

Bioactivity Assay of Microalgae: Antioxidant and Antidiabetic Potentials in a Transgenic Zebrafish Model

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Abstract

Background and Objective: Microalgae with antioxidant, alpha-amylase and alpha-glucosidase inhibition and NF-κB activation abilities have shown promising anti-diabetic characteristics. The present study aimed to assess effects of microalgae extracts of *Arthrospira platensis* and *Chlorella vulgaris* on β-cell regeneration using Tg (ins: CFP-NTR) in a transgenic zebrafish type-1 diabetic model.

Material and Methods: After 15 d of cultivation of microalgae, cells were extracted from the biomass. Biochemical assays such as assessments of carbohydrates, proteins, lipids, phenolic compounds and photosynthetic pigments were carried out. Transgenic zebrafish larvae were treated *in vivo*, with five various concentrations of the extract (500, 125 31.25, 7.81 and 1.95 μg.ml⁻¹).

Results and Conclusion: Results showed that aqueous extracts of *Arthrospira platensis* and *Chlorella vulgaris* included significant effects on β-cell regeneration in concentrations of 31.25, 7.81 and 1.95 μg.ml⁻¹ and 125, 31.25, 7.81 and 1.95 μg.ml⁻¹, respectively. The highest regeneration rates for *Chlorella vulgaris* and *Arthrospira platensis* extracts were observed at 125 and 31.25 μg.ml⁻¹, respectively (60 against 92%) ($P < 0.05$). Additionally, aqueous extracts of *Chlorella vulgaris* showed the highest antioxidant activity (93.77 ± 2.39) at 2500 μg.ml⁻¹. Regarding significant inhibitory and antioxidant effects of these microalgae, promising use of their extracts can be suggested. Therefore, optimizing natural extracts and carrying out further studies are necessary to verify the current results.

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1. Introduction

Microalgae are rich sources of vitamins and minerals, including vitamins A, B₁, B₂, B₃, B₅, B₇, C and E, nicotine, folic acid, iodine, potassium, iron, magnesium and calcium [1]. In recent decades, microalgae biomass and their extracts have been used to enrich drinks (e.g. fruit juices and milks) and foods [2] (e.g. breads, confectionaries and yogurts) [3,4]. Interestingly, a significant advantage of cyanobacteria is the absence of polysaccharides in their cell walls, which not only enhances their biocompatibility but also facilitates their easier digestion for humans [1]. The species of *Arthrospira* (*A.*) *platensis* of the Oscillatoriaceae family includes a group of filamentous cyanobacteria characterized

by cell chains (trichomes) enclosed in a thin sheath [5]. The *A. platensis* contains large quantities of proteins, all essential amino acids, essential fatty acids, minerals, vitamins and photosynthetic pigments [6]. Moreover, *A. platensis*, due to its high protein and nutritional value, and *Chlorella* (*C.*) *vulgaris*, due to its β-1,3-glucan which is an active immune stimulator that can eliminate free radicals and decrease blood lipids, have nutritional uses for humans, used as food supplements [7]. The *C. vulgaris* microalgae are unicellular and grow in freshwater, which contain proteins, polysaccharides, lipids, unsaturated fatty acids and carotenoids (mainly lutein) as well as immune stimulants,



vitamins and minerals [8,9]. Moreover, phycocyanin is a phycobiliprotein in cyanobacteria and algae such as *A. platensis* and is a pigment-protein complex in the phycobiliprotein family of light-absorbing proteins that exist with allophycocyanin and phycoerythrin. It is a light blue pigment that absorbs orange and red lights at 620 nm and emits fluorescence at nearly 650 nm. Absorbance at 620 nm indicates the maximum absorption of phycocyanin [10].

Type 1 or insulin-dependent diabetes is caused by autoimmune destruction of pancreatic β -cells and accounts for nearly 5–10% of all diabetic patients. Chronic diabetes can damage multiple organs and lead to their dysfunctions and hence high mortalities [11]. Islet transplantation as a promising treatment option includes limitations due to the lack of available donors [12]. For the first time, formation of new islets through budding of pancreatic ductal cells was demonstrated [13]. Furthermore, assessment of β -cell ablation by chemical treatments in rodents showed a significant improvement in β -cell mass, suggesting that the mature pancreas can partially be regenerated [14]. Pancreatic β -cells include a weaker antioxidant defense system (enzyme catalase, superoxide dismutase and glutathione peroxidase), compared to other tissues and organs, which makes these cells further sensitive to oxidative stresses [15]. Because of chronic hyperglycemia, oxidative stress can trigger various signaling pathways and thus worsen β -cell dysfunction. Furthermore, oxidative stress can cause death of β -cells due to an abnormal increase in the level of free radicals [16]. The antioxidant effect of algae can prevent destruction of β -cells and prevent type 1 diabetes. Several studies have reported that antioxidant characteristics of microalgal carotenoids [17], phycocyanins [18] and polysaccharides [19].

To assess effects of microalgae on diabetes, various animal models such as mice, rats and zebrafish have been used in previous studies. Zebrafish is an excellent model for *in vivo* designed studies; small size, transparency of larvae, ease of collecting large numbers of embryos, possibility of *in vivo* assessed candidate molecules and/or rapid screening, as well as possibility of live imaging at the level of organisms are advantages of this model [20]. While zebrafish can regenerate their pancreatic β -cells during their life, regenerative ability of the adult mammalian β -cells is limited [21]. In *Tg(ins:CFP-NTR)* transgenic zebrafish model, nitroreductase gene of *Escherichia coli* is expressed under the control of insulin promoter, which is harmless to the organism under normal physiological conditions. However, NTR converts metronidazole (MTZ) into a toxic product; hence, treatment of this transgenic model with MTZ leads to β -cell apoptosis. In *Tg(ins: CFP-NTR)* zebrafish larvae, destruction of β -cells occurs within 24 h. Importantly, β -cell mass and function are restored within a few days after MTZ washout. In adult fish, high blood sugar

and absence of β -cells are observed 3 d after MTZ treatment and recover by 2 w. The β -cells of transgenic larvae express a cyan fluorescent protein (CFP) and therefore tracking of these cells can be carried out easily using fluorescent microscope [22]. Due to the increase in the incidence of diabetes worldwide, various studies in the field of prevention and treatment of diabetes have been carried out. It is important to use responsive models such as transgenic zebrafish to investigate food and drug sources. This study aimed to investigate potentials of *A. platensis* and *C. vulgaris* extracts in the regeneration of pancreatic β -cells using *Tg(ins: CFP-NTR)* transgenic zebrafish model. This is a novel model for studying type 1 diabetes used in various medicinal formulations.

2. Materials and Methods

2.1. Cultivation and assessment of the growth rate of microalgae

A cell suspension of *A. platensis* was cultivated in Zarrouk's media (24×10^4 cell.ml⁻¹) at 28 °C and pH 9 $\pm$ 1, as well as 24 h exposure to light [23]. Sorokin and Krauss culture medium [24] was used for the cultivation of *C. vulgaris* and the culture conditions included 24-h exposure, 20 °C and pH 6 $\pm$ 1. To assess growth of the cells, cell counting and absorbance reading of the cultures were carried out. Absorbance readings for *C. vulgaris* and *A. platensis* cultures were carried out at 750 and 680 nm, respectively. Furthermore, samples were collected from the cultures every 3 d and growth rate and number of cells per milliliter were calculated using hemocytometer slide and Eqs. 1 and 2 for *A. platensis* [25] and *C. vulgaris*, respectively.

$$K = \ln N_1 - \ln N_0 / t \quad \text{Eq. 1}$$

Where, K was number of the cells per milliliter, N_1 was number of the cells at time t, N_0 was number of the cells at 0 time and t was the time (d).

$$\text{Cell count per milliliter} = \frac{\text{total number of counted cells}}{\text{number of counted blocks}} \quad \text{Eq. 2}$$

Suspension of *A. platensis* was harvested after 15 d of culture and separated using Nylon membranes with 25- μ m pore sizes and suspension of *C. vulgaris* was centrifuged at 3773 g for 15 min to collect the biomass [9]. Biomasses were dried under biosafety hood at laboratory temperature for a w and stored at 4 °C until use.

2.2. Extraction of microalgae

2.2.1. Extraction with water as solvent without pretreatment using ultrasonic device (US)

A mixture of 5 g of *A. platensis* and 5 g of *C. vulgaris* powder were dissolved in 50 ml of distilled water (DW), transferred to a glass container with a lid and then incubated at 70 °C for 24 h using water bath. They were filtered



through filter papers and lyophilized at -55 °C for 24 h with a pressure of 0.6 mbar for long-term storage, using Christ Alpha 1-4 device [26].

2.2.2. Extraction with water solvent and treatment using ultrasonic device (US+)

First, 2 g of *A. platensis* dry powder and 2 g of *C. vulgaris* powder were added to 200 ml of DW with pH 6 and treated for 15 min at 4 °C using ultrasonic device with an intensity of 50 MHz. This product was centrifuged at 966 g for 15 min at 4 °C. Then, supernatant was collected and lyophilized for long-term storage [10].

2.2.3. Extraction with methanol as solvent

For preparing methanolic extract of *A. platensis* and *C. vulgaris* biomasses, 1 g of dry powder of each species was dissolved in 100 ml of 80% (v/v) methanol and agitated for 24 h at 20 °C. Then, samples were filtered using filter papers and supernatant was lyophilized for long-term storage [27].

2.3. Quantitative phytochemical analysis

2.3.1. Assessment of carbohydrates

Phenol-sulfuric acid method was used for total carbohydrate assessment. Briefly, 0.001 g of *A. platensis* and *C. vulgaris* dry powders was added to 1 ml of DW, then 1 ml of 5% (w/v) aqueous phenol and 5 ml of 96% sulfuric acid were added to this solution. Samples were set at room temperature (RT) for 25 min. Using Unico 2100 UV-VIS spectrophotometer, total reducing sugars were estimated at 490 nm. Glucose in the range of 0–100 µg.ml⁻¹ was used to draw the standard curve [28].

2.3.2. Photosynthetic pigments estimation

In general, 1 g of dry *A. platensis* and *C. vulgaris* powder was added to 50 ml of 100% acetone and samples were homogenized for 1 min at 15 g using Wise HG-15D cube homogenizer device. Then, samples were filtered using cotton and centrifuged at 94 g for 10 min at 4 °C and then supernatant was collected. To measure the chlorophyll content, absorbance of the samples was measured using microplate reader at 662, 645 and 470 nm, which were the maximum absorbance for chlorophyll a, chlorophyll b and total chlorophyll, respectively. In this study, Eqs. 3, 4 and 5 were used to calculate these chlorophyll values. Acetone 100% was used as blank in this experiment [29].

$$C_a = 11.75 A_{662} - 2.350 A_{645} \quad \text{Eq. 3}$$

Where, C_a was the quantity of chlorophyll a, A_{662} was the absorbance at 662 nm and A_{654} was the absorbance at 654 nm.

$$C_b = 18.61 A_{645} - 3.960 A_{662} \quad \text{Eq. 4}$$

Where, C_b was the quantity of chlorophyll b, A_{654} was the absorbance at 654 nm and A_{662} was the absorbance at 662 nm.

$$C_{x+c} = 1000 A_{470} - 2.270 C_a - 81.4 C_b / 227 \quad \text{Eq. 5}$$

Where, C_{x+c} was the quantity of total chlorophyll, A_{470} was the absorbance at 470 nm and C_a was the quantity of chlorophyll a and C_b was the quantity of chlorophyll b.

2.3.3. Assessment of phenolic compounds

Total soluble phenolic content of the algae extracts was assessed using a method by Yucetepe et al. [30]. Content of the phenolic compounds in the extracts was assessed using Folin-Ciocalteu reagent. Thus, 0.2 ml of the extract and 0.2 ml of diluted Folin-Ciocalteu reagent (1:15) were mixed and after 5 min, 2.5 ml of 7% calcium carbonate solution were added to the mixture. Using DW, volume of the samples was adapted to 5 ml and after 90 min, absorbance of the samples was read at 750 nm. Total phenols were expressed as Gallic acid equivalents per gram dry weight of the sample.

2.3.4. Assessment of proteins

To measure the protein content, proteins must be extracted from the cells. Therefore, 15 ml of DW were added to 1 g of dry *C. vulgaris* and *A. platensis* powder and transferred onto a magnetic stirrer for 60 min at RT. These samples were transferred to an ultrasonic machine for 30 min at 4 °C with an intensity of 70 Hz. These were centrifuged at 4000 g for 30 min at 4 °C. The supernatant was collected and pH was adjusted to 3 using 0.1 M hydrochloric acid and 0.1 M sodium hydroxide. To collect sediments, samples were centrifuged at 4000 g for 30 min at 4 °C. Then, 2.5 ml of Bradford reagent were added to 250 µl of protein and the absorbance was read at 595 nm using spectrophotometer [31]. Using bovine serum albumin as the standard curve, protein concentration was assessed.

2.3.5. Assessment of lipids

For lipid assessment, 0.5 g of dry powder of *A. platensis* and *C. vulgaris* was transferred into a Soxhlet machine with 150 ml of n-hexane and incubated at 145 °C for 3 h [32]. Then, GC Agilent-6820 gas chromatography machine with column specifications of 30 m × 0.25 mm × 0.25 µm and nitrogen carrier gas was used to characterize fatty acids of the samples. The injection volume was 1 µl, the injection mode was split and the temperature started from 100 °C. After 2 min, temperature increased to 240 °C using Rate 3 and to 280 °C using Rate 10.

2.3.6. Assessment of phycocyanin content in aqueous extracts of *A. platensis*

The evaluation of the amount of phycocyanin and antioxidant tests after the treatment of zebrafish larvae with different concentrations of extracts and the evaluation of their effectiveness has been done. The quantity of phycocyanin in aqueous extracts of *A. platensis* was assessed by reading absorbance of the samples at 620 and 652 nm using spectrophotometer and Eq. 6 [10]:

$$\text{Phycocyanin (mg.l}^{-1}\text{)} = (\text{Abs}_{620} - 0.474 \times \text{Abs}_{652}) / 5.34 \quad \text{Eq. 6}$$



Where, A_{620} was the absorbance at 620 nm and A_{652} was the absorbance at 652 nm.

2.3.7. Assessment of antioxidants

Further analyses of the aqueous extracts and their antioxidant characteristics and phycocyanin contents were carried out using two methods of DPPH and iron chelation.

2.3.7.1. Antioxidant assessment of DPPH method

Briefly, 100 μ l of 0.5 M DPPH were added to 100 μ l of the sample by various concentrations, including 0.5, 1, 1.5, 2 and 2.5 mg.l^{-1} methanol. After complete pipetting, samples were set for 60 min at RT in dark. Then, absorbance of the samples was read at 517 nm. Methanol was used as the control and ascorbic acid as the positive control. Equation 7 was used to calculate the quantity of free radical inhibition as follows [33]:

$$\text{Inhibition (\%)} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}}{\text{Eq. 7}} \right] \times 100$$

Where, $\text{Abs}_{\text{control}}$ was absorption rate of the DPPH solution and methanol and $\text{Abs}_{\text{sample}}$ was absorption rate of the assessed sample.

Technically, IC_{50} is a number indicating the extract concentration, which can inhibit the oxidation process by 50%. The smaller the IC_{50} value, the higher the antioxidant activity [34].

2.3.7.2. Assessment of iron ion chelating activity

To carry out this assay, 50 μ l of the samples were mixed in various concentrations (0.5, 1, 1.5, 2 and 2.5 mg.l^{-1} DW) with 25 μ l of 2 mM FeSO_4 solution and then 50 μ l of 5 mM ferrozine solution were added to the mixture. Mixture was agitated well and absorbance was read at 562 nm after 10 min. Moreover, DW was used as negative control and EDTA was used as positive control. Equation 8 was used to calculate the quantity of iron ion chelation [35]:

$$\text{Chelating activity (\%)} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}}{\text{Eq. 8}} \right] \times 100$$

$\text{Abs}_{\text{control}}$ was the absorbance rate of DW and ferrozine solution and FeSO_4 and $\text{Abs}_{\text{sample}}$ was the absorbance rate of the assessed sample.

2.4. Fish care and breeding

Adult zebrafish were grown using Tecniplast recirculating system with 14/10 of light/dark photoperiods and water temperatures of 28 °C, Royan Zebrafish Core Facility, Tehran, Iran. The *Tg (ins: CFP-NTR)* transgenic line and TU wild-type strain were used in all experiments, carried out using protocols approved by the Ethical Committee of Royan Institute, Tehran, Iran. The hemizygous *Tg (ins: CFP-NTR)* embryos (to avoid the variability of fluorescent intensity between hemizygous and homozygous larvae)

were prepared by outcrossing of the transgenic *Tg (ins: CFP-NTR)* line with TU wild-type strain. Zebrafish embryos were cultured in E3 media and treated with 0.003% 1-phenyl-2-thiourea at 24 h post fertilization (hpf) till the end of the experiment. The 1-phenyl-2-thiourea completely suppressed pigmentation and enhanced trans-parency of the larvae, which was necessary for live imaging and quantification of a cyan fluorescent reporter.

2.4.1 Chemogenetic ablation

Zebrafish larvae at 72 hpf were exposed to a 10-mM solution of metronidazole (MTZ) for 24 h and then larvae were washed three times with E3 media. The MTZ treatment was adapted from the previously established protocol for near-full ablation of the β -cells in the pancreas of zebrafish larvae.

2.4.2. Treatment of larvae with the extracts

Treatment of larvae was carried out with five concentrations of each compound (500, 125, 31.25, 7.81 and 1.95 $\mu\text{g.ml}^{-1}$) from 4–6 d post fertilization (dpf). Thus, 15 β -cell ablated larvae were used for each concentration, including five larvae in 1.5 ml of E3 media in each well of 24-well plates. Three control groups were assessed, including none-treated control compromise larvae cultured in E3 media only, negative control consisted of larvae treated with MTZ for β -cell ablation without compound treatment in follow and positive control with larvae, which treated with NECA as a small molecule that increased β -cell regeneration after exposure to MTZ. At 6 dpf, live larvae were anesthetized with tricaine for imaging and images were captured using Olympus SZX16 fluorescence stereomicroscope equipped with a Canon digital camera. The effect of compounds on β -cell regeneration was assessed by measuring β -cell area (AU) in each larva using ImageJ software (NIH).

2.5. Statistical analysis

All experiments of this study were carried out three times and data were present as mean $\pm$ SD (standard deviation). One-way ANOVA was used to analyze statistical significances of data using GraphPad Prism 6 software. In general, p values less than 0.05 were recorded as significant.

3. Results and Discussion

3.1. Characteristics of *A. platensis* and *C. vulgaris* extracts

Algal growth and biomass productivity were assessed using cell counting and absorbance reading. Growth of *A. platensis* and *C. vulgaris* was assessed for 15 d under certain conditions and the highest cell growth was achieved on Day 10 for *A. platensis* and on Day 9 for *C. vulgaris* (Figure 1).



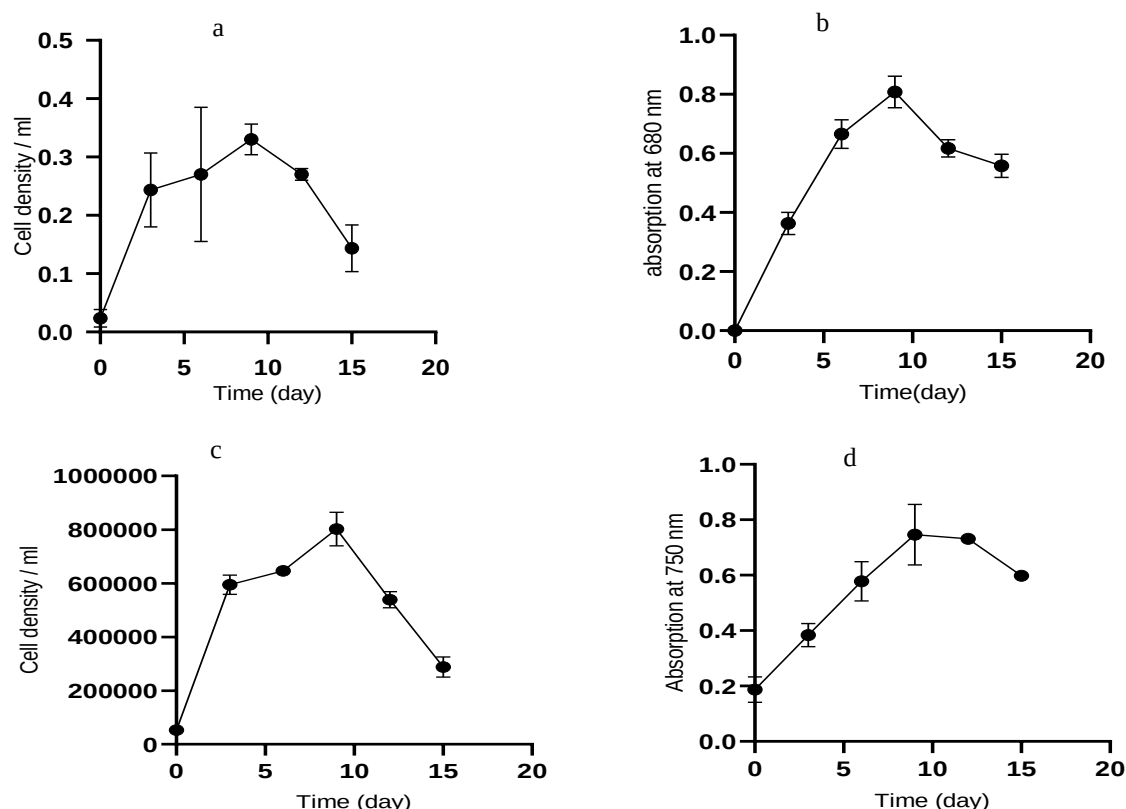


Figure 1. Growth curves of *A. platensis* and *C. vulgaris* in experimentally environmental conditions. Each point represents the mean value of three replicates. Bars indicate standard deviations. (a) Graph based on *A. platensis* cell counts; (b) graph based on *A. platensis* absorbance at 680 nm; (c) graph based on *C. vulgaris* cell counts; and (d) graph based *C. vulgaris* absorbance at 750 nm.

In the present study, it was detected that *A. platensis* and *C. vulgaris* included antioxidant characteristics and caused regeneration of pancreatic β -cells in *Tg(ins: CFP-NTR)* transgenic zebrafish. Zarrouk's medium was used for *A. platensis* cultivation and during the growth, temperature was set to a constant value of 28 °C, which resulted in the production of the highest biomass rate (3.27 g.l⁻¹). Effects of temperature on growth rate and active mass of *A. platensis* in Zarrouk's media under laboratory conditions were assessed in a study and it was reported that the highest quantity of microalgae active mass (2.74 g.l⁻¹) was produced at 24 °C in Zarrouk's media [36]. These results were similar to the present results. The pH value of the culture media was 8 on the initial days of *A. platensis* cultivation, which reached to 10 as cultivation time increased and at the beginning of the ascent stage. Alkaline pH (9 and 10) alone favored normal cell metabolism and the membrane function was not affected by the growth and chlorophyll content [37]. Additionally, it was reported that the maximum dry weight of the algal mass and contents of chlorophyll a and protein were produced at pH 10–9, [38]. Furthermore, it was demonstrated that the algae grown at pH 9 were able to decrease time of the lag phase, including the highest specific growth rate [39]. In this study, time of light exposure was set at 24 h without darkness. Tayebati et al. reported that the

maximum growth of *A. platensis* was under red light and 24-h light cycle without darkness [40]. During the growth of *C. vulgaris*, temperature was set to a constant value of 20 °C and pH was set in the range of 6 $\pm$ 2. In a study that investigated effects of various temperatures on the growth of *C. vulgaris*, it was shown that the maximum growth rate occurred at 25 °C [41]. Moreover, a study that investigated effects of pH (2, 5, 7, 9 and 11) and temperature (10, 20, 30 and 40 °C) on the growth and chemical composition of *C. vulgaris* biomass reported that the optimal range of environmental conditions for the cultivation of microalgae included pH 7–9 and temperature of 20–30 °C [42].

3.2. Compounds of *A. platensis* and *C. vulgaris*

Quantity of carbohydrates in *A. platensis* was 61.76 $\pm$ 2.44 μ g.ml⁻¹, which was almost 1.5 times higher than that in *C. vulgaris*. Quantity of total protein in *A. platensis* was approximately 1.5 times higher than that in *C. vulgaris* and quantity of phenolic compounds in *A. platensis* was higher than that in *C. vulgaris* with approximately 2.5 times. Quantities of carbohydrates, proteins and phenolic compounds are present in Table 1.

In the present study, quantity of total carbohydrates in *A. platensis* was 6.17%. Similarly, another study indicated that the quantity of total carbohydrate in this microalga was 6.46% $\pm$ 0.32 [42]. In this study, quantity of total lipid was



3% for *A. platensis*. In another study, quantity of total lipid in *A. platensis* was 2.45 ± 0.82 and $0.1\% \pm 0.01$, respectively [43]. Differences in the total lipid contents of the studies could be due to the differences in the types of solvent, time and temperature of the Soxhlet device. Quantity of total protein was 65.40% for *A. platensis*. In a previous study, it has been shown that quantity of *A. platensis* protein was 53.12% [44] possibly due to the various culture conditions and protein extraction methods. Quantity of photosynthetic pigments in micrograms per milliliter of solution volume is shown in Table 2. Quantity of total carotenoids in *C. vulgaris* was $0.912 \pm 0.15 \mu\text{g} \cdot \text{ml}^{-1}$, which was higher than that in *A. platensis*. Moreover, quantities of chlorophyll a and b in *C. vulgaris* were approximately 2.5 and 10 times more than that in *A. platensis*, respectively.

Oleic acid and linoleic acid ($\omega 6$) were the major fatty acids in *A. platensis* and *C. vulgaris*, respectively. Results of the fatty acid profiling are shown in Tables 3.

Based on the profiling results of *A. platensis* fatty acids, the predominant fatty acids included oleic acid (53.3%), palmitic acid (22.8%), linoleic acid (14.1%) and stearic acid (9.6%). In a previous study, palmitic, γ -linolenic and linoleic acids were dominant fatty acids [41]. In a report, palmitic acid was the most dominant fatty acid in *A. platensis* [45]. In another study, it was shown that palmitic acid with 30.1% of the total fatty acids was dominant within saturated fatty acids (SFAs), and oleic acid with 34% was dominant within unsaturated fatty acids [46]. Additionally, profile of *C. vulgaris* fatty acids included linoleic acid (30.1%), oleic acid (24.8%), palmitic acid (22.6%), stearic acid (16.7%) and linolenic acid (5.68%). In a previous study, it was shown that palmitic acid and linolenic acid accounted for 25.17 and 9.66% of the total fatty acids, respectively, in *C. vulgaris*. In addition, it was shown that palmitic acid, stearic acid, oleic acid and linolenic acid were dominant fatty acids [46].

3.3. Quantity of phycocyanin in aqueous extracts of *A. platensis*

Based on Figure 2, no significant differences were seen in the quantity of phycocyanin in *A. platensis* aqueous extracts.

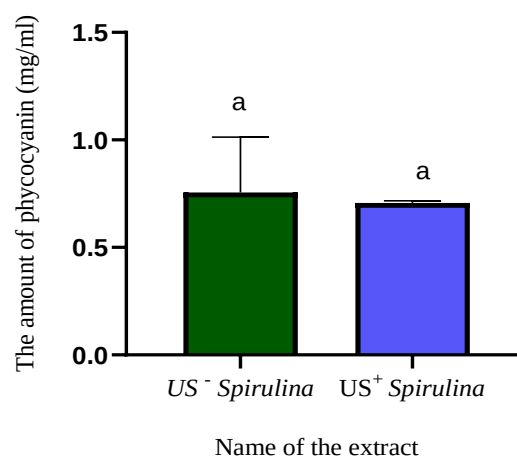


Figure 2. Phycocyanin content in the aqueous extract of *A. platensis* at 620 and 652 nm. Similar letters in the columns show insignificant differences between the groups.

3.4. Pancreatic β -cell regenerative capacity of microalgal aqueous extracts

The average values of β -cell mass in *Tg(ins: CFP-NTR)* transgenic zebrafish larvae for all treated and control groups are illustrated in Figure 3A. Results showed that aqueous extracts without ultrasonication of *A. platensis* and *C. vulgaris* significantly increased β -cell area (AIU), compared to negative controls. For *C. vulgaris*, effects on β -cell regeneration increased with concentration (Figure 3B).

In a study, alloxan-induced diabetic rats were treated with *A. platensis* supplementation and results showed that *A. platensis* treatment led to significant decreases in fasting blood glucose and increases in glycogen levels. The *A. platensis* supplementation prevented body weight loss and improved hepatotoxicity indices such as alkaline phosphatase and transaminase activities, bilirubin level and lipid peroxidation. Overall, this study showed that *A. platensis* treatment decreased hyperglycemia and oxidative stress in diabetic rats [47]. Additionally, histopathological studies of the pancreas in a study showed that *A. platensis* included antidiabetic effects by increasing secretion of insulin from β -cells of the pancreatic islets. Moreover, effects of *A. platensis* on regeneration of the pancreatic islets were observed with regeneration of the islet cells [48].

Table 1. Quantities of carbohydrates, proteins and phenolic compounds

Algae name	Carbohydrate	Protein ($\mu\text{g} \cdot \text{ml}^{-1}$)	Phenolic compounds
<i>A. platensis</i>	61.76 ± 2.44	2180.1 ± 0.29	111.51 ± 0.50
<i>C. vulgaris</i>	42.73 ± 3.80	1375.1 ± 0.02	40.28 ± 0.50

Table 2. Quantities of the photosynthetic pigments ($\mu\text{g} \cdot \text{ml}^{-1}$)

Species name	Chlorophyll a	Chlorophyll b	Total Carotenoids
<i>A. platensis</i>	5.404 ± 0.04	0.159 ± 0.14	0.711 ± 0.28
<i>C. vulgaris</i>	12.349 ± 0.11	1.722 ± 0.11	0.912 ± 0.15



Table 3. Fatty acid compounds (weight proportions of total fatty acids) in *A. platensis* and *C. vulgaris*

Fatty acid name	<i>A. platensis</i>	<i>C. vulgaris</i>
Palmitic acid (C16)	22.8	22.6
Stearic acid (C18)	9.6	16.7
Oleic acid (C18:1)	53.3	24.8
Linoleic acid (ω 6) (C18:2)	14.1	30.1
Linolenic acid (ω 3) (C18:3)	---	5.68

The hypoglycemic effect of *A. platensis* was assessed in diabetic male albino rats as a mammalian model treated with streptozotocin and it was reported that *A. platensis* included antioxidant and antidiabetic effects in streptozotocin-induced diabetic rats [49]. In the present study, *A. platensis* extract included antioxidant characteristics and regenerated pancreatic β -cells.

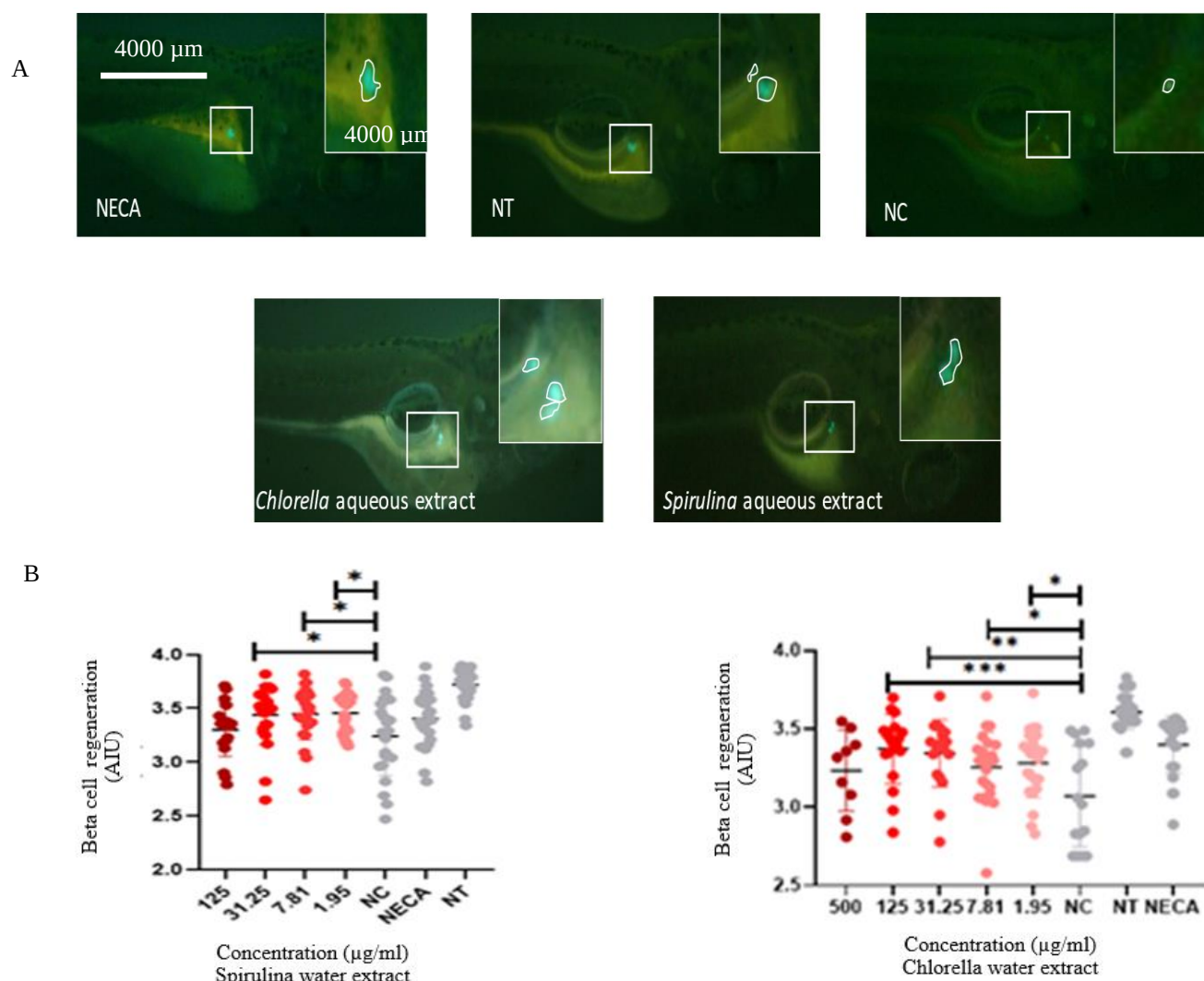


Figure 3. Pancreatic β -cell regenerative capacity of microalgal aqueous extracts. (A) The average values of β -cell mass in *Tg (ins: CFP-NTR)* transgenic zebrafish larvae treated with aqueous extracts of microalgae and control groups: transgenic larvae treated with metronidazole (NC), transgenic larvae (NT), and positive control (NECA). (B) Reconstructed mass of β -cells treated with aqueous extracts of *A. platensis* and *C. vulgaris* without ultrasound. Each dot represents one treated larva. Overall, 480 larvae were assessed using three repetitions of the experiments. One-way ANOVA was used for statistical analysis.

Lee et al. investigated the protective effects of *Spirulina maxima* extracts in a cytokine-mediated type-1 diabetes model *in vitro* and in streptozotocin-induced diabetic Wistar rats *in vivo*. Interleukin-1 beta (IL-1 β) and Interferon gamma (IFN- γ) caused significant cytotoxicity in mouse cells, increasing nitric oxide (NO), nuclear factor κ B (NF- κ B) and apoptosis associated with key genes. However, cytotoxicity of the cytokines significantly decreased using

Spirulina extract, which effectively inhibited NO production and suppressed apoptosis. These results showed that *Spirulina* extract might be effective for preserving viability and function of pancreatic β -cells against cytotoxic conditions. In addition, diabetic rats that consumed *Spirulina* extract orally showed decreases in glucose levels, increases in insulin and improvements in liver enzyme markers. Antioxidant effects of *Spirulina* extract may be beneficial in



type-1 diabetes treatment by increasing survival and decreasing or delaying cytokine-mediated destruction of β -cells [50]. In a study by Kulkarni et al. on *Tg(ins: Flag-NTR)s950* zebrafish models, the researchers detected that MTZ induced reactive oxygen species (ROS) production in presence of NTR. Furthermore, they reported that the level of produced ROS was proportional to the dose of prodrug added to the system. At high doses of MTZ, caspase 3 was rapidly cleaved and β -cells shifted to regulated cell death [51]. Based on the results, it can be concluded that the extracts of *A. platensis* and *C. vulgaris* include antioxidant characteristics and thus prevent death of pancreatic β -cells.

Cytokines alter and regulate the expression level of several genes via transcription factors (e.g. NF- κ B, STAT-1 and IRF-3) in β -cells [52]. The NF- κ B signaling pathway is an important pathway in the regulation of inflammation [53]. The NF- κ B is a significant transcriptional regulator in cells and normally binds to its inhibitor kappa B (I κ B) of the cytoplasm in the inactive form of p50-p65 heterodimer [54]. In β -cells, IL-1 β alone or in association with IFN- γ induces proteolysis of I κ B α and increases translocation of NF- κ B into the nucleus, where it can bind to specific κ B sites on DNA to regulate transcription of target genes [55]. The NF- κ B activation increases expression of downstream inflammatory mediators, including proinflammatory cytokines such as IL-1 β , interleukin-6 (IL-6) and TNF- α [56]. Cytokine-induced NF- κ B activation changes the gene

expression profile of β -cells by affecting several genes involved in β -cell differentiation, recruitment and activation of immune cells and β -cell apoptosis. Inhibition of NF- κ B activation or translocation leads to β -cell proliferation by suppressing β -cell damage or death induced by IL-1 β and IFN- γ ; therefore, NF- κ B is an important transcription factor in pancreatic β -cells [57]. Studies have shown that species of *A. platensis* inhibit cytokine-mediated cell apoptosis in pancreatic β -cells. Moreover, *A. platensis* extract effectively destroys cytokine-induced I κ B α to prevent the nuclear translocation of NF- κ B p65 subunits and downregulates downstream genes associated with β -cell apoptosis to protect cells against NF- κ B cytotoxicity [50].

3.5. Antioxidant activity of aqueous extracts and phycocyanin

Based on the DPPH assay, antioxidant activity of phycocyanin and water extracts of *A. platensis* and *C. vulgaris* showed that with increasing concentrations, the antioxidant activity increased and all extracts at concentration of 2.5 mg.l⁻¹ included the highest activity (Figure 4). Moreover, the highest activity belonged to aqueous extract treated with ultrasonic microalgae of *A. platensis* at the concentrations of 2 and 2.5 mg.l⁻¹. The IC₅₀ values for *A. platensis* (US⁻), *A. platensis* (US⁺), *C. vulgaris* (US⁻), *C. vulgaris* (US⁺) and phycocyanin extracts were 1.25, 1.53, 1.14, 0.95 and 0.90 mg.l⁻¹, respectively.

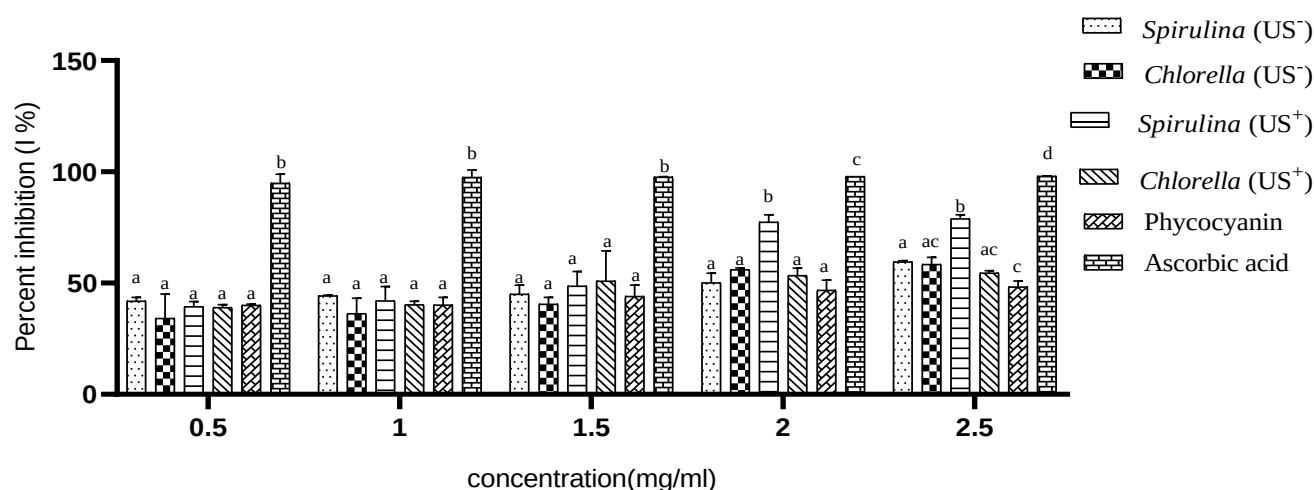


Figure 4. The DPPH radical scavenging ability in phycocyanin and aqueous extracts of *A. platensis* and *C. vulgaris*. Different English letters indicate significant differences. Results are reported as mean $\pm$ SD (standard deviation).

Antioxidant activities of phycocyanin and water extracts of *A. platensis* and *C. vulgaris* based on the iron chelation assay are shown in Figure 5. Results were similar to those of DPPH assay, 2.5 mg.l⁻¹ in each extract included the highest antioxidant activity and the highest activity was linked to the aqueous extract treated with ultrasonic *C. vulgaris* at 2.5 mg.l⁻¹. Results from the antioxidant assays showed that antioxidant activity increased with increases in

the concentrations of phycocyanin and aqueous extracts of the microalgae. A study investigated antioxidant characteristics of alcoholic extracts of *A. platensis* and *C.* [58] and reported similar results. At concentration of 2.5 mg.l⁻¹, the highest level of antioxidant activity with DPPH assay inhibition belonged to the aqueous extract of *A. platensis* (US⁺). This was significantly different from other extracts with similar concentrations. At high concentrations, the

level of DPPH inhibitory activity in *A. platensis* was higher than that of *C. vulgaris*. The major active component of *A. platensis* is the phycocyanin pigment, which scavenges free radicals and includes high antioxidant activities [58,59], showing that the antioxidant capacity of phycocyanin and its bioactive components (phycocyanopeptide and phycocyanobilin) is as follows: phycocyanin > phycocyanobilin > phycocyanopeptide. As concentrations of these component increase, their antioxidant activities increase [59]. Phycocyanins as antioxidants (soluble in water) can prevent ROS production or their destruction in producing cells (cyanobacteria) and in cells that consume these biochemical or are treated with them (animal cells). Researchers have shown that phycocyanins can remove hydroxyl radicals, alkoxyl radicals and superoxide anions. Furthermore, phycocyanins inhibit lipid peroxidation reactions and regenerate singlet oxygen, hypochlorous acid, peroxy radical, peroxy nitrite, NO and hydrogen peroxide [60].

In the present study, antioxidant activity increased with increases in the concentration of phycocyanins. This increase was not higher than that of other extracts with no significant differences. Antioxidant activity of the phenolic compounds is majorly due to their oxidation characteristics, reducing power and chemical structure, which can play

important roles in neutralizing free radicals, forming complexes with metal ions and quenching single and triple oxygen molecules [61]. A study was designed to investigate aqueous, ethanol and acetone extracts of several algae (*Spirogyra* sp., *Spirulina* sp., *Chlorella* sp. and *Chara* sp.) to assess antioxidant capacities of the extracts as well as their DPPH radical scavenging activities. It was concluded that *Spirulina* sp. included significant total phenolic composition and antioxidant activity, compared to other species. Results of the phenolic compounds in this study showed that *A. platensis* microalgae included higher phenolic compounds than that *C. vulgaris* did. Due to the lack of significant antioxidant activity of phycocyanins and presence of vitamins such as vitamins K and E and amino acids in *A. platensis*, its high antioxidant activity at high concentrations could be attributed to compounds other than phycocyanins. Presence of other bioactive factors such as vitamin C and proteins that are soluble in water increases antioxidant activity because comparison of various quantities of phycocyanins have shown that antioxidant activity is more significant in algal extracts at similar concentrations of phycocyanins. This could be linked to antioxidant activity of other factors in the aqueous extract of algae [62].

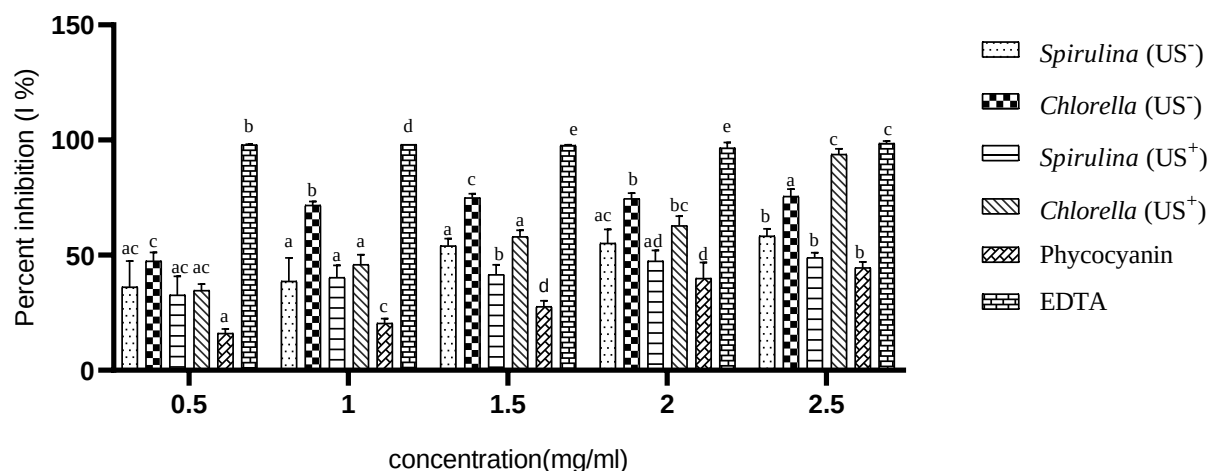


Figure 5. Iron ion chelating abilities in phycocyanin and aqueous extracts of *A. platensis* and *C. vulgaris*. Different English letters indicate significant differences. Results are reported as mean $\pm$ SD (standard deviation).

Based on the findings, increases in the concentration of aqueous extracts of microalgae could result in increases in metal chelation activity; thus, concentration of 2.5 mg.l⁻¹ included the highest antioxidant activity. A significant difference was detected in the concentration of 2.5 mg.l⁻¹ aqueous extract (US⁺) of *C. vulgaris* with other extracts. These results showed that use of ultrasonic devices could increase the chelating activity of *C. vulgaris* by extracting components that were effective in this procedure. In an experimental study, six various polysaccharides (RFPs, MAPs, UWP, AEPs, HWP and CEPs) were extracted

from *C. vulgaris* using repeated freeze-thaw, microwave, ultrasonic waves, alkaline treatment, hot water and cellulose. Then, antioxidant activities of the extracts were assessed and it was concluded that alkaline extraction method followed by ultrasonic extraction included the highest efficiency in the production of crude polysaccharides due to the complex and rigid cell walls of *C. vulgaris*. The polysaccharide extraction method was a key factor that affected the antioxidant activity of *C. vulgaris* polysaccharides. Additionally, extracts achieved by ultrasoni-



cation showed superior activities than those extracts achieved by other methods did [63].

4. Conclusion

The present study demonstrated that the zebrafish model could be used as a tool for algae screening and the aqueous extracts of *A. platensis* and *C. vulgaris* with their antioxidant characteristics and biologically active compounds at concentrations of 31.25, 7.81, 1.95 and 125, 31.25, 7.81 and 1.95 $\mu\text{g}\cdot\text{ml}^{-1}$, respectively, could regenerate pancreatic β -cells. In addition, it could be concluded that these extracts were possibly effective in inhibiting the NF- κ B pathway on β -cell regeneration and cell apoptosis. Since pure phycocyanin did not cause significant regeneration compared to the negative controls, this study demonstrated that compounds other than phycocyanins could be effective in regeneration of β -cells. Moreover, the present study demonstrated that the microalgal extracts of *A. platensis* and *C. vulgaris* could be used in foods and medicines.

5. Conflict of Interest

The authors report no conflict of interest.

6. Authors Contributions

Investigation, T.H; supervision, F.H, Y.T; writing review and editing, T.H, M.R. All authors have read and agreed to the published version of the manuscript.

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سنجش زیست فعالی ریزجلبک: پتانسیل های ضد اکسیدانی و ضد دیابتی در مدل گورخر ماهی تراریخته

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تاریخچه مقاله

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واژگان کلیدی

- خواص ضد اکسیدانی
- غذای دیابتی
- ریزجلبک
- بازسازی

نویسنده مسئول

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چکیده

سابقه و هدف: ریزجلبک ها با مهار ضد اکسیدان، آلفا امیلاز و آلفا گلوکوزیداز و توانایی های فعال سازی NF-kB ویژگی های ضد دیابتی امیدوار کننده ای را از خود نشان داده اند. مطالعه حاضر با هدف ارزیابی اثرات عصاره ریزجلبک های کلرلا ولگاریس و آرتروسیرا پلاتنسیس بر بازسازی سلول های β با استفاده از $Tg (ins: CFP-NTR)$ در مدل گورخر ماهی تراریخته مبتلا به دیابتی نوع ۱ انجام شد.

مواد و روش ها: پس از ۱۵ روز از کشت ریزجلبک ها، سلول ها از زی توده^۱ استخراج شدند. سنجش های بیوشیمیایی مانند ارزیابی کربوهیدرات ها، پروتئین ها، لیپید ها، ترکیبات فنلی و رنگدانه های فتوسنتزی انجام شد. لاروهای گورخر ماهی تراریخته تحت تیمار با پنج غلظت گوناگون عصاره ($500 \mu g.ml^{-1}$ ، 125 ، $31/25$ ، $7/81$ و $1/95$) در شرایط درون تنی^۲ قرار گرفتند.

یافته ها و نتیجه گیری: نتایج نشان داد که عصاره آبی جلبک های کلرلا ولگاریس و آرتروسیرا پلاتنسیس به ترتیب در غلظت های $31/25$ ، $7/81$ و $1/95$ میکروگرم در میلی لیتر و 125 ، $31/25$ ، $7/81$ و $1/95$ میکروگرم در میلی لیتر تأثیر معنی داری بر بازسازی سلول های β داشتند. بالاترین میزان باززایی عصاره کلرلا ولگاریس و آرتروسیرا پلاتنسیس به ترتیب در غلظت های 125 و $31/25$ میکروگرم در میلی لیتر (60 در برابر 92 درصد) مشاهده شد ($p < 0.05$). همچنین عصاره آبی کلرلا ولگاریس بیشترین فعالیت ضد اکسیدانی ($93/2 \pm 2/39$) را در غلظت $2500 \mu g.ml^{-1}$ نشان داد. با توجه به اثرات بازدارنده و ضد اکسیدانی قابل توجه این ریزجلبک ها، استفاده امیدوار کننده از عصاره آن ها را می توان پیشنهاد کرد. بنابراین، بهینه سازی عصاره های طبیعی و انجام مطالعات بیشتر برای تایید نتایج فعلی ضروری است.

تعارض منافع: نویسندگان اعلام می کنند که هیچ نوع تعارض منافع مرتبط با انتشار این مقاله ندارند.

^۱ biomass

^۲ *in vivo* آزمایش هایی که در درون بدن موجود زنده انجام می شود

