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Neuroprotective Efficacy of *Dunaliella salina* Against Paraquat-Induced Neurotoxicity in *Drosophila melanogaster*

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Abstract

Microalgae of *Dunaliella salina* possesses multiple biological properties which are mainly attributed to the active compounds such as polyphenol, chlorophyll and β-carotene. However, the utilization of *D. salina* as a neuroprotective agent in the management of Parkinson's disease is still questionable. Presently, we observed the potential neuromodulatory effects of *D. salina* extract against the toxicity exposed to paraquat in fruit fly (*Drosophila melanogaster)*. Male wild-type fruit flies were concomitantly induced by paraquat (3.5mM) and methanolic extract of *D. salina* (200µg/mL) in their diet during 4 days of observation. Paraquat-fed fruit flies exhibited a higher incidence mortality and severe influence on locomotor phenotypes (i.e. negative geotaxis) compared to the control. *D. salina* extract treatment that showed protection ability against these deleterious effects of paraquat. However, paraquat exposure caused a marked increase in malondialdehide (MDA) levels as indicating increase lipid peroxidation process in brains of fruit flies. Meanwhile, paraquat toxicity was also related to a significant decrease on dopamine levels in head of fruit flies, which were attenuated by *D. salina* extract treatment. Results revealed that *D. salina* extract significantly reduced the toxicity of paraquat compounds exposed to fruit flies and proved the usefulness of this model as a potential therapeutic strategy for symptoms that appear in Parkinson's disease.

Keywords: locomotor, survival, malondialdehide, dopamine.

1. Introduction

The presence of damage to dopaminergic neurons in the substantia nigra pars compacta as a hallmark of the onset of Parkinson's disease. Accordingly, the disease is predicted to double every 25 years and generally affecting the people above 65 years old with 1-2% of world's population (Siddique and Jyoti, 2017). Clinical disorders of the disease are locomotor symptoms such as resting tremor, muscle rigidity, postural imbalance and bradykinesia (Stephano *et al.*, 2018). While nonmotoric symptoms include depression, dementia and disturbed sleep (Soares *et al.*, 2017).

In recent times, researchers explained that the cause of Parkinson's disease remains unknown (idiopathic). However, it is estimated that the cause of this disease is more than 90% of environmental factors (Standaert *et al.*, 2016). One of environmental factors that cause the disease is the use of paraquat (herbicide) (Thakolwiboon *et al.*, 2017). Actually, paraquat is a free radical source which causes oxidative stress. Excessive oxidation on dopaminergic neurons reduces the production of dopamine compounds that function as neurotransmitters (Jhonsa *et al.*, 2016).

Remarkably, treatment of patients with Parkinson's disease is given by lepodova. The ability of synthetic drugs is quite helpful to replace the dopamine content that is lacking (Lazzari *et al.*, 2020). However, the use of lepodova is very risky because it has side effects that will aggravate the symptoms of Parkinson's disease including hallucinations, foot edema, nausea and vomiting (Wells *et al.*, 2019). Considering the side effects of lepodova, researchers continue to search for natural ingredients that possess the potential to treat this neurodegenerative disorder (Mohamed *et al.*, 2018). Therefore, the expected neuroprotective agent can at least reduce the symptoms or inhibit the development of Parkinson's disease.

Microalgae are photosynthetic microorganisms that contain many bioactive substances including antioxidant compounds (Sedjati *et al.*, 2020). β-carotene pigments contained in microalgae cells of *D. salina* are expected to function as neuroameliorative dopaminergic neurons that have been damaged (Wong *et al.*, 2016). Interestingly, other antioxidant compounds such as polyphenols and chlorophyll will also act against free radicals that cause excessive oxidation in the brain.

Actually, fruit flies have been known as alternative animals model with many advantages including being easy to maintain, fast breed (female flies can produce 55 eggs during their lifetime), and a short life cycle of around 12 days (Quintero-Espinosa *et al.*, 2016). Hence, this fruit fly is very suitable for use in medical research to look for potential therapeutic solutions for diseases. This can be done because fruit flies have about 75% homologous genes that regulate disease in humans (Nelson *et al.*, 2018). Associated with Parkinson's disease, these fruit flies have dopaminergic neurons scattered in all parts of the brain

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that allow a thorough study of the cellular mechanism of this disease (Jahromi *et al*., 2015).

Considering the promising potential of *D. salina* microalgae as a source of natural antioxidant compounds, it is hypothesized that this species of microalgae may offer protection against the toxicity of paraquat. Therefore, the study aimed to investigate the protective potential of *D. salina* against paraquat-induced locomotor damage and excessive oxidation using animal models of *D. melanogaster*.

2. Methods and Materials

2.1. Culture and Harvest of Microalgae D. salina

Microalgae *D. salina* was obtained from stocks owned by plant physiology laboratory, Biology Department of Universitas Islam Negeri Bandung of Indonesia. Furthermore, these microalgae were cultured using Walne medium on plastic containers with a capacity of 10 L. The culture room with a temperature of 23-26 ºC and lighting uses a 45 watt TL lamp with 2500 Lux intensity throughout the day for 12 hours. Microalgae were harvested using a centrifuge at 3000 rpm for 10 min and dried in an oven with a temperature of 50 ºC for 10 hours. Dry biomass was crushed with mortar and stored in a refrigerator before being used for subsequent analysis.

2.2. Extraction of D. salina

100 mL of methanol was added to conical flask that had 10 g of dry biomass of *D. salina* microalgae. Contents were shaken for 48 hours at room temperature. This mixture was filtered with filter paper and the solvent was evaporated until a crude extract was obtained. Finally, the crude extract was packaged in an airtight container and stored in the refrigerator for further analysis.

2.3. Total Phenolics

The test was carried out to measure the total phenol levels of *D. salina* extract using the procedure of Stockum *et al*. (2019) with a slight modification. 4 µL sample was dissolved with 35 µL of 1 N Folin Ciocalteu reagent. The solution was left to stand for 3 min, then 70 ml of 15% Na_2CO_3 solution was added, followed by the addition of 284 µL of aquadest. The mixture was kept in the dark for 2 h, after which the OD was read at 760 nm. As a standard, gallic acid was used (10 - 300 µg / mL). The obtained data were represented as mg gallic acid equivalents (GAE) per 100 g of extract.

2.4. DPPH (Diphenyl-picrylhydrazyl) Scavenging Assay

This assay was carried out to obtain antioxidant activity of *D. Salina* extract using the procedure of McCann *et al.* (2019). The stable purple colour of DPPH turns light yellow when it reacts with antioxidants from *D. salina* extract. Discoloration due to a mixture of 500 µL *D. salina* extract with 250 µL DPPH solution (0.3 mM) was measured spectrophotometrically. The mixture was shaken evenly and kept in a dark room at room temperature for 30 minutes. Furthermore, the inhibition ability of *D salina* extract against DPPH free radicals were preceded by OD measurements of the mixture which were read at 520 nm and its calculation made using the following equation:

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

2.5. Drosophila Stock

Wild type male fruit flies were obtained from the stock owned by the genetic laboratory, Department of Biology, Faculty of Science and Technology at State Islamic University (Universitas Islam Negeri) Bandung, Indonesia. Fruit flies were reared in agar medium consisting of dry yeast, sucrose, powdered milk, corn flour, and nipagin. Propionate acid was added as an anti-fungal and anti-bacterial agent. Fruit flies were reared in a 5.5 cm X 8.7 cm vial containing 10 ml of medium at constant temperature, humidity (70%) and under 12h dark/light cycle.

2.6. Survival Rates

Survival rate assay was performed to determine the number of flies still alive daily until the end of observation period (4 days). Therefore, 120 flies from each treatment group were included in survival data, and the total number of flies indicated the use of 4 replications per treatment group (30 flies/treatment replicate). In this survival rate observation, there was no change in the feed medium because it was to show its survival ability during the investigation.

2.7. Locomotor Phenotype

Negative geotaxis assay is a method used to determine the fly's locomotor phenotype. Fruit flies were anesthetized in brief ice, then carefully placed at the base of the glass column (14 cm high, 1.5 cm in diameter / 10 flies each). To start measuring, the fly's locomotor phenotype, flies gently were tapped down to the bottom of the column. Counting of climbing abilities was carried out on fruit flies that were able to pass the 5 cm in high during 6 s. The scores represent the sum of three independent replications (Sakai *et al*., 2019).

2.8. MDA (Malondialdehyde) Assay

Measurement of lipid peroxidation level of MDA was made based on Rzezniczak *et al*. (2017). In this assay, 90 µL homogenate of fly brain was mixed with 600 µL of 1% O-phosphoric acid, 5 µL of 10 mM butyl-hydroxytoluene (BHT), 200 µL of 0.6% thiobarbic acid and 105 µL of distilled water. Then, the mixture was immediately incubated at 100ºC for 10 min and spectrophotometrically the OD was read at 535 nm. The obtained data shows MDA levels as the end result of lipid peroxidation was expressed as µmol of TBARS formed / h / g tissue.

2.9. Dopamine Assay

This assay was performed to determine the dopamine content in the fly's brain using a procedure of Aryal and Lee, 2019. So, for this purpose a 40 head of flies from each group were prepared to be homogeneous in 500 µL of HCl-butanol. Next, the mixture was centrifuged at 2500 rpm for 7 min. The obtained supernatant was mixed with 250 µL of heptane and 100 µL of 0.1 M HCl and re-centrifuged at 2500 rpm for 7 min. The obtained supernatant was mixed again with 50 µL of 0.4 M HCl and 100 µL of iodine solution and incubated for 2 min. Thereafter, 100 µL of sodium sulphite and 100 µL of 10 M acetic acid were added to the mixture boiled at 100°C for 6

min. After the mixture cooled, OD was measured at 375 nm using a UV-Vis spectrophotometer.

2.10. Statistical Analysis

The obtained data was statistically analyzed using one way ANOVA followed by Duncan's multiple range test if there were significant differences at $p < 0.05$. Results were presented as mean and standard errors of mean. The statistical analysis was determined using SPSS software, version 16 for windows

3. Result

3.1. Phenolic Content and Antioxidant Activity

Phenolic content and antioxidant activity of microalgae *D. salina* extract performed by Folin-Ciocalteu protocol and DPPH assay respectively. Results of phenolic acid represented as gallic acid equivalent were 321.53 mg GAE/g extract, whereas the antioxidant strength of the *D. salina* extract can be seen from the IC_{50} value as measured by DPPH scavenging activity which is 74.49 µg / mL with a strong category compared to IC_{50} ascorbic acid which is 4.97 µg / mL with a very strong category.

3.2. Effectiveness of D. salina Extract on Survival Rate

The effect of *D.salina* extract treatment on the survival of fruit flies exposed to paraquat was observed for 4 days. As shown in Figure 1, the *D. salina* extract treatment had no effect on fruit fly survival when compared to the control group. Meanwhile, the survival rate of the fruit fly group that received paraquat treatment was seen to decrease since the first day of observation and continued to decrease until the end of the observation (day 4) with a survival rate of up to 40% (Figure 1). The administration of *D. salina* extract together with paraquat can increase the survival rate of fruit flies when compared to the fruit fly group that only received paraquat treatment (Figure 1).

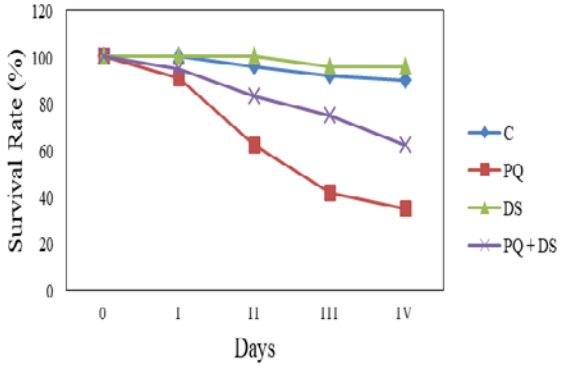

Figure 1. Effects of DS extract and PQ on survival rate (30 flies per replicate), of wild-type male fruit fly for 4 day observation. (C=control, PQ=3.5 mM paraquat, DS=200 µg/mL extract of *D. salina*).

3.3. Effectiveness of D. salina Extract on Locomotor Performance.

Negative geotaxis is a common measurement to determine the locomotor ability of fruit flies. The ability of vertical climbing of fruit fly inside the tube was observed by startling them at a distance of 5 cm in 6 s. The effect of *D salina* extract treatment not significantly change the locomotor performance of fruit flies when compared to control ($p > 0.05$). The fruit fly group exposed to paraquat significantly impaired locomotor ability when compared to controls ($p < 0.05$) (Figure 2). More than 50% of flies treated paraquat lag below the boundary line, showing locomotor deficit caused by paraquat. The fruit fly group treated with the *D. salina* extract and paraquat showed a significant increase in locomotor ability when compared to the fruit fly group that was treated only with paraquat (Figure 2, $p < 0.05$).

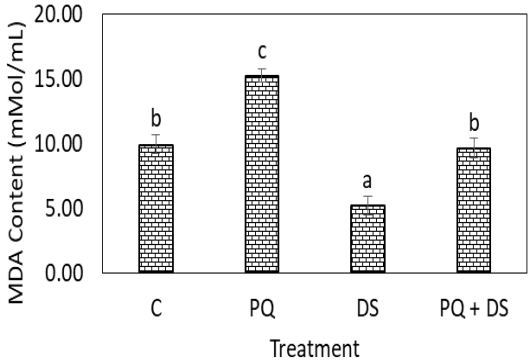

Figure 2. Effects of DS extract and PQ on negative geotaxis (10 flies per replicate), of wild-type male fruit fly at the end of observation. Error bars depict mean ± SEM. Different alphabets respect to significant difference ($p < 0.05$). (C=control, PQ=3.5 mM paraquat, DS=200 µg/mL extract of *D. salina*).

3.4. Effectiveness of D. salina extract on Lipid Peroxidation

The fruit fly group treated with *D. salina* extract significantly changed the MDA (malondialdehyde) content to be lower when compared to the control (Figure 3, $p < 0.05$). Meanwhile, the fruit fly group treated with paraquat showed a significant increase MDA content when compared to control ($p < 0.05$). However, MDA content of fruit fly group treated with *D. salina* extract and paraquat showed no significant difference when compared with the control ($p > 0.05$) (Figure 3). So, it can be seen from Figure 3 that the *D. salina* extract treatment was able to reduce the MDA content when compared to the fruit fly group that received PQ treatment (Figure 3).

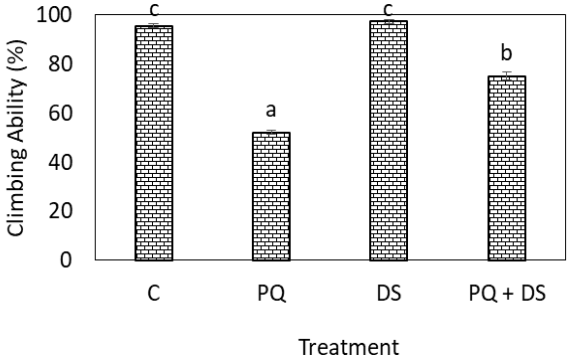

Figure 3. Effects of DS extract and PQ on MDA levels (40 flies per replicate), of wild-type male fruit fly at the end of observation. Error bars depict mean ± SEM. Different alphabets respect to significant difference ($p < 0.05$). (C=control, PQ=3.5 mM paraquat, DS=200 µg/mL extract of *D. salina*, MDA=malondialdehyde).

3.5. Effectiveness of D. salina Extract on Dopamine Levels

The fruit fly group treated with the *D. salina* extract produced almost the same dopamine levels and not

significantly different when compared to the control ($p > 0.05$). Meanwhile, the fruit fly group that received paraquat treatment produced lower dopamine levels and was significantly different when compared to the control ($p < 0.05$). For the fruit fly group treated with both the *D. salina* and paraquat extracts produced higher dopamine levels when compared to the fruit fly group that received PQ only treatment (Figure 4).

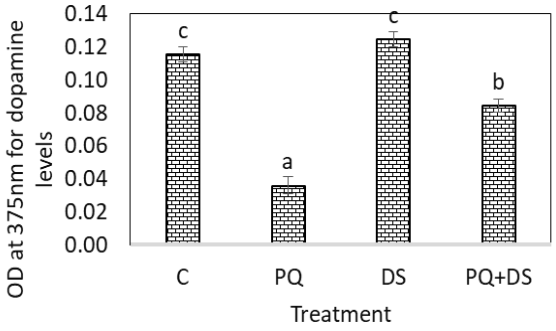

Figure 4. Effects of DS extract and PQ on dopamine levels (40 flies per replicate) of wild-type male fruit fly at the end of observation. Error bars depict mean ± SEM. Different alphabets respect to significant difference ($p < 0.05$). (C=control, PQ=3.5 mM paraquat, DS=200 µg/mL extract of *D. salina*, OD=optical density).

4. Discussion

At present, there is a major challenge for the medical world that there is no treatment for neurodegenerative disorders including Parkinson's disease. So, a continuous search for compounds that can prevent or inhibit damage to neurons is continually needed (Helena *et al.*, 2019). Oxidative stress is the main factor causing a variety of neurodegenerative disorders and other age-related degenerative disorders (Mohamed *et al.*, 2018). The incidence of lipid peroxidation, protein and DNA oxidation is increasing in the brain of the disease model (Xiong *et al.*, 2017). Concerning the above, various antioxidant compounds originating from natural sources have demonstrated neuroprotective abilities in models in-vitro and in-vivo which include polyphenols and β-carotene (Xiong and Yu, 2018).

According to studies of researchers in the health sector, there is a connection between Parkinson's disease and exposure to pesticides (Gupta *et al*., 2020). The study clearly shows that paraquat neurotoxins can damage locomotor performance of fruit flies and decrease survival. This effect can be observed when using *D.salina* extract in the fruit fly culture medium. The benefits of *D.salina* extract may be at least in part from the presence of potentially antioxidant compounds which include polyphenols and β-carotene (Sedjati *et al.*, 2020). Dopaminergic neurons in the brain will be damaged or even lost due to the occurrence of oxidative stress which stimulates degeneration in the onset of the disease (Guo *et al*., 2018). Paraquat as an herbicide is capable of damaging dopaminergic neurons which are indicated by locomotory disturbances in animal models used (Sanz *et al*., 2017). The concurrent time between the occurrence of dopaminergic neurodegeneration and locomotor disorders is thought to have a causal relationship (Niveditha *et al*., 2017). Although we did not specifically test for the dopaminergic neuron degeneration, it can be seen that fruit flies treated with paraquat will show permanent locomotory disruption which is certainly related to oxidative stress causing neuronal impairment in the brain.

Although the potential of antioxidant compounds in vitro has often been demonstrated, knowledge has not been found in vivo. Therefore, in this study the use of methanol extract from *D.salina* might increase endogenous antioxidant activity which has an effect on increasing dopamine levels and reducing MDA levels in the fruit fly brain. Likewise the presence of *D.salina* extract on fruit fly culture medium will be able to increase the survival rate and also the ability of negative geotaxis as an expression of its locomotor phenotype. The ability of the *D.salina* extract against the toxicity of paraquat compounds indicates the content of antioxidant compounds such as polyphenols (321.53 mg GAE/g extract) and has strong antioxidant activity (74.49 µg / mL).

5. Conclusion

In this study, we demonstrated that methanol extract of *D. salina* was able to protect against toxicity from paraquat as a neurotoxin agent. This capability was the contribution of potential antioxidant compounds from *D. salina* proven by its ability to increase survival rate, locomotor performance (in negative geotaxis), and ability to increase dopamine levels and reduce MDA levels as the end results of lipid peroxidation. The obtained data leads to the recognition that microalgae of *D. salina* as a neuroprotective agent have the potential to reduce symptoms found in Parkinson's disease.

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