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Short Communication

The Role of *Nannochloropsis* sp. Methanolic Extract in Reducing Hydrogen Peroxide-induced DNA Damage in L929 Cell Line

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ABSTRACT

Nannochloropsis sp., is one of the microalgae that produces important antioxidants. We postulate that the presence of phenolic compounds in the methanolic extract of *Nannochloropsis* sp. exhibits preventive effect against DNA damage induced by 100 µM concentration of hydrogen peroxide (H_2O_2) in L929 cells. High performance liquid chromatography (HPLC) analysis revealed that *Nannochloropsis* sp. methanolic extract contained antioxidant compounds such as *p*-coumaric acid, caffeic acid, naringenin and hesperitin. In this study, we utilised comet assay to measure the activity of DNA damage induced by H_2O_2 in L929 cells and the preventive effect of *Nannochloropsis* sp. methanolic extract and caffeic acid. After 24 hours of treatment, 100 µM of H_2O_2 induced 64.4 ± 4.6% of DNA damage in L929 cells compared with the untreated cells ($p < 0.05$). Interestingly, the scale of DNA damage induced by the H_2O_2 was reduced to 46.3 ± 12.7% when treated with 0.4 mg/mL of *Nannochloropsis* sp. methanolic extract ($p < 0.01$). The DNA damage was further reduced to 7.3 ± 2.9% when treated with 20 µM of caffeic acid ($p < 0.01$), a compound found in the microalgae extract. In conclusion, *Nannochloropsis* sp. methanolic extract which contained phenolic compounds, was able to protect L929 normal cells from oxidative DNA damage and therefore may confer protection from genetic mutation and cancer formation.

Keywords: Caffeic acid, DNA damage, microalgae, *Nannochloropsis*

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INTRODUCTION

Nannochloropsis sp. is a unicellular green microalga, which is normally found in marine and freshwater habitats. *Nannochloropsis* sp. produces important antioxidants such as omega-3 fatty acid and polyphenolic compounds (Azim et al., 2018). Antioxidant compounds found in microalgae, play a major role in preventing the formation of reactive oxygen species (ROS) and oxidative stress, which are involved in DNA damage (Goiris et al., 2012). Antioxidants such as polyphenolic compounds exert an effective antioxidant properties by quenching and neutralizing free radical chain reactions in biological systems (Zhao et al., 2014). This is due to their ability to donate electron and thus protect the cells from damaging effects (Zhao et al., 2014). Most species of unicellular green microalgae have ability in producing valuable secondary metabolites for different needs such as antioxidants, several different carotenoids, polyunsaturated fatty acids vitamins, anticancer and antiviral drugs (Skjånes et al., 2013). Most of the compounds produced (cyanovirin, oleic acid, linolenic acid, palmitoleic acid, vitamin E, B12, β-carotene, phycocyanin, lutein, and zeaxanthin) have antimicrobial antioxidant, and anti-inflammatory capacities, with the potential for the reduction and prevention of diseases (Markou & Nerantzis, 2013). In many cases, these secondary metabolites were produced when the algae are exposed to stress conditions linked to nutrient deprivation, light intensity, temperature, salinity and pH (Skjånes et al., 2013).

ROS form as products under normal physiological conditions due to the partial reduction of molecular oxygen. ROS, that is, superoxide anion (O_2^-), hydroxyl radical ($OH^•$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), arise in many ways, as a product of the respiratory chain in mitochondria, in photochemical and enzymatic reactions (Nita & Grzybowski, 2016). ROS are generated in large amount by excessive physical activities or environmental chemicals which creating oxidative damage to genomic DNA resulting in single and double strand breaks (Nimse & Pal, 2015). These damages can lead to changes in genetic sequence, which might transform cultured normal cells into rapidly proliferating, cancer-type of cells. In plants, ROS are always formed by the inevitable leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a by-product of various metabolic pathways localized in different cellular compartments (Sharma et al., 2012).

Previously, we had shown that *Nannochloropsis* sp. methanolic extract contained high total phenolic and flavonoid contents with strong anti-oxidant activities (Zainoddin et al., 2018). We postulate that methanolic extract of *Nannochloropsis* sp., contains phenolic compounds, which may exhibit preventive effects against hydrogen peroxide-induced DNA damage in the normal cells.

MATERIALS AND METHODS

Microalgae Sample

Nannochloropsis sp. was obtained from Fisheries Research Institute (FRI) Pulau Sayak, Kedah, Malaysia. The microalgae obtained were cultured in 1000 mL flask with three replicates using the sea saline water (10 ppt) with Walne (1970) medium according to protocols described in Zainoddin et al. (2018). Media for stock culture were replaced every two weeks and the cells were maintained under 25 ± 1°C under continuous lighting. The cells were cultured under continuous exposure of white fluorescent lamps (50.05 µmol photons m^{-2} s^{-1}) with aeration of normal gas. The cells were harvested for the extraction at the exponential phase of growth i.e. 1.5 × 10^6 cells/mL.

Microalgae Extracts

Cells of *Nannochloropsis* sp. were harvested according to the method as described in Pereira et al. (2015) with some modifications. Briefly, the cultured microalgae cells were dried using freeze drier for 72 hours. The dried microalgae were then subjected to sonication to disrupt the cell wall and extracted using 100% methanol at the ratio of 1 part of algae to 40 parts methanol (w/v). The extraction was performed overnight at room temperature (20°C) under continuous stirring. The extracted biomass was centrifuged at 10,000 × *g* for 10 minutes and supernatant was collected, filtered and dried by using rotary evaporator at 45°C under vacuum condition. Dried extracts were then resuspended in methanol to a final concentration of 20 mg/mL and stored at -20°C until further use.

High Performance Liquid Chromatography (HPLC) Analysis of *Nannochloropsis* sp.

Preparation of Standard Solutions. A total of 7 standards which include caffeic acid, *p*-coumaric acid, quercetin, naringenin, hesperetin, kaempferol and baicalin were prepared as a stock solution (15 ppm) in methanol and diluted with a final concentration of 5 ppm as a working solution. All the working solutions were stored in -20°C until further analysis.

Preparation of Mobile Phase. The analysis of HPLC involved 2 mobile phases as carrier solvent. In mobile phase A, a mixture of HPLC-grade methanol: acetonitrile: deionized water (40: 5: 55) and addition of 0.1% formic acid was prepared. For mobile phase B, same solvent with different ratio was mixed which includes HPLC-grade methanol: acetonitrile: deionized water (80: 5: 15) with addition of 0.1% formic acid. All the solvents were prepared according to the ratio and filtered with 0.22 µm nylon filter (Pall Gelman, Sigma Aldrich, USA), followed by degassing of the solvent (10 minutes sonication).

Chromatographic Conditions. The HPLC analysis was carried out using Varian Prostar HPLC, Germany. The separation method in this analysis was carried out using Eclipse Plus C18, 5 µm particle size and 4.6 mm ×

150 mm diameter (Agilent, Germany). Ten microliters of standards and samples were injected for analysis. The gradient mobile phase was run at a flow rate of 1.2 mL/min (Table 1).

Table 1
Gradient method for HPLC analysis

Time (min)	Flow-rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
Pre-run	1.2	100	0
2	1.2	92	8
4	1.2	91	9
12	1.2	90	10
18	1.2	81	19
20	1.2	0	100
20	1.2	100	0
30	1.2	100	0

Cell Culture and Treatments

L929 cell line which was derived from mouse fibroblast adipose tissue was used in this study. Cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 0.1% (v/v) penicillin-streptomycin (PenStrep) (Thermo Fisher Scientific, USA). A stock concentration of *Nannochloropsis* sp. methanol extract (10, 000 ppm) was prepared and working concentrations were diluted to 0.16 – 5.0 mg/mL prior to use.

Cell Viability Assay

Cell viability was measured using the [3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetra zolium bromide (MTT) (Sigma Aldrich, USA) assay. L929 cells were seeded at 5000-10000 cells for each well in 96-well plate, cultured for 48 hours until it reached 80-90% confluency. The cells were then treated with *Nannochloropsis* sp. methanol extract concentrations ranging from 0.16 mg/mL to 5.0 mg/mL and incubated for 24 hours. Ten (10) microliters of MTT solution (5 mg/mL) were added to each well and the plate was incubated for 4 hours at 37°C incubator. The solution in each well was then aspirated and the formazan product was thoroughly dissolved using 100 µL of DMSO. The optical density (O. D.) of each well was measured at 570 nm wavelength using microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA). The experiment was carried out independently three times.

Cell proliferation was determined using the following formula:

$$\frac{O.D.\ treatment - O.D.blank}{O.D.untreated\ cell - O.D.\ blank} \times 100\% \quad [1]$$

DNA Damage Analysis

Analysis of DNA damage activity was determined by using comet assay kit following the manufacturer's instructions (Trevigen, USA). L929 cells were seeded in T75 cm^2 flask in DMEM complete growth medium overnight to allow for cell attachment. The growth medium was then replaced with medium prior treatment. For this assay, L929 cells were treated with 100 µM of pre-chilled H_2O_2 and incubated for 20 minutes. After incubation with H_2O_2, cells were treated with the extract (0.4 mg/

mL) and caffeic acid (20 µM) for 24 hours. Cells were treated with H_2O_2 and serum-free medium for positive and negative controls, respectively. After 24 hours of incubation, cells were harvested from T75 cm^2 flask by trypsinization and washed with PBS. Thirty microliters of cells (1×10^5 cells/mL) were counted and mixed with 300 µL (ratio of 1: 10, v/v) of molten Comet LMAgarose and incubated at 37°C. Thirty microliters of mixture were pipetted and layered onto an area of the CometSlide™. The slide was incubated at 4°C for 10 minutes in the dark to accelerate gelling of the agarose disc. Then, the slides were transferred and immersed into pre-chilled lysis solution for 30 minutes at 4°C. The excess buffer from the slides was drained and the slides were concomitantly immersed in freshly prepared alkaline unwinding solution (0.2 M NaOH, 1 mM EDTA) at room temperature for 20 minutes, in dark condition. The slides were then transferred into pre-chilled alkaline electrophoresis solution for 10 minutes prior electrophoresis. Electrophoresis was run for 30 minutes. Once completed, the excess electrophoresis solution was drained from the slides. The slides were gently immersed twice in distilled water of 5 minutes each before being immersed in 70% ethanol for 5 minutes and air dried. All the cells were observed in a single plane after completely dried and stained with SYBR safe staining solution before being observed by fluorescence microscopy with a 490 nm filter. Scoring of Comet assay was carried out using Image J (NIH, USA).

Statistical Analysis

All results were presented as mean ± standard deviation. One-way ANOVA was used to compare the differences between the treatment groups. All analyses were conducted using SPSS software version 22.

RESULTS

High Performance Liquid Chromatography (HPLC) Analysis

The constituents present in *Nannochloropsis* sp. methanol extract were characterized using HPLC and each retention time was compared with the standard compounds. The major compounds that matched the retention time of the standard compounds (with the accepted deviation of less than + 0.1 min) found in *Nannochloropsis* sp. methanol extract were caffeic acid, *p*-coumaric acid, naringenin and hesperitin (Table 2).

Table 2
Retention times of the standards and the compounds found in the extract

Peak number	Name of compound	Retention time (min) (standards)	Retention time (min) (extract)	Area (mAU.min)
1	caffeic acid	1.984	2.002	159.4
3	*p*-coumaric acid	2.747	2.774	375.8
5	naringenin	8.172	8.236	9.7
6	hesperetin	9.529	9.594	17.2

Analysis of DNA Damage Activity

Treatment concentration for *Nannochloropsis* sp. methanol extract was determined using cell viability assay. L929 cells were treated with *Nannochloropsis* sp. methanol extract at concentration ranging from 0.16 mg/mL to 5.0 mg/mL) and the IC_{20} (inhibitory concentration which inhibits 20% of cell population) was extrapolated based on the proliferation curve of the MTT assay on L929 cells (Figure 1). We had chosen the IC_{20} as at this concentration, the cells were least toxic. As shown in Figure 1, the IC_{20} of *Nannochloropsis* sp. methanol was determined at 0.4 mg/mL.

The effect of treatments (*Nannochloropsis* sp. extract and caffeic acid) on H_2O_2-induced DNA damage on L929 cells was shown in Figure 2. The untreated cells (without any treatment) were

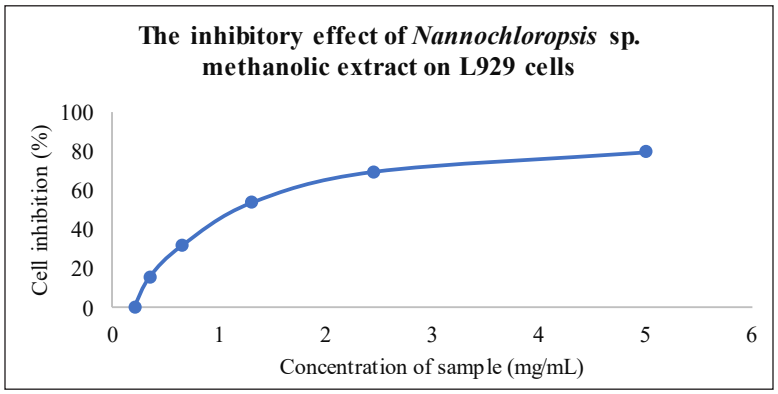

Figure 1. The effect of *Nannochloropsis* sp. methanol extract on the viability of L929 cell line and the IC_{20} value was determined after extrapolation from the graph

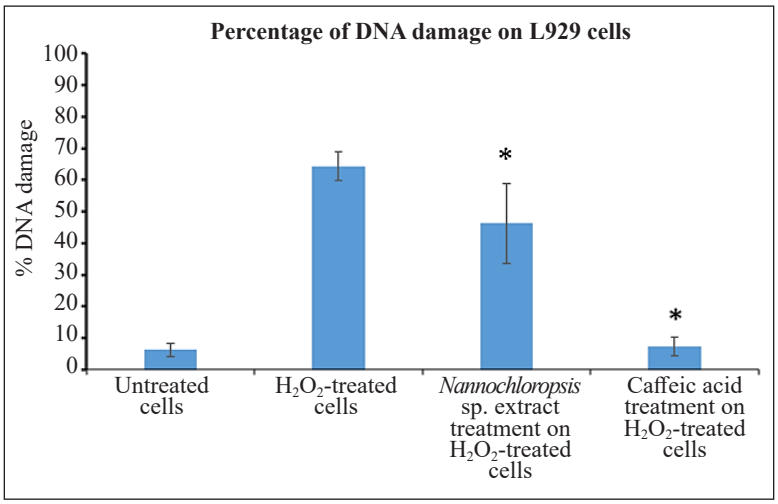

Figure 2. The effects of *Nannochloropsis* sp. methanol extract and caffeic acid treatments on hydrogen peroxide-induced DNA damage on L929 cells. * denotes significant difference when compared with H_2O_2-treated cells

used as a control. Treatment with 100 µM H_2O_2 had induced significant DNA damage (64.4 ± 4.6%, $p < 0.01$) in L929 cells when compared to the untreated cells, as measured by Comet assay. When H_2O_2 –treated cells were treated with the *Nannochloropsis* sp. methanol extract, the DNA damage induced by H_2O_2 was significantly reduced to about 46.3 ± 12.7% ($p < 0.01$). Treatment with a standard compound, caffeic acid at concentration of 20 µM on H_2O_2 –treated cells were able to further reduce the DNA damage to 7.3 ± 2.9% ($p < 0.01$).

DISCUSSION

Hydrogen peroxide induces oxidative stress and generates single and double strand breaks when the cells are exposed to it, causing oxidative DNA damage and decreased in cell viability (Driessens et al., 2009). DNA damage on the L929 cells was stimulated by the treatment with H_2O_2 at the concentration of 100 µM, which caused an average of 64.4% DNA damage when compared with the untreated cells. The concentration of H_2O_2 at 100 µM used in this study was shown to be sufficient in inducing the damage to the DNA in the normal cells, as previously shown in the other studies (Driessens et al., 2009; Luo et al., 2006; Miranda et al., 2008).

Our findings demonstrated that *Nannochloropsis* sp. methanolic extract at the concentration of 0.4 mg/mL was able to protect the DNA from H_2O_2-induced DNA damage for up to 21% in the L929 cells. We had postulated earlier that this preventive effect of *Nannochloropsis* sp. methanolic extract was attributed by the presence of phenolic antioxidant compounds. The high total phenolic content in the extract contributes to the high antioxidant capacity, measured by DPPH and ABTS assays in which we had published earlier (Zainoddin et al., 2018). Further analysis of the extract by HPLC showed that, based on the reference of retention time of the standards, there were 4 phenolic compounds identified such as caffeic acid, *p*-coumaric acid, naringenin and hesperitin (Table 2).

To demonstrate that these compounds, found in the extract might play an important role in the protection from H_2O_2-induced DNA damage, we had chosen one out of four compounds found, which was caffeic acid. Caffeic acid is commonly found in the microalgae species (Safafar et al., 2015) and it has been well-described to possess a strong antioxidant effect (Gülçin, 2006). Treatment with caffeic acid at concentration of 20 µM was able to markedly reduce the H_2O_2-induced DNA damage by 57.1%. The strong effect of caffeic acid in preventing the DNA damage in our study was not a surprise as caffeic acid has been shown previously to prevent DNA damage in human cell lines through the activation of ERK and lipoxygenase signalling pathways (Kang et al., 2006; Kim et al., 2016; Li et al., 2015). Activation of these pathways results in protective effects by decreasing in production of inflammatory cytokines and cytotoxicity (Kim et al., 2016), with an increase in cells differentiation and proliferation (Li et al., 2015).

Other important antioxidant compounds including *p*-coumaric acid, naringenin and hesperitin found in this study had been showed to protect the DNA damage from oxidative stress. We believed that these compounds may act synergistically to protect and prevent the cells from oxidative DNA damage induced by chemical and physical factors in various cells models such as in the eyes (Larrosa et al., 2008), brain (Ekinci-Akdemir et al., 2017), prostate (Gao et al., 2006) and liver (Thangavel et al., 2012). Their mechanism of actions involved various signalling pathways which protect the DNA damage such as inhibiting the activation xanthine oxidase activity (Larrosa et al., 2008), increasing the SOD and GSH enzymes (Ekinci-Akdemir et al., 2017) and enhance base excision repair, where the mutagenic process can be prevented (Gao et al., 2006).

Our study has shown that the methanolic extract of *Nannochloropsis* sp. contains phenolic compounds, which have been reported to be able to protect DNA damage in a wide range of cells and animals' models. The molecular mechanism exerted by the *Nannochloropsis* sp. methanol extract in protecting the normal cells from DNA damage and oxidative stress needs to be carried out in future study.

CONCLUSION

Methanolic extract of *Nannochloropsis* sp. contains many beneficial phenolic compounds such as caffeic acid, *p*-coumaric acid, naringenin and hesperitin. The presence of these compounds in the extract may protect cell from oxidative DNA damage and thus prevent genetic mutation and cancer formation.

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