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Antioxidant and anti-diabetic properties of *Spirulina platensis* produced in Turkey

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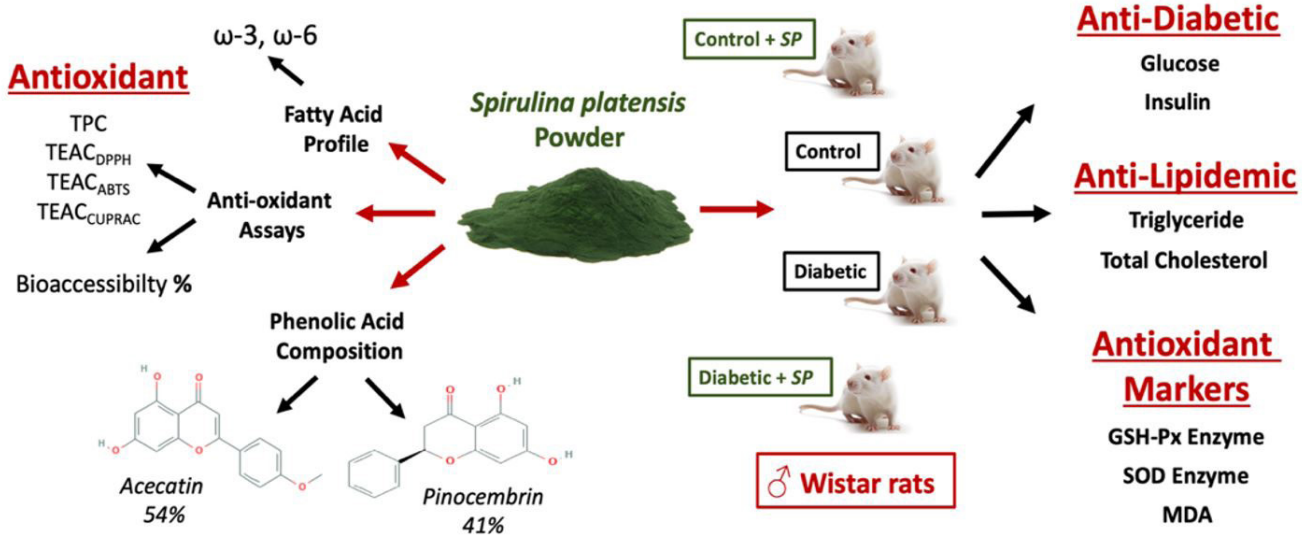
Abstract

Spirulina produced in Turkey has a high antioxidant capacity determined by the three common methods (ABTS, CUPRAC, and DPPH). The major phenolics found in *Spirulina* were acacetin (53.62%) and pinocembrin (41.28%). The bio-accessibility values of the phenolic compounds in *Spirulina* were approximately 60%. PUFA's are the significant antioxidant compounds to prevent lipid peroxidation. *Spirulina* has been found to reduce blood sugar and oxidative stress due probably to the high amount of *ω*-6 PUFA. By the treatment of *Spirulina*, the levels of antioxidant enzymes (GSH-Px and SOD) were increased 240 and 60% in the healthy rats, while 19 and 59% in the diabetics. In diabetic rats fed with *Spirulina*, glucose, triglyceride, total cholesterol levels in blood and malondialdehyde content in body tissues were decreased by 20, 31, 22 and up to 56%, respectively. *In-vitro* and *in-vivo* tests have shown that *Spirulina* has anti-hyperglycaemic, anti-hyperlipidaemia and antioxidative effects on diabetic rats.

Keywords: *Spirulina platensis*; antioxidant capacity; anti-diabetic; anti-hyperlipidaemic; oxidative stress.

Practical Application: Antioxidant, anti-diabetic and anti-hyperlipidemic properties of *Spirulina* produced in Turkey.



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1 Introduction

Diabetes mellitus is a systemic chronic metabolic disease characterized by clinical and biochemical indications of hyperglycaemia, dyslipidaemia, and glycosuria. There are a number of factors in the aetiology of *diabetes mellitus*. The prevalence of the disease mostly depends on genetic reasons, physiological factors, sedentary lifestyle, and obesity. Dietary habits such as high consumption of sugar and saturated fats are thought to be the main contributing factors to disease progression. There has been found a strong correlation between the several chronic diseases e.g. diabetes and consumption of polyunsaturated fatty acids (PUFA's) e.g. *ω*-6 and *ω*-3 in the diet (Neuman et al., 2017).

One of the main reflections of these chronic diseases in the body is hyperglycaemia that causes non-enzymatic glycation of proteins, glucose oxidation and oxidative degradation of these

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proteins. During the normal oxygen cycle or consumption in the body, free radicals are constantly produced by mitochondria. These free radicals are the initiation factors that can cause changes in the structure of lipids, proteins and nucleic acids. As a result of oxidation of those compounds that was caused by the free radicals, oxidative stress in the body increases and causes disruption of the balance between pro-oxidants and antioxidants in favour of pro-oxidants. Lipid peroxidation that causes oxidation of polyunsaturated fatty acids (PUFA) available in the biological membranes is initiated by reactive oxygen species that are responsible from the oxidative stress. In this cumulative reaction, MDA is formed as the final product of lipid peroxidation (Shinde et al., 2012). Lipid metabolism of diabetics also tends to deteriorate. When lipid profile of a diabetic person deteriorates; sensitivity to the lipid peroxidation and thus the risk of atherosclerosis increases. Therefore, hyperlipidaemia is a significant risk factor for atherosclerotic vascular disorders in diabetes. On the other hand, oxidative stress is closely related to the elevated sugar level in the blood. There has been found a close correlation between diabetes-based complications and the lipid profile in diabetic patients. In previous studies, *S. platensis* has been recommended as a food supplement that can reduce blood glucose and oxidative stress due to its antioxidant properties (Johansen et al., 2005). However, there is still a need to investigate its antioxidant effects on living organisms e.g. test animals. Antioxidant enzyme levels are among the parameters that have been used to evaluate antioxidant effects of some food components in the body. Biochemically, the activation of the antioxidant enzymes in the body and the balance between oxidants and antioxidants are strongly related to the anti-oxidative compounds consumed in diet. The most known antioxidant enzymes to evaluate oxidation stress in the body are glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) (Yegin & Mert, 2013). In recent years, many studies have been conducted to clarify the connection between consumed antioxidants in diet and chronic/metabolic diseases. For example, *Spirulina* is described as a superfood that can help to control metabolic syndrome (van den Driessche et al., 2018). In another research, *Spirulina* has been found to act as a free radical scavenger to inhibit lipid peroxidation and prevent DNA damage in fish (Abdelkhalek et al., 2015).

In nature, *Spirulina* is a microalgae growing in alkaline waters of the tropical regions and its industrial production has been developing very fast in recent years. *Spirulina* is blue-green algae and non-toxic species of cyanobacteria. *Spirulina* has been consumed as a food supplement due to its high protein content, essential amino acids, vitamins (vitamin B_{12}, B_6, B_2, A, and E), minerals (Fe, Ca, P, Mg, Zn, Cu, Cr, Mn, Na, K, and Se), enzymes, essential fatty acids, and other nutrients (Guldas & Irkin, 2010). It has been defined as a GRAS (Generally Recognized as Safe) food supplement by FDA in 2011. Although there have been conducted many studies related to *Spirulina*, limited number of studies regarding its effect on chronic and metabolic diseases has been done so far. In one of the newest studies, *S. platensis* has been found to contribute positively to glucose tolerance in the rats treated with fatty and high sucrose diet (Wan et al., 2019).

Phenolic compounds having antioxidant potential have been intensely investigated in recent years. As one of the phenolic constituents of *Spirulina*, pinocembrin (5,7-dihydroxyflavone) is a flavonoid that has hepatoprotective, anti-inflammatory, antibacterial effects (Shen et al., 2019). Pinocembrin as an anti-glycation agent has a suppressing effect on the enzymatically-induced glycation in the presence of high level of glucose (Potipiranun et al., 2018). Acacetin is another important phenolic compound available in *Spirulina* that has anti-peroxidative and anti-plasmodial properties. Acacetin is a phenolic that acts as an α-glucosidase inhibitor, radical scavenger, and glucose receptor (Sun et al., 2018).

Spirulina is a natural product that has high antioxidant properties due to its phenolic components and polyunsaturated fatty acids. In our study, in order to evaluate the antioxidant properties of *Spirulina*; total phenolic content and phenolic profile were investigated at first. It is obvious that bioavailability of food components into the systemic circulation differs due to many complex chemical and/or biochemical reactions seen during digestion. None of the food components is fully absorbed by the human gastrointestinal system and therefore, bioavailability that is a term that means the rate of absorption from the site of administration to the systemic blood circulation, is more important concept than the amount of food or food component consumed. But, determination of bioavailability is too difficult in vitro conditions. Thus, bioaccessibility that means the total amount of a food compound is potentially available for absorption was used to express rate of absorption for the food components in our research.

In recent years, functional and nutritional potentials of *Spirulina* have been investigated by many researchers. In this research, antioxidant capacity, phenolic contents (Hydrolyzable and extractable ones), bioaccessibility, phenolics and fatty acid profile of *Spirulina* that has been produced in the climatic conditions of Turkey, were determined. On the other hand, anti-hyperlipidemic, anti-hyperglycaemic and antioxidant effects of *Spirulina* on the selected blood parameters in the living organisms were examined.

2 Materials and methods

2.1 Materials and reagents

Spirulina was provided in powdered from Iskoc Corp., Mugla, Turkey. All reagents utilized in analyzing were in analytical-grade purity. High-quality water was used and obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Ethanol and methanol (Merck, CAS Number: 64-17-5, Darmstadt, Germany) were used in the analyses. Carbohydrate (glucose, fructose, and saccharose) standards were supplied from Sigma-Aldrich (St. Louis, USA). The phenolic acids (cafeic, chlorogenic, neochlorogenic and gallic) and flavonoids (kaempferol, quercetin, luteolin, apigenin, pinocembrin, pinoquercetin, 3,3-dimethylquercetin, sakuranetin, taxifolin, methylquercetin and acacetin) were purchased from Sigma-Aldrich (St. Louis, USA) and Fluka Chemie AG (Buchs, Switzerland). The other chemicals used for the analyses are as follows: Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, CAS Number: 53188-07-1), DPPH (1,1-Diphenyl-2-picryl hydrazine, CAS Number: 1898-66-4), ABTS (2,20-azinobis-(3-ethyl benzothiazoline-6-sulfonic acid, CAS Number: 30931-67-0), gallic acid (CAS Number: 149-91-7) pepsin (CAS Number:

9001-75-6) and pancreatin (Catalog Number: 1071301000) from Sigma-Aldrich (St. Louis, USA); gallic acid and neocuproin from Merck (CAS Number 484-11-7, Darmstadt, Germany) and bile salt from Thermo Scientific (Catalog number: LP0055J Waltham, Massachusetts, USA); ELISA kits for insulin YLA00337RA YL, GSH-Px YLA0119RA YL and SOD YLA0115RA YL from Biont, Shanghai were purchased.

2.2 The test animals

Thirty-six adult male Wistar rats were used. The weights of the rats were approximately 350-400 g. The rats were allowed free access to standard laboratory chow and three rats were housed in per cage. This study was conducted according to the ethical procedures and policies approved by the Committee for Animal Care and Usage of Bursa Uludag University, Bursa, Turkey (Licence number: 2019-02/06). Animals were randomized and divided into four groups as the healthy rats (control group) "C", the healthy rats fed with *Spirulina* "C+S", the diabetic rats "D", and the diabetic rats fed with *Spirulina* "D+S". Each group was contained nine rats. After the induction period (one week) that is a process to make rats diabetic was completed; the rats were divided into two groups as C+S and D+S and were fed with powdered *Spirulina* (50 mg/kg) during four weeks by means of the gavage application which means the administration of *Spirulina*-water mixture to the rats, through a tube leading down the throat to the stomach. The test animals were fed with the pre-determined dose of *Spirulina* as recommended by Joventino et al. (2012) and Garcia et al. (2018).

Induction process of the rats

The intraperitoneal injection of streptozotocin (Sigma-Aldrich, St. Louis, USA) was used to induce diabetes in the Wistar rats. Streptozotocin (65 mg/kg) that was dissolved in sodium citrate buffer (pH 4.5) previously, was used for the injection. The citrate buffer only was injected to the control rats (C). Blood glucose concentration was measured 48 h after the streptozotocin (STZ) injection. In our study, the rats have the blood glucose concentration is 200 mg/dL, were considered as diabetic and used for the tests.

2.3 Analyses

Sampling and measuring of blood parameters

Blood sampling was made under anaesthesia from the chest area. Blood samples were centrifuged (1500 rpm, Nuve NF 200, Turkey) to separate the serum and plasma for 10 min. Separated samples were stored -20 °C. The organ tissues including heart, muscle, skeletal muscle and liver were taken just after blood collection, washed with saline solution and stored at -20 °C to use for the analyses.

The parameters including insulin, glutathione peroxidases (GSH-Px) and superoxide dismutase (SOD) were determined by ELISA kit. Blood glucose level was measured with the blood sample taken from the rat tail once a week with a glucometer (Optima, Taiwan). For the lipid and the peroxidation assays, blood samples were transferred into EDTA-containing tubes at first, followed by centrifugation, and then the plasma phase was separated and analysed. Triglyceride (TG) and total cholesterol (TC) were measured in the autoanalyzer (TCHO-P 238608 kit for TG; TG-P 223104 kit for TC - Fuji Dri-Chem, Japan). The level of plasma malondialdehyde (MDA) which is an indicator of lipid peroxidation was determined spectrophotometrically (Nanocolor VIS II model, Macherey-Nagel, Germany) by Kamal et al. (1989) and expressed as *n*mole/mg MDA for each type of tissue. The fluid and the food consumptions on a daily base and the body weight with the blood glucose levels (Optima, Taiwan) on a weekly base were determined.

2.4 Physico-chemical analyses

Some physico-chemical properties of *Spirulina* such as pH, total titratable acidity, ash content, crude fiber and crude lipid were analysed. pH value was measured by pH-meter (S220-K Seven Compact, Mettler Toledo, Switzerland) and total acidity was determined by 0.1 N NaOH, expressed as malic acid equivalent. The moisture and ash content were determined according to Rebolloso Fuentes et al. (2000) and given as g/100g sample. Dietary fiber content was evaluated by AOAC method of 985.29 (Association of Official Analytical Chemists, 2007) and crude lipid content was determined according to D'Oca et al. (2011).

2.5 Total phenolics and antioxidant capacity

Antioxidant capacity and total phenolics of *Spirulina* samples were measured by three different extraction procedures (extractable, hydrolysable and bioaccessible) according to Vitali et al. (2009). For the extractable phenolics, 0.5 g of sample was mixed with HCl conc/methanol/water (1:80:10, v/v) solution. Then, the mixture was shaken in a water bath (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 20 °C and 250 rpm for 2 h. The extracts were centrifuged at 3500 rpm in 3 K 30 centrifuge (Sigma, Germany) at 4 °C for 10 min. The residue of extractable phenolics was mixed with methanol/H_2SO_4 (10:1) and kept in a shaking water-bath at 250 rpm and 85 °C for 20 h to obtain hydrolysable phenolics. Following, the extracts were cooled to the room temperature and then, they were centrifuged at 4 °C and 3500 rpm for 10 min. *In-vitro* digestion enzymatic extraction procedure (Bouayed et al., 2012) was used to determine bioaccessible phenolic by means of the model artificial digestion. For the gastric digestion, 0.5 g of *Spirulina* sample was treated with the pepsin enzyme (40 mg/mL in 0.1 M HCl) at 37 °C and 250 rpm for 2 h. Then, the intestinal digestion procedure was applied with porcine pancreatin enzyme (2 mg/mL) and porcine bile mixture (12 mg/mL) at 37 °C and 250 rpm for 2 h. The extracts were finally centrifuged at 15 °C and 3500 rpm for 10 min. The samples obtained by the three different extraction procedures were stored at -18 °C for the analyses.

Antioxidant capacity

The antioxidant capacity of the prepared extracts was determined by three common methods as ABTS (2,20-azinobis-(3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt), DPPH (2,2diphenyl-1-picrylhydrazyl) and CUPRAC (Cupric reducing

antioxidant capacity). The analytical procedures were slightly modified and performed as proposed by Apak et al. (2008). Absorbances of the extracts were read spectrophotometrically and expressed as µmole Trolox equivalent (TE) per g dry weight sample (mean ± SD for triplicates).

Total phenolic content

Total phenolic content was evaluated by the Folin-Ciocalteu method (Apak et al., 2008). Absorbances of the extracts were measured by spectrophotometer (UV Mecasys Optizen 3220, Daejeon, Republic of Korea). The results were determined as mg gallic acid equivalents (GAE) per 100 g dry weighed sample and expressed as the mean ± standard deviation (SD) for triplicates.

2.6 HPLC analysis of Spirulina

Sample preparation

The *Spirulina* samples dried were prepared for HPLC analyses according to Yousef et al. (2013). The *Spirulina* samples dried (2.5-3.0 g) were extracted with 0.3% acetic acid (30 mL) and prepared in MeOH/H_2O (70:30, v/v) using a high-speed homogenizer (IKA Ultra-Turrax) for 2 min. The contents were then transferred into 50 mL tubes and vortexed for 3-4 s. Then, they were centrifuged at 4000 rpm for 15 min at 20 °C. The collected supernatants were transferred into volumetric flasks (100 mL). Acetic acid solution (20 mL and 0.3%) was added on to the precipitated part in the flask and waited for 15 min at room temperature. During the period, the mixture was vortexed three times for 5 min of time intervals. The supernatant phases were collected in the flask and the final volume was adjusted to 100 mL with the extraction solvent pre-identified. The extracted phases were also mixed to obtain completely homogenized sample. The sample (3 mL) was filtered into the HPLC vials using 0.2 μm PTFE syringe filters (Fisher Scientific, New Hampshire, USA). The sample (10 μL) taken from each vial, was injected into the HPLC. If the sample was not used immediately, was stored at -20 °C.

Determination of phenolic profile

The extraction of phenolics was made according to Wahdan (1998). Phenolic composition of the samples was determined according to the method proposed by Socha et al. (2009). At first, the samples were weighed (5 g) and dissolved in 0.2 M of HCl solution. The prepared solution was saturated with NaCl and the phenolic compounds were extracted with ethyl acetate (Merck, Darmstadt, Germany). The sample solutions (20%, w/v) were acidified with HCl solution until the level of pH 2 was provided. After that, the solutions were saturated with approximately 10 mL of sodium chloride (Merck; Darmstadt, Germany) solution (30% w/v). The final solutions were extracted with the ethyl acetate solutions at the three stages (1x50 mL and 2x25 mL). Ethyl acetate was evaporated from the collected extracts at approximately 40 °C under vacuum. The residue was dissolved in 5 mL of methanol (Merck; Darmstadt, Germany) and stored at -18 °C. Then, the extracts were filtered by Millex-LCR syringe filters (PTFE) for the HPLC (High-performance liquid chromatography) equipment (Thermo ScientificTM DionexTM UltiMateTM 3000 RSLC system Waltham, Massachusetts, USA). The recovery rate of phenolic compounds was compared with the pre-defined phenolic standards as follows.

Recovery rate

To determine the efficacy of the extraction method, 1 mg of the standard phenolic acids was added into the sample. Ten different samples were used for spiking and 10 μL of each sample extract was injected into the HPLC instrument. Comparison of the spiked samples was made versus the samples extracted without the addition of commercial standards. The recovery of standard compounds was measured by comparing the difference between the sample and the sample contained the commercial standard. For each compound, the recovery was calculated with the following formula: (TCA-CA)/TCA × 100), where CA is amount of compound in the sample extract (μg) without spiking and TCA is the amount of total compound (μg) in spiked sample extract.

Phenolic acids such as chlorogenic, caffeic, neochlorogenic and gallic acid were detected at 280 nm absorbance value, some flavonoids such as apigenin, pinocembrin and acacetin were measured at 290 nm and while the other flavonoids (luteolin, quercetin, pinoquercetin, 3,3-dimethylquercetin, sakuranetin, taxifolin, methylquercetin and kaempferol) were determined at 330 nm. The mobile phase was eluted at a flow rate of 1 mL/min using the solutions of acetic acid (Sigma-Aldrich, St. Louis, USA) and 2.5 g/100 mL of acetonitrile (Merck, Darmstadt, Germany). The solvent circulation was conducted as following: It was initiated with 3% of acetonitrile and then, the concentrations were increased to 8, 15, 20, 30 and 40% for 10, 20, 30, 40 and 50 min, respectively. Finally, the column was eluted isocratically with acetonitrile before the following injection. The compounds were separated with a LiChrosorb column (C18, 250x4 mm) (Merck, Darmstadt, Germany) at a temperature of 25 °C. Individual phenolic acids and flavonoids were quantified by comparison of standards (Sigma-Aldrich, St. Louis, USA and Fluka Chemie AG, Buchs, Switzerland).

Determination of fatty acid composition

Lipid extraction procedure was made to determine the lipid content of *Spirulina*. Then, the lipid phase obtained from the extraction was used to analyse of fatty acid composition. The lipid extraction and determination of the lipid contents were determined according to AOAC (Association of Official Analytical Chemists, 2005). The fatty acids were converted to fatty acids methyl esters (FAME) by the method proposed by Matos et al. (2016). Fatty acids methyl esters (FAME) were determined by gas chromatography (GC-2014, Shimadzu, Kyoto, Japan). The gas chromatograph equipped with a split injection unit (SPL-2014), flame-ionization detector (FID-2014) and Restek capillary column (RTX) which has 105 m long and 0.25 mm internal diameter, was used. The column coated with 0.25 μm of 10% cyanopropyl phenyl and 90% bis-cyanopropyl siloxane, was used. The temperature for the detector and the injector was at 260 °C. The oven temperature was initially set at 140 °C and kept constant for 5 min. Then, it was increased to 260 °C with elevated

temperature steps (2.5 °C per min) and kept constant for 30 min at this temperature. The injection volume and the split ratio were 1μL and 10:1. The carrier gas was nitrogen and its flow rate was 2.2 mL per min at constant pressure (130.3 kPa). The fatty acids were determined to compare the retention times of the selected standards (Sigma, St. Louis, USA). Relative amounts of the fatty acids were calculated according to their chromatographic peak areas. The data of three independent samples was expressed as the mean value ± standard deviation.

2.7 Statistical analysis

SPSS statistical package (SPSS 16.0, Chicago, IL, USA) was used for the statistical analyses. Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Duncan's multiple range tests were carried out to compare means. Kruskal Wallis test, which is a rank-based non-parametric test, has been used if there is statistically significant difference between two or more groups of an independent variable on ordinal dependent or a continuous variable, was also used for data verification. Differences between the groups were identified by the Mann-Whitney U test in where required, too. The level of significance between means were evaluated according to $p < 0.05$.

3 Results and discussions

3.1 Physico-chemical properties

At first, the basic properties of *Spirulina* were determined as a raw material will be used in the research.

As seen in Table 1, it was verified that it is a rich source of the fiber and the crude lipid, when compared to the other algal sources (Tokusoglu & Uunal, 2003).

Table 1. Physico-chemical properties of the *Spirulina*.

Parameter	
pH	6.95±0.14[1]
Total titratable acidity (g/100 g)[2]	6.12±0.08
Moisture content (g/100 g)	5.33±0.18
Ash content (g/100 g)	7.23±0.14
Crude lipid (g/100 g)	7.39±0.17
Crude fiber (g/100 g)	7.45±0.22

[1]Mean values ± SD (n = 3); [2]Total titratable acidity expressed as malic acid equivalent.

3.2 Antioxidant capacity

Foods are the mixtures of many nutrients and chemicals, which have different single and/or complex chemical functions and antioxidant effects (Alavi & Golmakani, 2017). That is why, three different methods (CUPRAC, ABTS and DPPH) that have commonly been used to determine antioxidant capacity, were used in our research. The effects of *Spirulina* diet on the antioxidant capacity values are given in Table 2 and ranged from 1.28 to 4.33, 1.44 to 6.21 and 1.64 to 7.11 μmole trolox/g, respectively. Differences among the antioxidant capacity methods were probably due to the interactions between the compounds available in *Spirulina* and the active compounds used in the antioxidant capacity measurement method.

3.3 Total phenolics

The contents of the total phenolics (extractable, hydrolysable and bioaccesible phenolics) and antioxidant capacity were given in Table 2. In parallel to high total phenolic content, antioxidant capacities of the hydrolysable phenolics were higher than the extractable phenolics. Among extractable, hydrolysable and bioaccesible phenolics, the highest phenolic content was obtained from the hydrolysable phenolics. This was probably caused by experimental hydrolyzation procedure. It is thought that hydrolyzation causes to release some antioxidant compounds such as phenolics. Thus, microalgal matrix of the *Spirulina* was probably affected by the hydrolyzation procedure that causes to compose some derivate and ions that were released from the complex compounds (Ranneh et al., 2018).

During the extraction, phenolic compounds are generally affected by the chemical structure of the phenolics, extraction method, the particle size and presence of interfering substances. Therefore, it is difficult to identify a standard/uniform procedure that includes all types of extracts, are obtained from various food matrixes. In addition, as similar to typical digestion, some compounds available in food structure can be extracted easily, while some others such as glycosylated ester forms are needed to hydrolyse at first by the intestinal enzymes.

Food processing operations such as homogenization and heat treatment can lead to increase the bioavailability of these components (Abourashed, 2013). That's why; quantity and structure of the extracts can be changed and affected by the treatments used for sample preparation and extraction.

On the other hand, antioxidant capacity can be influenced by the molecular structure (free phenols, glycosides, and iron-phenol chelates e.g.) of polyphenols. In addition, the bioactive properties

Table 2. Total phenol, antioxidant capacity and bioaccessibility of *Spirulina*.

	Extractable Phenolics	Hydrolysable Phenolics	Bioaccessible Phenolics
Total Phenol (mg GAE/100g dw)	13.65 ± 0.03^{b1}	18.66 ± 0.63^{c}	10.11 ± 0.18^{a}
ABTS (µmole trolox/g dw)	1.28 ± 0.06^{a}	4.33 ± 0.46^{c}	3.29 ± 0.36^{b}
CUPRAC (µmole trolox/g dw)	1.44 ± 0.26^{a}	6.21 ± 0.02^{c}	4.53 ± 0.02^{b}
DPPH (µmole trolox/g dw)	1.64 ± 0.25^{a}	7.11 ± 1.23^{c}	3.94 ± 0.02^{b}

[1]Mean values ± SD (n = 3) with different superscripts in the same row are significantly different for extractable, hydrolyzable and bioaccessible phenolics ($p < 0.05$).

of food nutrients can be affected by the food matrix. Therefore, the availability of phenolics can be limited by the phenolic acids that can be interreacted with the other molecules or the food matrixes (Kasote et al., 2015). Strong linear correlations were found between the antioxidant capacity and the total phenolic content. The samples which have higher phenolic contents, also have higher antioxidant capacities (Table 2).

3.4 Phenolic profile

The phenolic profile of the *Spirulina* was given in Table 3. When it was compared to the standards, 15 different phenolic compounds were detected. The two most abundant phenolic compounds in the *Spirulina* are acacetin (35.37 μg/mg) and pinocembrin (27.23 μg/mg). The other phenolics determined are sakuranetin, luteolin, kaempherol, methylquercetin, quercetin and apigenin 0.78, 0.68, 0.53, 0.47, 0.26 and 0.25 μg/mg, respectively. The phenolics in the *Spirulina* have antioxidant properties (Andrade et al., 2018). Among these phenolics, apigenin is a compound that able to trigger autophagy in leukaemia cells.

The phenolic acids (gallic acid, caffeic acid, ferulic acid, *p*-coumaric acid, cinnamic acid, syringic acid, protocatechuic acid and chlorogenic acid) and some flavonoids (catechin and epicatechin) have previously found in microalgae and the cyanobacteria by Jerez-Martel et al. (2017). But, as seen from Table 3, it was found that the phenolic profile of *Spirulina* can be affected by regional differences such as climatic conditions and quantification of phenolics can change according to production and processing conditions.

3.5 Fatty acid profile

The polyunsaturated fatty acid (PUFA) contents are given in Table 4. *Spirulina* is a natural product that contains a significant amount of polyunsaturated fatty acids such as ω-3 and ω-6.

Table 3. Phenolic profiles of *Spirulina* using RP-HPLC.

Phenolics	RT (min)	λ (nm)	Conc. (μg/100 g)
Neochlorogenic acid	5	285	0.02 ± 0.01[1]
Chlorogenic	6	285	0.01 ± 0.01
Luteolin	7	330	0.68 ± 0.09
Caffeic acid	8	285	0.02 ± 0.01
Quercetin	9	330	0.26 ± 0.04
Apigenin	10	290	0.25 ± 0.06
Pinocembrin	12	290	27.23 ± 0.23
Gallic acid	13	285	0.03 ± 0.01
Pinoquercetin	17	330	0.08 ± 0.03
3,3-dimethyl quercetin	23	330	0.13 ± 0.03
Sakuranetin	24	330	0.78 ± 0.09
Taxifolin	28	330	0.11 ± 0.02
Methylquercetin	43	330	0.47 ± 0.07
Kaempherol	45	330	0.53 ± 0.04
Acacetin	47	290	35.37 ± 0.91

[1]Mean values ± SD (n = 3).

In the previous studies, different fatty acid compositions in *Spirulina* have been reported by the researchers. From the literature survey, it has been detected that the different results can be related to the solvent used for the extraction (Matos et al., 2016). Just as phenolic profile of *Spirulina*, its fatty acid profile can also be affected by the climate and processing conditions. It has been seen that *Spirulina* that was produced in another region, can have a different phenolic profile (Matos et al., 2016). In our research, the fatty acids that were found in higher amount were palmitic acid (C16:0) as saturated fatty acid, palmitoleic acid (C16:1) as monounsaturated fatty acid, alpha-linolenic acid (C18:3) as the ω-3 fatty acid and gamma linolenic acid (C18:3) as the ω-6 fatty acid. Among the polyunsaturated fatty acids (ω-3 and ω-6), the highest content was obtained from GLA (1866 mg/100 g). This was followed by the other polyunsaturated fatty acids such as ω-3 and ω-6 which are the important food components for human nutrition and health. These were LA (C18:2) with 144.81 mg/100 g

Table 4. Fatty acid profile of the *Spirulina*.

Fatty Acid	Conc. (mg/100 g)
Saturated Fatty Acids (SFA)	
C12:0	87.35 ± 3.11[1]
C14:0	94.27 ± 4.26
C16:0	3380.17 ± 5.41
C18:0	31.94 ± 4.23
Other SFA's[2]	323.33 ± 11.06
Total	3916.89 ± 36.12
Monounsaturated Fatty Acids (MFA)	
C15:1	378.45 ± 11.32
C16:1	546.14 ± 9.32
C18:1	69.25 ± 2.45
Other MFA's[3]	97.66 ± 6.29
Total	1091.50 ± 13.11
Polyunsaturated Fatty Acids (PFA)	
ω-3	
C16:4	36.14 ± 3.24
C18:3 (ALA[4])	144.76 ± 4.76
C20:5 (EPA[5])	32.65 ± 2.71
C22:6 (DHA[6])	67.49 ± 3.33
Total	281.04 ± 5.77
ω-6	
C18:2 (LA[7])	144.81 ± 18.76
C18:3 (GLA[8])	1866.27 ± 37.37
C20:4 (AA[9])	54.86 ± 4.91
C22:5	46.19 ± 5.54
Total	2112.13 ± 34.29

[1]Values are expressed as mean ± SD (n = 3); [2]Other SFA' s (Tridecanoic acid C13:0, Pentadecanoic acid C15:0, Behenic acid C22:0) were not considered due to minor purities; [3]Other MUFA' s (Margaroleic acid C17:1, Erucic acid C22:1, Nervonic acid C24:1) were not considered due to minor purities; [4]C18:3 (ALA): α-Linolenic Acid; [5]C20:5 (EPA): Eicosapentaenoic acid; [6]C22:6 (DHA): Docosahexaenoic acid; [7]C18:2 (LA): Linoleic acid; [8]C18:3 (GLA): Gamma-linolenic; [9]C20:4 (AA): Arachidonic acid.

and ALA (C18:3) with 144.76 mg/100 g (Table 4). The total amount of *ω*-6 fatty acids in *Spirulina* (2112 mg/100 g) is higher than the *ω*-3 fatty acids (281 mg/100 g). On the other side, the total content of the monounsaturated fatty acids is about 1/3 of the total saturated fatty acids in the *Spirulina*.

It was determined to have a considerable amount of GLA (88.35%) among the investigated PUFA, having a crucial anti-oxidant activity due to its three double bonds. The types of fatty acid composition may vary according to the production method and other external conditions like climate.

3.6 Antioxidant enzymes

When PUFA's are metabolized in the body, are converted to the sub-components having antioxidant capacity in the body. Thus, they play role to activate some antioxidant enzymes such as glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD).

The levels of GSH-Px and SOD in the blood are expressed in Figure 1. When the both group of test animals were fed with *Spirulina* diet; GSH-Px and SOD were found to increase 240 and 60% in the healthy rats, while they were increased 19 and 59% in the diabetic rats. It is obvious that the antioxidant components available in *Spirulina*, caused to a significant antioxidant effect in the rat bodies ($p \leq 0.05$). It was probably occurred due to cumulative effect of the phenolics and polyunsaturated fatty acids available in *Spirulina*.

3.7 Anti-hyperlipidemic and anti-glisemic effects

Oxidation is a very important reaction in the body in terms of many chronic diseases, e.g. diabetes. As a result of oxidation,

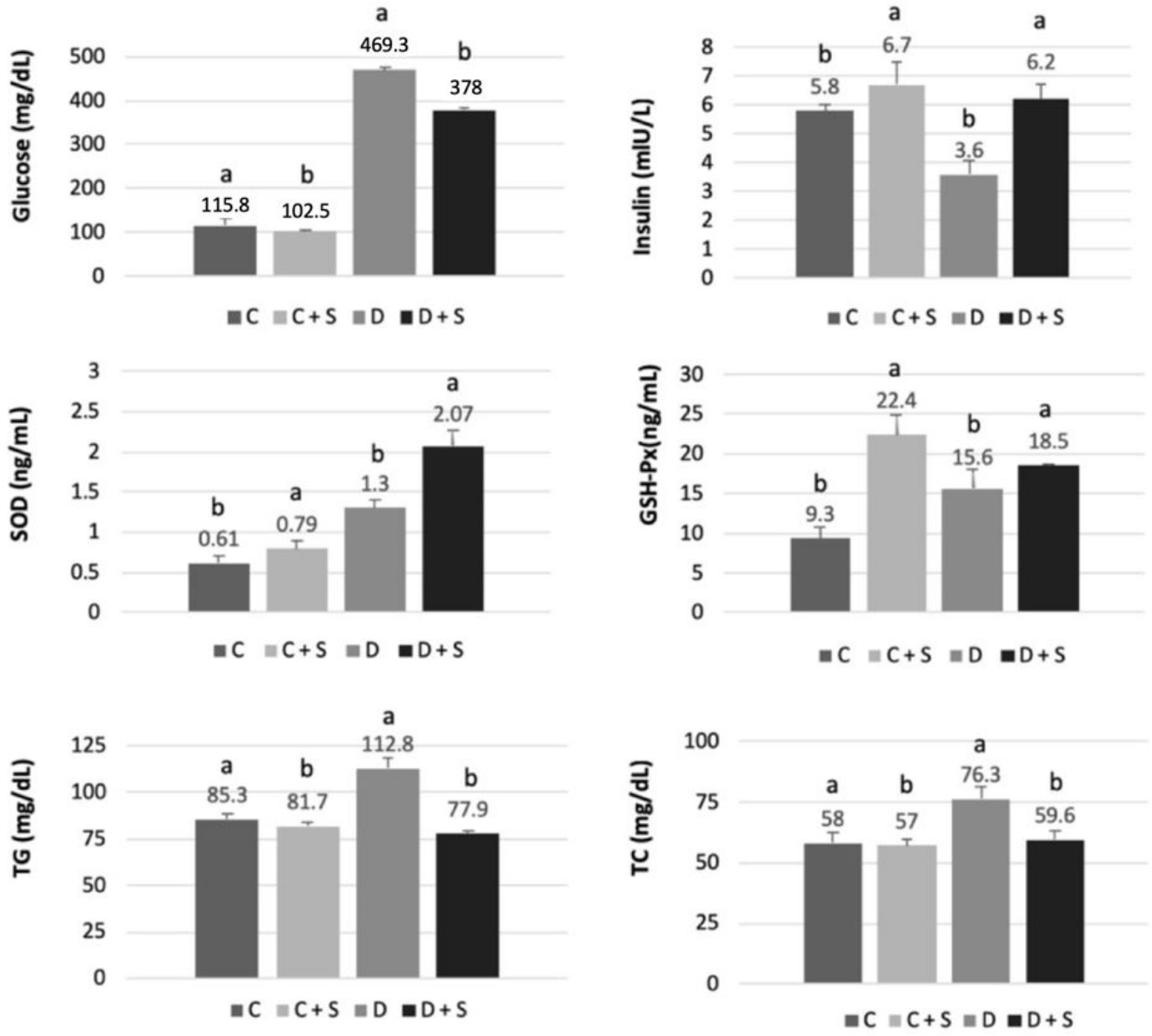

Figure 1. Changes in Glucose, Insulin, SOD, GSH-Px, TG and TC levels in rats. *C: Healthy rats as control group; C+S: Healthy rats fed with *Spirulina*; D: Diabetic rats; D+S: Diabetic rats fed with *Spirulina*.

glucose, which is the main energy source of the cells, is oxidized and causes oxidative stress or load in the cell.

In addition to glucose oxidation, hyperglycaemia causes some other reactions, such as oxidative degradation of the proteins and non-enzymatic glycation of proteins that may increase to the formation of free radicals in the body (Singh et al., 2014; Fournet et al., 2018). In our research, levels of triglyceride (TG) and total cholesterol (TC) were observed to increase significantly in the diabetic group versus to the control group, whereas the levels of TG and TC were decreased significantly in the diabetic rats fed with *Spirulina* (Figure 1). This decrease may be explained by the probable anti-hyperglycaemic effects of *Spirulina*. The *Spirulina* mixture has found to reduce body weight and serum lipids in the rats as an ameliorating agent as expressed by Chen et al. (2019).

Antioxidant activity in the body is an important process in terms of glucose oxidation and its damage to body tissues (Paiva et al., 2019). In this regard, interactions related to antioxidant capacity that limits of oxidation damage on the tissues in diabetes are important for health protection. It has been expressed that the glucose tolerance was improved and the hepatic enzyme NADPH oxidase was decreased in the rats treated by the obesogenic diet with *S. platensis* (Vidé et al., 2018). In our research, glutathione peroxidase enzymes (GSH-Px) were significantly increased in the healthy rats fed by *Spirulina* (C+S group) compared to the control group (Figure 1). Blood glucose, malondialdehyde (MDA) levels in plasma and tissue were reduced significantly in the diabetic rats fed with *Spirulina* (D+S group), while serum antioxidant enzyme activity (SOD) were significantly increased in the D+S group versus to the diabetic ones. According to our findings as expected; food consumption, fluid consumption, and blood sugar levels increased significantly in diabetic rats, while their insulin levels and body weight decreased significantly ($p \leq 0.05$).

On the other hand, oxidative stress causes to excessive increase on free radical concentration in body cells. Free radicals are the compounds formed in the cell as a result of normal metabolic processes, environmental factors and foods consumed. Elevated oxidative stress leads to cell, protein and DNA damage and thus, aging is accelerated. The concentration of malondialdehyde (MDA) is used as a biomarker to evaluate the level of oxidative stress causing DNA damage in an organism. MDA is one of the end products of polyunsaturated fatty acids' peroxidation in the cells. If the free radical concentration increases, excessive production of MDA is also increased. Therefore, plasma and tissue MDA levels are important indicators to evaluate the level of lipid peroxidation. If the concentration of free radicals as the end products of lipid peroxidation increases, the development of many health problems in the body such as atherosclerosis, ischemic and traumatic brain damage are provoked.

The plasma and the tissue MDA levels have also been mentioned to increase in the diabetic rats, previously (Das et al., 2002). In our research, *Spirulina* consumption was observed to reduce the elevated MDA levels in the tissues of diabetic rats (Table 5). However, tissue MDA levels were also found to show a tendency to decrease in the healthy rats fed with *Spirulina* (C+S group). Muscle, liver and kidney MDA levels significantly decreased in the C+S group as 8, 12 and 17% ($p \leq 0.05$). Plasma MDA levels tended to decrease in the C+S group and significantly decreased in the D+S group. This can be explained by hypoglycaemic effect of *Spirulina*. However, there have also been found controversial studies that antioxidant activity in diabetes can increase or decrease under certain conditions (Yegin & Mert, 2013; Ferrari, 2017). In this study, antioxidant enzyme (GSH-Px and SOD) activities significantly increased in the diabetic group versus to the control group. The elevated enzyme activity can be explained by the high lipid peroxidation levels in diabetes. In our study, SOD activity was significantly increased in the D+S group when compared to the diabetic group. In addition, it was seen that GSH-Px activity tended to increase in the D+S group compared to the diabetes only. It has found that there is strong correlation between the high enzyme activity and other antioxidant properties of *Spirulina* measured in our research, as seen from Table 2 and Figure 1. Our results are consistent with the other researchers who conducted a research on the antidiabetic effects of *Spirulina* on the rats (Gargouri et al., 2016).

As previously described, *Spirulina* is a natural food additive with high antioxidant potential. Compounds that have antioxidant

Table 5. Food and fluid intake, body weight and malondialdehyde (MDA) levels.

Parameter	C	C+S	D	D+S
Food intake (g/24h)	15.5 ± 0.3^{b1}	24.8 ± 1.0^{a}	40.0 ± 0.8^{a}	27.0 ± 0.8^{b}
Fluid intake (mL/24h)	29.3 ± 1.9^{b}	35.8 ± 3.6^{a}	119.8 ± 12.2^{b}	112.5 ± 8.3^{b}
Body weight (g)	412.3 ± 5.1^{b}	434.8 ± 1.9^{b}	377.3 ± 1.3^{b}	419.5 ± 1.1^{a}
Plasma MDA (nmole/mL)	2.3 ± 0.2^{a}	2.2 ± 0.8^{b}	9.1 ± 0.9^{a}	4.0 ± 0.9^{b}
Heart MDA (nmole/mg)	112.7 ± 1.1^{a}	97.1 ± 3.0^{b}	147.1 ± 1.6^{a}	119.0 ± 6.6^{b}
Muscle MDA (nmole/mg)	109.8 ± 1.0^{a}	101.5 ± 0.7^{b}	144.4 ± 2.0^{a}	125.8 ± 2.7^{a}
Liver MDA (nmole/mg)	106.7 ± 4.5^{a}	93.8 ± 3.1^{b}	174.2 ± 4.0^{a}	154.2 ± 5.3^{b}
Kidney MDA (nmole/mg)	118.9 ± 4.1^{a}	98.9 ± 2.0^{b}	161.8 ± 3.1^{a}	125.9 ± 9.2^{b}

[1]Values are expressed as mean ± SD for rats each group. The number, sex, and breed of test animals (rats) were 36 males and Wistar. The rats were divided into four groups and each group was included 9 rats. Healthy rats as control (C) with the rats fed with *Spirulina* (C+S) and the diabetic rats (D) with the diabetic rats fed with *Spirulina* (D+S) were compared in terms of statistical difference according to level of significance ($p < 0.05$).

potential in foods are generally bioactive components that contain number of double bonds such as antioxidant vitamins and minerals, flavonoids and phenolic compounds, and *ω*-3 and *ω*-6 polyunsaturated fatty acids (Maleki et al., 2019). As determined in our study, it is thought that the antioxidant potential of *Spirulina* is mainly caused by two components. In our research, *Spirulina* has found to have high amount of the phenolic compounds such as pinocembrin and acecatin, with *ω*-3 and *ω*-6 polyunsaturated fatty acids (Table 3 and 4). These substances have the number of double bounds in their molecular structure that are responsible from anti-oxidant effects (Prisacaru, 2016). Pinocembrin is also a phenolic available in propolis, has been found to have antioxidant, hypoglycaemic and anti-hyperlipidemic effects (Granados-Pineda et al., 2018). On the other hand, antioxidant effect of polyphenols is caused by their ability to bind proteins by means of covalent or non-covalent bonds and modifying proteins into insoluble or soluble compounds (Brudzynski & Maldonado-Alvarez, 2015). High levels of GSH-Px and SOD enzymes detected in *Spirulina* fed mice (Figure 1), which are commonly considered as the indicators of antioxidant activity in blood, are thought to relate with the high amounts of the phenolic compounds (Table 3) and *ω*-3 with *ω*-6 fatty acids (Table 4).

As monitored in the diabetic mice in our research, the main diagnostic characteristics of diabetes are high levels of blood glucose with excessive feed and fluid consumption and low body weight and insulin level (Table 5). *Polyphagia*, defined as persistent hunger and eating due to loss of energy in tissues and muscles due to insulin deficiency in diabetes, showed a tendency to recover 33% in D+S group. As a result of insulin deficiency, incomplete energy needs are provided from broken muscle-fat tissues instead of glucose and, this cause to weight loss as seen from the diabetic rats used in our research (Table 5). Type-I diabetes (insulin-dependent diabetes and juvenile diabetes), is a chronic disease in which the pancreas produces insufficient or no insulin hormone. Insulin is a significant hormone that allows glucose to enter cells to generate energy. *Spirulina* caused to decrease the glucose concentration 11.5% in control rats and 20% in diabetic rats. In parallel to changes seen in the glucose concentrations, the insulin levels were increased 15% in the healthy control rats and 72% in the diabetic rats due to the consumption of *Spirulina*.

Undesirable weight loss in the diabetic rat group due to increased insulin secretion was controlled to a certain level in the diabetic rat group fed with *Spirulina* (D+S) (Table 5). Low glucose level and high insulin level can be associated with the metabolic effects formed in the liver in the diabetic rats fed with *Spirulina*. Anti-hyperglycaemic properties of *Spirulina* have also been observed by Gargouri et al. (2016). *Spirulina* diet as monitored in our research caused to normalize feed and fluid consumption in the diabetic rats.

4 Conclusion

Spirulina, which has anti-hyperglycaemic, anti-lipidemic and antioxidant properties, has been found to be effective in protecting against oxidative stress in the rats with Type-I diabetes and can be used as a dietary supplement in the treatment of diabetes. However, these findings should be confirmed by further animal and human tests.

Spirulina, as an increasing food additive in recent years, has a rich phenolic profile, polyunsaturated fatty acids (PUFA) content and high antioxidant capacity. Beside the high antioxidant capacity that was caused by the number of components, e.g. phenolics, *Spirulina* has significant amount of polyunsaturated fatty acids that have anti-oxidant activity (Choopani et al., 2016). Under the climatic conditions of Turkey, basic quality parameters of *Spirulina* were investigated. It was determined to have a considerable amount of GLA (88.35%) among the investigated PUFA, having a crucial anti-oxidant activity due to its three double bonds. As determined in our research, the types of phenolics and fatty acid composition may vary according to the production method and other external conditions like climate.

Spirulina phenolics were found to have high bioaccesibility of approximately 60% in our study. It can be said that the rate of high bioaccessibility is also confirmed by animal experiments.

It has been mostly observed that MDA levels as an indication of oxidative stress in the diabetic patients tend to increase. In our study, it was observed that MDA levels of test animals decreased with *Spirulina* diet. In addition to its reducing effect on blood glucose, triglyceride (TG) and total cholesterol (TC); MDA were reduced in heart, muscle, liver, kidney, and plasma as 19, 13, 12, 22 and 56% in the diabetic rats treated with *Spirulina*.

Therefore, *Spirulina* is thought to play an active role to eliminate oxidative damage in diabetes including anti-hyperglycaemic and anti-hyperlipidemic functions.

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