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Antioxidant capacity of five microalgae species and their effect on heat shock protein 70 expression in the brine shrimp *Artemia*

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ABSTRACT

Oxidative stress caused by chemicals brings about mass mortalities in aquatic organisms and in this context, antioxidants and heat shock protein 70 (Hsp70) stimulating compounds represent potential alternatives to alleviate this problem during aquaculture. The antioxidant capacity and efficacies of five Malaysian indigenous microalgae, namely *Auxenochlorella pyrenoidosa*, *Chlorella vulgaris*, *Messastrum gracile*, *Desmodesmus subspicatus* and *Parachlorella kessleri* to regulate Hsp70 expression in the brine shrimp *Artemia*, an important live food species, were examined. *A. pyrenoidosa* contained the highest total phenol and flavonoid content (48.3 mg GAE/g extract and 34.4 mg QE/g) across all the microalgae examined in this study, whereas the lowest occurred with *C. vulgaris* (24.4 mg GAE/g extract and 14.0 mg QE/g). The ferric reducing antioxidant power of *A. pyrenoidosa, C. vulgaris, M. gracile, D. subspicatus and P. kessleri* respectively stood at 0.78, 0.62, 1.01, 0.77 and 0.68 ferrous equivalents (mM), with trolox equivalent antioxidant capacity assay revealed that *A. pyrenoidosa* possessed the highest scavenging activity at 1 mg/mL concentration. Interestingly, application of 1.0–2.0 × 10^6 cells/mL *C. vulgaris, M. gracile, D. subspicatus*, and *P. kessleri* as feed enhanced Hsp70 accumulation in *Artemia*, indicating that these microalgae consist stimulating compound(s) which potentially promote the synthesis of the 70-kDa stress protein in this crustacean species. Taken together, results from this study revealed that microalgae possess various antioxidant properties and coupled with their abilities to induce Hsp70, they are potential oxidative stress control alternatives in *Artemia* and perhaps other aquatic organisms used in aquaculture.

1. Introduction

Free radicals generated either through different biochemical reactions or caused by external sources (Dasgupta and Klein, 2014) destroy lipids, proteins, and nucleic acid in living organisms, ultimately resulting to death (Ahmed et al., 2014; Jerez-Martel et al., 2017; Li et al., 2007; Morowvat and Ghasemi, 2016). As one example, oxidative stress induced by water temperature rise killed at least 11 fish species in the Cocos Islands, Australia between 2007–2009 (Hobbs and McDonald, 2010). To date, it was revealed in many studies that, administration of antioxidants alleviate problems associated with free radicals, thus maintaining the well-being of aquatic organisms (Ahmad et al., 2018). In this context, natural antioxidants, such as those exist in plants and microalgae, are more superior than synthetic ones to suppress oxidative stress as the latter, such as hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT), are carcinogenic and toxic to animals (Li et al., 2007; Munir et al., 2013).

Microalgae are microscopic algae typically found in marine and freshwater environments, and they occur ubiquitously in sediments and water column. Microalgae tolerate harsh environments and like other higher plants, they perform photosynthesis, utilizing carbon source, light and water to produce food. Apart from carotenoids, fatty acids, enzymes, polymers, peptides and sterols, some microalgae species are rich in natural antioxidants and they serve as valuable alternatives to

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synthetic antioxidants, with β-carotene from *Dunaliella* and astaxanthin from *Haematococcus* as examples (Liu et al., 2016). In this context, it is therefore crucial to identify more microalgae species that are easy to culture and rich in antioxidants for use as alternatives to control stress brought upon by free radicals (Hajimahmoodi et al., 2010).

Heat shock proteins (Hsps), otherwise known as molecular chaperones or stress proteins, are found in all living organisms, from prokaryotes to eukaryotes, including microalgae. Hsps are classified into several families based on their molecular weight (kDa) and these include Hsp110, Hsp100, Hsp90, Hsp70, Hsp60 and the small heat shock proteins (sHsps), the latter less than 40-kDa (Sung et al., 2011). Heat shock proteins (Hsps) are synthesized in response to heat exposure and other forms of abiotic and biotic stressors to ensure the survival of organisms under stressful environmental conditions (Sung et al., 2008, 2014). Hsps, particularly Hsp70, protect organisms by assisting in the recovery of damaged proteins during stress (Sung et al., 2008). Additionally, they function as intercellular signalling molecules in the immune response of aquatic organisms during pathogen attack, activating immune cells to produce wide varieties of antimicrobial substances to battle infection (Pockley, 2003; Robert et al., 2010). It has been well documented that plants rich in natural bioactive compounds, such as curcumin, celastrol, gambogic acid and withaferin A among others, induce Hsps expression in living cells and/or whole organism (Sung and MacRae, 2011). Recent data also revealed that plant extracts enhance Hsps expression in organism (Sung et al., 2014). As one example, ethanolic *Jasminum sambac* leaf extract has been shown to activate and induce Hsp70 expression in rats (Alrashdi et al., 2012) whereas the combination of *Schisandra chinensis* berry extract, root extract of *Eleutherococcus senticosus* and *Rhodiola rosea* boosted Hsp70 accumulation in isolated human neurolgia cells (Asea et al., 2013).

Considering that both Hsps and antioxidants enhance tolerance of aquatic organisms against extreme environmental stresses (Troschinski et al., 2014), this study further examines the total content of phenolic and flavonoid in *A. pyrenoidosa, C. vulgaris, M. gracile, D. subspicatus* and *P. kessleri*, work which included determination of their antioxidant capacity. The efficacy of these microalgae species to regulate Hsp70 expression in the brine shrimp *Artemia*, an important crustacean live food used in aquaculture, were investigated. *Artemia* is good model organism for stress response studies because it has short life cycle and can be cultured in mass quantities (Ikhwanuddin et al., 2012; Sung et al., 2008). Outcomes generated from this study may assist in the formulation of strategies to protect aquatic organisms against stress, particularly when pollution caused by free radicals is becoming more rampant and severe nowadays.

2. Materials and methods

2.1. Microalgae collection, identification and culture

Five microalgae species namely *Parachlorella kessleri* (Fig. 1A1), *Auxenochlorella pyrenoidosa* (Fig. 1B1), *Desmodesmus subspicatus* (Fig. 1C1), *Chlorella vulgaris* strain UMT-M1 (Fig. 1D1) and *Messastrum gracile* (SE-MC4) (Fig. 1E1) were respectively collected from Setiu Wetland, Pulau Bayas, Kenyir Lake, mangrove area of Universiti Malaysia Terengganu (UMT) and Setiu Jetty in Terengganu, a state situated on the east coast of Peninsular Malaysia. These microalgae were identified based on their morphological characteristics, with observation of the cell structure performed by compound light microscopy and scanning electron microscopy (SEM). Fresh microalgae cells were fixed in 2.5 % glutaraldehyde with 0.1 M sodium cacodylate buffer (pH 7.2) and incubated at room temperature for 2 h. The upper portion of the solution was discarded and the pellets rinsed with 0.1 M sodium cocadylate buffer. Pellets were then submerged in 1 % osmium tetraoxide consisting 0.1 M sodium cocadylate buffer and incubated for 2 h. Pellets were rinsed with 0.1 M sodium cocadylate buffer, dehydrated with different ethanol concentrations and mounted on specimen stubs with hexamethyldisilazane (HMDS- Merk, ACS grade) prior to gold coating and observation with an analytical SEM (JEOL JSM-6360LA, Tokyo, Japan). Identification of the microalgae was based on cell shape and structure (Bock et al., 2011; Da Silva et al., 2017; Janse van Vuuren et al., 2006; Roy and Pal, 2015; Wu et al., 2013). The microalgae cells were isolated and grown to stationary phase with Bold's Basal Medium (BBM) (Nicholas and Bold, 1965) enriched with F medium-trace metals and vitamins (Guillard and Ryther, 1962) at 25 ± 2 °C. Aeration was provided and the cultures were exposed to constant illumination at 80 μmol m^{-2} s^{-1}. The microalgae cells were harvested by centrifugation 7 days upon inoculation.

The molecular identification of *P. kessleri* was performed according to the methods described by Jusoh et al. (2020). Briefly, DNA was extracted with GeneMATRIX Soil DNA Purification kit and amplified with PCR using eukaryotic primer set (Forward primer 5'à3': AACCTGGTT GATCCTGCC AGT; Reverse primer 5'à3':TGATCCTTCTGCAGGTTCAC CTAC). PCR was performed using GoTaq® PCR Core Systems (Promega, USA) at 95 °C for 5 min followed by 40 cycles of 95 °C for 45 s, 55 °C for 30 s and 72 °C for 2.5 min, followed by 72 °C for 10 min. The PCR products were sequenced and nucleotide sequences obtained were used as query against the BLASTN program in the NCBI nucleotide sequence database. The sequence revealed 99.53 % similarity with *P. kessleri.* Microalgae *C. vulgaris, M. gracile, A. pyrenoidosa* and *D. subspicatus* used in this study has been identified with similar method (Jusoh et al., 2020; Teh et al., 2019).

2.2. Microalgae collection and extraction procedure

Microalgae cells were collected by 3 min centrifugation at 16,470 *g* (Hitachi CR22N, Japan) and subsequently freeze dried with an EYELA FD-550R freeze dryer (Japan). Two grams of the microalgae cells were grinded into fine powder and transferred into a flask containing 100 mL analytical reagent grade methanol. Cells were mixed with an orbital shaker at 150 rpm for 24 h. Extract obtained were filtered through a Whatman No.1 filter paper and the solvent were evaporated and concentrated with a rotary evaporator (Buchi, R210A, Switzerland) at 35 °C. Extracts were collected and kept at −20 °C until further use (Goh et al., 2010).

2.3. Antioxidant activity

a) Trolox equivalent antioxidant capacity (TEAC) assay

The antioxidant capacity of microalgae was determined according to the TEAC method described by Farah Diyana et al. (2015). This assay measures the concentration of antioxidant compounds needed to stop or interfere the stable radical cation $ABTS^{\cdot+}$ by 50 %. $ABTS^{\cdot+}$ solution was prepared by mixing 7 mM ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt and 2.45 mM potassium persulfate (di-potassium peroxdisulfate) at a ratio of 1:1 (v/v). The $ABTS^{\cdot+}$ solution were stored in dark condition and at room temperature for 12–16 h. A dilution with methanol to reach an absorbance of 0.700 ± 0.050 at 734 nm was made prior to determining the sample absorbance value with a Varioskan Lux multiplate reader (Thermofisher Scientific, USA). A standard curve was constructed by using Trolox solution as reference. Approximately 20 μL of samples, ranging from 0.1 – 5 mg/mL, were then allowed to react with 200 μL of $ABTS^{\cdot+}$ solution in dark condition for 10 min. The absorbance was measured at 734 nm and the results were expressed as the percentage of ABTS scavenging activity following the formula below, where $A_{Control}$ is the absorbance of the control and A_{Sample} is the absorbance of the sample.

$$\text{ABTS scavenging activity (\%)} = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

The percentages were calculated based on the trolox equation of y = 0.3189x + 0.9545 where R^2 = 0.9929.

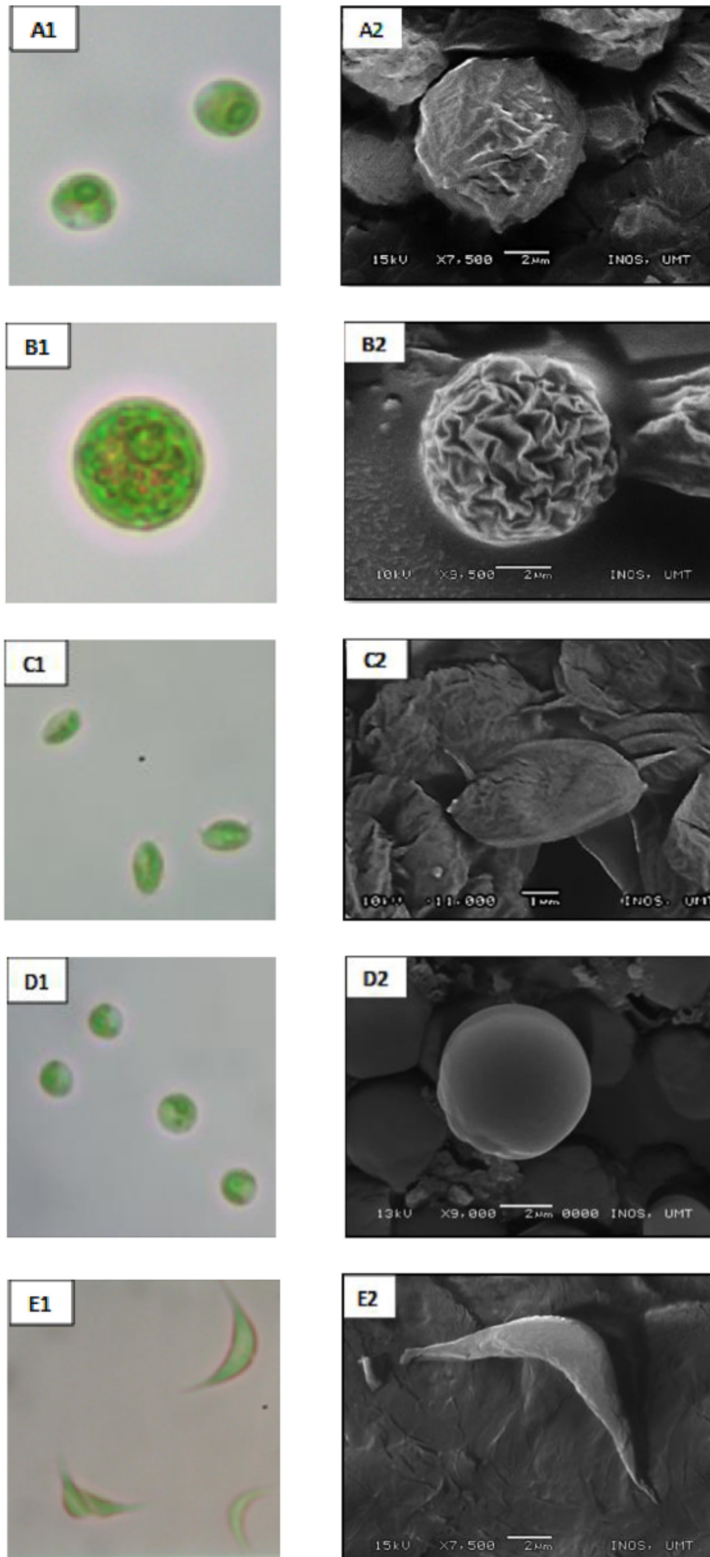

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Fig. 1. Microscope image of microalgae *Parachlorella kessleri* (A1, A2), *Auxenochlorella pyrenoidosa* (B1, B2), *Desmodesmus subspicatus* (C1, C2), *Chlorella vulgaris* (D1, D2) and *Messastrum gracile* (E1, E2). Figs A1, B1, C1, D1, and E1 represent images captured with a compound light microscope (1000 × magnification) and Fig. A2, B2, C2, D2, and E2 represent images captured with a scanning electron microscope, with white lines indicating the size of the microalgae (μm).

2.4. *Ferric reducing antioxidant power (FRAP) assay*

The FRAP values of microalgae were assessed according to the method described by Hajimahmoodi et al. (2010) and Heffernan et al. (2015) with minor modification. FRAP reagent was prepared by mixing 5 mL of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl with 5 mL of 20 mM $FeCl_3 6H_2 0$ and 50 mL of 0.3 mol/L acetate buffer at pH 3.6 and susequently heated to 37 °C. Twenty (20) μL of 1 mg/mL sample, standard and methanol (for blank) were allowed to react in dark condition with 180 μL of FRAP reagent for 40 min at 37 °C. The absorbance was measured with a Variouskan Lux multiplate reader (Thermofisher Scientfic, USA) at 595 nm. A standard curve was constructed by using Iron (II) sulphate solution and the results were expressed in mM Fe (II) equivalent.

b) Total phenolic assay

The total phenolic content was determined by Folin-Ciocalteu procedure (Goiris et al., 2012; Zhang et al., 2006) with minor modification. Twenty (20) μL of the standard and sample solution were separately mixed with 100 μL of Folin-Ciocalteu reagent and incubated for 5 min. Eighty (80) μL of 7.5 % sodium carbonate solution was added and the absorbance were measured with a Varioskan Lux multiplate reader (Thermofisher Scientific, USA) at 750 nm upon 2 h incubation at room temperature. Gallic acid was used as a standard to construct the calibration curve and the results obtained were expressed as gallic acid equivalent (GAE) per gram of microalgae extract.

c) Total flavonoid assay

Total flavonoid content in each microalgae extract was determined according to the method described by Herald et al. (2012) with minor modification. One hundred (100) μL distilled water was loaded into 96 wells, followed by 10 μL of 50 g/L $NaNO_2$ and 25 μL serial standard solution or sample solution. Fifty (15) μL of 100 g/L $AlCl_3$ was added into the mixture and incubated for 5 min. Fifty (50) μL of 1 mol/L NaOH and distilled water were subsequently added and the plate was then incubated on a shaker for 50 min at room temperature. The absorbance was measured with a Varioskan Lux multiplate reader (Thermofisher Scientific, USA) at 510 nm. A calibration curve was constructed with Quercetin as a standard and samples were expressed as quercetin equivalent (QE) per gram of microalgae extract.

2.4.1. *Artemia culture*

Artemia franciscana cysts from the Great Salt Lake (INVE Aquaculture, Inc., Ogden, UT, USA) were hatched in 30−32 mg/L autoclaved seawater. Vigorous aeration and constant illumination were provided continuously for 48 h during hatching. Nauplii were collected with a 100-micron net and subsequently grown to adults by feeding daily with PKC Nutri+®, a formulated feed developed at UMT for tank production of *Artemia*. Adult *A. franciscana* were collected 14 days upon culture and 30 animals were transferred into 18 separate containers, each containing 100 mL of seawater. *A. franciscana* adults were fed 24 h with 0.5, 1.0 and 2.0 × 10^6 cells/mL of microalgae prior to use in protein extraction. Each feeding treatment was performed in triplicate, with *A. franciscana* adults fed solely with PKC Nutri+® served as the control.

2.5. *Heat shock protein 70 determination in Artemia*

a) Protein extraction and quantification

Protein extraction of the adult *A. franciscana* was performed as essentially described (Sung et al., 2007, 2008). Animals were rinsed twice with distilled water to remove salt and residues, and placed on a clean tissue paper to dry. Five adults were homogenized in 60 μL cold buffer K (150 mM D-sorbitol, 70 mM potassium D-gluconate, 5 mM Magnesium chloride hexahydrate, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) containing 6 μL of protease inhibitor cocktail (Sigma-Aldrich, Inc. USA) with sterile microfuge pestles (Fisher Scientific, Ottawa, ON, Canada). The protein aliquots were centrifuged at 1902 *g* in cold condition for 5 min. The supernatant containing crude proteins were transferred into sterile microcentrifuge tube and protein concentration was determined using Bradford method, with bovine serum albumin (BSA) served as the protein standard. The protein extracts were stored at −80 °C prior to use in electrophoresis and western immunoblotting.

b) SDS-PAGE and western immunoblotting

Aliquots containing 200 ng protein were mixed with sample buffer at 1:1 (v/v) ratio and subsequently heated for 5 min at 95 °C. Samples were cooled at room temperature and centrifuged at 1902 *g* for 1 min. Twenty (20) μL protein samples were resolved in 12.5 % SDS polyacrylamide gels and electrophoresed at 120 V for 15 min followed by 150 V for 1 h. Two gels were run simultaneously, one stained with Coomassie blue (Bio-Rad Laboratories) and another were blotted to PVDF membranes (Bio-Rad Immun-Blot PVDF) with a Trans-Blot Turbo Transfer system (Bio-Rad, USA) for 30 min at 30 V following the manufacturer's recommendation. Blots were rinsed 3 times with Tris saline buffer for 5 min and blocked with a blocking buffer (50 mL of Tris-buffered saline containing 0.2 % (v/v) Tween 20 and 5 % (w/v) bovine serum albumin) overnight. Membranes were washed 3 times with tri saline for 5 min and subsequently incubated with a MA3-006 Hsp70 monoclonal antibody (Thermo Scientific, USA) for 2 h at 1:5000 dilutions. Membranes were rinsed thrice with Tris-saline buffer, each for 5 min and incubated with a horseradish peroxidase (HRP) conjugated Goat anti-mouse IgG polyclonal antibody at 1:5000 dilutions (Thermo scientific, USA). Detection was performed with 0.7 mM diaminobenzidine tetrahydrochloride dihydrate (DAB) (0.01 % (v/v) H_2O_2 in 0.1 M Tris-HCL (pH 7.6) (Sung et al., 2007, 2008).

c) Densitometry and statistical analysis

The blots were scanned with a GS-800 calibrated densitometer (BioRad Laboratories, USA) and the amount of Hsp70 was interpreted as density intensity/mm^2 with Quantity One software (BioRad Laboratories, USA). The data was presented as relative Hsp 70 expression in fold difference by comparing the densitometry analysis of sample with the control. Significant differences in antioxidant activities among five microalgae species were analysed with Kruskal-Wallis test and Dunn's Multiple Comparison test, and the strength of a relationship among the antioxidant assays was performed with Spearman correlation test. Both statistical analysis was analysed using IBM SPSS software version 25.0 (Armonk, NY: IBM Corp).

3. Results

3.1. *Morphological characteristics of microalgae*

Parachlorella kessleri cells appeared small and spherical, with diameter range from 3 − 8 μm. Scanning electron micrographs revealed the presence of reticulated cell wall, with furrows extended over the surface (Fig. 1A1, A2). *Auxenochlorella pyrenoidosa* cells appeared in either

solitary forms or in groups and they are spherical and green in colour, measuring approximately 5 − 8 μm. Scanning electron micrograph revealed ornamented cell wall patterned with uninterrupted ribs on the cell surface (Fig. 1B1, B2). *Desmodesmus subspicatus* cells appeared in either solitary forms or colonies consisting 2–4 cells arranged side by side, each measuring about 6 − 7 μm in length. Scanning electron micrograph revealed uninterrupted pattern of ribs on the cell surface and the presence of spines or dents (Fig. 1C1, C2). *Chlorella vulgaris* cells appeared spherical, subspherical or ellipsoid in shape. They occur either in single cell or colonies, each measuring approximately 3 − 6 μm in diameter. Scanning electron micrograph revealed smooth cell wall surface (Fig. 1D1, D2). *Messastrum gracile* (Fig. 1E1, E2) are found in loose, irregular bundles or tangled groups. Some of them lay over or twisted with one another. Cells appeared crescent in shape, with the length and width measuring approximately 26 μm and 6 μm, respectively. The cells are either curved or sigmoid, tapered gradually at both ends and consist single chloroplast which almost fills up the cell. Scanning electron micrograph revealed a smooth but unornamented cell wall pattern. All microalgae species belong to the class Chlorophyta.

3.2. Antioxidant activities

The antioxidant activities of microalgae determined with FRAP and TEAC assays were expressed in millimole of Fe (II) equivalent and IC_{50} (Inhibition concentration at 50 %) values (Table 1). The FRAP values varied from 0.62 ± 0.03–1.01 ± 0.06 mM Fe(II) eq/g extract weight. *M. gracile* provided the highest FRAP activity followed by *A. pyrenoidosa, D. subspicatus, P. kessleri* and *C. vulgaris*. *A. pyrenoidosa* provided approximately 70 % ABTS radical scavenging activity, the highest amongst the five microalgae species when examination was performed with 1 mg/mL of microalgae extract. The ABTS radical scavenging activity of *P. kessleri*, *D. subspicatus*, *M. gracile*, and *C. vulgaris* were 65.8, 65.1, 60.7 and 57.8 %, with values equivalent to 215.6, 203.3, 201.2, 187.3 and 178.2 μmol Trolox eq/g EW, respectively (Table 1). The percentage differences were insignificant ($p > 0.05$).

The total phenolic and flavonoid content of the microalgae were shown in Table 2. The total phenolic content varied significantly across the five microalgae species examined ($p < 0.05$), with *A. pyrenoidosa* accumulating the highest concentration while *C. vulgaris* the lowest. The total phenolic content of *A. pyrenoidosa* was approximately 50 mg, a value two-fold higher than *C. vulgaris*. It was noted that *A. pyrenoidosa* possessed the highest flavonoid content, accumulating approximately 35 mg, with the value two-fold more than *C. vulgaris*. The flavonoid content of other microalgae species stood at approximately 20 mg, with the differences insignificant among each other ($p > 0.05$). The total phenolic and flavonoid content as revealed by Spearman analysis, were positively correlated ($r = 0.602$, $p < 0.01$). Both FRAP and TEAC scavenging activity were also correlated with total phenolic content presence in the microalgae ($r = 0.539$ and $r = 0.429$, $p < 0.01$ respectively).

Table 1
Antioxidant activity of microalgae extracts at 1 mg/mL in FRAP and TEAC assays. FRAP value was determined based on the Iron (II) sulphate standard calibration curve: $y = 1.2036x - 0.024$, $r^2 = 0.9901$. The ABTS radical scavenging activity was determined based on the absorbance value at 734 nm and the percentages were calculated following the formula described in the methodology.

Microalgae species	FRAP (mMol Fe(II) eq/g extract weight)	ABTS radical scavenging activity (%)
Chlorella vulgaris	0.62 ± 0.03[d]	57.8 ± 2.3 [a]
Messastrum gracile	1.01 ± 0.06[a]	60.7 ± 2.5 [a]
Auxenochlorella pyrenoidosa	0.78 ± 0.08[b]	69.7 ± 2.2 [b]
Parachlorella kessleri	0.68 ± 0.04[c]	65.8 ± 3.4 [a]
Desmodesmus subspicatus	0.77 ± 0.05[b]	65.1 ± 4.7 [a]

Data represent mean and standard deviation for each species. [a-d]: Value with same superscripts letter for species meaning they are not significantly different (p > 0.05).

3.3. Heat shock protein 70

The expression of Hsp70 in adult *A. franscicana* was enhanced upon feeding with microalgae *M. gracile, P. kessleri, C. vulgaris* and *D. subspicatus* whereas the opposite occurred with *A. pyrenoidosa* (Table 3). It was noted that *D. subspicatus* enhanced *Artemia* Hsp70 accumulation two-fold and this condition was observed in all three microalgae concentrations tested in this study. A two-fold increase in Hsp70 expression were also observed in *Artemia* fed with 2×10^6 cells/mL of *M. gracile*. Hsp70 content in *Artemia* was augmented in a dose-dependent manner when *M. gracile, P. kessleri* and *C. vulgaris* were used as feed.

4. Discussion

Phenolic compounds are potent antioxidants and they break radical chains through hydrogen atom or single electron transfer (Goiris et al., 2012; Manivannan et al., 2012). Thus, methanolic extract of microalgae was used for analysis in this study because they primarily contain more phenolic compounds and antioxidative properties than other types of solvent extracts (Jerez-Martel et al., 2017; Manivannan et al., 2012; Safafar et al., 2015). The total phenolic content of *A. pyrenoidosa*, *C. vulgaris, M. gracile, D. subspicatus*, and *P. kessleri* ranged from 24.4–48.3 mg GAE/g, outcomes similar to *Tetraselmis* sp., *Nannochloropsis gaditana, Scenedesmus rubescens, Fischerella ambigua* and *Nostoc muscorum* in which the total phenolic content of these microalgae were found to accumulate between 25.5–48.6 mg GAE/g (Maadane et al., 2015; Morowvat and Ghasemi, 2016).

On other aspect, the total flavonoid content of the microalgae ranged from 14.0–34.7 mg QE/g extract. This concentration is considered high when comparisons were made with *Desmodesmus* sp., *Dunaniella salina*, *Nannochloropsis limnetica* and *Chlorella sorokinia*, where accumulation of this antioxidant compound in these microalgae merely reached 4.03 mg QE/g extract (Safafar et al., 2015). The differences might be attributed to the condition(s) used during cultivation since it has been noted in many studies that, temperature (Converti et al., 2009), light intensity (Gong et al., 2014; Jacob-Lopes et al., 2009; Rai et al., 2015), salinity (Rai et al., 2015) and nutrient composition (Converti et al., 2009; Goiris et al., 2015) greatly affect antioxidant accretion in microalgae. As revealed by Spearman correlation analysis, the total phenolic and flavonoid content in the microalgae were correlated.

Two assays were performed to determine the antioxidant activities of the microalgae in this study. The TEAC radical scavenging activity was high, indicating the presence of potential antioxidant compounds in microalgae to scavenge ABTS radicals, perhaps via single electron transfer or hydrogen atom transfer (Goiris et al., 2012; Prior et al., 2005). A study conducted by Goiris et al., 2012 revealed that the TEAC radical scavenging activity of *Phaeodactylum tricornutum, Tetraselmis* sp., *Botryococcus braunii, Chlorella vulgaris, Isochrysis* sp. ranged from 4.55 to 69.40 μmol Trolox eq/g dry weight, a value three-fold lower than what was obtained with the microalgae species examined herein. The FRAP value ranged from 0.62 mM Fe (II)/g to 1.01 mM Fe (II)/g. Comparison made with four antioxidant power groups indicated that the FRAP activities of these microalgae fall within the ‘extremely high’ category, with values exceeding 0.5 mM Fe(II)/g. As an applied note, many Chinese medicinal plants rich in natural antioxidant were grouped in this category and they are capable of eliminating free radicals build-up in an aquatic system, thus reducing oxidative stress (Wong et al., 2006). It was often stated that phenolic compounds are major components contributing to the antioxidant capacities of plants (Goiris et al., 2012). However, based on the correlation between FRAP

Table 2
Total phenolic and flavonoid content in the methanolic extract of different microalgae species. The total phenolic content of microalgae was determined with Folin Ciocalteu reagent based on Gallic acid equivalent (GAE) with the standard curve equation: $y = 0.0135x$, $r^2 = 0.9967$. Total flavonoid content was determined based on the quercetin standard calibration curve: $y = 0.004x + 0.0013$, $r^2 = 0.9856$.

Species	Phenolic content (mg of GAE/g of extract weight)	Flavonoid content (mg of QE/g of extract weight)
Chlorella vulgaris	24.4 ± 1.7^d	14.0 ± 3.1^c
Messastrum gracile	39.8 ± 6.0^b	19.8 ± 3.5^b
Auxenochlorella pyrenoidosa	48.3 ± 5.3^a	34.7 ± 6.5^a
Parachlorella kessleri	31.5 ± 6.5^c	21.2 ± 2.3^b
Desmodesmus subspicatus	26.6 ± 1.1^d	21.1 ± 7.8^b

Data represent mean and standard deviation for each species. $^{a-d}$: Value with same superscripts letter for species meaning they are not significantly different ($p > 0.05$).

Table 3
Relative Hsp70 expression of *Artemia* cultured with five microalgae samples at 0.5×10^6, 1.0×10^6 and 2.0×10^6 cells/mL (C1, C2, and C3). The relative expression of Hsp70 presented in fold difference was calculated based on the density ratio of sample: control.

Concentration (cells/mL)	Relative expression (Fold difference)		
Species	0.5×10^6 (C1)	1.0×10^6 (C2)	2.0×10^6 (C3)
Chlorella vulgaris	1.0	1.1	1.4
Messastrum gracile	1.0	1.6	1.9
Auxenochlorella pyrenoidosa	0.5	0.2	0.5
Parachlorella kessleri	1.1	1.2	1.4
Desmodesmus subspicatus	2.0	1.9	1.9

and TEAC scavenging activity with total phenolic content ($r = 0.539$ and 0.429 respectively), the antioxidant activities of microalgae might be associated with the presence of bioactive complexes other than phenolic compounds, amongst them include proteins, carotenoids and polysaccharides (Holtin et al., 2009; Mohamed, 2008). In this context, further work is necessary to identify whether these bioactive complexes exist in microalgae and how they function mechanistically as antioxidants during stress.

The expression of *Artemia franscicana* Hsp70 was enhanced upon feeding with microalgae *M. gracile, P. kessleri, C. vulgaris* and *D. subspicatus* but otherwise with *A. pyrenoidosa* (Fig. 2). It should be noted that antioxidants and chaperone-mediated protective mechanisms can be activated by oxidative stress either through similar or different pathways (Giraud-Billoud et al., 2013) and the presence of antioxidants may activate or inhibit Hsp70 production (Gorman et al., 1999; Troschinski et al., 2014). Like other shrimp species, the induction of Hsp70 in *Artemia* was found to be triggered by abiotic and biotic stress, as well as Hsp inducers, the latter predominantly found in plants (Baruah et al., 2012; Sung et al., 2018). At this point it is hard to conclude which of these factors boosted Hsp70 synthesis but based on the growth, survival and general well-being of the adult *Artemia* during culture (data not shown), Hsp70 expression was unlikely up-regulated by stress, but rather induced by potential Hsp70 stimulating compounds occurred in these microalgae. It should be noted that microalgae are common food for *Artemia* and they do not stress the organism. Many plant-based compounds have been identified to induce Hsp70 accumulation in humans and animals, including shrimp (Baruah et al., 2012; Niu et al., 2014; Roberts et al., 2010; Sung et al., 2011). For example, compounds isolated from medical plants namely alkannin, oxymatrine, osthole, palmatine chloride, and shikonin upregulated the expression of Hsp70 in human lymphoma U937 cells (Ahmed et al., 2012). TEX-OE®, an extract from the tropical cactus *Opuntia ficus indica*, effectively enhanced the production of endogenous Hsps in fish and shellfish. In similar study, a short term incubation of the model fish *Pterophyllum scalare* (Schultze) with TEX-OE® induced the synthesis of Hsp70 and Hsp90 in liver, muscle and gills, with circulating Hsps level higher than those induced by heat (Baruah et al., 2012; Roberts et al., 2010; Sung et al., 2012). Augmentation of Hsp70 in gills and muscle was apparent, demonstrating that TEX-OE® accelerated Hsp70 synthesis and enhanced

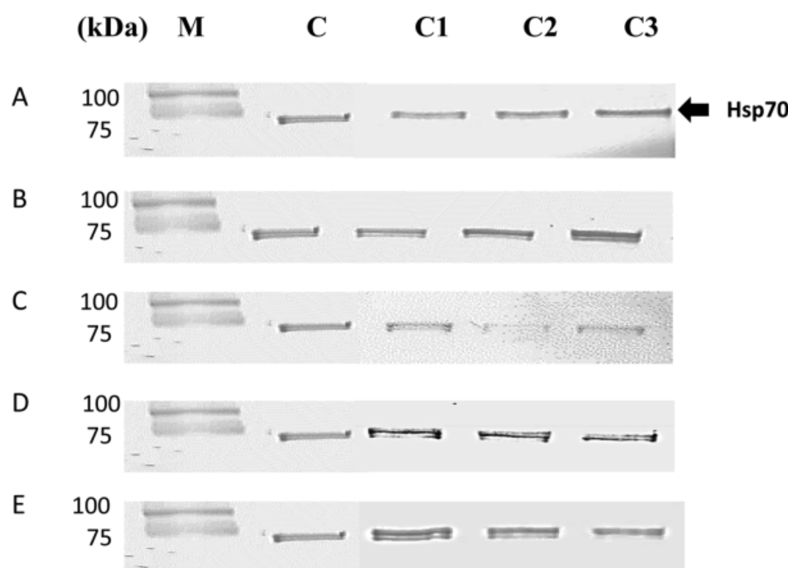

Fig. 2. Hsp70 expression of *Artemia* fed with A: *Chlorella vulgaris*, B: *Messastrum gracile*, C: *Auxenochlorella pyrenoidosa*, D: *Parachlorella kessleri*, E: *Desmodesmus subspicatus* at concentrations of 0.5×10^6 (C1), 1.0×10^6 (C2) and 2.0×10^6 cells/mL (C3). M, protein marker in kilodalton and C, Control (*Artemia* fed solely with PKC Nutri+®).

protection against bacterial pathogen. Other than this, paeoniflorin, metabolites of phenolic compounds from the herbal plant *Paeonia lactiflora* Pall, raised Hsp70 expression in cultured mammalian cells (Yan et al., 2004). Schisandrin B (Sch B), the dibenzocyclooctadien from the herb *Schisandra chinensis*, augmented the synthesis of Hsp25 and Hsp70 in rat heart (Ip et al., 2001). Furthermore, curcumin (diferuloylmethane) of the turmeric plant *Curcuma longa*, induces Hsp70 expression in K562 cells.

All of these compounds profoundly affect Hsp expression in humans and other animals and it should be possible that microalgae possess compounds which up-regulates Hsp70 expression in *Artemia*. It is therefore of fundamental and applied significance to determine the identity of Hsp70 stimulating compounds presence in these microalgae and examine their efficacies in controlling oxidative stress and perhaps other stresses in aquatic environments. Since microalgae is a promising source for antioxidant compounds, future studies should focus on identifying ways to enhance their accumulation as well as refine extraction protocols for plausible application in aquaculture and other sectors.

Author contribution

Irene Tiong Kai Ru: Data curation, Formal analysis, Interpretation of the data, Original draft, writing.
Thilahgavani Nagappan: Conceptualization.
Mohd Effendy Abdul Wahid: Funding acquisition, Validation, Critical revision of the article for important intellectual content.
Tengku Sifzizul Tengku Muhammad: Project administration, or logistic support, Critical revision of the article for important intellectual content.
Toda Tatsuki: Funding acquisition, Conceptualization.
Woro Hastuti Satyantini: Funding acquisition, Conceptualization.
Gunanti Mahasri: Funding acquisition, Conceptualization.
Patrick Sorgeloos: Validation, Critical revision of the article for important intellectual content.
Yeong Yik Sung: Conceptualization, Supervision, Validation, Critical revision of the article for important intellectual content.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2020.100433.

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