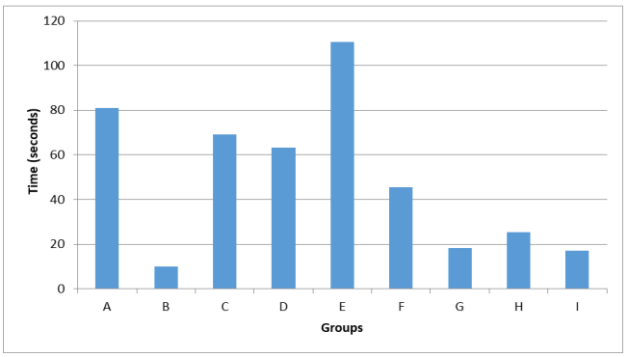

Fig. 5: Hanging wire test.

Key: Group A= control, Group B= PQ (PD rats), Group C= PQ+100 mg/kg methanol fraction of VA, Group D= PQ+200 mg/kg methanol fraction of VA, Group E= PQ+ 200 mg/kg levodopa, Group F= PQ+ 100 mg/kg ethyl acetate fraction of VA, Group G= PQ+ 200 mg/kg ethyl acetate fraction of VA, Group H= PQ+ 100 mg/kg n-hexane fraction of VA, Group I= PQ+ 200 mg/kg n-hexane fraction of VA. PQ= Paraquat, VA= vernonia amygdalina, PD= Parkinson's disease.

Results of the Immunohistochemistry

Tyrosine hydroxylase (TH): The substantia nigra (SNr) contains mostly dopaminergic neurons that express TH. These neurons project to several brain regions, particularly the striatum. Microscopic examination of the control rats revealed marked presence of TH-expressing neurons. Injection of paraquat caused a significant decrease in TH-expressing neurons. Treatment groups showed marked improvement except in groups E and H (fig 6).

Tyrosine expression in the striatum mostly appeared diffuse since the neurons expressing TH in the striatum have the neuronal bodies in the SNr, and project numerous axons into the striatum. There was an obvious expression of TH in the striatum across all groups. A noticeable decrease was observed in paraquat-injected rats. There was an improvement in the treatment groups except in group E (positive control).

Alpha-Synuclein: Alpha-synuclein activity in the striatum and the hippocampus were investigated. Microscopic examination showed more intense expression of alpha-synuclein in the striatum and hippocampus in paraquat-injected rats compared to control. Treatment groups appeared to show less α-synuclein expression compared to paraquat-injected rats (group B) (fig7)

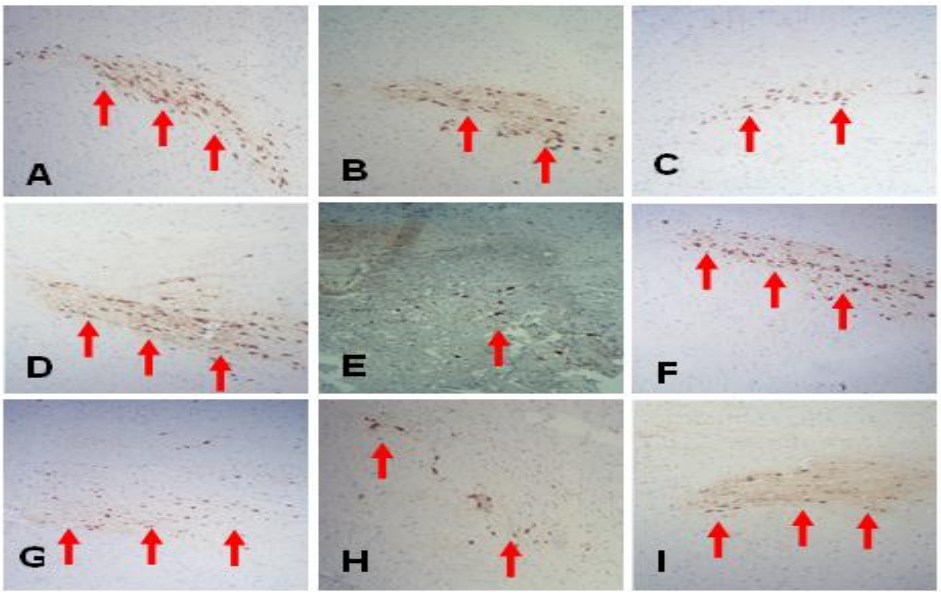

Fig 6: Shows immunohistochemistry (representative photomicrographs) of tyrosine hydroxylase (TH) in the substantia nigra. Red arrow= tyrosine hydroxylase (TH)-neurons.

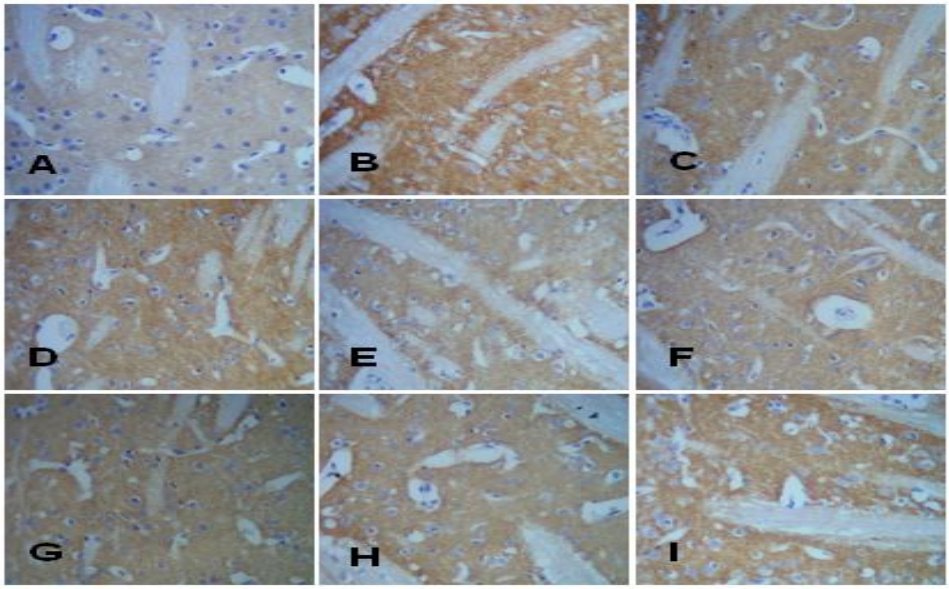

Fig 7: Shows immunohistochemistry (representative photomicrographs) of α-synuclein in the striatum.

Key: Group A= control, Group B= PQ (PD rats), Group C= PQ+100 mg/kg methanol fraction of VA, Group D= PQ+200 mg/kg methanol fraction of VA, Group E= PQ+ 200 mg/kg levodopa, Group F= PQ+ 100 mg/kg ethyl acetate fraction of VA, Group G= PQ+ 200 mg/kg ethyl acetate fraction of VA, Group H= PQ+ 100 mg/kg n-hexane fraction of VA, Group I= PQ+ 200 mg/kg n-hexane fraction of VA. PQ= Paraquat, VA= vernonia amygdalina, PD= Parkinson's disease.

Effect of *V. Amygdalina* on Serum Tyrosine Hydroxylase (TH) Concentrations.

The results of the effect of *V. amygdalina* on tyrosine hydroxylase (TH) concentrations are shown in fig.8 below. Paraquat (group B) caused a significant decrease in tyrosine hydroxylase levels. The value in the control rats was 245.88±8.34 pg/mL while the paraquat-injected rats had 180.82±10.36 pg/mL. *V. amygdalina* (notably the methanol fraction) was able to block paraquat and restored the value to normal level. This neuroprotective effect was similar to that caused by levodopa (group E). Among the treatment groups ethyl acetate fraction had the least effect, bringing the value to 196.76±6.42 pg/mL.This was significantly lower than that of the positive control (levodopa) (254.46±20.20). Therefore methanol fraction of *V. amygdalina* exerted a significant beneficial effect on tyrosine hydroxylase enzyme level.

Effect of *V. Amygdalina* on Serum α-Synuclein Activity

The results of the effect of *V. amygdalina* on α-synuclein levels are presented in fig.9 below. Paraquat significantly increased the α-synuclein levels. The value in control rats was 3.22±0.39 ng/mL, while paraquat-injected rats had significantly elevated value of 7.85±0.77. *V. amygdalina* (methanol, ethyl acetate, and n-hexane fractions) and levodopa significantly blocked paraquat and reversed the values. This neuroprotective effect, though exerted by the three fractions of *V. amygdalina* was best demonstrated by ethyl acetate fraction. 100 mg/kg and 200 mg/kg of ethyl acetate fraction had 2.92±0.28 and 2.86±0.32 ng/mL respectively. Therefore, all the three fractions of *V. amygdalina* (methanol, ethyl acetate, and n-hexane) showed significant beneficial effect by protecting against paraquat-induced injury and restoring the values to normal or near normal. This effect was similar to that of levodopa (4.53±0.42 ng/mL).

Effect of *V. Amygdalina* on Total Antioxidant Capacity (TAC) Levels

The results of the effect of *V. amygdalina* on total antioxidant capacity (TAC) levels are shown in fig.10 below. Control rats recorded 1025.60±62.05 µmol/L. In paraquat-injected rats the value significantly decreased to 329.40±19.66 µmol/L. *V. amygdalina* (notably n-hexane and methanol fractions) blocked paraquat. The value in n-hexane treated rats was 741.58±90.03 (72% of normal) while that of methanol fraction was 509.06±13.74 (49.7% of normal). The effect of methanol fraction was close to that of levodopa (584.00±30.76 µmol/L= 56.98% of normal). Thus the effect of n-hexane fraction was better than that of levodopa. Therefore, *V. amygdalina* (n-hexane and methanol fractions) significantly protected against paraquat-induced injury. This beneficial effect of n-hexane fraction of *V. amygdalina* was better than that of positive control, levodopa, which was better than that of methanol fraction. Ethyl acetate fraction did not have any significant effect on TAC and thus was not able to boost its level after paraquat-induced depletion.

Effect of *V. Amygdalina* on Malondialdehyde (MDA) Concentrations

The results of the effect of *V. amygdalina* on malondialdehyde (MDA) are shown in fig.11 below. The value in the control rats was 2.56±0.62 nmol/mL. It was significantly increased in paraquat-injected rats. The value was 4.82±0.25. All the three fractions of *V.*

amygdalina blocked paraquat. N-hexane, ethyl acetate, and methanol fractions (at 200 mg/kg) had the following values: 2.45±0.31, 2.75±0.23, 3.03±0.22 nmol/mL respectively. Levodopa significantly blocked paraquat. However, the n-hexane and ethyl acetate fractions demonstrated better effect than levodopa. The effect of levodopa was similar to that of methanol fraction. *V. amygdalina* (n-hexane, ethyl acetate, and methanol fractions) exerted beneficial effect on MDA by significantly reducing its serum concentrations after paraquat-induced elevation. It was dose-dependent.

Effect of *V. Amygdalina* on Glutathione Peroxidase (Gpx) Levels

The results of the effect of *V. amygdalina* on GPx levels are shown in fig.11 below. Control rats had 0.80±0.13 U/mL. This was significantly decreased by paraquat (0.30±0.04 U/mL). *V. amygdalina* (methanol and n-hexane fractions only) significantly blocked paraquat. The values in methanol and n-hexane fractions (at 200 mg/kg) were 0.66±0.09 and 0.66±0.08 U/mL respectively. Levodopa and ethyl acetate fraction did not exert any significant effect on GPx, having the following values: 0.34±0.01 and 0.46±0.08 U/ mL respectively. Therefore *V. amygdalina* (notably, methanol and n-hexane fractions) showed significant beneficial effect on GPx by increasing its levels when compared to paraquat-injected rats. The effect of n-hexane fraction (0.66±0.08 U/mL = 82.5% of normal) was equal to that of methanol fraction (0.66±0.09 = 82.5% of normal).

Effect of *V. Amygdalina* on Superoxide Dismutase (SOD) Activity

The results of the effect of *V. amygdalina* on superoxide dismutase (SOD) are shown in fig.11 below. Control rats had 16.10±3.83 U/mL. This was significantly decreased by paraquat (6.79±0.87 U/mL). *V. amygdalina* and levodopa partially blocked paraquat. The final value as seen in methanol fraction was 11.98±1.53; levodopa 13.66±2.36; ethyl acetate fraction 13.60±1.48; and n-hexane fraction 12.00±1.41 were not up to the normal value of 16.10±3.83 U/mL. *V. amygdalina* non-significantly reversed paraquat-induced decrease in SOD level.

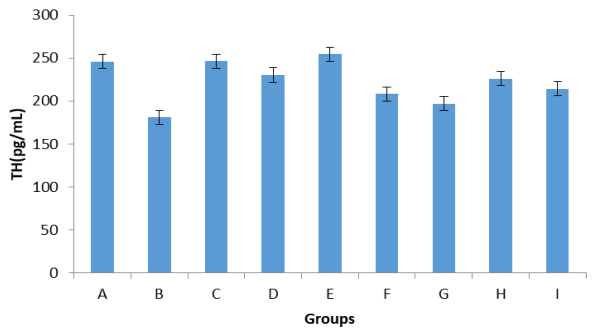

Figure 8: Effect of *V. amygdalina* on tyrosine (TH) concentrations.

Key: Group A= control, Group B= PQ (PD rats), Group C= PQ+100 mg/kg methanol fraction of VA, Group D= PQ+200 mg/kg methanol fraction of VA, Group E= PQ+ 200 mg/kg levodopa, Group F= PQ+ 100 mg/kg ethyl acetate fraction of VA, Group G= PQ+ 200 mg/kg ethyl acetate fraction of VA, Group H= PQ+ 100 mg/kg n-hexane fraction of VA, Group I= PQ+ 200 mg/kg n-hexane fraction of VA. PQ= Paraquat, VA= vernonia amygdalina, PD= Parkinson's disease. TH= Tyrosine hydroxylase.

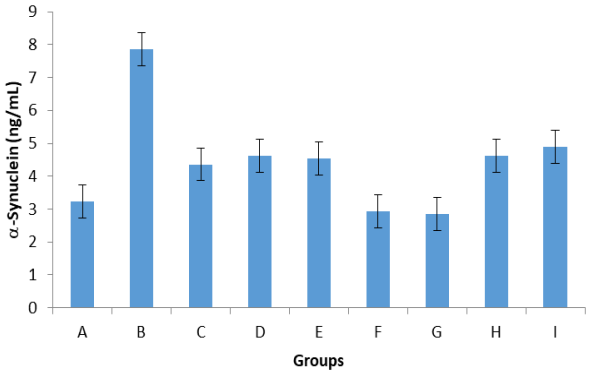

Figure 9: Effect of *V. amygdalina* on α-synuclein activity.

Key: Group A= control, Group B= PQ (PD rats), Group C= PQ+100 mg/kg methanol fraction of VA, Group D= PQ+200 mg/kg methanol fraction of VA, Group E= PQ+ 200 mg/kg levodopa, Group F= PQ+ 100 mg/kg ethyl acetate fraction of VA, Group G= PQ+ 200 mg/kg ethyl acetate fraction of VA, Group H= PQ+ 100 mg/kg n-hexane fraction of VA, Group I= PQ+ 200 mg/kg n-hexane fraction of VA. PQ= Paraquat, VA= vernonia amygdalina, PD= Parkinson's disease.

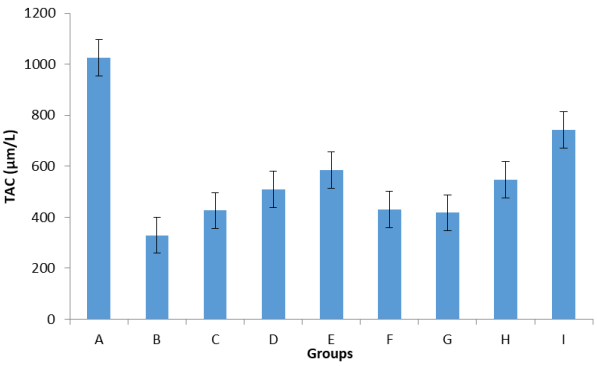

Figure 10: Effect of *V. amygdalina* on Total Antioxidant Capacity (TAC) levels.

Key: Group A= control, Group B= PQ (PD rats), Group C= PQ+100 mg/kg methanol fraction of VA, Group D= PQ+200 mg/kg methanol fraction of VA, Group E= PQ+ 200 mg/kg levodopa, Group F= PQ+ 100 mg/kg ethyl acetate fraction of VA, Group G= PQ+ 200 mg/kg ethyl acetate fraction of VA, Group H= PQ+ 100 mg/kg n-hexane fraction of VA, Group I= PQ+ 200 mg/kg n-

hexane fraction of VA. PQ= Paraquat, VA= vernonia amygdalina, PD= Parkinson's disease.TAC= Total antioxidant capacity.

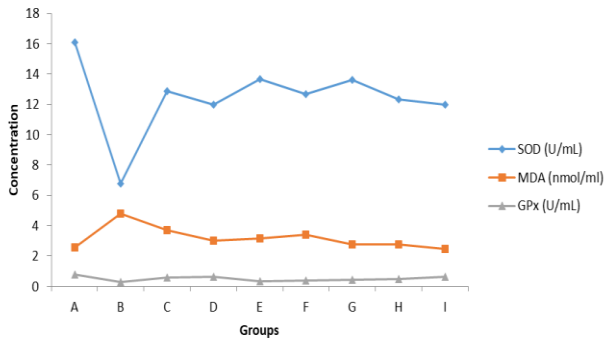

Figure 11: Effect of *V. amygdalina* on biomarkers of oxidative stress.

Key: Group A= control, Group B= PQ (PD rats), Group C= PQ+100 mg/kg methanol fraction of VA, Group D= PQ+200 mg/kg methanol fraction of VA, Group E= PQ+ 200 mg/kg levodopa, Group F= PQ+ 100 mg/kg ethyl acetate fraction of VA, Group G= PQ+ 200 mg/kg ethyl acetate fraction of VA, Group H= PQ+ 100 mg/kg n-hexane fraction of VA, Group I= PQ+ 200 mg/kg n-hexane fraction of VA. PQ= Paraquat, VA= vernonia amygdalina, PD= Parkinson's disease.SOD= Superoxide dismutase, MDA= Malondialdehyde, GPx= Glutathione peroxidase.

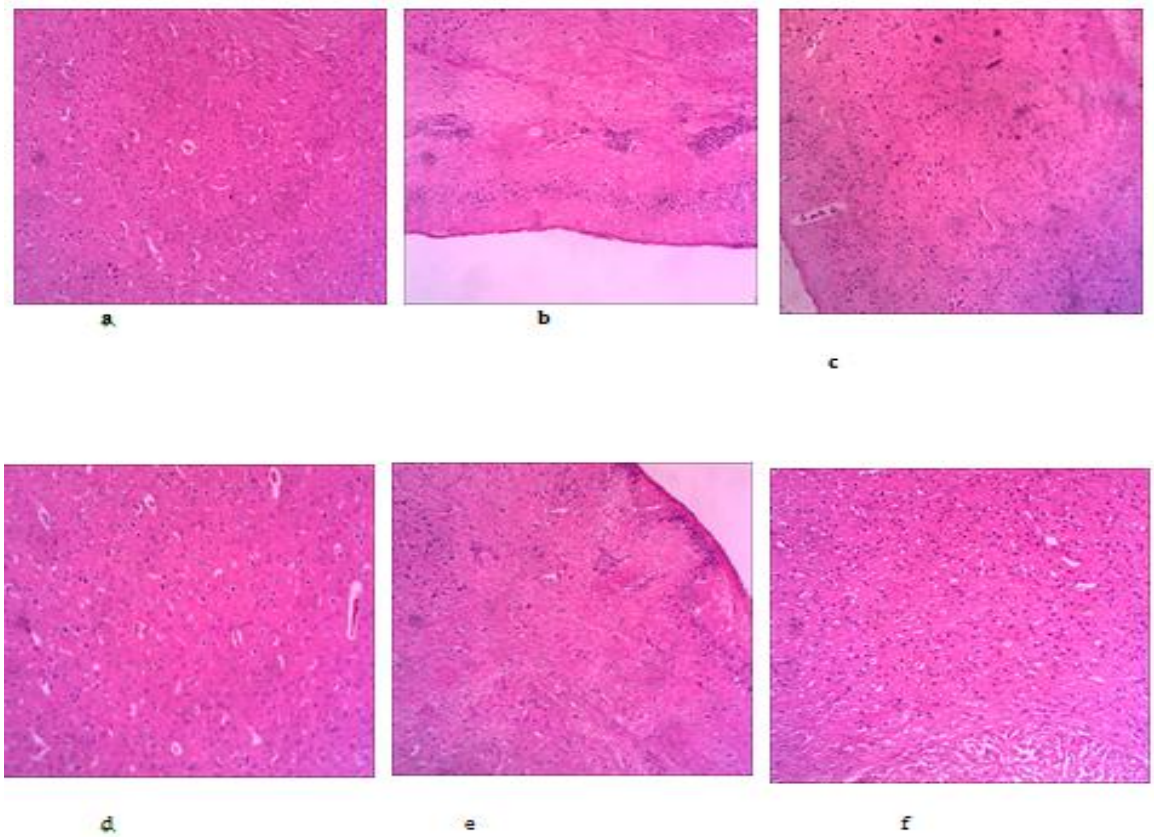

Fig 12: Shows histology of the substantia nigra.

KEY :(slide a) = (Normal or Control Rats): Showing Photomicrograph of Normal Brain Tissue (Substantia Nigra) With Neuron, Blood Vessel.(H&E)X100. (Slide b)= Showing Photomicrograph of Brain Tissue (Subtantia Nigra) With Infiltrating Inflammation.(H&E)X100. Paraquat-Injected Rats.(slide c)= Showing Photomicrograph of Brain Tissue (Substantia Nigra) with Less Hyperemic Vessels and Decreased Inflammation. (H&E)X100.Rats treated with methanol fraction of *V. amygdalina*. (Slide d)= Showing Photomicrograph of Brain Tissue (Substantia Nigra) With Hyperemic Vessels And Necrotic Neuron.(H&E)X100. Positive Control, Levodopa-Treated Rats.(Slide e)= Showing Photomicrograph of Brain Tissue (Substantia Nigra) With Less Heamorrhage And Infiltrating Inflammatory Cells.(H&E)X100. Rats treated with ethyl acetate fraction of *V. amygdalina*. (Slide f)= Showing Photomicrograph of Brain Tissue (Substantia Nigra) With Prominent Nucleoli And Astrocytes With Sharp Demarcated Nuclei.(H&E)X100. Rats treated with n-hexane fraction of *V. amygdalina*.

Histopathological Analysis of the Neuroprotective Effect of *V. Amygdalina*

The results of histopathological analysis of the neuroprotective effect of *V. amygdalina* are shown in Fig.12 above. In the substantia nigra, control rats showed normal brain tissue with neurons and blood vessels. In paraquat-injected rats there was large number of infiltrating inflammatory cells, confirming presence of neuroinflammation. There were some hyperemic vessels and necrotic neurons in levodopa-treated rats. Rats treated with methanol fraction of *V. amygdalina* showed less hyperemic vessels and decreased inflammation. Similarly, rats treated with ethyl acetate fraction of *V. amygdalina* showed decreased hemorrhage and infiltrating inflammatory cells. In rats treated with n-hexane fraction of *V. amygdalina*, there was scanty or no inflammation, presence of some cells with prominent nucleoli and astrocytes with sharp demarcated nuclei.

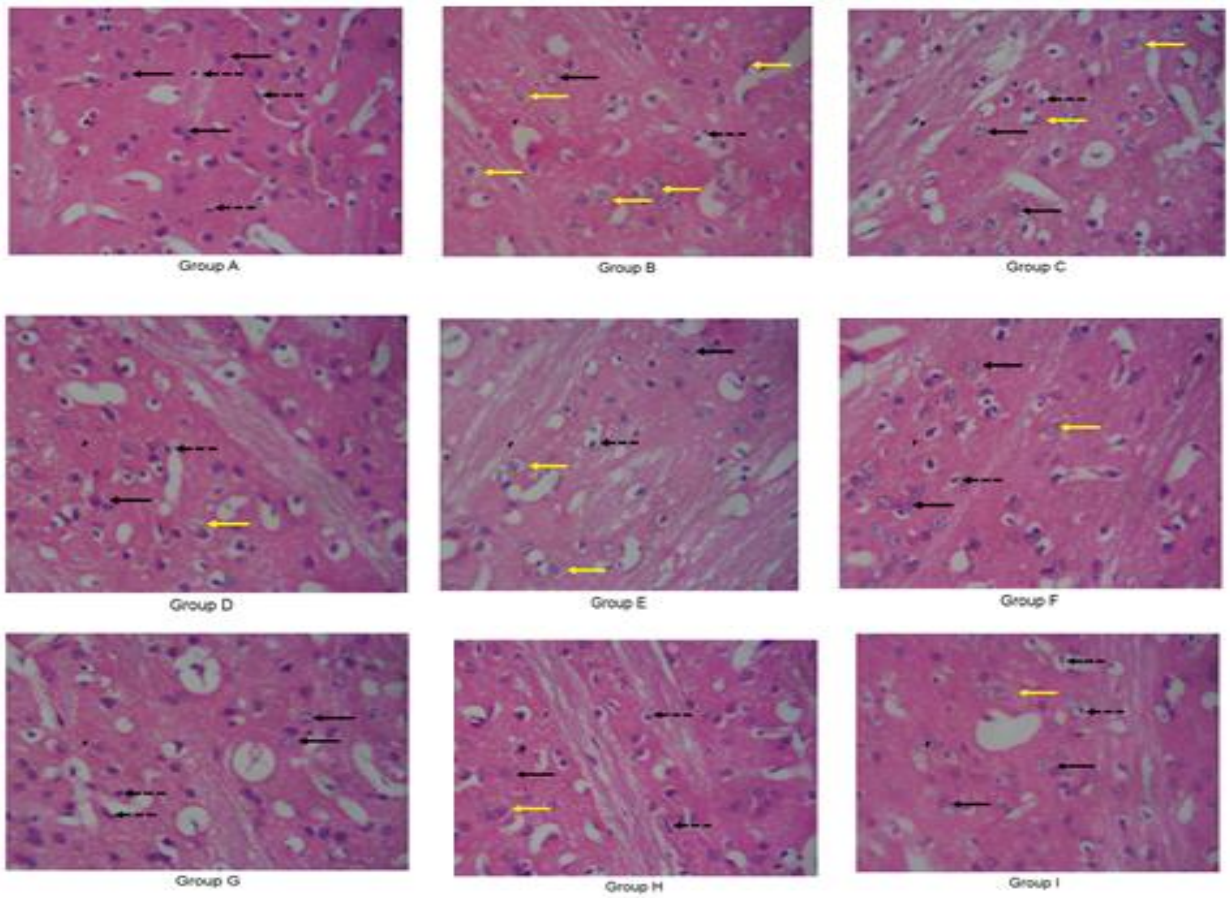

Fig 13: Shows histology of the striatum (representative photomicrographs).

Key: Group A= control, Group B= PQ (PD rats), Group C= PQ+100 mg/kg methanol fraction of VA, Group D= PQ+200 mg/kg methanol fraction of VA, Group E= PQ+ 200 mg/kg levodopa, Group F= PQ+ 100 mg/kg ethyl acetate fraction of VA, Group G= PQ+ 200 mg/kg ethyl acetate fraction of VA, Group H= PQ+ 100 mg/kg n-hexane fraction of VA, Group I= PQ+ 200 mg/kg n-hexane fraction of VA. PQ= Paraquat, VA= vernonia amygdalina, PD= Parkinson's disease. Black arrows= intact neurons. Dashed arrows= glia cells. Yellow arrows= neurons with degenerating features.

In the striatum, control rats showed mostly intact striatal histology, with several medium-sized neurons, and numerous glia. Group B rats (paraquat-injected rats) only showed several features of neurodegenrating neurons including neuronal swelling, cytoplasmic vacuolations and pyknotic nuclei. Degenerating neurons were also observed in treatment groups albeit to a lesser extent, with groups F, H, and I appearing to be least affected.

In the hippocampus, control rats showed mostly intact hippocampal histology in CA fields. Large pyramidal neurons with conspicuous nuclei and nucleoli were readily observed, as well as numerous glia. Group B (paraquat-injected rats) only showed several features of neurodegenerating neurons including neuronal swelling, cytoplasmic vacuolations and pyknotic nuclei. Additionally, parenchyma appeared particularly vacuolated. Degenerating neurons were also observed in treatment groups albeit to a lesser extent. Groups D, F, G, H, and I showed improved hippocampal histology.

DISCUSSION

The neurobehavioral activities of paraquat-induced Parkinsonian Wistar rats were investigated using elevated plus maze (EPM), pole test, and hanging wire/arm grip test. The results showed that Parkinson's disease was successfully induced with paraquat. The main features observed were tremor, bradykinesia, hunching, and derceased locomotor activities.

In this study the injection of paraquat, a neurotoxic agent, caused the PD rats to spend more time in the closed arm and less time in the open arm compared with the control (Groups B vs A). This showed mild anxiogenic property. However, this effects were not statistically significant. Group I rats showed a decrease in the time spent in the closed arm and less time still in the open. A decrease in the closed arm did not translate to an increase in open arm. This could be attributed to the fact that the rats spent some time in the center square which was regarded as neutral zone. Time spent in the center square was not counted for open or closed arm. There were some changes in other groups. However, these changes were not statistically significant.

The result of this study showed two opposing tendencies. Paraquat appeared to exert mild anxiogenic property. This was evidenced by the fact that the PD rats spent 285±5.96 seconds in closed arm compared with 277.6±13.89 seconds of control rats. In addition, PD rats spent 3.59±1.83 seconds in open arm compared with 13.29±5.88 seconds of control rats. The PD rats showed increased anxiety. The treatment group (especially n-hexane 200 mg/kg) showed a decrease (269.7±11.23 seconds) compared with control (277.6±13.81 seconds in closed arm) and 1.26±1.26 seconds compared with control (13.29±5.88 seconds in open arm). Therefore, n-hexane fraction of VA had mild anxiolytic effect. Thus the opposing effects of paraquat and fraction of VA resulted in non-significant effect overall. Equally, the methanol and ethyl acetate fractions of VA did not have any significant anxiolytic effects on parkinsonian Wistar rats.

This result is not in agreement with the findings of Onasanwo et al [28] who reported that ethyl acetate fraction of VA showed anxiolytic-like effect in mice.This could be due to the following reasons. First, their study was on mice, mine was on Wistar rat. Whether there is a specie difference in terms of pharmacological response needs to be investigated further by other researchers. Second, their study was on normal experimental animals, while mine was on drug-induced Parkinsonian rat model. Anxiety doesn't seem to be a key feature of Parkinson's disease. Parkinson's disease is mainly a movement disorder.

In the pole test, the effect of VA was dramatic. 200 mg/kg ethyl acetate fraction significantly decreased the time the rats spent on the pole (p=0.029). The control rats spent 186.79±51.91 seconds while group G spent 17.70±6.59 seconds. Thus 200 mg/kg ethyl acetate was able to reverse the paraquat induced bradykinesia. Pole test is primarily a test of bradykinesia. Bradykinesia is slowness of movement. Group B (PD rats) had 182.19±30.63 seconds while group G had 17.70±6.59 seconds. This decrease in time spent on the pole was statistically significant (p=0.036).

Therefore, ethyl acetate fraction of VA at 200 mg/kg significantly reversed paraquat-induced bradykinesia and improved movement in parkinsonian Wistar rats. This effect was dose-dependent. At 100 mg/kg the duration of time was 70.31±24.73 seconds while at 200 mg/kg it was 17.70±6.59 seconds. The effect of ethyl acetate was better than that of the levodopa (positive control).

Methanol and n-hexane fractions of VA decreased the time the rats spent on the pole test. However, this decrease was not statistically significant.

In the hanging wire test the PD rats performed poorly, spending 9.97±2.50 seconds compared with 80.89±49.15 seconds of the control rats. However, the change was not statistically significant. Methanol fraction of VA and levodopa groups performed better than other treatment groups. The overall results were not statistically significant. Several factors could influence results in the hanging wire test. The weight of the rats, for instance, and the behavior of the rats could tilt the balance towards lower time on the wire. Rats that have higher weights would find it difficult to sustain that weight on a wire. Furthermore, rats that are agitated cannot last long on the wire. Hanging wire test is a test of muscle strength and coordination. PD rats would definitely have very low scores. Since parkinsonian rats have tremor as one of its cardinal features coordination abilities would be impaired.

PD research is very challenging. It requires using appropriate neurotoxic agent and choosing suitable animal models that can recapitulate the major features of PD.[29] Rats and mice have been shown to fulfil this requirement. Several neurotoxic agents have been shown to replicate PD in the laboratory. They include MPTP, rotenone, and paraquat.[2] For this study I chose paraquat because of availability and ease of administration.

Several approaches have been adopted to buttress the fact that PD was actually induced in the experimental rats. They include: clinical observations involving neurobehavioral tests, blood based biomarkers such as serum tyrosine hydroxylase levels, α-synuclein levels,[30] markers of oxidative stress such as malondialdehyde (MDA) for lipid peroxidation, glutathione peroxidase (GPx), superoxide dismutase (SOD), total antioxidant capacity (TAC),[31] immunohistochemistry, histopathology.[32] All these investigative approaches provided scientific evidence for induction of PD and neuroprotective activity of *V. amygdalina*. Of the blood-based biomarkers, only α-synuclein and tyrosine

hydroxylase are specific for PD. The others are markers of oxidative stress. It is generally acknowledged that neurotoxicants induce PD through oxidative stress and there is a positive relationship between PD and oxidative stress.

Paraquat was able to replicate the major features, not all, of PD. Paraquat is an herbicide with toxic effects. The mechanisms of toxicity of paraquat in brain tissue and other tissues such as lung, kidney, and liver are similar. For its neurotoxicity, paraquat is taken up into the brain through the neutral amino acid transport system and then transported into striatal cells.[33] Once inside the cell it undergoes one electron reduction causing widespread oxidative stress.

The results of the immunohistochemistry showed convincingly that the three fractions of *V. amygdalina* had significant neuroprotective effects against paraquat-induced injury in the brain.

The control rats showed abundance of tyrosine hydroxylase (TH)-expressing neurons in both the substantia nigra and the striatum. Paraquat caused a decrease in these neurons.Treatment groups, especially D (200 mg/kg methanol fraction), F (100 mg/kg ethyl acetate fraction), G (200 mg/kg ethyl acetate fraction), and I (200 mg/kg n-hexane fraction) significantly blocked paraquat and restored TH-expressing neurons to control level. Thus the three fractions of *V. amygdalina* significantly protected the brain neurons in the substantia nigra and striatum against paraquat-induced injury. This neuroprotective effect was better than that of the positive control ─ E (levodopa-treated rats).

It appears that there is more to PD pathogenesis than just dopamine loss. Otherwise levodopa would have shown significant neuroprotection. However, this was not the case. Instead, the effect was minimal. This suggests that there are other mechanisms involved in PD at the biochemical level. Some researchers have suggested that neuroprotection against toxin-induced PD could be due to MAO-B (monoamine oxidase- B) inhibition.[32] The gas chromatography test I did on *V. amygdalina* showed it contained some bioactive compounds, one of which is propargylamine, a known MAO-B inhibitor.

This study confirmed that tyrosine hydroxylase was most strongly expressed in the nigrostriatal pathway (fig.6). It is important to note that there was no expression of TH in the hippocampus. I did not count TH-positive neurons. However, based on estimation, approximately 35% of TH-expressing neurons were destroyed by paraquat. This is similar to the 37% TH-positive neuron loss in adult male Wistar rats reported by Ossowska et al.,[34] However, my result differed from 60% and 65% TH-positive neuron loss in mice reported by Brooks et al.,[16] and Somayajulu-Ntu et al.,[35] respectively. The difference in the reported TH-positive neuron loss could be due to species differences. Those who worked on mice reported higher values than those who worked on Wistar rats. This could suggest that mice were more susceptible to paraquat toxicity than Wistar rats.
Alpha-synuclein expression in the striatum and hippocampus was investigated (fig. 7). Paraquat caused intense expression of alpha-synuclein compared to control. Treatment groups showed less α-synuclein expression. This indicates neuroprotection. Alpha-synuclein is a component of Lewy body which is pathognomonic for PD.

Alpha-synuclein activity is vital in the pathogenesis of PD. The injection of paraquat caused intense expression of this synaptic protein in the brain as well as causing increase in its activity in the blood.

In this study intra-peritoneal injction of paraquat weekly for three weeks caused a TH-positive neuron loss of about 35%. This was enough to induce the key features of PD without causing significant pulmonary toxicity. *V. amygdalina* (methanol, ethyl acetate, and n-hexane fractions) had significant neuroprotective effects by influencing TH-expression and α-synuclein activity. These are two important biomarkers that are specific for PD.

The result of the blood-based biomarker tyrosine hydroxylase revealed that paraquat significantly ($p=0.049$) decreased its levels. *V. amygdalina* (notably methanol fraction) was able to block paraquat and reversed its value to normal. In addition there was significant difference ($p=0.021$) between paraquat-injected rats and levodooa-treated rats.

Tyrosine hydroxylase is an important biomarker for PD. Tyrosine hydroxylase is a cytosolic enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to dihydroxyphenyalanine (L-DOPA). It does so using oxygen (O2) as well as cofactors (iron and tetrahydrobiopterin). L-DOPA is a precursor for dopamine, which in turn, is a precursor for the neurotransmitters noradrenaline and adrenaline. Tyrosine hydroxylase, therefore, catalyzes the rate-limiting step in the synthesis of catecholamines.

The decrease in tyrosine hydroxylase level was due to neurotoxic effect of paraquat. This confirmed that dopaminergic/catecholaminergic fibers were involved. Paraquat caused damage by generating reactive oxygen species which are injurious to dopaminergic neurons. In addition, tyrosine hydroxylase is an enzyme that is susceptible to oxidative stress.

A consistent abnormality in PD is degeneration of dopaminergic neurons in the substantia nigra leading to a reduction of striatal dopamine levels. As tyrosine hydroxylase catalyzes the formation of L-DOPA, the rate-limiting step in the biosynthesis of dopamine, tyrosine hydroxylase depletion may lead to PD.

Another important blood-based biomarker investigated was α-synuclein. A-Synuclein is pathognomonic for PD. Paraquat significantly (p=0.000) increased α-synuclein levels. This up-regulation of α-synuclein was significantly blocked by *V. amygdalina* (methanol, ethyl acetate, and n-hexane fractions). Furthermore, administration of levodopa significantly (p=0.003) reversed this paraquat-induced increase in α-synuclein activity.

Alpha-synuclein is unequivocally linked to the pathogenesis of PD. It is an abundant neuronal protein which localizes predominantly to presynaptic terminals. It is believed that α-synuclein expression secondary to neurotoxicants is protective. The initial response of neuronal cells to paraquat was to increase α-synuclein activity. This could be an attempt by neurons to counteract and limit injury caused by the neurotoxic agent. However, some researchers have suggested that overexpression of α-synuclein might actually delay cell death caused by toxic agents and protect against apoptotic stimuli.[36]

It should be noted that although α-synuclein is found in presynaptic terminals, not all terminals accumulate the protein in neurodegenerative disorders. Furthermore, although predominantly present in the nervous system, some amount of α-synuclein have been detected in red blood cells.[37]

V. amygdalina (methanol, ethyl acetate, n-hexane fractions) demonstrated very significant neuroprotective effect by blocking paraquat-induced injury and reversing α-synuclein overexpression. Paraquat toxicity is known to involve the generation of free radicals via redox reaction. This oxidative stress is believed to trigger α-synuclein aggregation by altering the biophysical properties of the protein and/or impairing mechanisms of protein degradation within the neurons.[38]

The homeostatic mechanism of the body functions in such a way as to maintain a balance between prooxidants and antioxidants. Any disruption of this balance can lead to cellular injury and possibly degeneration and death.

Paraquat significantly (p=0.000) decreased total antioxidant capacity (TAC). *V. amygdalina* (notably, n-hexane and methanol fractions) significantly (p=0.000 and p=0.018, respectively) blocked paraquat and reversed the TAC levels to 70% and 49.66% of the normal value respectively. N-hexane fraction of *V. amygdalina* demonstrated higher neuroprotective effect than methanol fraction. Furthermore, n-hexane fraction showed efficacy better than positive control, levodopa (56.98% of normal).

Total antioxidant capacity (TAC) is the measure of the amount of free radicals scavenged by a test solution. It is used to assess the antioxidant capacity of biological samples.

That TAC was significantly decreased in paraquat-injected rats confirms the strong role oxidative stress plays in the pathogenesis of PD. *V. amygdalina* was able to counteract this due to abundant antoxidants it contains.

Paraquat significantly (p=0.003) increased malondialdehyde. *V. amygdalina* (methanol, ethyl acetate, and n-hexane fractions) significantly (p=0.027, p=0.007, p=0.001 respectively) blocked paraquat and restored the MDA to normal value.

Malondialdehyde results from lipid peroxidation of polyunsaturated fatty acids. It is a marker of oxidative stress. The degree of lipid peroxidation can be estimated by the amount of malondialdehyde in tissues.[39] Paraquat significantly increased MDA, suggesting that this could be another way paraquat causes injury to cells. This is because increased lipid peroxidation disrupts the membrane of tissues and results in breakdown of homeostatic mechanisms leading to cell injury and possibly death. *V. amygdalina* was able to counteract this due to its high antioxidant activities.

V. amygdalina had significant beneficial effect on glutathione peroxidase (GPx) and superoxide dismutase (SOD). These are enzyme antioxidants that are very important for normal functioning of the body. Paraquat significantly ($p<0.001$ and $p<0.05$ for GPx and SOD respectively) decreased their levels. *V. amygdalina* (notably methanol and n-hexane fractions) counteracted paraquat-induced injury and protected GPx. Furthermore *V. amygdalina* partially blocked paraquat and non-significantly counteracted the effect of paraquat on SOD. SOD appeared to be more susceptible than GPx to paraquat-induced injury.

Clearly, *V. amygdalina* has shown strong neuroprotective activities through multiple mechanisms. It significantly relieved oxidative stress and strongly counteracted lipid peroxidation. In addition it boosted antioxidant enzymes and has antioxidant activities.

Earlier, phytochemical analysis on *V. amygdalina* showed the presence of alkaloids, tannins, saponins, flavonoids, phenols, steroids, and glycosides. Flavonoids are shown to have antioxidant activities.[40]

Gas chromatograph-mass spectrometry (GC: MS) result showed that *V. amygdalina* contained some bioactive compounds with antioxidant properties e.g. oxazole.[23] Some have antiparkinsonian properties such as propargylamine,[21,22] and muscle relaxant properties such as mephenesin.[24] All these bioactive compounds can best explain its mechanism of action. For instance, it has been reported that novel (hetero) arylalkenyl propargylamine compounds showed neuroproptective effect against MPTP-induced models of PD. The authors hypothesized that the mechanism of action could be due to simultaneous inhibition of monoamine oxidase-B (MAO-B) and oxidative stress-induced pathological

dopamine release by the novel propargylamines.[32] Interestingly, *V. amygdalina* contains significant amount of this compound, propargylamine, as shown by GC: MS result in this study.

Similarly, *V. amygdalina* contains mephenesin which is a centrally acting muscle relaxant. It is used for the treatment of spasticity, muscle spasms, and dyskinesia.[24]

The neuroprotective effect of *V. amygdalina* is also supported by histopathological evidence (figs.12 & 13). The slide from control rats appeared normal. However, slide from paraquat-injected rats showed signs of neuroinflammation and degeneration as evidenced by the presence of large number of infiltarating inflammatory cells, hyperemic vessels and necrotic neurons, and signs of pyknosis. *V. amygdalina* effectively protected the brain tissue from this damage. This neuroprotective effect was more demonstrated by n-hexane fraction of *V. amygdalina* than methanol and ethyl acetate fractions. The positive control did not offer adequate protection. Thus *V. amygdalina* protected better than the positive control (levodopa-treated rats).

Neuroinflammation and degenerative changes are pathological hallmarks of PD. That paraquat induced these changes confirmed PD or PD-like pathological process. The extent of *V. amygdalina* limited the spread of this paraquat-induced damage is seen as a measure of its neuroprotective activity. In methanol and ethyl acetate fractions of *V. amygdalina*, the slides showed some areas of focal inflammation. This suggested that the inflammation was resolving and these fractions were able to limit its spread. In n-hexane fraction of *V. amygdalina*, the protection offered was very good to the extent that the slide of the brain tissue appeared like that of the control rats.

CONCLUSION

In this study we investigated the neuroprotective effects of *V. amygdalina*. Solvent fractionation of the leaves of *V. amygdalina* yielded methanol, ethyl acetate, and n-hexane fractions. Gas chromatography revealed the presence of some bioactive compounds which contributed to its pharmacological actions. Indeed, paraquat, an herbicide, which served as a neurotoxic agent was used to induce PD. We were able to recapitulate the key features of PD after intraperitoneal injection of paraquat weekly for three weeks. *V amygdalina* demonstrated significant neuroprotective effect against paraquat-induced PD in Wistar rats. This was strongly supported by the results of several blood tests for biomarkers specific for PD (such as α-synuclein, tyrosine hydroxylase [TH]); immunohistochemical evaluation, and histopathological findings. The mechanisms of action of *V. amygdalina* in causing neuroprotective effect could be by increasing TH levels, decreasing α-synuclein, counteracting oxidative stress, and increasing antioxidant activities.

REFERENCES

1. Lees AJ, Hardy J, Revesz T.Parkinson's disease. The lancet, 2009; 373: 2055-2066.
2. Hallas BH.Parkinson's disease update: treatments and animal models. Kopf Carrier, 2015; 85.
3. Dauer W, and Prrzedborski S.Parkinson's disease: mechanisms and models. Neuron, 2003; 39(6): 889-909.
4. Carlsson A, Lindqvist M, Magnusson T. 3, 4, Dihydroxyphenylalanine and 5-hyroxytryptophan as reserpine antagonists. Nature, 1957; 180: 1200.
5. Carlsson A.The occurrence, distribution and physiological role of catecholamines in the nervous system. Pharmacol. Rev, 1959; 11(2): 490-493.
6. Erasto P, Grierson DS, Afolayan AJ. Bioactive sesquiterpene lactones from the leaves of *vernonia amygdalina*. J Ethnopharmacology, 2006; 106: 117-120.
7. Njan AA, Adiza B, Agba AG, Byamgaba D, Diaz-liera S, Bansberg DR. The analgesic and antiplasmodial activities and toxicology of vernonia amygdalina. J Med Food, 2008; 11: 574-581.
8. Moundipa PF, Kamini G, Flore M, Bilong CF, Bruchhaus I. In vitro amoebicidal activity of some medicinal plants of the Barmin region (Cameroon). Afr J Trad CAM, 2005; 2: 113-121.
9. Gidey M, Aslaw Z, Aimqvist T, Woidu Z. An ethnobotanical study of medicinal plants used by the Zay people in Ethiopa. J Ethnopharmacol, 2003; 85: 43-52.
10. Melsin. Rat tyrosine hydroxylase ELISA KIT (CAT. NO.: EKRAT-0756), Human α-synuclein (SNCA) ELISA KIT (CAT. NO: EKHU-1285). Product leaflet. Melsin Medical Co., Limited, China, 2019. Available at: https://www.meslin.com.
11. Cragg GM, Newmann DJ. Natural product drug discovery in the next millennium. Pharm Biol, 2001; 139: 8-17.
12. Abubakar AR, Haque M. Preparation of medicinal plants: basic extraction and fractionation procedures for experimental purposes. J Pharm Bioall Sci, 2020; 12:1-10.
13. Ingle KP, Deshmukh AG, Padole DA, Dudhare MS, Moharil MP, Khelurker VC. Phytochemicals: extraction methods, identification, and detection of bioactive compounds from plant extracts. J Pharmacogn Phytochem, 2017; 6:32-6.
14. Lorke D. A new approach to practical acute toxicity testing. Arch Toxicol, 1983; 54(4): 275-287.
15. Buss AD and Butler MS, (Eds.). Natural product chemistry for drug discovery. The Royal Society of Chemistry, Cambridge, 2010; 153.
16. Brooks AI, Chadwick CA, Gelbard HA, Cory-Slechta DA, Federoff HJ. Paraquat elicited neurobehavioral syndrome caused by dopaminergic neurone loss. Brain Res, 1999; 823(1-2): 1-10.
17. Matsura K, Kabuto H, Makino H, Ogawa N. Pole test is a useful method for evaluating the mouse movement disorder caused by striatal dopamine depletion. J Neurosci Methods, 1997; 73(1): 45-8.

18. Pellows S, Chopin P, File SE, Briley M. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods, 1985; 14(3): 149-67.
19. Ijonome OM, Olatunji SY, Owolabi JO, Naicker T, Aschner M. Nickel-induced neurodegeneration in the hippocampus, striatum and cortex; an ultrastructural insight, and the role of caspace-3 and α-synuclein. Journal of trace elements in medicine and biology, 2018; 50:16-23.
20. Asif M. Biological overview on thiirane derivatives. SOP Transactions on Applied Chemistry, 2014;1: 1.
21. Bar-am O, Amit T, Weinreb O, Youdim MBH, Mandel S. Propargylamine containing compounds as modulators of proteolytic cleavage of amyloid protein precursor: involvement of MAPK and PKC action. J Alzheimer's Dis, 2010; 21(2): 361-371.
22. Chen JJ, and Swope DM. Clinical pharmacology of rasagiline: a novel second-generation propargylamine for the treatment of Parkinson's disease. The Journal of Clinical Pharmacology, 2005; 45: 878-894.
23. Parveen M, Ali A, Ahmed S, Malla AM, Alam M, Silva PSP, Silva MR, Lee DU. Synthesis, bioassay crystal structure and ab initio studies of Erlenmeyer azlactones. Spectrum Acta A Mol Biomol Spectrosc, 2013; 104: 538-545.
24. Kaada BR. Site of action of myanesin in the central nervous system. J Neurophysiol, 1950; 13(1): 89-104.
25. Pokhodylo N, Shyyka O, Matiyechuk V. Synthesis of 1, 2, 3-Triazole derivatives and evaluation of their anticancer activity. Sci Pharm, 2013; 81(3): 663-676.
26. Noolvi M, Agrawai S, Patel H, Bodiger A, Garba M, Zambre A. Synthesis, antimicrobial and cytotoxic activity of novel azetidine-2-one derivatives of 1H-benzimidazole. Arabian J Chem, 2014; 7(2): 219-226.
27. Busch R, Graubaum HJ, Gruenwald J, Faergemann J. Therapeutic effect of 1, 5-pentanediol for herpes simplex labialis: a randomized, double-blind placebo-controlled clinical trial. Adv. Ther, 2009; 26(7): 719-27.
28. Onasanwo SA, Aitokhuehi NG, Ajayi TO, and Faborode OS. The anxiolytic properties of vernonia amygdalina (Asteraceae) in laboratory mice. Afr J Biomed Res, 2016; 19: 229-233.
29. Blesa J, Phani S, Jackson-Lewis V, and Przedboorski S. Classic and new animal models of Parkinson's disease. J of Biomedicine and Biotech, 2012; 845618: 10. doi:10.1155/2012/845618.
30. Chahine LM, Stern MB, and Chen-Plotkin A. Blood-based biomarkers for Parkinson's disease. Parkinsonism Relat Discord, 2014; 20(0 1): 99-103.
31. Saracchi E, Fermi S, Brghina L. Emerging candidate biomarkers for Parkinson's disease: a review. Aging and Disease, 2014; 5(1): 27-34.
32. Baranyi M, Porceddu PF, Goloncser F, Kulcsar S, Otrokocsi L, Kittel A, Pinna A, Frau L, Huleatt PB, Khoo M, Chai CLL, Dunkel P, Matyus P, Morelli M, Sperlagh B. Novel (hetero) arylalkenyl propargylamine compounds are protective in toxin-induced models of Parkinson's disease. Molecular Neurodegeneration, 2016; 11: 6. Doi: 10.1186/s13024-015-0067-y.
33. Shimizu K, Ohtaki K, Matsubara K, Anyama k, Uezono T, Sarto O, Suno M, Ogawa K, Hayase N, Kumura K, et al. Carrier-mediated processes in blood-brain barrier penetration and neural uptake of paraquat. Brain Res, 2001; 906: 135-142.
34. Ossowska K, Wardas, J, Smialowska M, Kuter K, Lenda T, Wieronska JM, Zieba B, Nowak P, Dabrowska J, Bortel A. A slowly developing dysfunction of dopaminergic nigrostriatal neurons induced by long-term paraquat administration in rats: an animal model of preclinical stages of Parkinson's disease. Eur J Neurosci, 2005; 22: 1294-1304.
35. Somayajulu-Ntu M, Sandhu JK, Cohen J, Sikorsta M, Sridhar TS, Matei A, Borowy-Borowski H, Pandey S. Paraquat induces oxidative stress, neuronal loss in substantia nigra region and Parkinsonism in adult rats: neuroprotection and emelioration of symptoms by water-soluble formulation of coenzyme Q10. BMC Neurosci, 2009; 10: 88.
36. Lee M, Hyun D, Halliwell B, Jenner P. Effects of the overexpression of the wild-type or mutant alpha-synuclein on cell susceptibility to insult. J Neuro Chem, 2001; 76: 998-1009.
37. Nakai M, Fujita M, Waragai M, Sugama S, Wei J, Akatsu H, Ohtaka-Maruyama C, Okado H, Hashimoto M. Expression of alpha-synuclein, a presynaptic protein implicated in Parkinson's disease, in erythropoietic lineage. Biochem Biophys Res Commun, 2007; 358: 104-110.
38. Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA. The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice─ Prarquat and alpha-synuclein, J Biol Chem, 2002; 277: 1641-1644.
39. Guyton AC, Hall JE.Textbook of medical physiology.12th ed. Elsevier, Philadelphia, 2011; 693.
40. Igile GO, Olszek W, Jurzyysta M, Burda S, Fantunso M, Fasnmade AA. Flavonoids from *Vernonia amygdalina* and their antioxidant activites. J Agric Food Chem, 1994; 42: 2445-2448.