



## The bioactive component and DNA protective capability of cultured *Spirulina* in Turkey (Marmara Region)

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### Abstract

*Spirulina* is a filamentous microalgae composed of microscopic cells and is a member of the class Cyanobacteria. In this study, antimicrobial and antioxidant activities of *Spirulina* produced in Alg Production Unit of Yalova University Armutlu Vocational School by using the 2500 L volume of transparent polycarbonate green house at temperatures between 28-30 °C were determined. *Spirulina platensis* produced and harvested intensively in June, 2015 and was filtered and washed with 45 micron plankton gland and then dried at -60 °C. Total amount of phenolic and total flavonoid substance contents of *Spirulina platensis* were determined by using spectrophotometric methods. Antibacterial activity tests were determined by disk diffusion method using 4 different standard bacterial strains. For DNA-protecting activity, pBR322 plasmid DNA and UV-C method were used. Total oxidant status was determined using Rel-Assay Diagnostics (TAL, TOL) and DPPH methods. Antibacterial properties were not determined against the determined strains in *Spirulina* samples prepared at different concentrations. *Spirulina* samples are well-known to have antioxidant and antiradical activity. *Spirulina platensis* has been found to have good DNA protective activity. Better DNA protection has been identified, especially with samples at higher concentrations.

**Keywords:** *Spirulina*, pBR 322, UV-C, DNA, TAL, TOL.

### Introduction

Microalgae, which have great importance as primary producers in the nutrient cycle in aquatic ecosystems, are photosynthetic and microscopic organisms that work to establish the CO<sub>2</sub> balance between the oceans and the atmosphere and are sources of O<sub>2</sub> for the atmosphere. Microalgae biotechnology increases the mass of photosynthetic cells that produce the first organic matter in the aquatic ecosystem, adds products to biomass in the collection stage so as to render the biomass valuable, and enhances the commercial value of these species. In addition to genetic studies, researches that affect algae physiology also enable the production of beneficial metabolites for humans. It is an established fact that there are more than 40,000 different microalgae species (Chamarro *et al.*, 1996). It has been determined that microalgae having a high growth potential exhibit photosynthetic activity, which is 10-20% higher than that of land plants due to their simple structure. Consequently, they grow faster and can generally triple or quadruple in weight within just a few hours. Considering all these features, algae are more ideal in terms of producing lipids or other

metabolites in comparison to land plants. Microalgae cells increase the production of some metabolites within the cell in order to protect themselves and enhance their strength when they are under unsuitable conditions. They store some materials in their cells when the light is intense or some nutrients are lacking in the medium. This storage system may include pharmaceutical and nutraceutical substances such as nutrients, pigments, proteins, fat and glycerol (Guiry, 2012). *Spirulina* is a blue-green alga, that is used as a dietary protein supplement or food additive. It is highly rich in proteins, carotenoids, polyunsaturated fatty acids, vitamin B complex, vitamin E and minerals. *Spirulina* contains two types of biliproteins, which have antioxidant properties, i.e. C-phycocyanin and allophycocyanin. Approximately 20% of the microalgal protein fraction consists of phycocyanin, which is a water-soluble blue pigment (Kalafati *et al.*, 2010; Mishima *et al.*, 1998). Especially in recent years, it has been shown that the active ingredients in natural products could have important roles in terms of offering protection from diseases associated with oxidative stress. It is believed that these biologically active substances eliminate the harmful effects of free radicals on the cell with their antioxidant character.

Here with, identifying the antioxidant activity of a natural product under testing is considered a starting point for more extensive studies (Kaur and Kapoor, 2001). Many methods have been defined to identify the antioxidant activity of herbal extracts. Total antioxidant level, total oxidant level and radical scavenging are antioxidant measurement methods based on electron transfer.

These methods enable us to determine the ability of an antioxidant agent in reducing an oxidant compound added to the medium based on color change. The darkening of the color, hence higher absorbance values, is proportional to the antioxidant power of the agent (Dai and Mumper, 2010). They are also frequently used in studies that assess the antioxidant character of natural products due to being fast, reliable and cost-efficient (Kaur and Kapoor, 2001).

Therefore, in this study, the antioxidant characteristics of extracts prepared from spirulina biomass were identified. One of the most effective radicals that appears in the metabolism is the hydroxyl and hydrogen peroxide radical. This radical can easily attack nucleotides and cause permanent damage to the structure of DNA, which is the most important genetic nucleotide (Gutteridge and Halliwell, 2000). In the presence of H<sub>2</sub>O<sub>2</sub>, DNA exposure to UV rays causes open-circular DNA fragmentation and formation of linear DNA (linDNA: one or more fragmentations in both chains) (Tepe, Degerli, Arslan, Malatyali, & Sarikurku, 2011). Meanwhile, genetic disorders may develop as a result of fragmentations in the DNA chain. Irreversible DNA damage can cause carcinogenesis, aging and other degenerative diseases (Russo *et al.*, 2000). Therefore, we evaluated the oxidant effects in conjunction with the DNA protective activity of spirulina in this study.

## Materials and Methods

### Production of Spirulina and Production Conditions

The *Spirulina (Arthrospira) platensis* employed in this study was produced at an average temperature of 29 °C and pH 11.1 in raceway algae ponds with 2500 L volume within a polycarbonate greenhouse under summer season conditions and then harvested in the Algae Production Unit at Armutlu Vocational High School, Yalova University. Spirulina powder was obtained from the harvested fresh spirulina biomass using the freeze-drying method for 22 hours at -60 °C. The total oxidant status of the spirulina samples that were dried and ground under suitable conditions were determined using Rel Assay Diagnostics kits (TAS, TOS) and DPPH methods. The total phenolic content and total flavonoid content in spirulina were determined using spectrophotometric methods. Phenolic content was specified in terms of

mg gallic acid/100g weight. The results of flavonoid content assay were given in terms of mg catechin/L. Antibacterial activity tests were conducted using four different standard bacterial strains with the disk diffusion method. The pBR322 plasmid DNA and UV-C method were used for DNA protective activity.

### Determination of DPPH Free Radical Scavenging Capacity and Antioxidant Activity

The free radical scavenging capacity of spirulina was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. When DPPH solution is added to the extracts it results in a decrease in the absorbance value at 517 nm optical density and if the extracts become lighter in color it indicates that they are radical scavengers (Hatano, Kagawa, Yasahara, & Okuda, 1988). DPPH solution added to two different spirulina samples at different concentrations results in a decrease in the absorbance value at 517 nm optical density and the color of the compound becoming lighter indicates that the spirulina component is a radical scavenger. Results were evaluated by assessing DPPH radical scavenging activity % followed by the calculation of the IC<sub>50</sub> value, and it was seen that the level of anti-radical activity was quite high in both concentrations.

DPPH radical scavenging activity was calculated according to the formula given below:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

### Determination of Total Antioxidant Activity Using the ABTS Method

Commercially available kits were used to determine the antioxidant capacity of spirulina extract. Antioxidant agents in the sample transform the dark blue-green ABTS radical to reduced ABTS form and the change in absorbance at 660 nm indicates the total antioxidant level of the sample. ABTS (2,2-azino-bis (3-ethyl benzo thiazoline-6-sulphonic acid) was used as the radical and vitamin E analogue Trolox was used as the positive control (Halliwell, 1994). The Rel Assay Diagnostics- TAS Assay Kit was used in this study.

### Determination of Total Oxidant Status (TOS) and Oxidant Status

This test is calibrated using hydrogen peroxide. In the presence of oxidant, ferrous ion oxidizes the chelator complex into ferric ion. The density of oxidation reaction increases depending on the presence of oxidants in the reaction medium. Ferric ion forms a colored complex in an acidic environment. Color intensity is measured spectrophotometrically. The absorbance value

indicates the oxidant value in the sample. The results obtained were compared with  $\mu\text{mol H}_2\text{O}_2$  equiv./L to determine the TOS value (Tarpey, Wink, & Grisham, 2000). This study employed the Rel Assay Diagnostics - TOS Assay Kit.

### Oxidative stress index (OSI)

The oxidant level in the spirulina samples with different concentrations was calculated following total antioxidant and total oxidant value measurements (Ulas *et al.*, 2013). Oxidative stress index (OSI) is an indicator of oxidative stress level and is calculated as follows:

$$\text{OSI} = (\text{TOS, } \mu\text{mol H}_2\text{O}_2 \text{ equivalent/L}) / (\text{TAS, } \mu\text{mol Trolox equivalent/L}) \times 10$$

### Total Phenolic and Total Flavonoid Content Assay

#### Phenolic Compound Assay

Total phenolic compound assay was performed using Folin-Ciocalteu's reagent. Distilled water was added to 0.1 mL stock solution prepared from spirulina until a volume of 4.6 mL was reached. 0.3 mL  $\text{Na}_2\text{CO}_3$  solution (2%) and 0.1 mL Folin-Ciocalteu's reagent were added and the resulting mixture was stirred using a vortex. Spectrophotometry absorbance at 760 nm was measured using a spectrometer after allowing the mixture to sit under ambient conditions for 2 hours. The results were calculated in terms of gallic acid equivalent (GAE) (Slinkard and Singleton, 1977).

#### Flavonoid Compound Assay

Methanol was added to 0.1 mL spirulina stock solution until a volume of 4.8 mL was reached. 0.1 mL Al ( $\text{NO}_3$ ) (10%) and 0.1 mL  $\text{NH}_4\text{CH}_3\text{COO}$  solutions (1 M) were added. The resulting mixture was allowed to sit under ambient conditions for 40 minutes after the vortex process, and absorbance at 415 nm was measured using a spectrophotometer and recorded. The obtained results were calculated as catechin equivalent (Ravishankar, Rajora, Greco, & Osborn, 2013).

### DNA Protective Activity of Spirulina

pBR322 plasmid DNA (vivantis) was used in order to determine the DNA protection activity of spirulina from damages caused by UV and oxidative stress. Plasmid DNA was exposed to damage by applying  $\text{H}_2\text{O}_2$  and UV in the presence of capsaicin in the component. According to the method specified by Russo *et al.* (2000) imaging was performed on 1.25% agarose gel (Russo *et al.*, 2000). pBR322 plasmid DNA was used for this purpose. In the medium containing plasmid DNA samples, after exposure to

$\text{H}_2\text{O}_2$  and UV, which have a mutagenic effect on DNA and cause DNA fragmentation, the samples were run on 1.5% agarose gel (Kumar, Devasagayam, Jayashree, & Kesavan, 2001). 3.0  $\mu\text{l}$  pBR322 plasmid DNA (172 ng. $\mu\text{l}$ ) and 1  $\mu\text{l}$  30%  $\text{H}_2\text{O}_2$  were placed in tubes. A UV transilluminator (DNR-IS) device that generates light at 302 nm wavelength and 8000  $\mu\text{W}/\text{cm}$  intensity at ambient temperature was used as the light source. After gel electrophoresis was conducted for 45 minutes, the photograph was obtained through imaging in the gel documentation system (DNR-IS, Mini BISa Pro).

### Antimicrobial Activity Assay

In order to conduct the antimicrobial activity assay, the disk diffusion test was performed in accordance with the European Committee on Antimicrobial Susceptibility Testing's (EUCAST) criteria (CLSI, 2016). The spirulina samples prepared for the test were dissolved in distilled water. 1 ml distilled water was used for a 50 mg sample. In the study, the test group was set at two concentrations. A vortex device was used in order to ensure the homogeneity of the mixtures, followed by impregnating sterile blank disks of 6mm diameter with 20  $\mu\text{l}$  of mixture. Some amount of *S. aureus* (ATCC 29213), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25322) and *S. maltophilia* bacterial strains that were previously checked for reproduction in their medium were placed separately in SF broth and evaluated using a Mcfarland device based on the 0.5 Barium Sulfate Turbidity Standard. Samples collected using sterile swabs from these suspensions, which were suspended and vortexed, were inoculated onto Mueller-Hinton agar surface in an amount of 100  $\mu\text{l}$  and seeded for disk diffusion purposes. After these steps, disks impregnated with samples were placed on an agar surface using sterile tongs. The mediums were incubated for 24 hours at 35 °C. After the incubation was complete, the diameters of the zones formed were measured using a ruler. In the study employing *S. aureus* (ATCC 29213), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25322), and *S. maltophilia* bacterial strains, zone diameters were not observed in any of the different concentrations. In light of these results, it was determined that these concentrations do not have an antibacterial effect on this bacterial strain.

### Results & Discussions

Lower OSI values indicate lower oxidative stress. The OSI values of spirulina were found to be at very low levels in our study. These low OSI levels indicate very low oxidant activity, and the fact that antioxidant levels are very high in both of the different concentrations can be seen when compared to the reference values in Table 1.

Throughout the world, especially in America and Japan, spirulina is used in powder, capsule or

tablet form as a dietary supplement. With an annual production of over 2000 tons globally, it is also consumed by being added to dairy products, confectionaries and some dishes (Oğuz, 2008). In Turkey it has started to be consumed in recent years. In this study, we evaluated some biological activities of spirulina produced within the scope of the TAGEM project conducted at Yalova University, Turkey. Today, antioxidants are added to human food in order to prevent the damages caused by free radicals on cells. There is an increase in antioxidant use as a dietary supplement. It has been determined in phycobilin studies constituting a major part of phycocyanin which is one of the active ingredients in spirulina, has a role in eliminating hydroxyl radicals (Zhou *et al.*, 2005). Studies have shown that Spirulina extract eliminates free radicals (hydroxyl and peroxy) and reduces microsomal lipid oxidation. It has been determined that this antioxidant activity stems from phycocyanin, which is a biliprotein. It has also been reported that phycobiliprotein and phycocyanin are important for antioxidant activity (Estrada, Bescos, & Fresno, 2001). Unsuitable drying methods used during spirulina processing lead to approximately 50% phycocyanin loss. Therefore, in this study, spirulina biomasses were obtained by freeze-drying. Hence, cases involving the loss or reduction of active ingredients that have biological activity in spirulina samples were eliminated in this study. The study revealed that the antioxidant level of spirulina was significantly high, whereas oxidant levels were significantly low. It has also been reported in the literature that it plays a critical role in antioxidants in eliminating the harmful effects of UV rays that cause

DNA damage (Hitoshi, 2010). In fact, human skin has several mechanisms to reduce the harmful effects of VIS (visible rays) and UV rays (especially UV-C). However, high levels of exposure to UV rays can lead to a reduction in the number of cellular antioxidants and ultimately lead to UV-induced oxidative DNA damage caused by reactive oxygen species (André *et al.*, 2017).

In addition to UV rays, free radicals can cause DNA damage. For example, a free radical species, and hydrogen peroxide can cause DNA damage by converting guanine to 8 - hydroxy-guanine (Cadet, Sage, & Douki, 2005). For this reason, we would like to evaluate the protective activity of DNA against oxidative conditions and UV rays in our study. In this study, the DNA protector analysis demonstrated the strong anti-oxidant properties of spirulina. Recently, detailed studies have been conducted on extracts from medical and aromatic plants to investigate new compounds that control oxidative DNA damage causing cancers (Oguzkan *et al.*, 2016). DNA damage can arise from the harmful effects of UV rays leading to a destruction of the stratosphere layer, and negative effects on living organisms. Moreover, UV rays can cause serious diseases, such as include skin cancer and skin aging. Topical application of both enzymatic and non-enzymatic skin antioxidants is an effective approach to protect skin against harmful effects of UV rays (Lien, Pham, Hua, & Chuong, 2008). For this reason, the use of alternative agriculture products in healthcare sector, especially in cosmetics and medicine should be assessed by testing the protective activity on DNA. In this study, spirulina extracts have been demonstrated to be a good protective

**Table 1.** Antioxidant (TAS), oxidant (TOS), DPPH, IC<sub>50</sub> and OSI values of spirulina

| Spirulina                | 1/10 Concentration | 1/20 Concentration | Reference Range  |
|--------------------------|--------------------|--------------------|------------------|
| TAS (mmol/L)             | 4,142              | 3,195              | TAS>2 Good Value |
| TOS (mmol/L)             | 1,54               | 1,23               | TOS<5 Good Value |
| OSI (µmol/L)             | 0,37               | 0,37               | OSI<1 Good Value |
| IC <sub>50</sub> (µg/ml) | 1,53               | 1,35               |                  |



**Figure 1.** DNA protective activity bands of spirulina.

K1: Plasmid DNA (3 µl) + dH<sub>2</sub>O (6 µl); K2: Plasmid DNA (3 µl) + dH<sub>2</sub>O (6 µl)+ UV+ H<sub>2</sub>O<sub>2</sub> (1 µl); Sp 1\10: Plasmid DNA (3 µl) + spirulina (5 µl)+ UV+ H<sub>2</sub>O<sub>2</sub> (1 µl); Sp 1\20: Plasmid DNA (3 µl) + spirulina (5 µl)+ UV+ H<sub>2</sub>O<sub>2</sub> (1 µl)

activity as shown in Figure 1. It was seen that among the spirulina samples at different concentrations, the spirulina sample with a higher concentration had higher activity in comparison to the control group. Thus, we can say that the effect of spirulina on DNA conservation is very good.

The IC<sub>50</sub> results obtained using DPPH radical scavenging activity assay, which is applied to determine the antioxidant status, were found to be significantly high, wherein it was determined that the spirulina produced in Turkey has extremely good antioxidant properties. The antimicrobial activity of *S. platensis* varies depending on the different ingredients contained within.

The synergistic effect of the  $\gamma$ -linolenic acid (Demule, Decaire, & Decano, 1996), active fatty acid (Xue et al., 2002) and lauryl contained in *S. platensis* varies and their antimicrobial activity changes depending on the palmitoleic acid. In the literature, studies concerning the antimicrobial activity of *S. platensis* have reported significantly different *S. platensis* antimicrobial activities depending on the microorganisms tested. For example, it was significantly effective against *Candida albicans* yeast strain, but not effective against gram-negative bacteria (Mostafa et al., 2014). In our study, no antibacterial activity against *S. aureus* (ATCC 29213), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25322) and *S. maltophilia* strains was identified. On the other hand, some bacterial species did not respond to extracts of *S. platensis*, whereas the purified fractions showed a broad-spectrum of activity against different test organisms. This might be due to the masking of antibacterial activity through the presence of some inhibitory compounds in the extract as observed by Sastry & Rao (1994). Phenolic and flavonoid substances are secondary metabolites generally found in fruits and vegetables, wherein they exhibit free radical scavenging, enzymatic activity regulating, cell proliferation inhibiting, antibiotic, antiallergic, anti-diarrheal, anti-ulcerative and anti-inflammatory effects (Hajimahmoodi et al., 2010). Some studies on spirulina have reported that spirulina contains very low amounts of phenolic and flavonoid substances found in some plants (Onofrejova et al., 2010). The spirulina we produced also exhibited very low amounts of flavonoid and phenolic substances (Table 2).

In recent years, some researchers have found that microalgae contain extremely important bioactive molecules. Microalgae produce a wide range of both

primary and secondary metabolites. Furthermore, it has been reported that algae produce significantly lower amounts of secondary metabolites, which have pharmacological potential, in comparison to many plants (Rastogi and Sinha, 2009). In addition, microalgae are not appealing as they contain very low amounts of phenolic compounds. Some studies have reported that some compounds contained in microalgae under ambient conditions are effective in producing phenols and flavonoids (Grace and Logan, 2000). It is aimed to increase the phenolic substance content with the development of alternative production methods, such as the use of natural reactors for producing spirulina that contains specific bioactive ingredients (Hajimahmoodi et al., 2010). The spirulina produced within the scope of our study also exhibited significantly low amounts of flavonoid and phenolic substances.

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**Table 2.** Phenolic, flavonoid results of spirulina

|                                 | Spirulina<br>(1/10) | Spirulina<br>(1/20) |
|---------------------------------|---------------------|---------------------|
| Flavonoid (catechin/ $\mu$ g)   | 0,0022              | 0,0014              |
| Phenolic (gallic acid/ $\mu$ g) | 0,0020              | 0,0025              |

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