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Effect of Celery (*Apium graveolens* L.) Microgreens on *Drosophila melanogaster*

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Abstract:

Celery (*Apium graveolens* L. ; family : Apiaceae) was often used as a spice in daily food. However, this plant contains many antioxidant compounds useful for attenuating neurodegenerative disorders including Parkinson's disease. Planting celery in the form of microgreens harvested 15 days was expected to increase the content of bioactive compounds. In the current study, we intended to evaluate the neuromodulatory potential of methanol extract of celery microgreens on fruit flies (*Drosophila melanogaster* Meigen : family Drosophilidae ; ordo : Diptera) which were exposed to paraquat. Neuroprotective capacity was assessed by survival rate, locomotor performance, lipid peroxidation and dopamine content after being treated with 120 µg/mL extract of celery microgreens and 3.5 mM paraquat for 4 days. Phytochemical constituents from extract of celery microgreens were measured including total polyphenol content and antioxidant activity using the radicals scavenging method. Exposure of adult fruit flies to paraquat will cause a decrease in the survival and locomotor phenotype improved by extract of celery microgreens treatment. In parallel, increased malondialdehyde content from lipid peroxidation and decreased dopamine content can be improved by the presence of celery microgreens extract. Neuroprotective capacities indicate a high content of antioxidant compounds of celery microgreens extract. Our study concludes that celery microgreens exhibited to retard the effect of oxidative stress that causes Parkinson's disease.

Keywords: celery, fruit fly, microgreens, Parkinson, paraquat.

Introduction:

Parkinson's disease is the second important neuronal disturbance after Alzheimer's disease. This disease usually attacks people over the age of 50 but now the disease can be suffered by younger people[1]. According to World Health Organization, the number of people with Parkinson's disease in the world is around one billion[2]. Symptoms of Parkinson's disease include motor disorders such as resting tremor, postural instability, rigidity and bradykinesia, while non-motor disorders include constipation, anxiety, depression and fatigue[3]. The cause of Parkinson's disease is still uncertain (idiopathic), but some researchers argue that besides genetic factors as well as environmental factors are the causes of this neurodegenerative disease. One herbicide is known to be the cause of Parkinson's disease is Paraquat (N, N,-dimethyl-4,4′-bipyridinium dichloride)[4]. These compounds are closely related to the emergence of oxidative stress because of excessive reactive oxygen species (ROS). Oxidative reactions can cause damage to dopaminergic neurons in the Substantia Nigra producing dopamine compounds which function as neurotransmitters[5].

Presently, treatment is carried out on Parkinson's disease patients with Levodopa. However, the efficacy of this drug is only limited in reducing symptoms caused by Parkinson's disease, by temporarily replacing the role of dopamine compounds that have been reduced even lost in people with Parkinson's disease[6]. The use of these synthetic drugs in the long term can cause symptoms of side effects such as nausea, vomiting, foot edema, dyskinesia and hallucinations[7]. Potential drugs that can reduce symptoms of Parkinson's disease and

protect nerve tissue are very important because living nerve cells are only once and cannot be regenerated[8]. The use of drugs from natural ingredients in the form of medicinal plants and herbs to delay or retard the progression of Parkinson's disease has been carried out by many researchers. From our knowledge, the use of microgreens, especially from celery species is still very rarely found. The potential of the antioxidant content of these microgreens has been widely investigated. Celery has many active compounds such as polyphenols, various pigments (chlorophyll and β-carotene) which function as antioxidants that can reduce oxidative stress[9].

The use of fruit fly as an animal model in this study is based on several advantages including: easy maintenance, small body size can reduce the place of culture, produce many eggs (30-50) and growth from eggs to mature flies about 10 days, and the ethics committee permission are not required[8]. Likewise, this fruit fly has an orthological genetic similarity of about 77% with genes that cause disease in humans[10]. Adult fruit flies have dopamine-producing neurons that are very feasible for use in the study of Parkinson's disease[11]. In this paper, all that has been done during the research is to reveal the role of celery microgreens extract as a neuroprotective agent and paraquat compounds as neurotoxic agents that correlate with neurodegenerative diseases, especially Parkinson's disease. The methodology shows how to conduct a preliminary study to find the lowest concentration of celery microgreens extract which can reduce the toxicity of paraquat as seen from measuring locomotor activity of fruit flies. On the other hand, the paraquat concentration used was 3.5 mM referring to the study of Soares *et al.*[12]. Observation of Parkinson's symptoms is carried out in live and dead fruit flies (head). *In vivo* observations include survival and locomotor activity through negative geotaxis. Whereas *in vitro* observations are measurements of dopamine and malondialdehyde (MDA) levels as the end result of lipid peroxidation reactions on the head of fruit flies.

Materials and Methods:

Culture and harvest of microgreens celery

Celery (*Apium graveolens* L. ; family : Apiaceae) seeds grown as microgreens were purchased from farm shops in Bandung city of Indonesia. Seeds were sown on zeolite substrate by sowing evenly on the surface. Microgreens planting uses small plastic containers with many holes at the bottom (Fig. 1). Microgreens planting is carried out in the Plant Physiology laboratory room at a temperature of 24-27ºC and a relative humidity of 70% with watered an alternate day till 15 days. Harvesting is done on the 16 th day, by cutting the microgreens just above the surface of the medium using sharp scissors. Furthermore, fresh biomass microgreens are dried in the oven at 50ºC for 24 hours for further processing.

Figure 1. Celery microgreens

Preparation of celery microgreens extract

For obtaining celery microgreens extract, 4 g of dry biomass was macerated on 20 mL of methanol p.a. Then it was left for 24 h and continued to stir with a shaker for 5 h. This maceration was done three times until the residue became pale. Then the filtrate and residue were separated using filter paper. The filtrate was concentrated by evaporating the methanol solvent in the water bath 65 °C until a crude extract in the form of a concentrated paste was obtained.

Determination of total polyphenol content.

Ten µl of celery microgreens extract was dissolved in the methanol: aquadest (1: 1) up to 5 ml mixture. The 300 µl solution was homogenized with 2 ml Folin Ciocalteau reagent and left for 3 min. Furthermore, Na_2CO_3 was added to the solution to be allowed to stand for 60 min at room temperature. Likewise, the same method was carried out on gallic acid with concentrations of 25, 50, 75, 100, 125, 150, 175 and 200 µg/ml. Measurement of total polyphenol content in both microgreens extract and gallic acid using a spectrophotometer at a wavelength of 765 nm which was repeated three times. As a standard, a curve is made between the concentration of gallic acid and its absorbance [13]. The total polyphenol content is measured by the equation:

$$TPC = \frac{C.V.Fp}{g} \qquad \text{……..1}$$

Notes:

C - polyphenol concentration.
V - volume of extract (mL).
FP - Dilution factor.
g - weight of sample (g).

DPPH radical scavenging activity assay.

Measurement of antioxidant activity performed by using *2,2 diphenyl-1-picrylhydrazyl* (DPPH) and measurement of vitamin C existing in references are utilized as control[14]. Celery microgreens extract to be tested was mixed with 20 mg/L DPPH solution so the final concentrations were 10, 50, 100, 200, 400 and 800 µg / mL. The test tube of the mixture was covered with aluminum foil and left for 25 min at room temperature. Furthermore, the absorbance value was determined at a wavelength of 517 nm and the DPPH scavenging activity was measured using the following equation:

$$(\text{inbibition } (\%)) = \frac{\text{Absorbance control} - \text{Absorbance extract}}{\text{Absorbance control}} \times 100\% \quad \text{.....2}$$

Inhibition concentration 50 (IC50) value is expressed as the amount of extract concentration which can reduce 50% DPPH free radicals.

Culture and treatment of *Drosophila*

Wild type male fruit flies were obtained from stocks owned by the genetic laboratory of science and technology faculty of UIN Bandung. The composition of the medium used is a mixture of weight/volume of 1% yeast, 1% agar, 2% sucrose, 1% milk powder, and 0.08% nipagin. All treatments were given along with the diet to flies aged three days from hatching for four days of observation. There were four treatment groups including: the first group of flies were not treated (control). The second group, flies treated with 120 µg / mL celery microgreens extract, the third group, flies treated with 3.5 mM paraquat and the fourth group, flies received both celery microgreens extract and paraquat. Bottles of fruit flies culture were placed in a room at 21 ± 1 °C and 70% of relative humidity, with photoperiod 12 hours in dark / 12 hours in light.

Survival rates

Survival rate determined from the number of fruit flies that are still alive for four days of observation. Then 30 fruit flies each treatment was observed every day[3]. This survival data is used to calculate survival rate using the following formula:

$$SR = \frac{Nt}{N0} \times 100\% \quad \text{.....3}$$

Notes:

SR - Survival rate;

N0 - Number of *D. melanogaster* at the beginning of study.

Nt - Number of *D. melanogaster* still alive at the end of study.

Negative geotaxis assay

The locomotory ability of fruit flies was identified by a negative geotaxis assay (Fig. 2). Fruit flies were chosen when anesthetized on ice blocks and then placed at the base of the glass column (length 12 cm, diameter 2 cm). Approximately 15 min fruit flies' recovery from cold exposure, were tapped gently to the bottom of the column. Then the fruit flies were allowed to climb with a distance of 5 cm for 6 sec[15]. The locomotory ability of fruit flies was calculated using the following formula:

$$\frac{1}{2}\left[\frac{N_{tot} + N_{top} - N_{bot}}{N_{tot}}\right] \times 100 \quad \text{.....4}$$

Notes:

Ntop - the number of fruit flies that have reached above 5 cm.

Nbot - flies that remain below 5 cm

Ntot - show the number of flies in this study

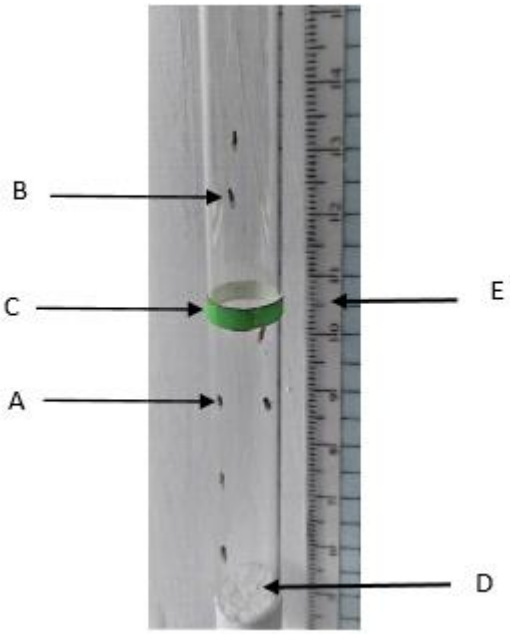

Figure 2. Negative geotaxis assay of fruit flies (a) unable to pass (b) able to pass (c) boundary mark, 5 cm high (D) base of coloum tube (E) ruler scale

Lipid peroxidation Assay

The initial procedure, 30 heads of fruit flies from each treatment were homogenized by adding 0.6 ml 50 mM sodium phosphate buffer and 10% trichloroacetic acid at pH 6. Then, mixture was centrifuged at 5,000 rpm for 20 min. The resulting supernatant was divided into two parts. The first part, 0.3 ml supernatant mixed with 0.1 ml 0.1 M EDTA and 0.6 ml solution (1% tiobarbituric acid and 0.05 M NaOH) was incubated at 100 ° C for 7 min. The second part, 0.3 ml the supernatant and 0.5 ml H_2O is carried out the same procedure as the first supernatant. Then all samples were cooled on ice and centrifuged at 8,000 rpm for 2 minutes. The absorbance of the sample was measured by a

spectrophotometer at a wavelength of 535 nm[16]. The levels of malondialdehyde (MDA) as the end result of lipid peroxidation was calculated by the following equation:

$$\text{Content of MDA (nMol/ml)} = \frac{0{,}2422+\text{absorbance}}{0{,}0241} \ldots\ldots\ldots 5$$

Estimation of dopamine content

About 40 heads of fruit flies were crushed in a solution of 500µL HCl-butanol. The resulting suspension was centrifuged at 3000 rpm for 5 min and removed from the supernatant. Furthermore, the supernatant was mixed with 250 µL heptane and 100 µL 0.1 M HCl, then was centrifuged again at the same speed and time as before. The final supernatant will be used for estimating dopamine content. Then 100 µL supernatant were mixed with 50 µL 0.4 M HCl and 100 µL iodine solution, then incubated for 2 min. The mixture was added 100 µL sodium sulfite and 100 µL 10 M acetic acid, then boiled at 100 °C for 5 min. The supernatant was cooled at room temperature, absorbance measurements were carried out with a wavelength of 375 nm [17]. Measurements were repeated three times from each treatment.

Statistical analysis

All data from the study were expressed as mean ± SEM (standard error of mean). The measurement was performed triplicate. The data was analyzed by one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) with P-value less than 0.005 was considered as statistically significant. Statistical analysis was conducted using software of SPSS version 16.

Results:

Total Polyphenol Concentration and Antioxidant Activity

The total amount of phenolic compounds was determined by testing thiobarbituric acid at 255 mg GAE/g extract, while the antioxidant activity measured by the DPPH test showed that celery microgreens extract had good DPPH scavenging activity. Value of IC50 for celery microgreens extract was 77.23 µg/mL whereas ascorbic acid was 4.95 µg/mL.

Celery microgreens extract improves the survival rate of *D. melanogaster*

Fruit flies exposed to 3.5 mM paraquat exhibited the high mortality after four days of observation compared to controls (Fig. 3). Whereas celery microgreens extract treatment shows a number of fruit fly deaths but very few, so it is not significantly different from controls. The treatment of celery microgreens extract can significantly reduce mortality due to paraquat toxicity.

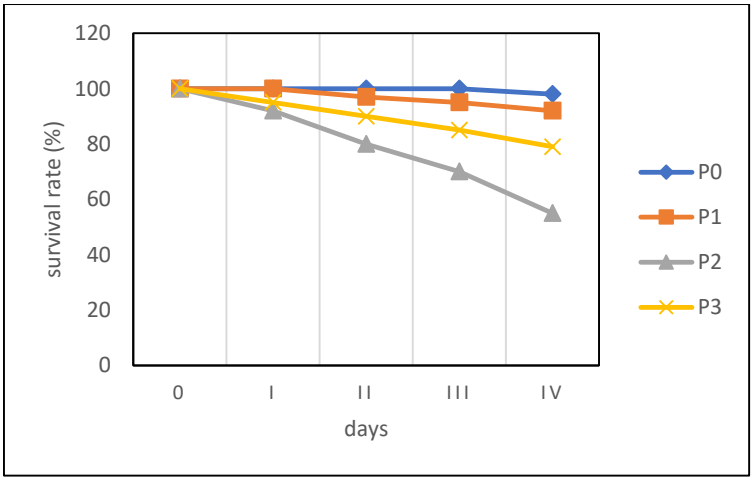

Figure 3. Effect of celery microgreens extract on survival rate on the 4th day of observation (P0 = control;
P1 = 120 µg/mL celery microgreens extract; P2 = 3.5 mM paraquat; P3 = 120 µg/mL celery microgreens extract + 3.5 mM paraquat).

Celery microgreens extract improves locomotor performance of *D. melanogaster*

Paraquat-induced fruit flies showed limitations in locomotor performance as seen from a reduction in climbing ability when compared with controls. The treatment of celery microgreens extract shows an increase in climbing ability in fruit flies that have been exposed to paraquat. Even the climbing ability of fruit flies treated only with celery microgreens extract was not significantly different from the controls (Fig. 4).

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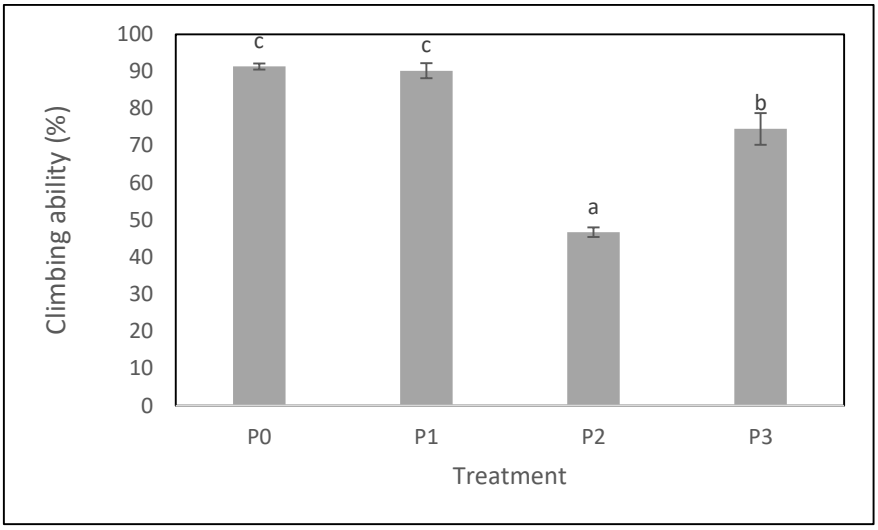

Figure 4. Effect of celery microgreens extract on climbing ability on the 4th day of observation (P0 = control; P1 = 120 µg/mL celery microgreens extract; P2 = 3.5 mM paraquat; P3 = 120 µg/mL celery microgreens extract + 3.5 mM paraquat). Data is the average ± standard error. Value with different letters are significantly different at p < 0.05 (DMRT)

Effect of celery microgreens extract on lipid peroxidation

MDA content in fruit flies treated with celery microgreens extract are lower when compared to the control (Fig. 5). Likewise, celery microgreens extract can reduce MDA content in fruit flies that have been exposed to paraquat poison. MDA content as substances produced due to oxidative stress show a lipid peroxidation process in the head of fruit flies.

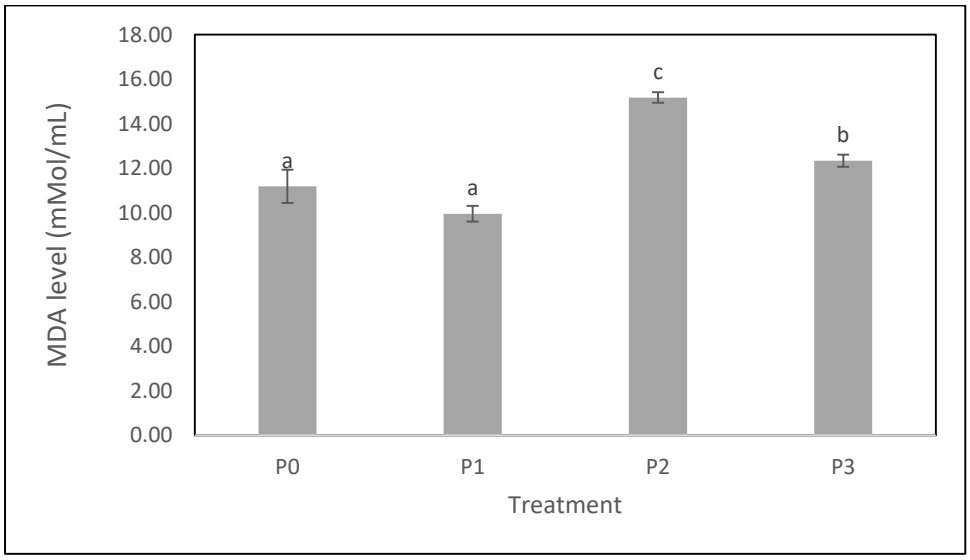

Figure 5. Effect of celery microgreens extract on MDA levels on the 4th day of observation (P0 = control; P1 = 120 µg/mL celery microgreens extract; P2 = 3.5 mM paraquat; P3 = 120 µg/mL celery microgreens extract + 3.5 mM paraquat). Data is the average ± standard error. Value with different letters are significantly different at p < 0.05 (DMRT)

Effect of celery microgreens extract on dopamine levels of flies exposed to paraquat

From this study it can be seen that fruit flies treated with celery microgreens extract had the same dopamine content as controls (not statistically significantly different) (Fig. 6). Likewise, fruit flies exposed to paraquat in the presence of celery microgreens extract were able to be repaired by increasing their dopamine levels even though they were not as high as those given a single treatment of celery microgreens extract.

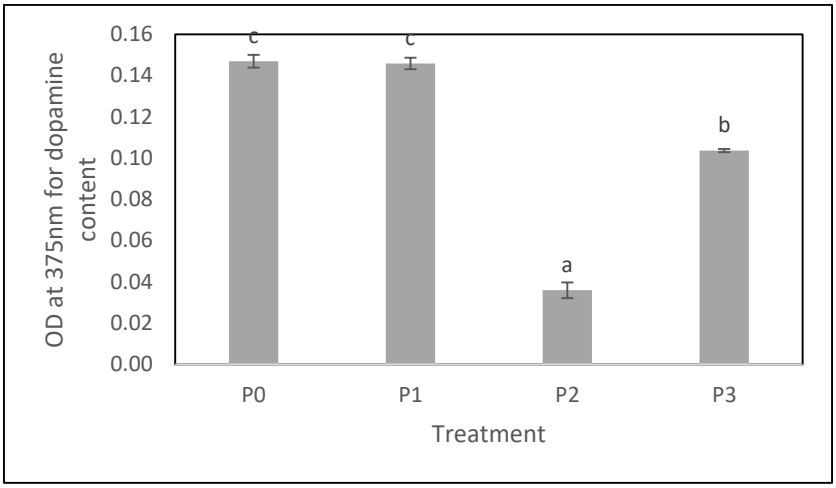

Figure 6. Effect of celery microgreens extract on dopamine level on the 4th day of observation (P0 = control; P1 = 120 µg/mL celery microgreens extract; P2 = 3.5 mM paraquat; P3 = 120 µg/mL celery microgreens extract + 3.5 mM paraquat). Data is the average ± standard error. Value with different letters are significantly different at p < 0.05 (DMRT)

Discussion:

The study aims to identify the potential of methanolic extract of celery microgreens in reducing paraquat toxicity by observing Parkinson's symptoms in fruit flies. Paraquat is an herbicide that is often used by farmers and is also used by researchers in its research to generate oxidative stress. The use of paraquat increase excessive oxidation, which is known to cause deleterious effects to DNA, lipids and proteins that leads to cell death[18]. The poisons from paraquat have long been known to damage important organs in humans such as kidneys, liver, heart and brain. Actually the mechanism of paraquat destruction of nerve tissue is not fully understood, but now several ways are proposed such as: induction of oxidative stress, mitochondrial dysfunction, apoptosis or autophages[19].

Organisms exposed to paraquat will show an increase in MDA (malondialdehyde) as a result of increased lipid peroxidation and cause damage to the dopaminergic nerve where dopamine is produced. Furthermore, dopamine levels will decrease which causes movement disorder similar to the symptoms caused in Parkinson's disease patients[20]. In this study, it can be shown that *D. melanogaster* is exposed to paraquat has Parkinson's disease. It was seen that paraquat-exposed fruit flies showed increased mortality (Fig 3), damage to locomotor tissue (Fig 4) and also a decrease in dopamine content in the head (Fig 6). From the existing parameters, it can be concluded that the fruit flies used in this study have suffered from neurodegenerative diseases, especially Parkinson's disease. Decreased dopamine levels can be caused by two factors: the loss or damage of the dopaminergic nerve and/or increased dopamine oxidation[12].

The toxicity of paraquat seems to be directly related to the onset of oxidative stress. In this study there was an increase in lipid peroxidation (Fig. 5) on the heads of fruit flies exposed to paraquat. So, it can be seen clearly that the administration of celery microgreens extract which has antioxidant strength (IC50 = 77.23 µg/mL with strong categories) and polyphenol compounds (255 mg GAE/g extract) is able to prevent the toxicity of paraquat through scavenging of free radicals. The use of various herbal extracts such as *Zedoariane Rhizoma* (family: Zingirebaceae), *Sanguisorba officinalis* (family: Rosaceae) and *Decalepis hamiltonii* (family: Apocynaceae) roots and several bioactive compounds such as quercetin, curcumin can protect fruit flies from paraquat exposure which causes increased mortality, failure of locomotor function and oxidative damage[21], but the use of celery microgreens extract for neurodegenerative disorder such as Parkinson's disease, in our knowledge has not been found. At present the use of natural ingredients such as microgreens containing antioxidant compounds is very important to reduce oxidative stress causing various degenerative diseases in the human body. Actually, the function of dopamine as a neurotransmitter is involved in regulating the movement, cognitive influence and neuroendocrine secretion. Paraquat exposure to organisms can reduce dopamine levels which can affect one of several symptoms such as bradykinesia, stiffness and tremor [22].

The data of the study indicate that celery microgreens extract was able to prevent the decrease of dopamine levels in paraquat-exposed fruit flies. It is evident that celery microgreens extract has potential as a neuroprotective agent that can improve the locomotory appearance of paraquat-exposed fruit

flies. These results are consistent with observations that there is close correlation between failure of locomotor function and reduce of dopamine levels [23].

Conclusion:

From several measurement parameters carried out in this study it appears that celery microgreens extract have the potential as a neuroprotective agent. This potential is inseparable from the presence of a large content of antioxidant compounds such as polyphenol compounds. Thus, celery microgreens can be used as a potential agent to prevent or reduce the rate of onset of neurodegenerative diseases, especially Parkinson's disease. Applying celery microgreens as functional food may be expected to reduce oxidative stress which results in increased dopamine levels in brain of fruit fly.

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Authors' Declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in UIN Sunan Gunung Djati Bandung.

Author Contributions:

Conceptualization, M.A.S.; Methodology, M.A.S. and M.S.; Investigation, M.A.S. and M.S.; Writing—Original Draft Preparation, M.A.S and Y.Y.; Writing—Review and Editing, M.A.S.; Resources, M.A.S.; Data Curation, M.A.S. and Y.Y.; Supervision, M.A.S.; Project Administration, M.A.S. and Y.Y.; Funding Acquisition, Y.Y. All authors have read and agreed to the published version of the manuscript

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تأثير نبات الكرفس (Apium gravyolens L.) على ذبابة الفاكهة السوداء

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[3]تعليم الرياضيات بجامعة بنديديكان إندونيسيا.

الخلاصة:
غالبًا ما يستخدم الكرفس (***Apium gravolens* L**. ؛ العائلة: **Apiaceae**) كتوابل في الطعام اليومي. ومع ذلك ، يحتوي هذا النبات على العديد من المركبات المضادة للأكسدة المفيدة لتخفيف الاضطرابات التنكسية العصبية بما في ذلك مرض باركنسون. كان من المتوقع أن يؤدي زرع الكرفس على شكل نباتات صغيرة تم حصادها لمدة 15 يومًا إلى زيادة محتوى المركبات النشطة بيولوجيًا . في الدراسة الحالية ، كنا نهدف إلى تقييم إمكانات التعديل العصبي لمستخلص الميثانول من الكرفس الصغير على ذباب الفاكهة (**Drosophila melanogaster Meigen**: عائلة **Drosophilidae** ؛ **ordo: Diptera**) التي تعرضت للباراكوات. تم تقييم القدرة الوقائية العصبية من خلال معدل البقاء على قيد الحياة ، والأداء الحركي ، وأكسدة الدهون ومحتوى الدوبامين بعد معالجتها بمستخلص 120 ميكروغرام / مل من الكرفس المجهرية و 3.5 ملي باراكوات لمدة 4 أيام. تم قياس المكونات الكيميائية النباتية من مستخلص نبات الكرفس الصغير بما في ذلك محتوى البوليفينول الكلي والنشاط المضاد للأكسدة باستخدام طريقة مسح الجذور. سيؤدي تعرض ذبابة الفاكهة البالغة للباراكوات إلى انخفاض معدل البقاء على قيد الحياة وتحسين النمط الظاهري الحركي عن طريق استخراج الخضر الصغيرة من الكرفس. في موازاة ذلك ، يمكن تحسين محتوى **malondialdehyde** المتزايد من بيروكسيد الدهون وانخفاض محتوى الدوبامين من خلال وجود مستخلص الكرفس الصغير. تشير القدرات الوقائية العصبية إلى نسبة عالية من المركبات المضادة للأكسدة من مستخلص الكرفس الصغير. خلصت دراستنا إلى أن الخضر الصغيرة من الكرفس تعمل على تأخير تأثير الإجهاد التأكسدي الذي يسبب مرض باركنسون.

الكلمات المفتاحية: الكرفس ، ذبابة الفاكهة ، الخضار الصغيرة ، باركنسون ، الباراكوات.