Effect of *Chlorella vulgaris* extract on high sucrose diet-induced diabetes in *Drosophila melanogaster*

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**ABSTRACT.** Microalgae *Chlorella vulgaris* is a microscopic green alga known to have potential as a source of food and medicine (functional food). However, the pharmacological capacity of these microalgae species against disease due to metabolic disorders is not understood. Therefore, this study aims to evaluate the effect of *C. vulgaris* extract on animal models of *Drosophila melanogaster*, which are fed with a high-sucrose diet (HSD). In this study, the wild-type *Drosophila* used for seven days of observation was divided into four groups. The first group was used as a control without treatment, the second was treated with HSD (30%), the third was treated with *C. vulgaris* extract (120 g/L), and the fourth group had double treatment (HSD and *C. vulgaris* extract). Meanwhile, the parameters observed included fecundity, hatchability, hemolymph glucose, and triglyceride levels. The results showed that the *C. vulgaris* extract was able to reduce the negative effects of administering HSD. In addition, the single and double treatment of *C. vulgaris* extract and HSD significantly decreased the levels of hemolymph glucose as well as triglycerides of *Drosophila*. At the same time, the fecundity parameters and hatchability significantly increased compared to *Drosophila* that received only HSD treatment. Based on these results, *C. vulgaris* extract has potential to be used as an antihyperglycemic agent. However, further study is recommended to prove it.

**Keywords:** antihyperglycemic; fecundity; green alga; hatchability; hemolymph glucose

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**INTRODUCTION**

Currently, people often consume foods and drinks with high sugar levels which leads to the emergence of various deadly diseases (Kumar et al., 2014; Bai et al. 2018; Kandinasti & Farapati, 2018). Meanwhile, sugar in the body is digested, absorbed, and converted into monomer compounds, monosaccharides, then transported by the blood for target cells and tissues to be metabolized (Dashty, 2013; de Aquino Silva et al., 2020). The long-term consumption of foods with high sugar levels disrupts the homeostasis of glucose metabolism in the body. This metabolic disorder is in the form of hypertension, coronary heart disease, stroke, cancer, and especially, type 2 diabetes mellitus (T2DM) (Rizos & Elisa, 2014; Fernández-Real et al., 2015; Harshavardhana & Krishna, 2019).

As a disease with metabolic disorders, T2DM has distinctive symptoms such as high blood glucose and triglyceride levels (Musselman et al., 2019). When the development of T2DM becomes uncontrollable, it leads to the emergence of various serious problems such as ocular morbidity and blindness, kidney failure, amputation, and permanent disability (Bertram et al., 2013; Manu et al., 2018). Meanwhile, new cases of the disease are on the increase, characterized by its occurrence in children which was previously found in adults only. Moreover, the treatment of T2DM uses insulin as a synthetic hypoglycemic agent which is administered orally or by injection (Peyrot et al., 2012; Wong et al., 2016). This treatment is very risky with more severe side effects such as damage to the eyes, kidneys, heart, and others (Oyeniran et al., 2020).

Microalgae have been known for various therapies against degenerative disease. Meanwhile, *C. vulgaris* is a microscopic green...
alga that is used as a supplement and functional food (Gui et al., 2019). The content of phenolic compounds from this species strengthens its function as a source of antioxidant compounds which improve body metabolism (Tugcu et al., 2017). Furthermore, the antioxidant ability of C. vulgaris extract is high enough to stabilize free radicals that damage DNA, mitochondria, and other organelles (Zakaria et al., 2017). However, the regulation of C. vulgaris against metabolic disorders due to high sugar levels consumption is not fully understood.

Drosophila or fruit flies have been known as animal models that are used to study diabetes. Drosophila has 74% homologous genes with human genes that control various diseases such as diabetes mellitus (Westfall et al., 2018). In addition, the mechanism of glucose homeostasis is fully observed through these fruit flies. Based on the observations of Wang et al. (2020), Drosophila shows the characteristics of T2DM patients such as symptoms of hyperglycemia.

In this study, the effect of microalgae C. vulgaris extracts was observed on the appearance of T2DM symptoms in animal models of HSD-induced Drosophila. The observations conducted include fecundity, hatchability, hemolymph glucose, and triglyceride levels. Moreover, C. vulgaris extracts were observed for total phenolic level and antioxidant activities using the diphenyl picryl hydrazyl (DPPH) method. The results of this study serve as the basis for preclinical drug discovery using microalgae C. vulgaris.

MATERIALS AND METHODS

Culture and treatment of Drosophila. Drosophila used was the wild type from the collection of Laboratory of Plant Physiology, Department of Biology, UIN SGD Bandung. Drosophila were reared in a culture vial containing 10 ml of solid medium with the following composition: 1% weight/volume (w/v) of yeast, 2% w/v sucrose, 1% w/v powdered milk, 1% w/v agar, and 0.08% w/v nipagine (Brookheart et al., 2017). The treatment started from Drosophila in the 3rd instar phase and observations were carried out for seven days of treatment. Furthermore, the four treatment groups were prepared as follows: the first group was used as a control without treatment, the second group received 30% of HSD treatment, the third group was treated with 120 g of C. vulgaris extract in 1 L medium, and the fourth group received HSD and C. vulgaris extract treatments. The environmental conditions around the Drosophila culture site were temperature of 26 ± 1°C, relative humidity of 60 ± 5%, and light intensity of 2000 lux while photoperiod was 12 h of light and 12 h of darkness.

Extraction of C. vulgaris. A total of 5 g dry biomass from C. vulgaris was dissolved in 25 ml of methanol p.a and stored for approximately 24 h at room temperature. The solution was centrifuged at 4000 rpm for 15 min and the supernatant obtained was concentrated using a rotary evaporator at a temperature of 40-50°C to form a concentrated extract (Chen et al., 2018).

Measurement of total phenolic levels. Measurement of total phenolic levels in C. vulgaris extract was conducted following the procedures from McCann et al. (2007), with slight modifications. The extract of C. vulgaris was added with 35 µL of Folin Ciocalteu 1 N reagent. After leaving for 3 min, 70 ml of 15% Na2CO3 solution and 284 µL of aquadest were added to the solution. The mixture was stored in a dark room for 2 h and measured on a UV-vis spectrophotometer 760 nm. Gallic acid was used as a standard with a concentration range of 10 to 300 µg/mL. Meanwhile, the data obtained were expressed as mg gallic acid equivalents (GAE) per 100 g of extract.

DPPH test. The test for antioxidant activity of C. vulgaris extract was carried out according to the procedure of Von Stockum et al. (2019). The stable DPPH solution has a purple color that immediately changes to a light yellow color when it reacts with antioxidant compounds derived from C. vulgaris extract. Meanwhile, 500 µL of C. vulgaris extract was mixed with 250 µL of 0.3 mM DPPH solution. This mixture was shaken until homogeneous and stored in a dark room for 30 minutes. Furthermore, the OD resistance of C. vulgaris extracts against DPPH free radicals was measured by a spectrophotometer at a
wavelength of 520 nm. The inhibition was calculated using the following equation:

\[
\frac{\text{Inhibition radical scavenging (\%)} \times 100}{\text{Control absorbance} - \text{Sample absorbance}} = \frac{\text{Control absorbance}}{\text{Control absorbance}}
\]

**Fecundity and hatchability test.** A total of 20 female *Drosophila* aged three days from each group were placed in a culture vial that contains a standard medium and yeast as the only food source. The *drosophila* was transferred every 3 h to a new culture bottle that contains standard medium and yeast. This was followed by the calculation of the fecundity of total eggs produced by 20 *Drosophila* per h. After 22 h, all culture bottles were examined and the 1st instar larvae were counted. The hatching rate was calculated from the total number of larvae divided by the total number of eggs produced (Gáliková *et al.*, 2017).

**Measurement of hemolymph glucose levels.** This test used 35 individuals (one male and female *Drosophila*) for each group after seven days of treatment. A total of 2 µL of colorless hemolymph was placed on a 96-well microplate (Thermo-Scientific) which contained 0.1% N-phenylthiourea in 50 µL of PBS (Phosphate-Buffered Saline). Furthermore, 150 µL of Glucose Autokit (Wako) reagent was added to each well and incubated at room temperature for 20 minutes before measuring the absorbance at a wavelength of 505 nm. The calculation of glucose concentrations was conducted using standard curves (Navrotskaya *et al.*, 2016).

**Measurement of hemolymph triglyceride levels.** About 35 male and female *Drosophila* from each treatment group were crushed to obtain their homogenate. At the initial stage, homogenate in PBS was mixed with Tween 0.1%. The mixture was later heated to 65°C for 5 min to inactivate the lipase. After the mixture cools down, the vortex was added to the triglyceride infinity test reagent (ThermoFisher TR22421) and the mixture was incubated at 37°C for 5 min. The mixture absorbance was measured on a spectrophotometer with a wavelength of 540 nm and the results were compared against a standard curve (Ecker *et al.*, 2017).

**Data analysis.** The data were analyzed statistically using one-way analysis of variance (ANOVA) and when the test showed a significant difference at a 95% confidence level, further tested was conducted using Duncan’s Multiple Range Test (DMRT). Moreover, all data were presented as mean ± standard deviation while various letter labels on each accompanying data showed significant differences in the 95% confidence interval (P <0.05) (Steel & Torrie, 1984).

**RESULTS AND DISCUSSION**

*Drosophila* is a model animal that is often used for research on metabolic diseases such as diabetes due to its advantages which include a short life cycle, ease of food treatment manipulation, and a well-known methodology of metabolic observation. Meanwhile, HSD consumption stimulates metabolic homeostasis disharmony and causes uncontrolled inflammation that damages organelles associated with T2DM. Our study showed for the first time that *C. vulgaris* extract has a protective effect against HSD-induced diabetes symptoms in *Drosophila* by preventing oxidative stress and restoring its endogenous antioxidant defenses. The effect of *C. vulgaris* extract includes increased satiety from *Drosophila* due to the intake of organic compounds. Meanwhile, a previous study by Bortolatto *et al.* (2015), stated that adding organic compounds to the diet of *Drosophila* increased their satiety process. Furthermore, the extracts of *C. vulgaris* act by regulating appetite in the hunger center or through certain mechanisms that correlate with food entry pathways in *Drosophila*. There is sufficient evidence that the inductors causing hyperglycemia have induced oxidative stress followed by the auto-oxidation process of glucose which triggers oxidative damage.

Previous studies showed that antioxidant compounds such as phenolic acid have the potential to delay and prevent complications associated with diabetes. Similarly, antioxidant compounds as phytochemical substances reduce oxidative damage by free radicals (Forni *et al.*, 2019). Moreover, oxidative stress
damages vital organs and compounds such as lipids, proteins, DNA, and cell membranes (Oyeniran et al., 2020). The potential of C. vulgaris extract includes inhibiting glucose absorption in Drosophila's body, followed by utilizing existing glucose through the increased activity of enzymes involved in glucose metabolism. Meanwhile, the consumption of HSD is correlated with the development of a hyperglycemic state in Drosophila which is responsible for the increased oxidative stress (Ecker et al., 2017). The extract of C. vulgaris is a compound with highly relevant functions in the antioxidant defense system. There is a correlation between hyperglycemia and oxidative stress in Drosophila through the emergence of an imbalance between free radical production and endogenous antioxidant physiological mechanisms (Robson et al., 2018). These results showed the defensive role of antioxidant compounds in the extract of C. vulgaris against the adverse effects of oxidative stress on Drosophila.

Table 1. The analysis results of the biomarker of HSD-induced Diabetes in Drosophila.

| Parameters            | Sex | Treatments | C      | S      | V      | S+V   |
|-----------------------|-----|------------|--------|--------|--------|-------|
| Fecundity (number/h)  |♀   | Control    | 40.60 ± 1.20a | 9.60 ± 0.80a | 41.60 ± 1.02a | 25.40 ± 0.49³ |
| Hatchability (%)      |♀   | Control    | 98.60 ± 0.80b | 78.20 ± 0.75a | 96.40 ± 1.02b | 87.80 ± 1.17h |
| Hemolymph glucose     |♀   | Control    | 22.48 ± 0.32a | 77.44 ± 0.34a | 26.52 ± 0.43a | 50.72 ± 0.79h |
| (mg/dL)               |♀   | Control    | 24.18 ± 0.53a | 88.06 ± 0.40a | 26.02 ± 0.70a | 55.22 ± 0.54h |
| Hemolymph triglyceride|♀   | Control    | 42.78 ± 0.68a | 86.34 ± 0.83c | 56.34 ± 0.56c | 76.48 ± 0.85c |
| (µg/mg)               |♀   | Control    | 45.9 ± 0.55a  | 96.56 ± 0.45c | 62.88 ± 0.74b | 81.16 ± 0.57c |

Notes: C = Control; S = HSD; V = C. vulgaris extract; S + V = HSD + C. vulgaris extract. The different alphabets for each data value shows significantly different at DMRT test with α = 0.05).

According to DMRT, Drosophila group treated with C. vulgaris extract produced the number of eggs was not significantly different from the control Drosophila group. Similarly, the number of eggs hatched in the Drosophila group treated with C. vulgaris extract was not insignificantly different from the group with C. vulgaris extract and HSD treatments (Table 1). Moreover, the ovary of Drosophila is the largest among the organs, while observation of the organ showed that various treatments affect reproductive ability, including the number of eggs produced (Diop et al., 2015). Furthermore, it is acceptable to use female Drosophila affected by HSD due to the positive correlation between ovarian size and the number of eggs (Mendes & Mirth, 2016). Disorders of the reproductive organs of Drosophila due to HSD treatment are shown from decreased egg production and fertility as well as the accumulation of triglycerides (Buescher et al., 2013). In this study, the data correlated with Werthebach et al. (2019), which showed that HSD treatment of Drosophila successfully decreased egg production. Moreover, oxidative stress is caused by the entry of dietary components such as carbohydrates which continues with changes in metabolite structure and affects Drosophila reproduction. The female Drosophila with HSD treatment produced few mature eggs, which lead to decreased egg hatching. Furthermore, the consumption of HSD in female Drosophila cause decreased viability of eggs to hatch. The HSD treatment also decreased the hatching of Drosophila eggs, while excess glucose led to increased cellular degeneration and decreased fertility associated with low hatchability of eggs.

Based on DMRT calculations for hemolymph glucose and triglyceride levels, the female and male Drosophila groups in the C. vulgaris extract treatment were insignificantly different from the Drosophila control group. Meanwhile, the female group generally showed higher hemolymph glucose and triglyceride levels than the male group (Table 1). The intolerance mechanism of glucose and T2DM in mammals is caused by metabolic changes such as enzymatic dysregulation and increased glucose as well as triglycerides (Ecker et al., 2017). Meanwhile, Drosophila administered a double treatment of C. vulgaris extract and HSD showed improvements in glucose and
triglyceride levels due to its work in regulating appetite and reducing HSD intake. Furthermore, the results showed a close relationship between increased glucose and triglycerides as well as increased oxidative stress in Drosophila. Excessive sucrose consumption inhibits the formation of satiety signals in the brain, leading to the release of more glucose into the body (Rovenko et al., 2015). The researchers observed that the effect of dietary manipulation such as HSD in Drosophila response according to gender differences (Castellanos et al., 2013). Therefore, HSD treatment of female Drosophila clearly shows an increase in hemolymph glucose and triglyceride levels (Buescher et al., 2013).

CONCLUSION

C. vulgaris extract has various actions in Drosophila metabolism. It acts mainly by increasing satiety, responding to the decrease in glucose and triglyceride levels in HSD-induced hyperglycemic Drosophila. The results also showed the pharmacological potential of C. vulgaris extracts against metabolic disorders such as T2DM. Therefore, further studies are recommended to analyze the cellular and physiological mechanism of C. vulgaris extract against HSD, which causes T2DM disease.

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