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Antioxidant and Neuroprotective Activity of Wheat Microgreen Extracts against Rotenone Induced Neurotoxicity in *Caenorhabditis elegans*

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Abstract

Background: Wheatgrass (*Triticum*, Family: Poaceae) juice is considered as a living food and often consumed due to its countless health benefits. Wheatgrass juice contains many bioactive compounds which can be useful for ameliorating neurogenerative diseases. Wheat in the form of microgreen (7th day after sowing) have been reported to have higher level of bioactive compounds compared to the mature wheatgrass or grain. **Objective:** To evaluate the protective effects of wheat microgreen extract against rotenone induced neurodegeneration in *Caenorhabditis elegans*. **Methods:** Worms were exposed to the different concentrations of rotenone to determine the concentration that can induce neurodegeneration without causing any mortality. Wheat microgreen extract was exposed to worms along with rotenone to determine the effective concentration. Neuroprotective potential of wheat microgreen extract was assessed by foraging and locomotory performance, free radical generation, cytotoxicity assay and dopamine content. **Results:** 4 μM concentration of rotenone was found non lethal but able to induce behavior changes after 48 h of exposure. Wheat microgreen extract at 1 mg/mL concentration was found minimum and effective when exposed along with rotenone to worms. Rotenone exposed *C. elegans* were observed to have reduced locomotory and foraging behaviors along with dopamine content, while an increased level of free radical and cytotoxicity. Wheat microgreen extract improved behavior performance and dopamine content, also reduce free radical generation and cytotoxicity in the rotenone exposed worms. **Conclusion:** Our study concludes that wheat microgreen exhibits neuroprotective and antioxidant potential and can be considered as a possible treatment for neurodegeneration.

Keywords: Wheat microgreen; *Caenorhabditis elegans*; Neurodegeneration; Rotenone

1 Introduction

Neurological disorders are the second leading cause of death and affecting more than 9 million individuals, worldwide[1,2]. There are approximately 600 neurological disorders that majorly include epilepsy, stroke, Alzheimer's disease, and Parkinson's disease. The World Health Organization reports that during the past several years, the burden of neurological disorders has increased globally and is now acknowledged as a significant public health concern[2]. Unfortunately, specific cause of neurological disorders remained unclear, but researchers believe that genetic and environmental factors contribute majorly along with poor lifestyle in the initiation of neurological diseases.

Rotenone, a plant extract derived from *Lonchocarpus* and *Derris* species plants, used as a broad-spectrum insecticide and pesticide and a well-known neurotoxin. Rotenone is a strong inhibitor of mitochondrial complex I, leads to oxidative stress with limited ATP synthesis[3,4]. Animal models exposed to rotenone develop behavioral and pathological symptoms resemble to Parkinson's diseases that include motor deficit, variety of non-motor-symptoms and dementia[4–7]. Conventional methods for the treatment neurological diseases are palliative and only provide symptomatic relief such as reducing pain and stress of a serious illness[8]. However, long term use of these synthetic medicines can causes symptoms of adverse side effect such as- nausea, diarrhea, insomnia, vomiting, foot edema, chronic daily headache, severe dyskinesia and hallucination[8–10].

Drugs with natural origin are gaining more attention of scientific community as they are very safe and devoid of adverse effects. Many medicinal plants and herbs have been examined to prevent, delay or retard the progression of neurological diseases[11,12]. Wheatgrass juice works as potent antioxidant and regulate expression of neuroprotective markers such as brain-derived neurotrophic factor and cAMP-response element binding protein, inflammatory markers like TNF-α and tau genes[13–15]. Previous studied reported that wheatgrass exert antidepressant effects in cells, mice and rats, also helps to recover from various cognitive functions and lower the risk of conditions like Parkinson and Alzheimer's[15,16]. Several studies claimed that wheatgrass juice is also a safe and effective treatment for ailments such as obesity, high blood pressure, cancers, anaemia, diabetes, infertility, eczema[17,18]. Wheat microgreen, newly sprouted leaves, contains more than 90 minerals, sodium, many essential enzymes, vitamins, antioxidants and amino acids[19,20]. Now a day, microgreens are defined as a superfood due to its high nutritional potential than the mature plants. However, only few studied has been performed to investigate the therapeutic potential of microgreens[21–24] and from our knowledge, none of the study has been reported on the neuroprotective properties of wheat microgreen.

Caenorhabditis elegans is used as animal model in the present study as it is a small transparent animal model provides an excellent, fast and inexpensive platform to screen number of the compounds in a limited period of time with the visible inspection. *C. elegans* has well define nervous system, numbers of mutant and models available for many human diseases including Parkinson's and Alzheimer's diseases with highly conserved signal transduction pathways across the taxa[25,26].

2 Materials and methods

2.1 *Caenorhabditis elegans* strains

Nematode growth medium (NGM) plates were used to maintained the Bristol N2 (wild type) strains of the worms with *Escherichia coli* strain OP50 as a food sources, at 20°C±1.5°C[27].

2.2 Culture and harvest of wheat microgreen

Wheat (*Triticum*, Family: Poaceae) HI1605 variety seeds were procured by ICAR-Indian Agricultural Research Institute, Regional station, Indore. Prior to sowing, the seeds were soaked in clean water for several hours. We used small plastic container with many holes at the bottom side. The soaked seeds are sprinkled densely over growing media i.e. coco peat of about 2.5 inches. Then again, a thin layer of growing media is covered over the seed layer. Now the prepared tray is sprinkled over with water. Microgreen was grown in plant culture room at the temperature of 24-27°C with relative 70% humidity and alternate day watering. We have not used any sort of chemical such as pesticides, fungicide or fertilizers as it is an organic nutrient rich food so any nutrient should not be compromised. Wheat microgreen were harvested after 7 days of sowing using a sharp pair of scissors and then washed under running water[28] (Figure 1).

Fig 1. Wheat microgreens

2.3 Preparation of WME

Freshly harvested wheat microgreens were used to prepare extract. For that, 3 grams of microgreen were crushed in motor pastel then chloroform and methanol were added in 1:1

ratio. The crushed leaves were further homogenized and sonicated to ensure that majority of the cells breaks and releases most of their phytocompounds. The samples were centrifuged at 4000 rpm for 10 min. The supernatant was collected in separate tube and lyophilized. Lyophilized samples were dissolved in DMSO to prepare a stock concentration of 20 mg/ml of WME.

2.4 Determination of the concentration of rotenone and WME

L1 stage worms were exposed to the different concentrations (2, 4, 6, 8 and 10 μM) of rotenone every day for 48 h and observed under stereomicroscope for survival and growth.

In order to choose concentrations for microgreen extract on *C. elegans*, we have taken 2 parameters in consideration 1st visual observation and 2nd behavior assay. We have exposed worms to the 0.5, 1 and 2 mg/ml concentration of microgreen extract along with the 4 μM of rotenone for 48 h and then observe worms under the stereomicroscope for clear vision and behavior changes.

2.5 Behavior assays

Behavior assays were performed as reported in [29]. Briefly, after 48 h treatment worms were washed, harvested and transferred individually on an unseeded NGM plate. After 1 min recovery period individual worm was manually scored under the microscope, 1 min for head trashes and 20 sec for body bends. Change in the direction of bending at the midbody is defined as head thrash that is useful to observe the effect of compounds on motor neuron. A body bend is referred as a change in the direction of the part of the nematodes corresponding to the posterior bulb of the pharynx along the Y-axis, assuming that the nematode was traveling along the X-axis. Both the assays have been performed in triplicate with 30 replicates (Randomly selected) for each group.

2.6 Quantification of reactive oxygen species (ROS)

Level of ROS was determined as reported in [30]. Briefly, after the treatment worms were washed with 1XPBS and about 1000 worms from each group were separately transferred into the each well of black 96-well plate with transparent bottom in triplicates. 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Sigma, U.S.A) dye (0.05 mM working concentration) was added individually and incubated for 30 min on an orbital shaker at 20°C. Inside the cell nonfluorescent dye oxidized into highly fluorescent 2′,7′-dichlorofluorescein and was measured at 485/535 nm in Spectrophotometer Spectramax (Molecular Devices, UK).

2.7 MTT assay

Tetrazolium MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is used to assess cellular metabolic activity. MTT is a cell-membrane permeable dye and intracellularly reduced by NAD(P)H-dependent cellular oxido-reductase enzyme to an insoluble purple colored formazan product. This product is soluble in organic solvent and thus, measured calorimetrically. Hermaphrodite worms (~1000 worms) in triplicate were transferred to each well of transparent 96-well microtitre plate along with 50 μl of MTT (10 mg/ml) dye and incubated at 20°C for 3 h. the plate was centrifuged at 2000 rpm for 10 min, and the supernatant was aspirated. Formazan formed in worms was solubilized in 100 μl DMSO and measured absorbance at 595 nm in Spectrophotometer (Spectramax, Molecular Devices, UK). MTT gives a measure of viable cells and is shown in results section as fold change in comparison to control.

2.8 Estimation of dopamine associated behavior using 1-Nonanol assay

A nonanol repulsive assay is an established and indirect method to estimation of dopamine level in *C. elegans*. Level of dopamine is related to the recognition, motivation, memory, adaptation and motor activity in worms. Nonanol assay has been performed as reported in [31]. Briefly, worm after the treatment were washed 3 times with 1X PBS buffer and left tabletop to settle down. A drop of worm suspension was placed at 90 mm NGM plates and let it dry. An eye lash attached with a toothpick dipped in 1- nonanol (Sigma Aldrich; 131210; 0.01% 1-nonanol dissolved in DMSO) and was placed near the head of worm. Time taken by worm to exhibit the repulsive behavior was calculated using a stopwatch.

3 Results

3.1 Rotenone exposure to *C. elegans*

We observed delayed development in the worms those were exposed to the 6, 8 and 10 μM of rotenone (Figure 2). Thus, these concentrations were excluded since these are very high concentration to analyze the rotenone induced neurodegeneration in *C. elegans*. 4 μM concentration of rotenone was chosen to work with which was also supported by literature [32,33].

3.2 WME exposure to *C. elegans*

We found that 0.5 mg/ml microgreen extract were not able to recover from rotenone (4 μM) induced significant changes in the behavior of worms compared to control (data not shown). While plates seeded with 2 mg/ml microgreen extract were more greenish and harder to observe in microscope.

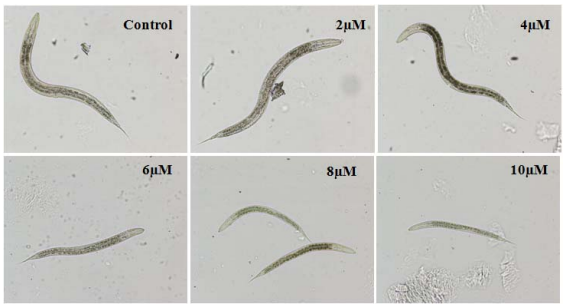

Fig 2. Exposure of rotenone at different concentration affects the growth of worms

Worms exposed to the 1 mg/ml microgreen extract along with rotenone were easy to observe in the stereomicroscope as well as ameliorate rotenone induce behaviors changes (Figure 3). So, we have chosen 1 mg/ml of microgreens concentration to work further.

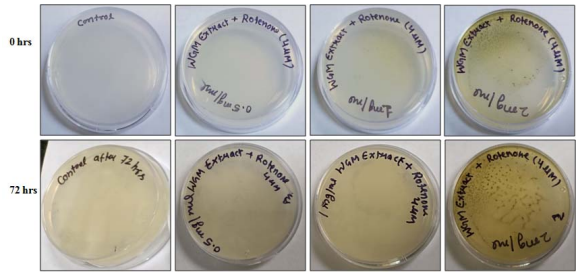

Fig 3. Exposure of WME at different concentration along with rotenone (4μM) to determine the concentration of WME

3.3 WME improves locomotory and foraging performance of *C. elegans*

It was observed that rotenone induces significant ($p<0.05$) change in the body bend (Mean value 6.4±0.3) and head trashes (Mean value 60±2.5) behavior of *C. elegans* at 4 μM concentration (Figure 4 **a, b**). While, when WME was exposed along with the rotenone treatment worms were observed to have normal body bend and head trashes behaviour with the mean value 18.25±0.35 and 143.35±0.85 respectively, comparable to control (Mean value 17.4±0.2 and 135.85±1.15) (Figure 4 **a, b**). So, wheat microgreens extract can ameliorate rotenone induced behavior changes in worm. 61-68% recovery was observed in behaviour of WME+rotenone treated worms compared to rotenone exposed worms.

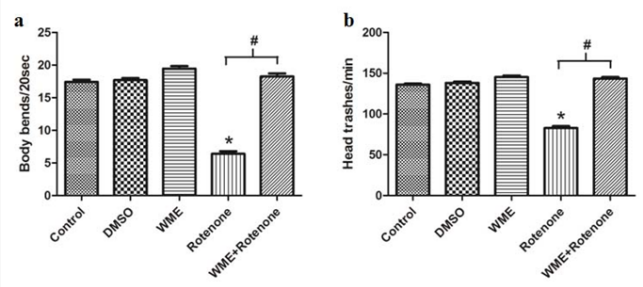

Fig 4. Effect of WME on the rotenone induced behaviour changes in worms (a) Body bends (b) Head trashes. n=30, bar = mean±SEM of three independent experiments; Bonferroni corrected *p<0.05 = Significant against control; #p< 0.05 = Significant recovery (in presence of WME) against rotenone exposure

3.4 WME confers protection against rotenone induced ROS generation

ROS production in organisms exposed to rotenone significantly increased in comparison to control. 4-fold increase in ROS generation was observed after 48 h exposures of rotenone at 4 μM concentration, respectively (Figure 5). Microgreen treatment at 1 mg/ml concentration along with rotenone was found to completely attenuate ROS generation in case of rotenone treatment (Figure 5; Control=1).

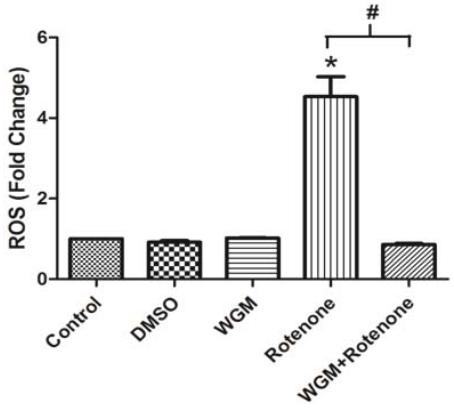

Fig 5. Effect of WME on rotenone induced ROS generation in *C. elegans*. Bar = mean±SEM of three independent experiments; Bonferroni corrected *p<0.05 = Significant against control: #p<0.05 = Significant recovery (in presence of WME) against rotenone exposure

3.5 WME reduced rotenone induced cytotoxicity

Mitochondrial activity reduces significantly ($p<0.05$) in rotenone exposed organisms compared to controls. We

observed 33% reduction in mitochondrial activity of the organism exposed to rotenone, respectively. When WME was given along with rotenone mitochondrial activity was found comparable to the control (Figure 6; Control=1).

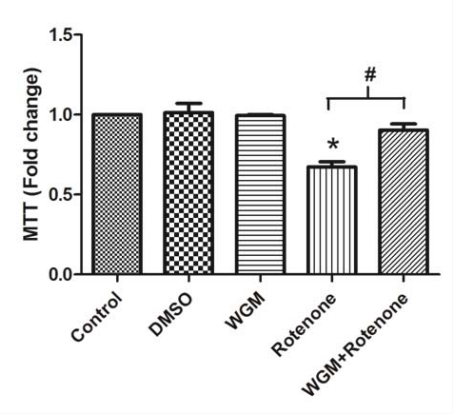

Fig 6. Effect of WME on rotenone induced cytotoxicity in worms. Bar = mean±SEM of three independent experiments; Bonferroni corrected *p<0.05 = Significant against control: #p<0.05 = Significant recovery (in presence of WME) against rotenone exposure

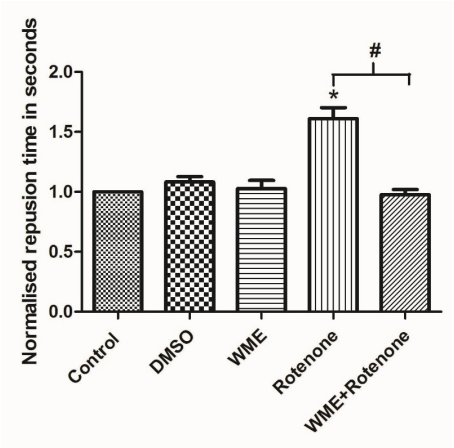

Fig 7. Effect of WME on rotenone induced reduction in dopamine content in worms. Bar = mean±SEM of three independent experiments; Bonferroni corrected *p<0.05 = Significant against control: #p< 0.05 = Significant recovery (in presence of WME) against rotenone exposure

3.6 Effect of WME on dopamine associated behavior of *C. elegans* exposed to rotenone

Effect of the dopamine level on the behavior of worms was assayed through 1- nonanol assay. 1- nonanol responsive movement of *C. elegans* is associated with the dopamine levels, where higher repulsive time related with lower the dopamine level and vice versa. Control was used to normalize the repulsive time. Compared to control, a significant increase ($p<0.05$) in the repulsive time was observed in the worm treated with rotenone. While, in comparison to control when worms were exposed to rotenone along with WME no significant change was observed (Figure 7; Control=1).

4 Discussion

The present study is performed to analyze the efficiency of WME in reducing the rotenone induced toxicity and behavior changes in *C. elegans*. Rotenone is widely used by the farmers as herbicide and pesticide and researcher to make symptomatic diseases model to study Parkinson's diseases. The action mechanism of rotenone induces neuronal damage in different organism is remained a mystery and now several ways are proposed such as free radical induced oxidative stress, inhibition of mitochondrial complex I, reduced ATP synthesis, upregulation of pro-inflammatory factors, autophagy[3,6,7].

Exposure of rotenone inhibit mitochondrial complex-I and generate free radical leads to the oxidative damage and apoptosis in neuronal cell. Furthermore, degeneration of neuronal cell affects the ability to produces neurotransmitters causes movement disorder similar symptoms caused by neurodegenerative diseases. In the present study, *C. elegans* were exposed to the pesticide rotenone showed growth retardation at the higher concentrations (Figure 2), increased ROS generation (Figure 4), increased cell mortality (Figure 5), altered locomotory and foraging behaviors (Figures 6 and 7) and decreased dopamine content (Figure 7). Experimental parameters used in this study helped be concluded that the worms exposed to the rotenone suffered with neurodegenerative diseases specially Parkinson's disease. Rotenone toxicity can be related to the oxidative stress and in the present study there was significant free radical generation was observed (Figure 4) along with increased cell mortality (Figure 5) in the worms exposed to rotenone. On the other hand, it has been clearly seen the WME has antioxidant potential and prevent the rotenone induced toxicity through scavenging of free radicals (Figure 4). Further, 1-Nonanol assay indicates that rotenone exposure can reduce dopamine level which further affect the behavior of the worms, are the classic sign of Parkinson's diseases while WME ameliorated rotenone induced changes in dopamine level and behavior changes in the worms. Various medicinal plants in the form of herbal extract such as *Malva parviflora, Scutellaria baicalensis, Ginkgo biloba, Camellia sinensis, Panax ginseng, Grewia tiliaefolia* etc. were observed to provide protection against neurodegenerative diseases in many in-vitro and in-vivo model systems[34–38] but use of WME against neurodegenerative diseases, in our knowledge is not been explored. In the present scenario where long term use of synthetic medicine causes severe side effects and affects the life expectancy, the use of

natural ingredients such as microgreen is safe and effective with almost no side effects. Studies suggests that microgreens are rich in antioxidants, vitamins, minerals and polyphenolic compounds help to reduce oxidative stress, a common cause of various degenerative diseases in humans.

Thereby, the study indicates that WME was able to prevent and reduce the symptoms of rotenone induced neuronal damage in *C. elegans*. The data of study evident that WME has potential to ameliorate rotenone induced alteration in locomotors and foraging behavior of worms and therefore can be consider as a neuroprotective agent. The results also highlighted the antioxidant potential of wheat microgreen as the oxidative stress known to participate in a wide range of diseases including neurodegenerative diseases.

5 Conclusion

WME extract displayed potential neuroprotective activity as illustrated by various important behavior parameters performed in this study. Biochemical parameters exhibit strong antioxidant activity of WME extract as it reduces free radical generation and cytotoxicity in rotenone exposed worms. This potential of WME extract is inseparable and largely dependent on the polyphenolic compounds. So, using WME as a functional food reduces the oxidative stress this may result in the increased level of neurotransmitters such as dopamine in the present study. Thus, WME can be considered as potential neuroprotective agent in order to prevent or reduces the incidence of neurogenerative disorders.

Abbreviations

Wheat microgreen extract: WME; Neurodegeneration: ND; *Caenorhabditis elegans*: *C. elegans*

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Authors' Contributions

MS conceptualization, Methodology, Investigation, data analysis, writing work; SS Investigation, Conceptualization. Both the authors have seen and approved the content of written work.

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