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Original Research Article

Amaranthus viridis improves relative anxiety behaviour and cognitive deficit in rotenone induced Parkinsonism in albino rats

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ABSTRACT

Background: Parkinson's disease (PD) is typically characterised by motor shortfalls. However, non-motor symptoms like mood disorders (anxiety, depression) and impaired cognition are also associated features. Previous studies have demonstrated a neuroprotective effect a plant against a disease. Consequently, this current study was focused on assessing its efficacy in extenuating non-motor shortfalls such as anxiety-like behaviour and impaired cognition induced by rotenone.
Methods: PD was induced in rats by administering rotenone (10 mg/kg BW orally) for 28 days. The vehicle and the test drug were given orally daily for one hour prior to rotenone administration. The protective effect of methanol extract of *A. viridis* (500 mg/kg BW) was assessed through an array of tests; elevated plus maze test, Morris water maze test, and novel object recognition test. The rats were sacrificed on day 28th and neurobiochemical analyses of the hippocampus were performed using HPLC.
Results: The findings of this study showed that co-administration of *A. viridis* reversed the rotenone-induced anxiety-like behaviour and cognitive shortfalls to a significant extent ($p<0.001$). It also restored the hippocampal neurotransmitters [(5-hydroxytryptamine (5-HT), 5-hydroxy indole acetic acid (5-HIAA), and dopamine (Da)] significantly ($p<0.001$).
Conclusions: *Amaranthus viridis* offered neuroprotective effects that ameliorate non-motor symptoms in PD. This could be a novel insight into the therapy of PD. This study provides scientific evidence that *A. viridis* attenuates non-motor symptoms like anxiety-like behaviour and cognitive deficits in Parkinsonism. This extract can be a potential candidate in herbal formulations as a neuroprotectant against PD.

Keywords: Depression, Neuroprotection, Dopamine, Norepinephrine

INTRODUCTION

It is important and worthy to note that of all, neurological diseases known that PD is rated as the second major regular brain degeneration disease which affects elderly people.[1] This disease does surface progressively due to disturbances posed by autonomic behaviours, motor and cognitive impairment. The fundamental pathology of PD is very complex. However, at the moment, there is no satisfactory pharmacotherapeutic intervention.[2] Consequently, plants' bioactive components have been areas of interest in the treatment and management of PD due to minor side effects on protracted treatment. On the other hand, rotenone: a pesticide is used to induce PD in rodents.[3] Decrease of brain monoamines that is: dopamine (DA), 5-hydroxytryptamine (5-HT), and nor-adrenaline (NA), oxidative stress, and neuroinflammation induced by rotenone are the features attributed to the motor and non-motor deficits connected to PD.[4]

Amaranthus viridis which belongs to the family of *Amaranthaceae* is a plant that has great medicinal value in Nigeria. Certain reports about the plant showed that the plants have demonstrated pharmacotherapeutic activities on some ailments such as inflammation, boils, gonorrhoea, orchitis, hemorrhoids, and purgative. Interactions with traditional healers and testimonies from their patients showed that *Amaranthus viridis* has pharmacotherapeutic effects on anxiety-like behaviour and cognitive deficit in PD.[5] These herbal claims have not been investigated scientifically. Hence, addressing this issue, the present research study was carried out to investigate and evaluate the protective role of *Amaranthus viridis* against anxiety-like behaviour, cognitive impairments, and neurobiochemical deficits in PD rats induced with rotenone.

METHODS

Plant material collection and authentication

The fresh leaves of *Amaranthus viridis* plant were collected in the early morning hours in Jos Plateau State Nigeria. The plant was identified and authenticated by a plant taxonomist: Mr. Joseph Jeffrey Azila of herbarium unit, department of forestry technology, Federal College of Forestry, Jos Plateau State Nigeria with the herbarium number "FHI3081".

Preparation of plants and extraction procedure

Freshly collected leaves of *Amaranthus viridis* were washed with clean tap running water to get rid of any dirt. Thereafter, the leaves were dried under room temperature. The dried leaves were pounded into powdered form locally by the use of mortar and pestle. The coarsely powdered materials were later extracted with methanol (absolute) using the Soxhlet apparatus. The extract was later evaporated to dryness under reduced pressure and the residue was preserved for future use.

Phytochemical screening

The preliminary phytochemistry of *Amaranthus viridis* methanol leaf extract was done in order to ascertain different plant's secondary metabolites like, tannins (ferric chloride test), alkaloids (Mayer's and Dragendorff reagent test), saponins (Froth test), steroids (Liebermann-Burchard test), terpenoids (Salkowski test), flavonoids (ammonia and sulphuric acid test) and anthraquinones (Borntrager's test).

Animals

Albino rats of both genders weighing between 150 gm and 200 gm body weight were sourced from the animal house of the University of Jos. They were housed under regulated temperature (24±1° C), relative humidity (45-55%), and 12 ratio 12 light and dark cycle with free access to standard pellet diet (Grandcereal Nigeria Limited Jos) and drinking water. After the ethical Committee approval (UJ/FPS/F-17-00379) of the University of Jos where the laboratory works were done, all experiments performed on laboratory animals were in accordance with the Alex Ekwueme federal university animal research ethical committee.

Drugs and chemicals

Rotenone, dopamine hydrochloride, and 5-hydroxytryptamine (5-HT), 5-hydroxyindolaceticacid (5-33HIAA) was purchased from Sigma Aldrich, Germany. Octyl sodium sulphates, EDTA, sodium metabisulphite, all the chemicals were of analytical grade and purchased from standard manufacturers: Sigma Aldrich, Germany.

Acute toxicity test

The acute toxicity test of methanol leaf extract of *Amaranthus viridis* was done by using the OECD Guidelines 423 [Limit test] was followed.[6] At doses of 5, 50, 1000, and 2000 mg/kg, the extract was given orally in a stepwise manner. No mortality or behavioral changes were observed during the 24 hours of observation.

Experimental design

Randomized selected Wistar albino rats were divided into three groups (n=6) to be given treatments as follows; Group-I served as normal control and was administered with vehicle olive oil (1 ml/kg p.o). Group II was treated with Rotenone (10 mg/kg p.o.) daily. Group III (AMV (*Amaranthus viridis* 500 mg/kg + rotenone 10 mg/kg) was considered as the test group.

All the treatments were given orally for a period of 28 days. The Drugs and vehicle were given one hour before rotenone administration. Behavioural tests were performed from day 14 of the study period. After the last behavioural assessments on day 28, the rats were sacrificed by cervical dislocation, and the hippocampus was dissected out quickly for the neurobiochemical analyses.

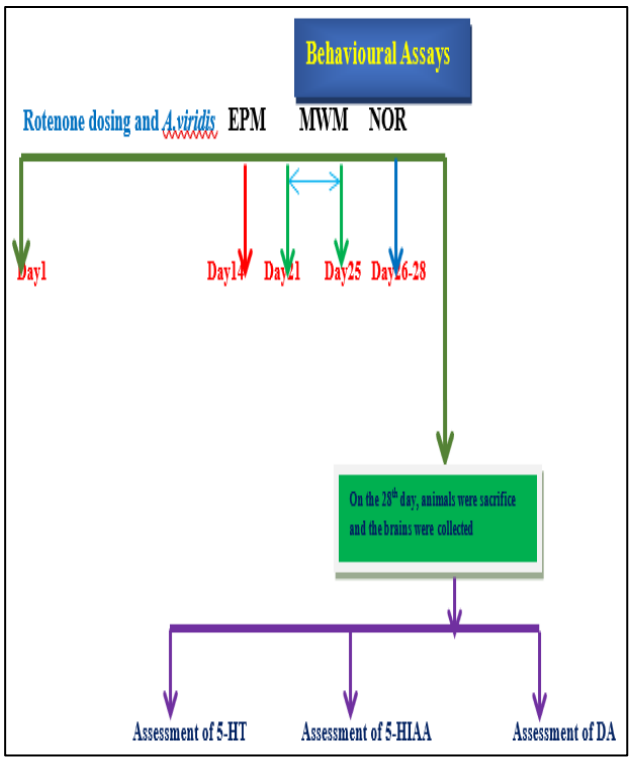

Figure 1: Behavioural assay.

Behavioural assay

Elevated plus maze test

Here, a system of experimental model known as the elevated plus maze test was employed to assay behaviours that look like anxiety which was highlighted by Anseloni et al.[7] The apparatus is made up of two open arms (50×10cm) and two closed arms (50 ×10 ×40 cm) with a middle (central) platform (10×10 cm). This was elevated to a height of 50 cm more than the ground. Each animal was placed in the central (middle) platform opposite the open arm and was allowed to walk around them for 5 minutes. The behavioural measures such as a number of entries into the open and closed arm, and time spent in the open and closed arm were recorded. The index of anxiety like-behaviour was determined from the time spent in open and closed arms and the number of entries was taken as an indicator of locomotive activity.[7]

Morris water maze test

Similarly, another experimental model called "Morris water maize test" was adopted to study the learning and memory of the animal under investigation.[8] This was designed to know if the animal learns to escape from swimming in the water and stay on the hidden platform. The water maze contains a spherical pool 150 cm in width and 45 cm in height containing water to a level of 30 cm height at a temperature of 28±1°C, the pool was demarcated into four equal quadrants with the help of threads; a platform of (10×10 cm) is submerged 2 cm below the water level known as target quadrant. Initially, the rats were trained to locate and climb on the hidden platform for 2 minutes, and this was done by placing them at random in one of the four quadrants. They were allowed to wait on the platform for 20 seconds. During each session, the time taken to locate and climb on the hidden platform was recorded as the escape latency time (ELT), which was considered as the index of acquisition or learning. Whereas, on the 5th day, during the spatial probe test, the platform was removed and the animal was allowed to explore the pool for 120 seconds, mean time spent in the target quadrant (Q4) served as an index of retrieval or memory.

Novel object recognition (NOR) test

The method demonstrated by Cohen et al was employed to assess the cognitive ability of animals in distinguishing the novel surroundings.[9] The apparatus used in this test was a grey-coloured wooden box having two identical containers (Glass and metallic) of the same size. The test was performed in three phases as habituation phase (day 1) training phase (day 2) and the test phase (day 3).

During the day 1 activity, the animals were free to explore the environment (NOR apparatus) for 10 minutes while during the day 2 activity; the rats were collated into the box again with a similar glass container for 10 minutes. In the test phase (day 3), one of the glass containers was substituted with a similar metallic container (Novel object) and the animals were presented with a Novel environment for 3 minutes. The time spent by rats facing the novel object and sniffing or touching it was carefully observed.[10]

Neurobiochemical assessment

As this behavioural estimation was brought to a conclusion, the rats were demobilised and quickly the brains were removed. The level of dopamine, 5-HT and 5-HIAA in hippocampus were evaluated with of HPLC fluorescence detector method Schlumpf et al.[11] Prior to the sample estimation, the HPLC system was calibrated using 100 ng/ml of each standard concentration of amines, the known weight of brain samples were homogenised in 0.1ml hydrochloric acid-n butanol for 1 min in the cool surrounding. Then the sample was centrifuged at 2000 rpm for 10 minutes. 0.08 ml of supernatant was removed and added to an Eppendorf tube containing 0.2 ml of heptane and 0.025 ml of 0.1M HCl. Shaking for 10 minutes, the tube was centrifuged as above to separate two phases, the upper organic phase and the aqueous phase were used for estimation of dopamine and serotonin.[12]

Assessment of dopamine

To the aqueous phase of the sample 0.02 ml, 0.005 ml of 0.4 M of HCl and 0.01 ml EDTA were added followed by 0.01ml iodine solution for oxidation, the reaction was stopped after 2 minutes by adding 0.01ml of Na_2SO_3 in 5 M NaOH (0.5 gm Na_2SO_3 in 2 ml H_2O + 18 ml 5M

NaOH) and 0.01 ml of 10M acetic acid was added after 1.5 mins. The solution was heated to 100°C for 6 minutes and was allowed to cool up to room temperature. The readings were taken at 330-375nm.[13]

Assessment of serotonin (5-HT)

The serotonin was determined by the method of Vatassery et al into an ascorbic acid solution by freezing and sonicating, ascorbic acid stabilizes the serotonin in the sample and ethanol was added to enhance the final fluorescence of the serotonin, which was measured in concentrated HCl medium.[14]

Assessment of 5-HIAA

The 5-hydroxyindolacetic acid was determined by Bearcroft et al the mobile phase methanol 14%, octyl sodium sulphate 0.023% and EDTA 0.0035% pH maintained at 2.9 by adding 0.1M phosphate buffer. The solution was heated to 100°C for six minutes and cooled down to room temperature. The readings were taken for excitation at 295nm and emission at 345nm.[15]

Statistical analysis

Data analysis was done by applying one-way ANOVA with post hoc Tukey's multiple comparison tests using GraphPad Prism 7.0. The minimum significance level was set at ($p<0.05$).

RESULTS

This study was focused to evaluate the effect of *Amaranthus viridis* on non-motor deficits such as Anxiety-like behaviour and cognitive impairments associated with PD.

Behavioural indices

Elevated plus maze test

In the elevated plus maze test, animals treated with rotenone showed highly significant increase in the number of entries and time spent in closed arms as compared to the vehicle-treated control rats. ($p<0.001$). The above parameter revealed that anxiety was associated with rotenone-induced PD. Moreover, we found that increased anxiety induced by rotenone was attenuated by *A. viridis* (500 mg/kg) to highly significant extent. ($p<0.001$) when compared with the rotenone control group (Table1).

Morris water maze test

In this test, rats in the control group exhibited a normal spatial learning aptitude and recognition memory. However, rotenone has significantly prolonged the escape latency time to a highly significant extent ($p<0.001$) in comparison to the control group, indicating impaired spatial learning tasks. The spatial probe test on 5th day, displayed reduced time spent in the target quadrant indicating impaired memory ability. Notably, treatment with *Amaranthus viridis*, exhibited a significant reversal of these effects of rotenone, indicating improved spatial learning and memory function. ($p<0.001$) (Table 2).

Novel object recognition test

In this test, rotenone administration, significantly decreased the time spent with the novel object ($p<0.001$) that explained the recognition memory impairment (Figure 1B). Animals treated with *A. viridis* spent a significantly higher time ($p<0.001$) in exploring the novel object compared to control rats, indicating enhanced recognition memory against rotenone-induced memory impairment.

Table 1: Effect of drugs on elevated plus maze test.

Groups	Mean time spent in opened arm (Sec)	Mean time spent in closed arm (Sec)	Mean number of entries in opened arm	Mean number of entries in closed arm
I (Control)	52.6±8.3	115.7±2.0	3.6±0.5	2.4±0.2
II (Rotenone 10 mg/kg)	15.1±3.1$^{\#}$	204.7±9.7$^{\#}$	1.6±0.4$^{\#}$	4.3±0.4$^{\#}$
III (AMV 500 mg/kg)	45.8±0.0***	102.8±8.2***	3.8±0.3***	4.1±0.1***

Results are expressed as Mean ± SEM (n=6). One-way ANOVA with post hoc Turkey's multiple comparison tests was used. #:p<0.001when compared with the control group while ***:p<0.001 when compared with the rotenone control group.

Table 2: Effect of *Amaranthus viridis* on rotenone induced changes in spatial working memory assessed through Morri's maze test and time spent with novel object in recognition test.

Groups	Time spent in day1 (Sec)	Time spent in day4 (Sec)	Time spent in day 5 (Sec)	Time spent in 4th quadrant (Sec)	Time spent with novel object (Sec)
Control	45.20±2.3	16.30±1.6	11.30±3.0	67.80±0.1	34.00±2.5
Rotenone (10 mg/kg)	90.40±3.2	56.50±2.0$^{\#}$	50.90±0.5$^{\#}$	22.60±1.0$^{\#}$	19.00±1.1$^{\#}$
AMV (500 mg/kg)	50.80±2.8	22.60±1.3***	17.00±2.4***	55.50±1.2***	32.00±0.6***

Values are expressed as Mean ± SEM, (n=6) and data were analysed by one-way ANOVA followed by Turkey's multiple comparison test. #:p<0.001when compared with the control group while ***:p<0.001 when compared with the rotenone control group.

Table 3: Effect of different drugs on 5-HT, 5-HIAA and dopamine levels in hippocampal tissue.

Group	5-HT (ng/gm of tissue)	5-HIAA (ng/gm of tissue)	DA (ng/gm of tissue)
Control	360	320	150
Rotenone	200$^{\#}$	150$^{\#}$	50$^{\#}$
AMV	345***	280***	100***

Values are expressed as mean ± SEM, (n=6.) Data were analysed by One-way ANOVA followed by Tukey's multiple comparison test. #: p<0.001 when compared to normal control group. ***: p<0.001; test group vs rotenone control group. ns: The test group vs control group.

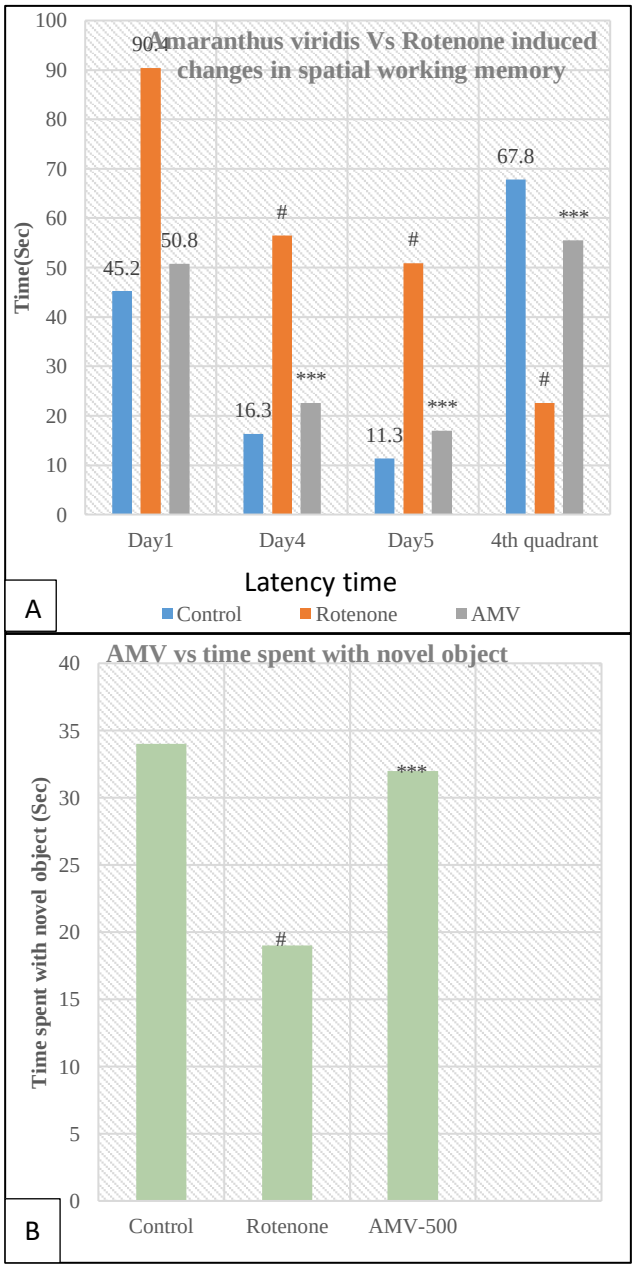

Figure 2 (A and B): Effect of *Amaranthus viridis* on rotenone induced changes in spatial working memory assessed through Morri's maze test and time spent with novel object in recognition test. Time spent with novel objects in novel object recognition test.

Values are expressed as Mean ± SEM, (n=6) and data were analysed by one-way ANOVA followed by Turkey's multiple comparison test. #:p<0.001when compared with the control group while ***:p<0.001 when compared with the rotenone control group. AMV (Amaranthus viridis

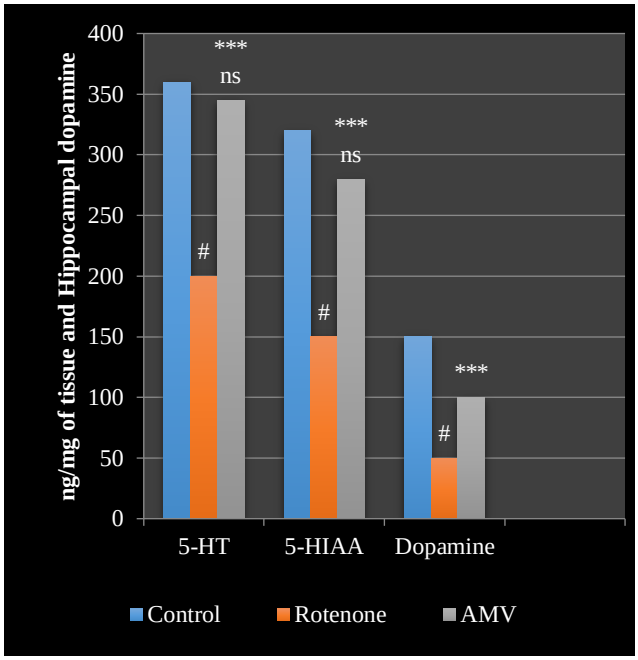

Figure 3: Effect of different drugs on 5-HT, 5-HIAA and dopamine levels in hippocampal tissue.

Values are expressed as Mean ± SEM, (n=6.) Data were analysed by One-way ANOVA followed by Tukey's multiple comparison test. #: p<0.001 when compared to normal control group. ***: p<0.001; test group vs rotenone control group. Ns (No significance): The test group vs control group.

Neurobiochemical analyses

In this experiment, it was noted a highly significant decrease in dopamine, serotonin and its metabolite 5-hydroxy indole acetic acid level in the hippocampus with rotenone treatment in comparison to the control group (p<0.001). *Amaranthus viridis* treatment restored these rotenone-induced biochemical deficits to a highly significant extent when compared to rotenone control rats (p<0.001). Again, these monoamine levels with *Amaranthus viridis* treatment were comparable to that of vehicle-treated control animals (p>0.05) (Figure 2).

DISCUSSION

Numerous factors of the pathogenesis of PD is a key challenging issue in its present therapeutic approach. Additionally, recent evidence on the neuroprotective effect of bioactive compounds derived from plant sources is in frontline research in exploring the benefits with minimal risk in the management of neurodegenerative diseases. In

this study, we evaluated the protective effect of methanol extract of *Amaranthus viridis* on non-motor symptoms developed in rotenone-induced PD rats. Rotenone, a neurotoxic pesticide is highly lipophilic in nature and crosses the blood-brain barrier. Rotenone-induced PD in Wistar albino rats is a well-documented model and was selected in the present investigation.

In this study, an elevated plus maze test was used as an anxiety model. It was carefully noted that *Amaranthus viridis* exhibited the anti-anxiety effect in rotenone-induced PD rats as evident from increased time spent and a number of entries into the open arms of plus-maze. Morris water maze test is a widely accepted model for screening spatial working memory and recognition memory and performance in this test is reported to be associated
with the effect of drugs on neurotransmitters.[16]
Considering Morris water maze test, treatment group displayed an extended flight latency period to get to desired quadrant showing a damaged spatial learning function.

Furthermore, in the spatial probe test, a reduced time spent in the target quadrant showed damaged memory ability. The outcomes of this study concur with that of Sivandzade et al.[17] It is of paramount importance to note that with *Amaranthus viridis* treatment, there was a reversal of these effects induced with rotenone, explai ning the recovery of learning and memory.

The novel object recognition test is another well-characterized test for the assessment of memory function, especially episodic memory.[18] It was noted in this study that, rotenone impaired recognition memory ability in rats while exploring novel objects and *Amaranthus viridis* ameliorated this recognition shortfall as measured by the time spent exploring novel objects.

On neurobiochemical analyses, the biochemical alterations such as decreased hippocampal serotonin and dopamine level observed in this experiment concurred with anxiety-like behaviour and cognitive decline asserted by rotenone. Modification in neurotransmitter homeostasis in striatal and
extrastriatal parts of the brain in PD models is well docu mented.[19] The evidence on the link between 5-HT and level of anxiety confirms the outcomes from the elevated plus maze test.[20] The observations of this study tally with that of Gameiro et al who have also reported similar effects with rotenone.[21] Interestingly, in this study, *Amaranthus viridis* (500 mg/kg) restored these neurotransmitter deficits induced with rotenone indicating its anti-anxiety and memory-enhancing effects.

In previous studies, rotenone was found to decrease antioxidants (SOD, GSH) and increase lipid peroxides (MDA) in brain tissue homogenate.[22] *Amaranthus viridis* amended those level biochemical alterations induced by rotenone. It was also discovered that the methanol extract of *Amaranthus viridis* possesses strong anti-oxidant and anti-inflammatory properties in an in-vitro test.[23] Hence, this can further explain the anti-anxiety-like behaviour and cognitive improvement shown by *Amaranthus viridis*. Thus, oral administration of methanol extract of *Amaranthus viridis* abrogated the anxiety-like behaviour and impaired cognition induced with rotenone by replacing serotonin and dopamine in the hippocampus.

CONCLUSION

From this study, it can be concluded that, *Amaranthus viridis* improved non-motor symptoms of PD. This novel finding suggests further investigations in line of investigating the role of specific neurochemicals and receptors involved in this neuroprotective effect.

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