



## Abiotic stresses induce total phenolic, total flavonoid and antioxidant properties in Malaysian indigenous microalgae and cyanobacterium

Nur Husna Azim<sup>1</sup>, Atiqah Subki<sup>1</sup>, Zetty Norhana Balia Yusof<sup>1,2\*</sup>

<sup>1</sup>Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

<sup>2</sup>Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

Email: [zettynorhana@upm.edu.my](mailto:zettynorhana@upm.edu.my)

Received 2 February 2017; Received in revised form 14 June 2017; Accepted 20 June 2017

### ABSTRACT

**Aims:** The use of microalgae as source of natural antioxidants is under explored in Malaysia. Previous studies have shown that microalgae contain minerals, polysaccharides, amino derivatives, carotenoids and phenolic compounds. This study aimed to determine total phenolic and flavonoid compounds and antioxidant activity when microalgae (*Nannochloropsis oculata* and *Tetraselmis* sp.) and cyanobacterium (*Anabaena* sp.) were subjected to abiotic stresses.

**Methodology and results:** Treatment of sodium chloride (NaCl), sodium hypochlorite (NaOCl) and copper (Cu<sup>2+</sup>) were given when the cultures reached the exponential phase of growth and were collected at three different time points. Non-treated cultures were used as controls. Total phenolic and flavonoid contents were determined using Folin-Ciocalteu phenol reagent and aluminium chloride colorimetric assays. Antioxidant activities were measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay. *Tetraselmis* sp. exhibited the highest phenolic content under copper stress (10.35 ± 0.33 µg GAE/mg extract). *Nannochloropsis oculata* showed the highest total flavonoid content under copper stress (33.85 ± 3.16 µg QE/mg extract). *Anabaena* sp. showed the highest radical scavenging activity under NaOCl stress (96.42 ± 0.26%).

**Conclusion, significance and impact of study:** This study showed that total phenolic, flavonoid and antioxidant activities in treated cultures were high compared to non-treated cultures. These microorganisms could be utilized as a source of useful bioactive compounds while exploiting its abundance.

**Keywords:** microalgae, cyanobacteria, antioxidant, stress

### INTRODUCTION

Antioxidants are molecules that inhibit free radicals that are produced from the oxidation of other molecules. Most foods and pharmaceutical industries use synthetic antioxidants to prolong product shelf life and to enhance the stability of therapeutic agents. Synthetic antioxidants are widely used as it is cheaper than natural antioxidants. The two most common synthetic antioxidants used today are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). However, recent studies have shown that these synthetic antioxidants are suspected to be carcinogenic (Maadane *et al.*, 2015) and will consequently result in DNA damage (Abd El-Aty *et al.*, 2014).

Primary source for natural antioxidants are plants, but the production of antioxidants from plants are costly and time consuming. Recently, interests in the utilization of microalgae have increased due to their high secondary metabolites activities and also because they have been

recognized as a new source of antioxidants apart from plants (Maadane *et al.*, 2015). Microalgae are photosynthetic unicellular microorganism that can be cultivated in a large-scale system at fast pace. Recent study by Klejdus *et al.* (2010) showed that several classes of flavonoids can be found in microalgae and cyanobacteria and they showed capabilities in inhibiting lipid peroxidation. Studies by Klejdus *et al.* (2010) and Kováčik *et al.* (2010) showed that a variety of phenolic compounds are present in microalgae. Natrah *et al.* (2007) also revealed that some microalgae such as *Isochrysis galbana* and *Chaetoceros calcitrans* have the potential to be utilized as natural sources of antioxidants with high nutritional value.

Interestingly, the adaptability towards stress in algae was found to be associated with accumulation of several organic solutes which includes phenolic, flavonoid and antioxidant compounds (Hasegawa *et al.*, 2000). This may

\*Corresponding author

be due to the increase in production of reactive oxygen species (ROS) radical molecules under stress conditions which leads to oxidative damage (Kreslavski *et al.*, 2007). The growing interest in the search of natural antioxidants to replace the synthetics is currently ongoing. Nevertheless, the exploration of antioxidant compounds from microalgae is lacking in Malaysia. This project was carried out in order to determine the amount of phenolic and flavonoid compounds in microalgae (*N. oculata* and *Tetraselmis* sp.) and cyanobacterium (*Anabaena* sp.) and the effects of abiotic stresses towards the antioxidant activities in these organisms.

## MATERIALS AND METHODS

### Microalgae samples

Cyanobacterium *Anabaena* sp. was kindly given by Dr. Japareng Lalung from Universiti Sains Malaysia (USM), Penang while microalgae (*N. oculata* and *Tetraselmis* sp.) were obtained from Centre of Marine Science (COMAS), Universiti Putra Malaysia (UPM), Port Dickson. *Anabaena* sp. was grown in 250 mL Erlenmeyer flask that was placed on an open orbital shaker at 29±2 °C and continuously shaken at 150 rpm for 24 h under 50 µmol photons m<sup>-2</sup>s<sup>-1</sup> illumination. The medium used was modified BG-11 at pH 7.2.

*N. oculata* and *Tetraselmis* sp. were grown in 1000 mL Erlenmeyer flask at 26±2 °C in an incubator shaker at Agro-Biotechnology Institute (ABI), Selangor. Both species were grown under 50 µmol photons m<sup>-2</sup>s<sup>-1</sup> illumination for 24 h and continuously shaken at 150 rpm. The medium used was TAP/2 seawater at pH 7.1.

### Treatment application

Treatments were applied when the growth of the organisms reached exponential phase (OD<sub>1000 nm</sub> = 0.8). The experiments were carried out by supplementing the media with increasing concentrations of stress inducers namely, copper [(Cu<sup>2+</sup>) (0.5, 2 and 5 ppm)], sodium hypochlorite [(NaOCl) (0.5, 10 and 5 µM)] and sodium chloride [(NaCl) (50, 150 and 250 mM)]. Samples were collected at day 0 (D<sub>0</sub>), day 3 (D<sub>3</sub>) and day 6 (D<sub>6</sub>) to see the effects immediately after the application and the next few days up to day 6 which were predicted to be the points where the full effects have taken in.

### Preparation of sample extracts

Crude extraction was carried out using HPLC grade methanol (Merck, Germany) as an extraction solvent. A volume of 200 mL of each culture was centrifuged at 8000 x g for 30 min at 15 °C. The supernatant was discarded while the pellet was retrieved and dried at 60 °C for 2 h. The dried pellet was weighed and mixed with methanol (1:40, w/v). Extractions were performed overnight with continuous stirring. The supernatant was recovered after centrifugation of the mixture at 8000 x g for 30 min at 15 °C by filtering the supernatant through Whatman filter

paper. The supernatant was dried using rotary evaporator. The dried extracts were resuspended with 1 mL methanol to a specific final concentration and stored at -20 °C until further use (Guedes *et al.*, 2011).

### Total phenolic content assays

Total phenolic content was determined using spectrophotometric method by Slinkard and Singleton (1977). The extracts were prepared with a concentration of 100 µg/mL. A volume of 0.5 mL of each extract was added with distilled water to up to 3 mL and 0.5 mL of Folin-Ciocalteu's phenol reagent. After 5 min, 2 mL of 2 % sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) solution was added and left for 60 min in the dark before the absorbance was taken. The calibration curve was extrapolated to determine the total phenolic content using gallic acid solution (10-70 µM) and the results were expressed in gallic acid equivalents per mg of extracts, GAE/mg.

### Total flavonoid content assays

Total flavonoid content was determined by a calorimetric method (Zishen *et al.*, 1999). Approximately 0.5 mL of each extract with the concentration of 100 µg/mL was added with 1 mL 100 % methanol to make up to 3 mL. The mixture was left for 5 min after the addition of 0.4 mL distilled water and 0.3 mL of 5 % sodium nitrite (NaNO<sub>2</sub>). Approximately 2 mL of 1 M sodium hydroxide (NaOH) and distilled water was added to make up to 10 mL after 0.3 mL of 10% aluminium chloride (AlCl<sub>3</sub>) was added and left at room temperature for 1 min. The mixture was left for 15 min after being shaken. The absorbance was measured at 510 nm and the concentrations of total flavonoids were determined as quercetin equivalents per mg of extracts, QE/mg.

### Antioxidant assay

The antioxidant activity (AA %) of samples were assessed by DPPH free radical assay according to method described by Blois (1958). The samples were prepared with a concentration of 100 µg/mL. The reaction mixture consisted of 3 mL of sample and 1 mL of DPPH radical solution. The changes in colour (from deep violet to light yellow) were measured at 517 nm after 60 min of reaction. The capacity of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was calculated using following formula:

$$DPPH \text{ scavenging effect (\%)} = \frac{(ADPPH - AS)}{ADPPH} \times 100$$

ADPPH is the absorbance of the DPPH solution without any addition of extracts while, AS is the absorbance of the DPPH solution when the sample extract was added.

**Statistical analysis**

Collected data were analysed using analysis of variance or t-test procedure. Significant differences between treatments were tested using Duncan's multiple range test (DMRT). Statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS) 10.0.

**RESULTS**

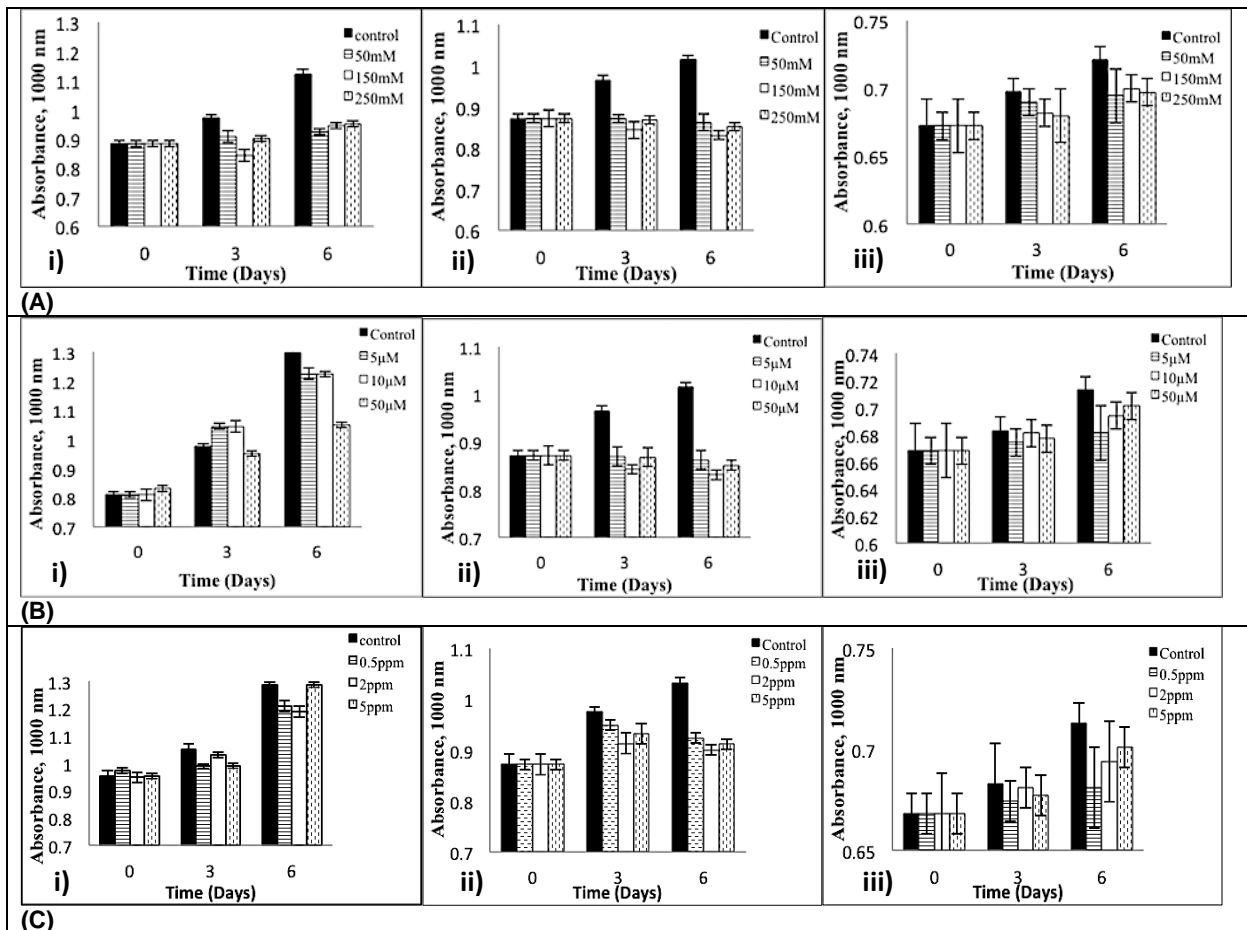
**The effect of NaCl, NaOCl and Cu<sup>2+</sup> on the growth of *N. oculata*, *Tetraselmis* sp. and *Anabaena* sp.**

Effects of abiotic stresses (NaCl, NaOCl and Cu<sup>2+</sup>) on the growth of *N. oculata*, *Tetraselmis* sp. and *Anabaena* sp. are shown in Figure 1. Growth of *N. oculata* and *Anabaena* sp. after NaCl treatment increased (Figure 1A) with time but not more than control and high at 250 mM (OD<sub>1000nm</sub>= 0.951) and 150 mM (OD<sub>1000nm</sub>= 0.701) respectively. Growth of treated *Tetraselmis* sp. was

decreased while control was increased with time. Higher concentration of the treatment inhibited the growth of most of the cultures at D<sub>6</sub> (OD<sub>1000nm</sub>= 0.8309).

As for NaOCl treatment (Figure 1B), growth of *N. oculata* and *Anabaena* sp. after treatment were increased with time but not more than control and high at 10 µM (OD<sub>1000nm</sub>= 1.224) and 50 µM (OD<sub>1000nm</sub>= 0.701) respectively. Growth of treated *Tetraselmis* sp. was decreased while control was increased with time and inhibited the most at 10 µM (OD<sub>1000nm</sub>= 0.8309) respectively.

Figure 1C shows the growth of *N. oculata* and *Anabaena* sp. after treatment was increased over time but not more than control and high at 5 ppm (OD<sub>1000nm</sub>= 1.287, 0.701) respectively. Growth of treated *Tetraselmis* sp. was decreased while control (OD<sub>1000nm</sub>= 0.853) was increased with time and inhibited the most at 2 ppm (OD<sub>1000nm</sub>= 1.015) respectively.



**Figure 1:** Growth before and after treatment of (A) NaCl, (B) NaOCl and (C) Cu<sup>2+</sup> in microalgae i) *N. oculata* ii) *Tetraselmis* sp. iii) *Anabaena* sp. at different time points. The growth was monitored by measuring optical density at 1000 nm. Each value in the graph is represented as mean ± SD (n=3).

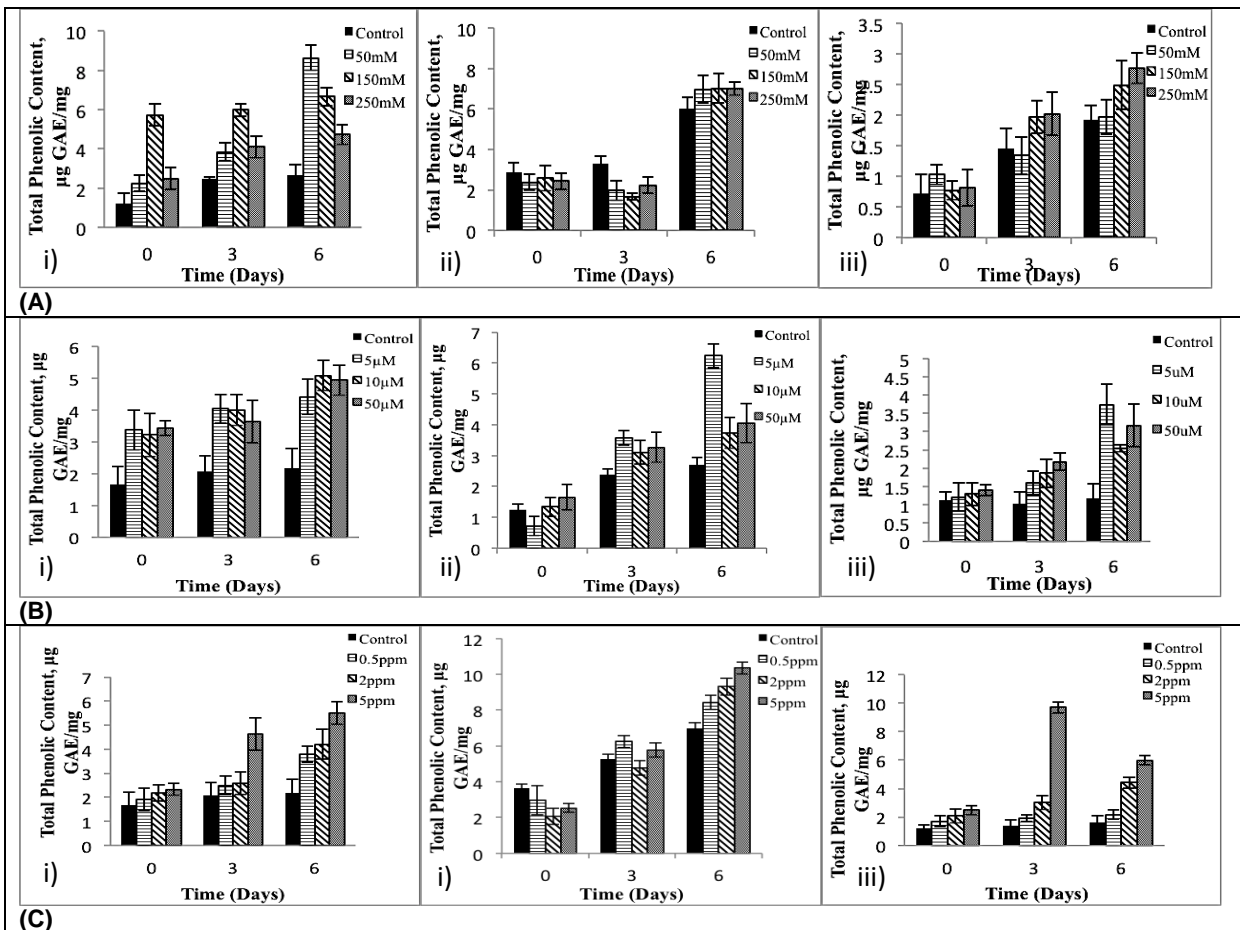
**The effect of NaCl, NaOCl And Cu<sup>2+</sup> on the total phenolic content of *N. oculata*, *Tetraselmis sp.* and *Anabaena sp.***

The results of the effect of abiotic stresses (NaCl, NaOCl and Cu<sup>2+</sup>) on total phenolic content in *N. oculata*, *Tetraselmis sp.* and *Anabaena sp.* are shown in Figure 2. In NaCl treatment (Figure 2A), the total phenolic content in all treated cultures were increased compared to non-treated cultures at all time points and all species exhibited the highest phenolic content at D<sub>6</sub>. The highest amount of phenolic compounds in treated *N. oculata* was at 50 mM (8.64 ± 0.6 µg GAE/mg extract) as shown in Figure 2A (i). The highest amount of phenolic compounds in treated *Tetraselmis sp.* and *Anabaena sp.* were at 250 mM (7.02 ± 0.33, 2.77 ± 0.55 µg GAE/mg extract).

Total phenolic content in all treated cultures were also increased compared to non-treated cultures for NaOCl treatment (Figure 2B) and exhibited the highest phenolic

content at D<sub>6</sub> for all species. The highest amount of phenolic compounds in NaOCl treated *N. oculata* was at 10 µM (5.09 ± 0.68 µg GAE/mg extract). The highest amount of phenolic compounds in treated *Tetraselmis sp.* and *Anabaena sp.* were at 5 µM of NaOCl (6.24 ± 0.39, 3.74 ± 0.55 µg GAE/mg extract).

Finally, for the Cu<sup>2+</sup> treatment (Figure 2C), total phenolic content in all treated cultures were increased compared to non-treated cultures at each time point and exhibited the highest phenolic content at 5 ppm of Cu<sup>2+</sup>. The highest amount of phenolic content in Cu<sup>2+</sup> treated *N. oculata* and *Tetraselmis sp.* was at D<sub>6</sub> (5.51 ± 0.68, 10.35 ± 0.33 µg GAE/mg extract) while the highest phenolic content in Cu<sup>2+</sup> treated *Anabaena sp.* was at day 3 (5.98 ± 0.33, µg GAE/mg extract). All in all, among the three species, the highest amount of phenolic compounds was seen to be produced by *Tetraselmis sp.* when induced with 5 ppm Cu<sup>2+</sup> [Figure 2C (ii)].



**Figure 2:** Total phenolic content before and after treatment of (A) NaCl, (B) NaOCl and (C) Cu<sup>2+</sup> in i) *N. oculata* ii) *Tetraselmis sp.* iii) *Anabaena sp.* at different time points. All treated and non-treated cultures initial concentration for the assay was 100 µg/mL. Each value in the graph is represented as mean ± SD (n=3).

**The effect of NaCl, NaOCl and Cu<sup>2+</sup> on the total flavonoid content of *N. oculata*, *Tetraselmis* sp. and *Anabaena* sp.**

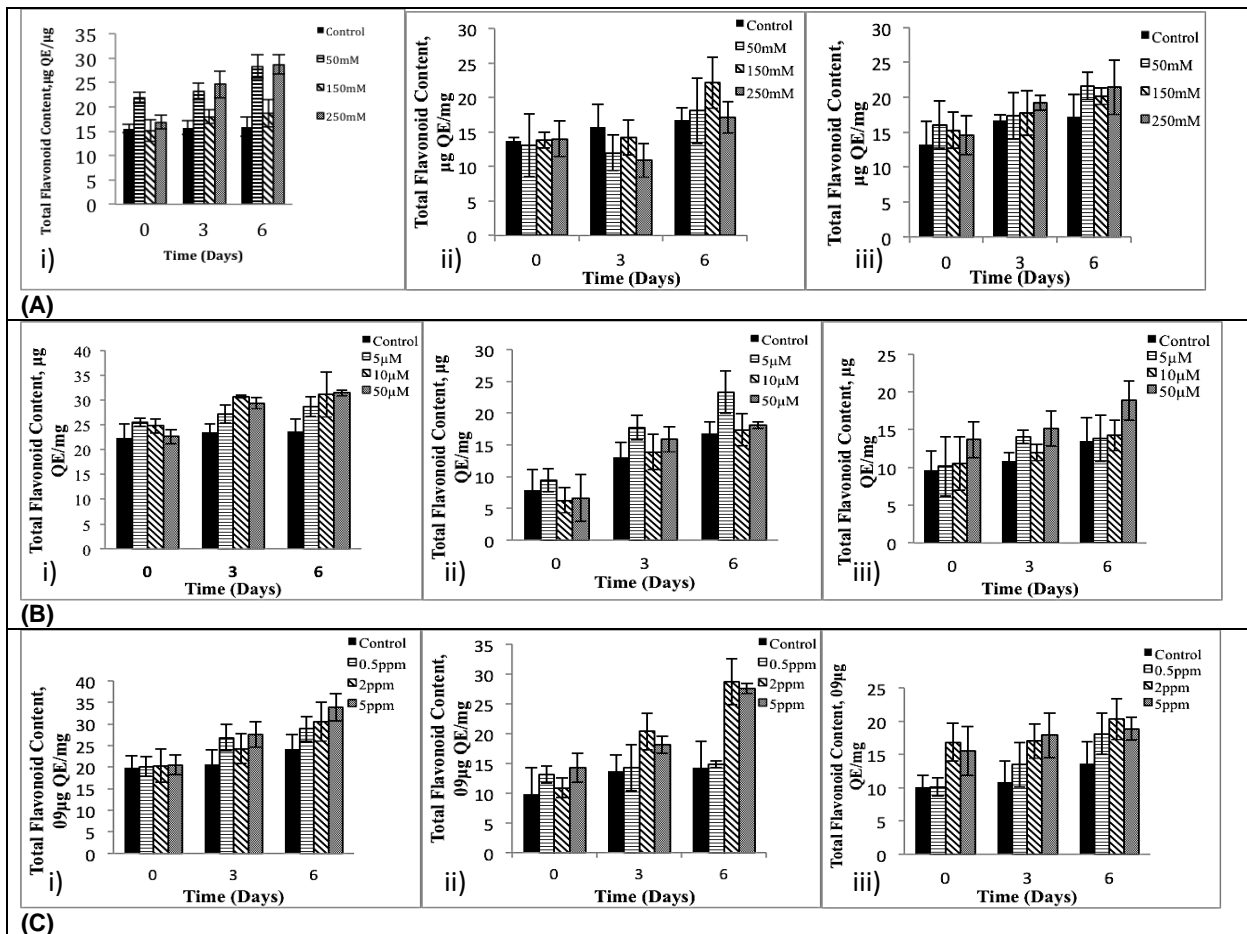
The results of the effect of abiotic stresses (NaCl, NaOCl and Cu<sup>2+</sup>) on total flavonoid content of *N. oculata*, *Tetraselmis* sp. and *Anabaena* sp. are shown in Figure 3. Total flavonoid content in all NaCl treated cultures were increased compared to non-treated cultures at each time point and exhibited the highest amount of flavonoid compounds at D<sub>6</sub> for all species (Figure 3A). The highest amount of flavonoid compounds in NaCl treated *N. oculata* and *Anabaena* sp. were at 250 mM (28.67 ± 2 and 21.63 ± 3.89 µg QE/mg extract). The highest amount of flavonoid compounds in treated *Tetraselmis* sp. was observed at 150 mM NaCl (22.17 ± 3.70 µg QE/mg extract).

In NaOCl treatment (Figure 3B), the highest amount of flavonoid compounds in *N. oculata* and *Anabaena* sp.

were at 50 µM (31.54 ± 0.56, 18.85 ± 2.63 µg QE/mg extract). On the other hand, the highest flavonoid content accumulated in treated *Tetraselmis* sp. was at 5 µM NaOCl (23.29 ± 2.63 µg QE/mg extract) (Figure 3B (ii)).

Based on Figure 3C, the total flavonoid content in all Cu<sup>2+</sup> treated cultures were increased compared to non-treated cultures at each time point and exhibited the highest flavonoid content at D<sub>6</sub> for all species. The highest flavonoid content observed in treated *N. oculata* was at 5 ppm Cu<sup>2+</sup> (33.85 ± 3.16 µg QE/mg extract) while *Tetraselmis* sp. and *Anabaena* sp. were at 2 ppm Cu<sup>2+</sup> (28.67 ± 3.59, 20.33 ± 3.09 µg QE/mg extract).

*N. oculata* was seen to produce the most number of flavonoid compounds when induced with all three types of stresses and the highest flavonoid content was induced by Cu<sup>2+</sup> [Figure 3C (i)].



**Figure 3:** Total flavonoid content before and after treatment of (A) NaCl, (B) NaOCl and (C) Cu<sup>2+</sup> in i) *N. oculata* ii) *Tetraselmis* sp. iii) *Anabaena* sp. at different time points. All samples and control concentrations were 100 µg/mL. Each value in the graph is represented as mean ± SD (n=3).

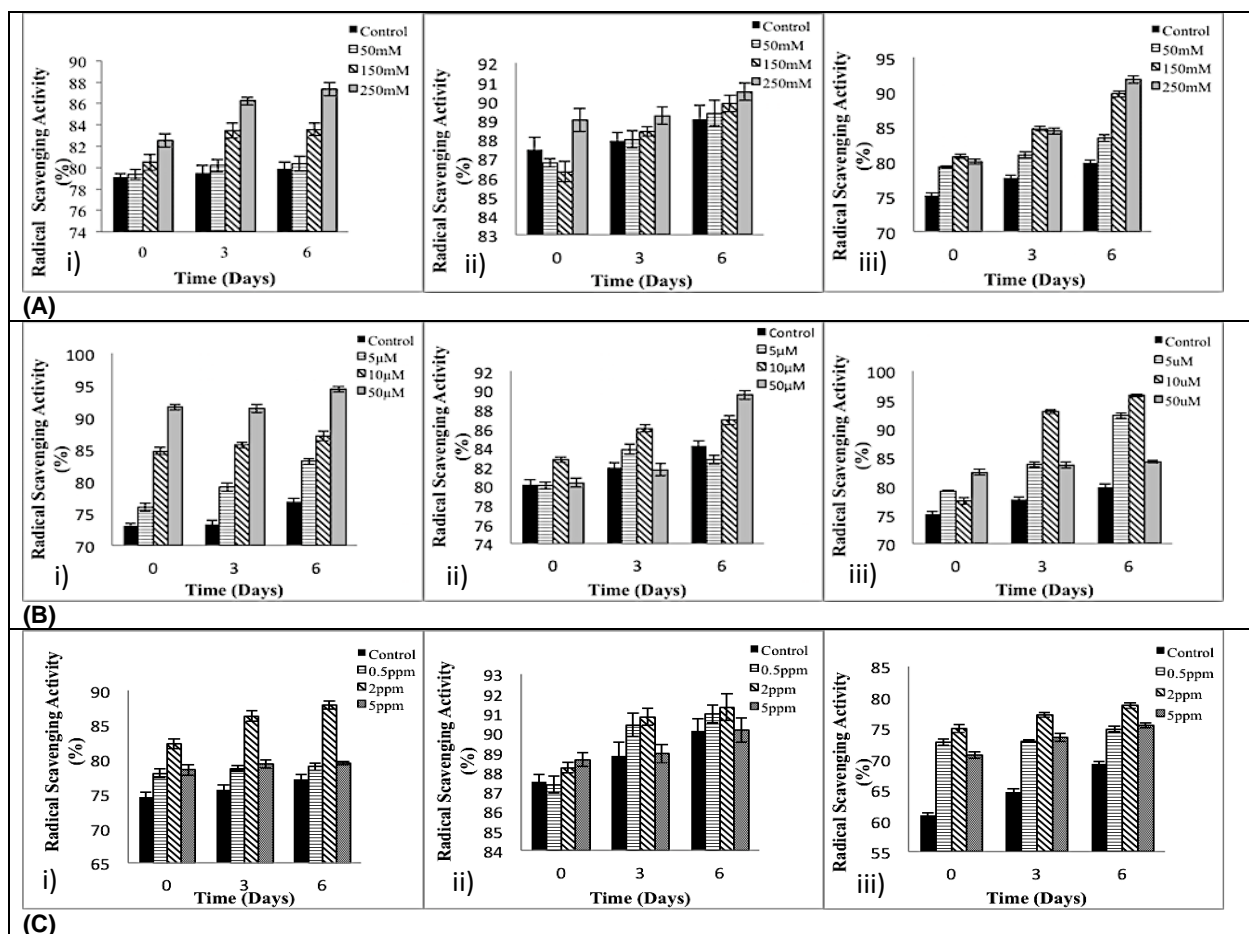
**The effect of NaCl, NaOCl and Cu<sup>2+</sup> on the scavenging activity of *N. oculata*, *Tetraselmis* sp. and *Anabaena* sp.**

The radical scavenging activities in all NaCl treated cultures were increased compared to non-treated cultures at each time point and the most activity was at D<sub>6</sub> for all species as shown in Figure 4A. The highest antioxidant activity in treated *N. oculata*, *Tetraselmis* sp. and *Anabaena* sp. were 87.95 ± 0.62, 85.96 ± 0.46, 93.07 ± 0.42 % inhibition of DPPH radical at 250 mM respectively.

Radical scavenging activities in all NaOCl treated cultures (Figure 4B) were also increased compared to non-treated cultures at each time point and the most activity was at D<sub>6</sub> for all species. The highest antioxidant

activity in treated *N. oculata* and *Tetraselmis* sp. with 90.41 ± 0.44, 91.10 ± 0.44% of inhibition of DPPH radical at 50 μM respectively while in treated *Anabaena* sp. was at 10 μM with 96.42 ± 0.46% of inhibition of DPPH radical respectively.

Last but not least, radical scavenging activities in all Cu<sup>2+</sup> treated cultures (Figure 4C) showed an increase compared to non-treated cultures at each time point and the most activity was at D<sub>6</sub> for all species. The highest antioxidant activity for all three species was recorded at 2 ppm Cu<sup>2+</sup> treatment with 89.67 ± 0.61, 87.18 ± 0.69, 78.68 ± 0.62 % of inhibition for *N. oculata*, *Tetraselmis* sp. and *Anabaena* sp. respectively.



**Figure 4:** Radical scavenging activity before and after treatment of (A) NaCl, (B) NaOCl and (C) Cu<sup>2+</sup> in i) *N. oculata* ii) *Tetraselmis* sp. iii) *Anabaena* sp. at different time points. All samples and control concentrations were 100 μg/mL. Control is DPPH in methanol only. Radical scavenging activity was calculated after subtracting absorbance of samples from that of control. Each value in the graph is represented as mean ± SD (n=3).

## DISCUSSION

This project was carried out in order to determine the amount of phenolic and flavonoid compounds as well as antioxidant activities in microalgae (*N. oculata* and *Tetraselmis* sp.) and cyanobacterium (*Anabaena* sp.) under normal and under abiotic stress conditions. Cultures were treated with various concentrations of NaCl (salinity/osmotic stress), copper ion ( $\text{Cu}^{2+}$ ) and NaOCl (oxidative stress). Results from this study have shown to be in agreement with a study reported by Hasegawa *et al.* (2000) where antioxidant compounds were found to be associated as an adaptability strategy against stress conditions.

According to Gu *et al.* (2012) *N. oculata* growth was increased when treated with NaCl during 19 days of cultivation. Srivastava *et al.* (2005) stated that *Anabaena* sp. growth treated with 150 mM NaCl grew faster than control. In contrast, growth of treated *Tetraselmis* sp. was decreased over time for each treatment. Kirst (1990) explained that microalgae might lose dry biomass productivity or reduction in growth because they will expend energy while attempting to maintain turgor pressure under high salinity. Under oxidative stress, Galindo *et al.* (2010) reported that NaOCl have caused growth of microalgae *Dunaliella salina* to be inhibited after 24 h. This previous work correlates with the growth of NaOCl treated *Tetraselmis* sp. in this study. Apart from that, growth of green microalgae was shown to be inhibited due to  $\text{Cu}^{2+}$  accumulation and toxicity by Anurag *et al.* (2014) but growth of treated *N. oculata* and *Anabaena* sp. in this study did not show the same trend. This might be due to the adaption of the organisms to  $\text{Cu}^{2+}$  stress.

In this study, *N. oculata*, *Tetraselmis* sp. and *Anabaena* sp. were extracted using 100% methanol as it was reported as the most efficient solvent for phenolic extraction and radical scavenging compounds in microalgae (Abd El-Aty *et al.*, 2014). Total phenolic content was determined using Folin-Ciocalteu reagent to measure the total concentration of phenolic hydroxyl groups. Polyphenols react with sodium carbonate form a blue complex that can be read by visible-light spectrophotometry at 650 nm (Slinkard and Singleton 1977). This study explored total phenolic content in microalgae and cyanobacterium culture when treated with three different stresses. The first stress was NaCl to mimic salinity stress. *N. oculata* and *Tetraselmis* sp. and *Anabaena* sp. can be found both in fresh and marine water system. When water level drops, salinity will increase thus will cause the microalgae and cyanobacterium to experience stress. Total phenolic content in NaCl treated microalgae and cyanobacterium was increased proportionally with time and all were higher consistently than control. Kawasaki *et al.* (2001) and Ozturk *et al.* (2002) reported that salinity stress can cause a series of changes in photorespiration, photosynthesis, osmotic adjustment, amino acid and carbohydrate synthesis. Previous studies have also proven that phenolic compounds accumulate under salt stress

conditions followed by enhanced antioxidative activity in *Plectonema boryanum*, *Hapalosiphon intricatus*, *Anabaena doliolum* and *Oscillatoria acuta* (Singh *et al.*, 2014). Biosynthesis of phenolic compounds come from carbohydrate metabolism before they enter shikimate pathway and form phenylalanine and phenolic acid. The increase in carbohydrate content will increase total phenolic content. This was proved by Kirrolia *et al.* (2011) that reported carbohydrate content in microalgae *Scenedesmus quadricauda* was increased in all NaCl treatment as compared to control. Singh *et al.* (2014) also reported that salt stress induces the accumulation of phenylpropanoid and antioxidant activity in cyanobacteria and microalgae. However total phenolic content in treated *Anabaena* sp. showed no significant difference. This study showed that phenolic contents in NaOCl treated cultures were increased. Research done by Markou *et al.* (2012) showed decrease in phenol concentration and turbidity in *A. platensis* after NaOCl treatment. This might be due to concentration of NaOCl (5  $\mu\text{M}$ , 10  $\mu\text{M}$  and 50  $\mu\text{M}$ ) used in this treatment was not enough to cause any decrease in phenolic content and turbidity. However, according to Ip and Chen (2005) *Chlorella zofingiensis* treated with 0.0001 mM of NaOCl gave high chlorophyll and carotenoid content.

Flavonoid content was determined using calorimetric method. Aluminium chloride forms acid stable complexes with the C-4 keto groups and either hydroxyl group of flavones and flavonols, orthodihydroxyl groups in the A- or B-ring of flavonoids which can be detected at wavelength 510 nm (Kalita *et al.*, 2013). This research showed that total flavonoid content in treated cultures is higher than control at each time point. It is because flavonoids are the largest group of naturally occurring phenolic compounds such as flavonols, flavones, anthocyanidins, flavans and flavonols (Ohshima *et al.*, 1998). *N. oculata* contains higher concentration of flavonoids compared to *Tetraselmis* sp. and *Anabaena* sp. Previous study by Barreiro *et al.* (2016) stated that accumulation of polyphenol was higher than control under copper stress in *Dunaliella tertiolecta*.

There are numerous methods used to evaluate antioxidant activity of natural compounds with varying results. 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical that is usually used to measure antioxidant activity. DPPH is reduced by antioxidant and can be measured at 510 nm (Abd El-Aty *et al.*, 2014). Discoloration of DPPH showed that free radical scavenging potentials of the sample by their hydrogen donating ability (Pallab *et al.*, 2013). When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced (Kalita *et al.*, 2013). Goiris *et al.* (2012) reported that both carotenoid and phenolic content significantly contributed to the antioxidant capacity of microalgae. However, according to Hajimahmoodi *et al.* (2009), there was a positive correlation between antioxidant capacity and phenolic content only in FRAP (Ferric Reducing Antioxidant Potential) assay and not in 2,2-diphenyl-1-picrylhydrazyl High performance liquid chromatography (DPPH-HPLC). Maadane *et al.* (2015) stated that phenolic and

carotenoids compounds might not be the major contributors to the antioxidant capacities of these microalgae. Winter *et al.* (2008) reported that NaOCl will form hypochlorous acid (HOCl) when reacted with water which will interrupt protein folding and inhibit glucose oxidation thus cause biochemical damages to exposed organisms. The biocidal effect of NaOCl involves the action of free radicals generated which lead to oxidative stress (Leichert *et al.*, 2008) and lipid peroxidation (Ebenezer and Ki 2014). Thus, NaOCl is suitable as oxidative stress inducer to generate ROS by increasing the percentage of radical scavenging activity in all treated cultures. Manivannan *et al.* (2012) stated that antioxidant activity of methanolic extract in the presence of copper is higher than control. Srivastava *et al.* (2005) hypothesized that anti-oxidative defence system is stimulated in *A. doliolum* by copper. This study has showed that total phenolic and flavonoid contents in treated cultures were increased compared to non-treated cultures. This proved that abiotic stress induces the accumulation of phenolic and flavonoid contents thus increases free radical scavenging activity in microalgae and cyanobacterium.

Percentage of increase in *N. oculata* that showed highest total phenolic content under treatment with NaCl was 226 % ( $8.64 \pm 0.6 \mu\text{g GAE/mg extract}$ ). Percentage of increase in the highest total flavonoid content under NaCl, NaOCl and  $\text{Cu}^{2+}$  stresses were 78, 32 and 39 % ( $28.67 \pm 2$ ,  $31.4 \pm 0.56$  and  $33.85 \pm 3.16 \mu\text{g QE/mg extract}$ ) respectively. Percentage of increase in *Tetraselmis* sp. that exhibit high phenolic content under NaCl and  $\text{Cu}^{2+}$  stresses were 15% and 48% ( $7.02 \pm 0.39$ ,  $10.35 \pm 0.33 \mu\text{g GAE/mg extract}$ ) respectively. Percentage of increase in *Tetraselmis* sp. that exhibit high percentage of radical scavenging activity under  $\text{Cu}^{2+}$  treatment was 72% ( $91.31 \pm 0.69\%$ ). Percentage of increase in *Anabaena* sp. that gave high percentage of radical scavenging activity under NaCl and NaOCl stresses were 12 and 16% ( $91.8 \pm 0.52$  and  $96.42 \pm 0.26\%$ ).

## CONCLUSION

As a conclusion, high phenolic and flavonoid content and increased percentage of free radical scavenging activities were observed after induction of stress in local microalgae and cyanobacterium. *Tetraselmis* sp. and *Anabaena* sp. showed that they have the potential to be developed as a natural source of these useful compounds. However, further studies should be carried out to identify and quantify these bioactive compounds alongside looking at the best stress inducer for further studies. It is hoped that this study will contribute to the search for a natural source of useful bioactive compounds.

## ACKNOWLEDGEMENT

This work was carried out with the support of "Geran Putra Universiti Putra Malaysia (Project No.: 9425900)", Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

## REFERENCES

- Abd El-Aty, A. Mohamed, A. A. and Samhan, F. (2014). *In vitro* antioxidant and antibacterial activities of two fresh water cyanobacterial species, *Oscillatoria agardhii* and *Anabaena sphaerica*. *Journal Application Pharmacy Science* **4**, 69-75.
- Anurag, T. and Bafila, G. (2014). Copper stress on cellular contents and fatty acid profiles in *Chlorella* species. *Journal of Biological Sciences* **14**, 209-217.
- Barreiro, D., Prins, W. and Ronsse, F. (2016). Hydrothermal liquefaction (HTL) of microalgae for biofuel production. *Journal of Biomass and Bioenergy* **53**, 113-127.
- Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature* **26**, 1199-1200.
- Ebenezzer, V. and Ki, J. (2014). Biocide sodium hypochlorite decreases pigment production and induces oxidative damage in the harmful *dinoflagellate cochlodinium polykrikoides*. *Journal of Algae* **29**, 311-319.
- Galindo, C., Garrido, M. C., Casanueva, J. F. and Nebot, E. (2010). Degradation models and ecotoxicity in marine waters of two antifouling compounds: Sodium hypochlorite and an alkylamine surfactant. *Science of the Total Environment* **408**, 1779-1785.
- Goiris, K., Muylaert, K., Fraeye, I., Foubert, I., De Brabanter, J. and De Cooman, L. (2012). Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. *Journal Application Phycology* **24**, 1477-1486.
- Gu, N., Lin, Q., Li, G., Tan, Y., Huang, L. and Lin, J. (2012). Effect of salinity on growth, biochemical composition and lipid productivity of *Nannochloropsis oculata* CS 179. *Engineering in Life Sciences* **12**, 631-637.
- Guedes, A. C., Amaro, H. M., Pereira, R. D. and Malcata, F. X. (2011). Effects of temperature and pH on growth and antioxidant content of the microalga *Scenedesmus obliquus*. *Biotechnology Progress* **27**, 1218-1224.
- Hajimahmoodi, M., Faramarzi, M. A., Mohammadi, N., Soltani, N., Oveisi, M. R. and Nafissi Varcheh, N. (2009). Evaluation of antioxidant properties and total phenolic contents of some strains of microalgae. *Journal Application Phycology* **22**, 43-50.
- Hasegawa, P. M., Bressan, R. A., Zhu, J. K. and Bohnert, H. J. (2000). Plant cellular and molecular response to high salinity. *Plant Molecular Biology* **51**, 463- 499.
- Ip, P. and Chen, F (2005). Employment of reactive oxygen species to enhance astaxanthin formation in *Chlorella zofingiensis* in heterotrophic culture. *Journal of Process Biochemistry* **40**, 3491-3496.
- Kalita, P., Barman, T. K., Tapas, P. K. and Ramen, K. (2013). Estimation of total flavonoids content (tfc) and antioxidant activities of methanolic whole plant extract of *biophytum sensitivum* linn. *Journal of Drug Delivery and Therapeutics* **3**, 33-37.



- Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D. and Bohnert, H. J. (2001).** Gene expression profiles during the initial phase of salt stress in rice. *Journal of Plant Cell* **13**, 889–905.
- Kirrolia, A., Bishnoi, N. and Singh, N. (2011).** Salinity as a factor affecting the physiological and biochemical traits of *Scenedesmus quadricauda*. *Journal of Algal Biomass Utilization* **2**, 28-34.
- Kirst, G. O. (1990).** Salinity tolerance of eukaryotic marine algae. *Annual Review of Plant Physiology and Plant Molecular Biology* **41**, 21-53.
- Klejdus, B., Lojkova, L., Plaza, M., Šnoblova, M. and Štěrbová, D. (2010).** Hyphenated technique for the extraction and determination of isoflavones in algae: Ultrasoundassisted supercritical fluid extraction followed by fast chromatography with tandem mass spectrometry. *Journal of Chromatography* **1217**, 7956-7965.
- Kováčik, J., Klejdus, B., Bačkor, M. (2010).** Physiological Responses of *Scenedesmus quadricauda* (Chlorophyceae) to UV-A and UV-C Light. *Photochemistry and Photobiology* **86**, 612-616.
- Kreslavski, V. D., Carpentier, R., Klimov, V. V., Murata, N. and Allakhverdiev, S. I. (2007).** Molecular mechanisms of stress resistance of the photosynthetic apparatus. *Membrane and Cell Biology* **1**, 185-205.
- Leichert, L. I., Gehrke, F., Gudiseva, H. V., Blackwell, T., Ilbert, M., Walker, A. K., Strahler, J. R., Andrews, P. C., and Jakob, U. (2008).** Quantifying changes in the thiol redox proteome upon oxidative stress *in vivo*. Proceedings of the National Academy of Sciences USA **105**, 8197-8202.
- Maadane, A., Merghoub, N., Ainane, T., Arroussi, H. E., Benhima, R. Amzazi, S. and Wahby, I. (2015).** Antioxidant activity of some Moroccan marine microalgae: Pufa profiles, carotenoids and phenolic content. *Journal of Biotechnology* **21**, 13-19.
- Manivannan, K., Ananthraman, P. and Balasubramanian, T. (2012).** Evaluation of antioxidant properties of marine microalga *Chlorella marina*. *Asian Pacific Journal Tropical Biomedical* **2**, 42-46.
- Markou, G., Chatzipavlidis, I. and Georgakakis, D. (2012).** Cultivation of *Arthrospira (Spirulina) platensis* in olive-oil mill wastewater treated with sodium hypochlorite. *Bioresource Technology* **11**, 234-241.
- Natrah, F. M., Yusoff, F. M., Shariff, M., Abas, F. and Mariana, N. S. (2007).** Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value. *Journal of Applied Phycology* **19**, 711-718.
- Ohshima, H., Yoshie, Y., Auriol, S. and Gilibert, I. (1998).** Antioxidant and pro-oxidant actions of flavonoids: Effects on DNA damage induced by nitric oxide, peroxy nitrite and nitroxyl anion. *Free Radical Biology and Medicine* **25**, 1057-1065.
- Ozturk, Z. N., Talame, V., Deyholos, M., Michalowski, C. B., Galbraith, D. W., Gozukirmizi, N. and Bohnert, H. J. (2002).** Monitoring large-scale changes in transcript abundance in drought and salt stressed barley. *Plant Molecular Biology* **48**, 551-573.
- Pallab, K. T., Barma, K., Pal Tapas, K. and Ramen (2013).** Research article estimation of total flavonoids content (TFC) and anti-oxidant activities of methanolic whole plant extract of Biophytum. *Journal of Drug Delivery and Therapeutics* **3**: 33-37.
- Singh, D., Prabha, R. and Meena, K. (2014).** Induced accumulation of polyphenolics and flavonoids in cyanobacteria under salt stress protects organisms through enhanced antioxidant activity. *American Journal of Plant Sciences* **5**, 726-735.
- Slinkard, K. and Singleton, V. L. (1977).** Total phenol analyses: Automation and comparison with manual methods. *American Journal of Enology and Viticulture* **28**, 49-55.
- Srivastava, A. K., Bhargava, P. and Rai, L. C. (2005).** Salinity and copper-induced oxidative damage and changes in the antioxidative defence systems of *Anabaena doliolum*. *World Journal of Microbiology and Biotechnology* **21**, 1291-1298.
- Winter, J., Ilbert, M., Graf, P. C. F., Özcelik, D. and Jakob, U. (2008).** Bleach activates a redox-regulated chaperone by oxidative protein unfolding. *Cell* **135**, 691-701.
- Zhishen, J., Mengcheng, T. and Jianming, W. (1999).** The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry* **64**, 555-559.