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Evaluation of Antioxidative Activity of Phenolics in Methanolic Extracts of Blue Green Algae

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ABSTRACT

A phenolic rich extract has been isolated from the algae species, *Arthrospira platensis*. The objectives of this study were to determine the total amount of phenolics extracted from blue green alga and to evaluate the antioxidative activity of phenolic extracts in different concentrations of methanolic solvent using free radical scavenging assay. This study began with extraction of the Arthrospira with liquid nitrogen into powder after filtration and overnight drying in the oven. This is followed by determination of total phenolics in different concentrations of methanolic solvent and studied for free radical scavenging activity using 2, 2-diphenyl-picrylhyldrazyl assay. The concentrations of total phenolics determined by the folin-ciocalteu method was found to be 252.72 $mg.l^{-1}$ gallic acid equivalent in aqueous extracts which showed that phenolic compounds can dissolve more in water medium. The 100% methanolic extracts showed significantly higher antioxidative activities in all assays while in different concentrations of 100% methanolic extracts, the concentration of 120 $mg.l^{-1}$ showed the highest free radical scavenging activity. This study showed that blue-green algae is rich in phenolic compounds, which are natural antioxidants and may help reduce the problem of climate change by absorption of CO_2 from the atmosphere.

Key words: Antioxidant, antioxidative activity, free radical, phenolic, algae, *Arthrospira platensis*.

INTRODUCTION

Blue-green algae are single-celled and filamentous organisms that have been traditionally regarded as simple plants called as cyanobacterium. These algae are usually found in damp places or bodies of water and thus are common in terrestrial as well as aquatic environment. However, terrestrial blue green algae are usually rather inconspicuous and far more common in moist, tropical regions than dry ones, because they lack vascular tissues and other adoptions to live on land. As mentioned above, algae grow in almost every habitat in every part of the world (Woolfe, 1992).

Like plants, algae require sunlight and carbon dioxide to grow. They absorb carbon dioxide (CO_2) from the atmosphere and use the sunlight for photosynthesis. Photosynthesis is an important biochemical process in which plants, algae, and some bacteria convert the energy of sunlight to chemical energy. This chemical energy is used to drive chemical reactions such as the formation of sugars or the fixation of nitrogen into amino acids, the building blocks for protein synthesis (Xiang *et al*., 2001).

Athrosphera platensis species are rich in protein dietary fiber, minerals, vitamins and antioxidants such as phenolic acids, anthocyanins, tocopherol and beta carotene (Woolfe, 1992). In recent years, several reports have indicated that the polyphenols found in algae have antioxidant activity and exert several health-promoting functions in humans (Islam *et al*., 2003). Among the phenolic compounds, various flavonoids have recently been shown to be effective scavengers of peroxyl radicals (Sawa *et al*., 1999).

Polyphenols are categorized as phytochemicals and their sources are black tea (Dreosti, 2000), green tea (Cheng, 1999), rosemary (Frankel *et al*., 1996) and grape extracts (Gehm *et al.,* 1997). Studies have indicated that these polyphenols have high antioxidant activities that helps reduce oxidative damage due to excess of reactive oxygen species (ROS) which is implicated in the risk of cardiovascular disease, cancer, and age-related neuronal degeneration, such as alzhimer's and parkinson disease (Ames *et al*.,1993).

The free radicals are generated in the human body through aerobic respiration and exist in different forms mainly superoxide, hydroxyl, hydroperoxyl, peroxyl and alkoxyl radicals. Generally, natural antioxidant enzymes in healthy individuals neutralize these free radicals (Rimbach *et al*., 2005). However, dietary antioxidants are helpful in assisting the body to neutralize free radicals. Therefore, it is important to consume a diet rich in antioxidants to reduce the harmful effects of oxidative stress (Xiang *et al*., 2001).

Several methods are available for evaluating antioxidant activities of natural compounds in food or biological systems. The most commonly used procedure is 2,2-diphenyl 1-picrylhydrazyl (DPPH) assay which used DPPH as free radical generators. The mechanism is that the absorption spectra of the stable free radicals changes when the molecule is reduced by an antioxidant or a free radical species. Several studies have reported that there were correlations among different antioxidant analysis methods and various phytochemicals concentrations in algae (Awika *et al*., 2003). The objectives of the present study were to determine the total amount of

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phenolics from *Arthospira platensis* using different concentrations of methanol and to evaluate the antioxidative activities of various concentrations using free radical scavenging assay. Blue-green algae is potentially rich in phenolic compounds and with the growing interest in natural antioxidants it is possible to extract high value compounds while reducing the problem of climate change with the absorption of CO_2 from the atmosphere.

MATERIALS AND METHODS

Organisme and Culture Condition

Arthrospira plantensis was obtained and cultured at Marine Biotechnology Lab. and the phytochemical analysis was carried out at the Biochemistry Lab University Industry Selangor (UNISEL). The *algae* was cultured in zarouk medium for two weeks before being harvested using a vacuum filter (Buchi). The filtrated *Arthrospira* was dried overnight at 60ºC in the oven (Optic Technology) and then grinded with liquid nitrogen into powder form.

Chemicals

Chemicals used all from Merck were gallic acid (3, 4, 5-trihydroxybenzoic acid) (99.9% purity), DPPH (2, 2-diphenyl-picrylhyldrazyl), folin-ciocalteu phenol reagent, sodium carbonate (98.7% purity) and methanol (99.9% purity).

Free Radical Scavenging Assay

The reaction of phenols with folin-ciocalteu reagent produces a blue color and the intensity of the color can be measured using Thermo-Spectronic spectrophotometer at 765 nm. These forms of solution are the basis reaction for the quantification of phenolics concentration in samples using gallic acid as the standard (Gao *et al*., 2000). A stock solution of gallic acid at the concentration of 1 mg in 100 ml was prepared in distilled water. This stock solution was then diluted with water to 0, 5, 10, 50, 75, 100, 250, 300, 375, and 500µgml^{-1}. The test tubes were labeled according to the concentrations of the solution contained.

One hundred micro liter of sample extracts were piped into a clean test tube, and 2 ml of water, 0.2 ml of folin-ciocalteu reagent and 1 ml of 15% sodium carbonate (Na_2SO_3) were added in the same test tube. The mixture was vortexed to ensure uniform mixing and then left to incubate for 2 hours at room temperature. These tubes were vortexed again after 2 hours of incubation and the absorbance was measured by spectrophotometer at wavelength 765 nm. A standard curve was plotted for the absorbance obtained at different concentrations of gallic acid. The concentrations of phenolics in the sample extracts were expressed as µg gallic acid equivalent (GAE) per gm of sample, respectively. GAE can be defined as the concentration of gallic acid which contains the same amount of phenolics in the sample under investigation (Gao *et al*., 2000).

The solution of free radical of DPPH is purple in color and absorbs maximally at a wavelength of 515nm. Antioxidants such as certain phenolics are able to scavenge the free radicals of DPPH resulting in decrease in intensity of the purple color, which can be measured by spectrophotometer (Porto *et al*., 2000). A stock of DPPH solution was prepared by weighing 0.25 mg of DPPH powder and dissolved it in 100 ml of 50% methanol. The solution was stored in a dark glass bottle wrapped in aluminum foil since DPPH is sensitive to light. Gallic acid was prepared at the concentration of 300mg.l^{-1}. After setting the spectrophotometer program to automate assay, the sample cell cuvette was filled with 1800 µl of DPPH and added with 200µl of sample. Absorbance at the wavelength 515 nm was recorded at 15 second interval for 3.5 min. The procedure was repeated using DPPH in 50% methanol as a control. The data was statistically analyzed using one-way ANOVA.

RESULTS

The reaction of different concentrations of phenols with the folin-ciocalteau reagent produced a blue color with different intensities. A linear graph was obtained when absorbance of gallic acid was plotted against different concentrations (R^2 = 0.9985). The total concentrations of phenolics were based on the solvent proportions. Aqueous extract of algae in lower concentrations of methanol showed higher amounts of phenolics. It decreased in the order of 100% water > 10% > 20% > 25% > 50% > 75% > 100% of methanol solvent. The highest total phenolics were in the 100% water with 252.72 mg.l^{-1} GAE while the lowest was in the 100% methanol with 123.27 mg.l^{-1} GAE as shown in Figure (1).

Based on the different concentrations of methanolic extraction as shown in Figure (2), gallic acid showed the highest free radical scavenging activity with 91% reduction followed by 100% methanolic extract with

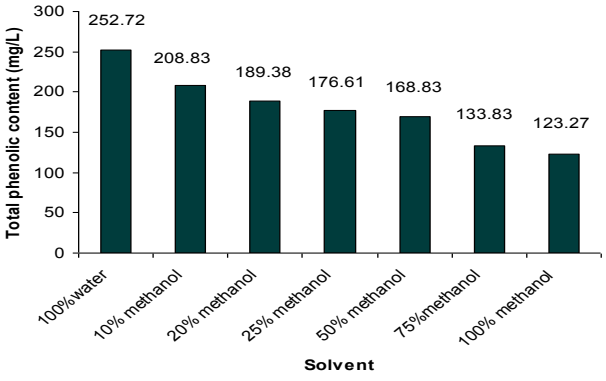

Figure (1): Total phenolics in different percentage of methanolic solvent. All the values were expressed as the mg.l^{-1} GAE ($p \leq 0.05$).

69% while the lowest free radical scavenging activity was shown by 100% water (59%).

The free radical scavenging assay of different concentrations of 100% methanolic extract as shown in Figure 3, the concentration of 120 mg.l^{-1} showed the highest free radical scavenging activity with 53% reduction while 20 mg.l^{-1} showed the lowest activity with 19% reduction.

DISCUSSION

The content of total phenolics in different percentage of solvents extraction decreased significantly ($p \leq 0.05$) in the order of 100% water > 10% > 20% > 25% > 50% > 75% > 100% of methanol solution. The highest concentration of total phenolics was in the 100% water with 252.72 mg.l^{-1} GAE while the lowest was in the 100% methanol with 123.28 mg.l^{-1} GAE. The concentration of total phenolics increased with the polarity of the solvent. This means that the phenolics are more soluble in aqueous than solvent solution. This may be due to hydroxyl groups existing in the chemical structure of phenolics that can provide the necessary component and influence with the concentrations of total phenolics (Prior *et al*., 2005). Tea contains approximately more phenolics as compared to blue green algae as measured by folin-ciocalteau reagent method with 118 600 mg.l^{-1} GAE in ethanolic solvent (Ooi, 2001). However, this does not rule out the usefulness of blue green algae extracts as another potential source of phenolic compounds.

The control result, the 50% methanol, did not show any free radical scavenging activity when compared with all concentrations of methanolic algal extract. Only a small change in absorbance was observed for the control due to the dilution effect when water (50% of total volume) was added to the DPPH solution. In contrast, gallic acid at 300 mg.l^{-1} showed significantly ($p \leq 0.05$) higher changes in absorbance compared to the control for the first 10 seconds and achieved equilibrium after 50 seconds of incubation time. This suggests that gallic acid was able to scavenge free radicals of DPPH at any concentrations of methanolic extract tested. However, the efficacy of gallic acid to scavenge free radicals was dependent on the concentration of the solvent extraction (Sawa *et al*., 1999).

The absorbance of different concentrations of methanolic extract showed a significant decreasing trend ($p \leq 0.05$) of DPPH reduction. Gallic acid has the best antioxidative activity among all the samples followed by 100%, 75%, 50%, 25%, 20%, 10% methanolic extracts and 100% water. The reaction between antioxidant and DPPH occurs very rapid for the first 20 min, with the transfer of the most labile hydrogen (H) atom to the free radicals and the disappearance of the purple color of DPPH occurs almost immediately upon contact between reactants. Therefore, they reduce faster and then become static or

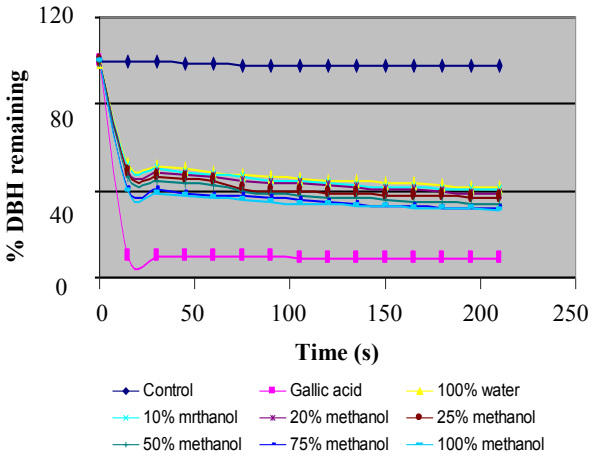

Figure (2): The percentage of DPPH remaining in different concentrations of methanolic solvent and time at 515 nm. The absorbances were measured at 15 second interval for 3.5 min ($p \leq 0.05$).

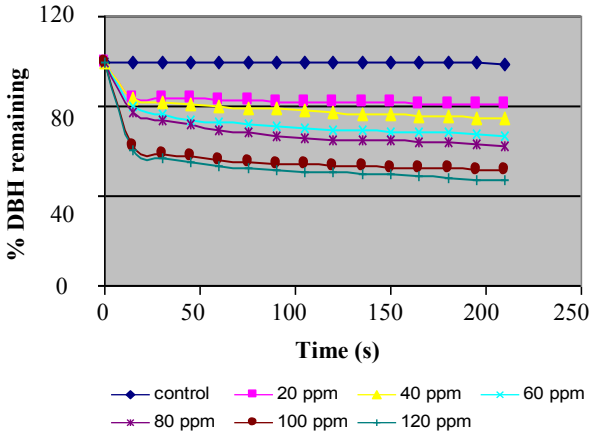

Figure (3): The percentage of DPPH remaining in different concentrations of 100% methanolic extract for 3.5 min ($p \leq 0.05$).

stationary while the subsequent slow step depends on the residual H donating capacity of antioxidant degradation products (Sanchez-Moreno *et al.,* 1999).

The antioxidative activity of phenolics in 100% water was the weakest activity compared to the samples extracted in methanolic solvent. It was found that the percentage remaining of DPPH radicals were achieved at 100% methanolic extracts. The total phenolics are higher in aqueous but low activity in 100% methanolic extract. The different concentrations of *Arthosphera* extract give different reactive oxygen species scavenging activities, and these may be a result of presence of different kinds of phenolic compounds that either hydrophilic or hydrophobic in the sample (Awika *et al*., 2003).

The same condition occurred for the absorbance in samples with different concentrations of 100% methanolic extracts. All the samples showed a significant ($p \leq 0.05$) decreasing trend based on the concentrations of methanolic extract. The efficacies were 20 > 40 > 60

> 80 > 100 > 120 $mg.l^{-1}$, respectively. The antioxidative activity of phenolics at concentration of 120 mgl^{-1} was 53% reduction compared to control. At this concentration, the reaction between antioxidant and DPPH occur rapidly followed by 100 $mg.l^{-1}$ (48%) > 80 $mg.l^{-1}$ (38%) > 60 $mg.l^{-1}$ (33%) > 40 $mg.l^{-1}$ (25%) > 20 $mg.l^{-1}$ (20%). (Sanchez-Moreno *et al,* 1999). observe on the consistent value of scavenging rates of gallic acid and classified these compounds as displaying, intermediate and rapid kinetic behavior, respectively.

The disadvantage of using the folin-ciocalteu reagent method, it does not differentiate the different phenol types. Substances such as sugar, ascorbic acid, aromatic amines, sulfur oxide, iron and other compounds can interfere with the folin-ciocalteu assay. Therefore, correction for interfering substances should be made to accurately measure total phenolics of samples. (Prior *et al*., 2005).stated that non-phenolic substances and inorganic substances may interact with folin-ciocalteu reagent, thus giving an inaccurate and higher than actual amount of phenolics in sample.

Other studies by various researchers have shown that polyphenols are good scavengers of free radicals. Yen and (Chen 1995). showed that tea extracts are good scavengers of DPPH radicals. Gallic acid was shown to have higher free radical scavenging capacity as compared to tannic acid, caffeic acid, quercetin, butylated hydroxyanisole, ferulic acid and resveratrol (Sanchez-Moreno *et al.,* 1999). Free radical scavenging activity capacity of polyphenol was also confirmed in grape products (Sanchez-Moreno *et al.,* 1999), commercial cognacs (Porto *et al*., 2000), olive oil (Visioli and Galli, 1998) and anion skin extracts (Suh *et al.,* 1999).

CONCLUSION

The results of the present study confirmed that blue green algae (*Arthrospira platensis*) contained high significant amount of phenolics. Concentrations of total phenolics extracted decreased from 100% water to 100% methanolic solvent where the highest total phenolics were in the 100% water with 252.72 $mg.l^{-1}$ GAE while the lowest was in the 100% methanol with 123.28 $mg.l^{-1}$ GAE. The phenolic compounds are more soluble in aqueous as compared to methanolic extracts. Blue green algae had potent antioxidative activity, tested by free radical scavenging. As scavengers of free radicals in different percentage of methanolic extracts, the antioxidative activity increased from 100% methanolic solvent to 100% water. One hundred percent methanol showed the highest free radical scavenging activity with 69% while the lowest free radical scavenging activity was showed by 100% water with 59% reduction. DPPH of free radical scavenging activities increased with an increase in total phenolics of methanolic fraction. Based on the results obtained from this study, future studies are proposed to get a better understanding of the characteristics and properties of the antioxidants present in blue green algae; purification of the phenolics of blue green algae using high performance liquid chromatography (HPLC) and identify the nature of the individual phenolic compounds in the samples.

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