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# HYPOGLYCEMICANDANTIOXIDANT EFFECT OF THE ETHANOL EXTRACT OF *CHLORELLA VULGARIS* INALLOXAN-INDUCED DIABETES MICE

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**ABSTRACT : The present study aimed to reveal the effect of ethanol extract of *Chlorella vulgaris* on the levels of both blood sugar and Malondialdehyde (MDA) in the laboratory alloxan-induced diabetes malemice. 160 mice have been divided into five groups, each group contains 8 mice that weretreated at four intervals (7, 14, 21 and 28 days), respectively. The results showed that there was a significant increase (P≥ 0.05) of both sugar and MDA in the blood serum of the laboratoryalloxan-induced mice treated at a concentration of 150 mg/kg in the second positive control group (diabetes induced and injected with 0.1 concentration of physiological solution) compared to the negative control group (injected with the physiological solution). On the other hand, the alloxan-induced diabetes male mice treated with algae extract showed a significant decrease in the levels of both sugar and MDA in the high- and low-dose groups (40 and 80) mg/kg compared to the second positive control group.**

***Key words :*** Diabetes, hyperglycemia, malondialdehyde, *Chlorella vulgaris*.

## INTRODUCTION

Diabetes represents a widespread global health problem resulted from changes in people's lifestyles, such as the nature of city life, eating habits and lack of movement. These factors have contributed to the global spread of this chronic disease. Diabetes leads tomany serious complications, including renal failure, Hyperlipidemia and Hypercholesterolemia, which are among the risk factors that cause Coronary heart diseases and Atherosclerosis (IDF, 2015). Therefore, several efforts have been devoted to overcoming this disease, such as weight andfood control, exercise and therapeutic drugssuch as insulin or oral treatments for anti-hyperglycemia (Hanafeld, 2007).

The use of chemical drugs is considered less safe because of their multiple side effects, such as weight gain and an increase in the problemsof the digestive system (Stein *et al*, 2014). Their use also may not slow down the development of the disease (Wolfson *et al*, 2007). Therefore, many researchers have tried to find alternatives that are less toxic and harmful to the body, on the one hand,and have a powerful effecton controlling the treatment of the disease on the other. The optimal choice was medicinal plants and herbs (Chauhan and Dixit, 2007), which can be more useful in reducing both blood sugar and side effects compared to chemical drugs (Souza *et al*, 2012). Interest has also increased nowadays in obtaining antioxidants from natural sources, including algae extracts, as it is recommended to use natural instead of the carcinogenic and toxic chemically manufactured compounds.Experimental studies have indicated the potential benefit of antioxidants for the prevention and treatment of diabetes. Microalgae are a renewable energy source for active chemicals, especially antioxidants (Tavakoli *et al*, 2017). *Chlorella vulgaris* is a single-celled green alga used in the prevention of diabetes and lipid oxidation in food (Elsheikh *et al*, 2018). Sun *et al* (2011) have tested different extracts of *Chlorella zofingiensis*. Each extract contained different concentrations of carotenoids and astaxanthin. They have noticed that extracts rich in astaxanthin are powerful anti-diabetic drugs. In another study of a group of patients with non-alcoholic lipid liver disease (NAFLD) and high blood sugar, patients were given 300mg of *Chlorella* sp every day in addition to vitamin E for eight weeks. A decrease at the probability level (P≤0.05)was observed in their blood sugar and lipid profile (Ebrahimi-Mehrangiz *et al*, 2014).

In light of the medicinal importance of algae and its widespread use in the medical and pharmaceutical preparations and food industries and to contribute to

studies that are concerned with the effect of some algae species in reducing the level of sugar in the blood of laboratory animals, this study aimed to investigate the effect of algae extract of *C. vulgaris* on blood sugar and MDA levels in the alloxan-induced diabetes mice.

## MATERIALS AND METHODS

### Experimental animals

The experiment was carried out on male laboratory mice of the *Musmusculus* - Balb/c species, whose weight ranged between (23-25g). The mice were placed in plastic cages equipped with a metal lid with a clip containing a water bottle and a place dedicated to laying the feed. The floor was covered with clean sawdust replaced every week to keep the mice clean. The animals were given their food and put in the animal house of the Department of Biology, College of Education for Pure Sciences - University of Basrah.

### Experiment design

The experiment used 160 male laboratory mice whose ages ranged (10-12) weeks and weighed (23-25) g. It was divided into four intervals (7, 14, 21 and 28 days). Each intervalcovered five groups, each group containing eight mice. These are:

1. The negative control group was injected with0.1 ml of physiological solution, the normal saline.
2. The first positive control group (algae extract) was injected with a 0.1 ml of the algae extract at a concentration of 80 mg/kg.
3. The second positive control group (induced diabetes) was injected with 0.1 ml of the physiological solution.
4. The low-dose treatment group (induced diabetes) was injected with 0.1 mlof algae extract at a concentration of 40 mg/kg.
5. The high-dose treatment group (diabetic induced) was injected with 0.1 ml of algae extractat a concentration of 80 mg/kg.

### Diagnosis of active compounds of an alcoholic extract using a Spectrometry Gas Chromatography-Mass Apparatus

Biologically active compounds were diagnosed in alcohol extract isolated from the algae using the GC-mass (Agilent 7890B GC with 5977A MSD) device available in the Directorate of Environment and Water - Environmental Research Center, Baghdad. The following conditions were followed in isolation and diagnosis:

Column type 5% phenyl methyl siloxane, pressure 100kpa, inert gas flow rate (inert gas used is helium 1 ml/ min), while the temperature of column stove was 70ºC, after that it was raised to 280ºC at a speed rate of 10ºC / min, injection degree 280ºC, solvent cutting time was 4 minutes, injection pattern was Pulsed splits, examination range of molecular weight was 40-600 m/z and test time was 20 minutes, injection volume was 1µl. After obtaining the mass spectrum for each compound, the results were processed by the GCMS Solution program and the separated peaks were defined based on the NIST Library.

### Induce diabetes mellitus in animals

Diabetes mellitus was induced in laboratory mice after the food was blocked for 12 hours. Later on, the animals were injected with the alloxanin the intraperitoneal (IP), at a concentration of 150 mg/kg of body weight, with a volume of (0.1ml) per animal. After seven days of injection, the blood sugar level was checked in some animals, it was around 250 mg/dl. The animals were then regarded as diabetic (Jothivel *et al*, 2007).

### Preparing doses

Two doses of *C. vulgaris* algae extract (40 and 80) mg/kg, were prepared depending on the lethal dose of half of the experiment animals $LD_{50}$. Their value was more than 5,000 mg/kg (Himuro *et al*, 2014).

### The method and duration of injection

Male mice were injected into the intraperitoneal (I.P) for four intervals (7, 14, 21 and 28 days) one injection per day. Low-dose group mice were injected at a concentration of 40 mg/kg, while the high-dose group injected at a concentration of 80 mg/kg of algae extract.

### Collection of blood samples

After anesthetizing with chloroform, the animals were dissected. The blood was then drawn from the hearts of male laboratory mice directly (Cardiac puncture) using a 1 ml medical syringe at four intervals, as blood was drawn seven days after the laboratory animals were treated with the algae extract. Blood was also drawn after 14, 21 and 28 days of treatment. Blood samples were then placed in Jell tubes free of the anticoagulant. They were left for 30 minutes for clotting to obtain the serum that was separated by Centrifuge equipment with a speed of 3000 rpm for 10 minutes. Later, they were placed in Eppendorf tubes and stored at -18°C until biochemical tests were performed (Thavasu *et al*, 1992).

### Determination of serum glucose level

The serum glucose level was measured following the Tietz (1995) enzymatic method by using the equipment supplied by the British company, Randox. The absorption of the serum sample was measured at 500nm wavelength.

### Determination of Serum Malondialdehyde level (MDA)

The Beuge and Aust (1978) method modified by Wysocka *et al* (1995), was adopted as Thiobarbituric acid (TBA) interacted with MDA and the reactive material was read at wavelength 532nm.

### Statistical analysis

The Statistical Package for Social Science (SPSS) V.20 program was adopted to analyze data statistically by using the table of variance analysis Anova at the probability level P≤0.05 (SPSS.2001).

## RESULTS

### The chemical diagnosis of the algal extract under study

Table 1 and Fig. 1 represent the absorption spectrum of the algae extract, which showed that it is composed of 29 compounds. Several compounds formed a high percentage among other compounds.

### The effect of algae extract on the glucose level in the blood serum of male laboratory mice

The results showed a significant increase at (p≤0.05) in the sugar level in the second positive control group compared to the negative and first positive control groups and the low- and high- dose groups throughout the four intervals.

The statistical analysis also indicated a significant decrease in both the low- and high-dose groups compared to the second positive control group during the four intervals. A significant decrease was observed in the first positive control group compared to the negative control and the second positive and the low-and high-dose group during the 7-day interval. The results of the 14-day interval demonstrate a significant decrease in this group compared to the negative control and the second positive and high-dose groups and a significant decrease compared to the low-dose and negative control groups during the 21-day interval. Besides, the results reflected that in the 28-day interval, there was a significant decrease in the blood sugar compared to the high- and low-dose and negative control groups (Table 2, Fig. 6).

### The effect of the algae extract on the Malondialdehyde level (MDA) in the serum of male laboratory mice

The results summarized in Table 3 present that the treatment of male laboratory mice with alloxan indicated a significant increase at (p≤0.05) in the concentration of Malondialdehyde (MDA) in the second positive control group compared to the negative control group and other treated groups during the four intervals.

The statistical analysis also revealed a significant decrease in the level of MDA in the high-and low-dose groups compared to the second positive control group during the intervals of 7, 14, 21 and 28 days and a significant decrease in the first positive control group

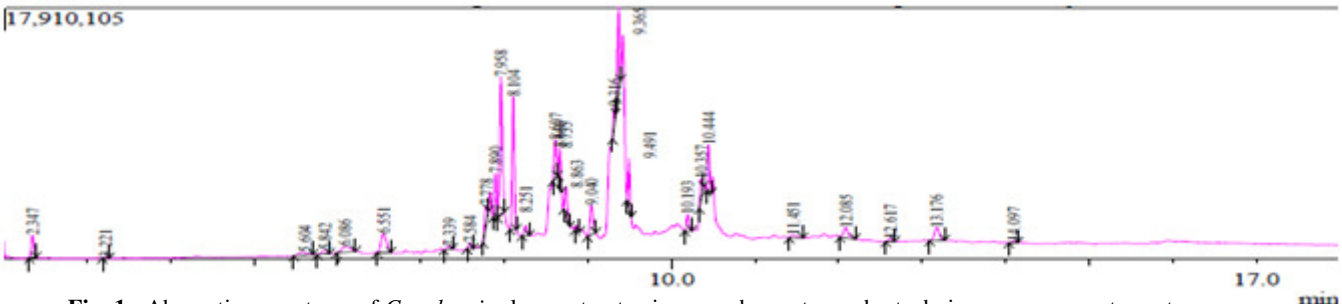


**Fig. 1 :** Absorption spectrum of *C. vulgaris* algae extract using gas chromatography technique - mass spectrometry

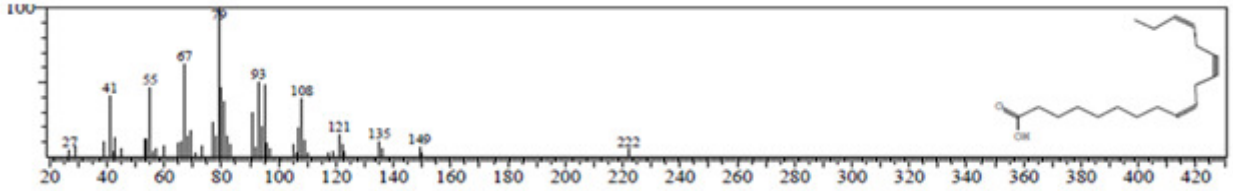

**Fig. 2 :** Mass spectrometry of the compound 9,12,15-Octadecatrien-1-ol, (Z, Z, Z) using gas-mass spectrometry technology.

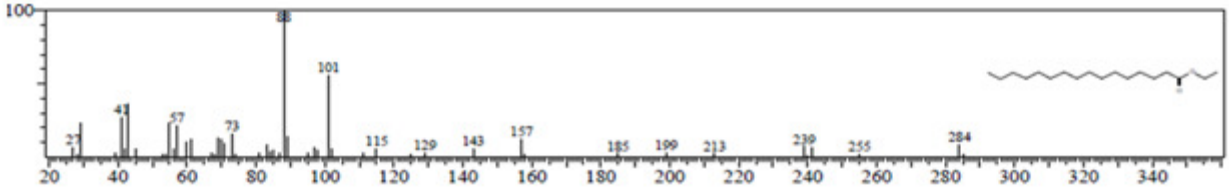

**Fig. 3 :** Mass spectrometry of Hexadecanoic acid, ethyl ester using gas chromatography technique- mass spectrometry.

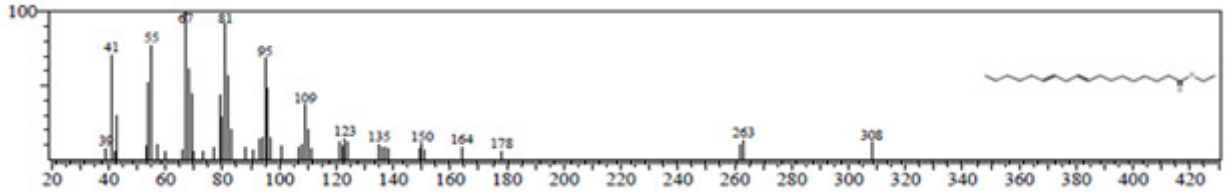

**Fig. 4 :** Mass spectrometry of the compound 9, 12-Octadecadienoic acid, ethyl ester using gas chromatography technique - mass spectrometry.

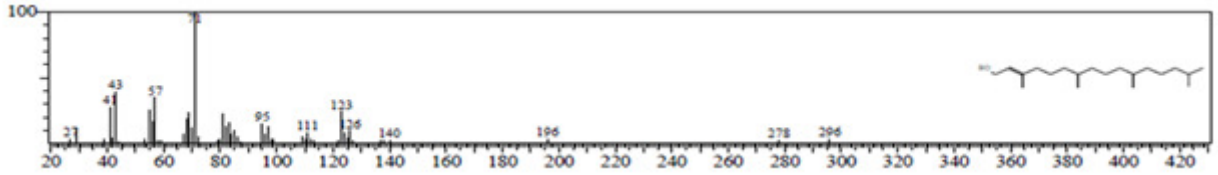

**Fig. 5 :** Mass spectrometry of Phytol compound using gas chromatography technique - mass spectrometry.

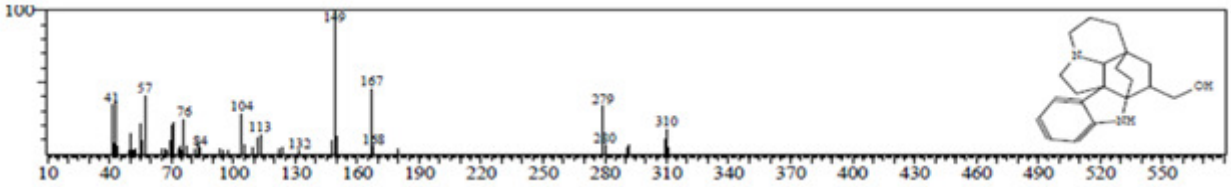

**Fig. 6 :** Mass spectrometry of Aspidofractinine -3-methanol compound (2.alpha, 3.beta, 5.alpha) using gas chromatography technique-mass spectrometry.

**Table 1 :** Mass spectrum of compounds isolated from *C.vulgaris* algae extract.

| Compound name | Molecular weight | Molecular formula | Percentage | Retention time |
|---|---|---|---|---|
| 9,12,15-Octadecatrien-1-ol, (Z,Z,Z) | 246 | C18H32O | 18.09% | 7.958 |
| Hexadecanoic acid,ethyl ester | 284 | C18H36O2 | 14.51% | 8.104 |
| 9,12-Octadecadienoic acid, ethyl ester | 308 | C20H36O2 | 7.74% | 9.365 |
| Phytol | 296 | C20H40O | 4.52 % | 9.040 |
| Aspidofractinine-3methanol, (2.alpha, 3.beta, 5.alpha) | 310 | C20H26N2O | 3.15 % | 13.176 |

**Table 2 :** Effect of *C.vulgaris* algae extracts on blood sugar level in male laboratory mice (mg/dl). (Rate ± standard deviation).

| Periods / Treatment | 7 days | 14 days | 21 days | 28 days |
|---|---|---|---|---|
| Negative control | $121.23^{c}$± 6.91 | $107.28^{cb}$± 11.28 | $105.75^{bc}$± 8.47 | $104.25^{cbd}$± 14.86 |
| Positive control 1 | $46.37^{e}$± 6.58 | $78.96^{ed}$± 12.45 | $75.33^{ed}$± 9.54 | $56.62^{e}$± 9.97 |
| Positive control 2 | $272.12^{a}$± 17.72 | $282.35^{a}$± 25.36 | $288.33^{a}$± 8.94 | $323.79^{a}$± 45.17 |
| Low-dose treatment | $101.44^{d}$± 13.52 | $87.05^{d}$± 14.22 | $95.91^{cd}$± 12.07 | $98.06^{db}$± 14.58 |
| High-dose treatment | $190.00^{b}$± 15.08 | $111.37^{b}$± 16.99 | $83.33^{d}$± 15.62 | $108.70^{b}$± 13.02 |

a, b, c, d, e different letters indicate significant differences ($p < 0.05$) among groups.

**Table 3 :** Effect of the algae extracts on the level of MDA in the serum of male laboratory mice (mg/dl). (Rate ± standard deviation).

| Periods / Treatment | 7 days | 14 days | 21 days | 28 days |
|---|---|---|---|---|
| Negative control | $14.75^{bc}$± 1.56 | $12.65^{cd}$± 2.50 | $4.83^{ed}$± 1.33 | $8.30^{becd}$± 1.82 |
| Positive control 1 | $6.05^{e}$± 0.79 | $9.07^{e}$± 1.52 | $5.86^{d}$± 0.91 | $7.00^{dec}$± 1.38 |
| Positive control 2 | $34.90^{a}$± 2.22 | $29.21^{a}$± 3.88 | $34.76^{a}$± 3.42 | $34.03^{a}$± 5.65 |
| Low-dose treatment | $10.80^{d}$± 1.58 | $11.68^{d}$± 1.74 | $9.26^{cd}$± 1.93 | $9.64^{c}$± 0.99 |
| High-dose treatment | $13.90^{c}$± 1.08 | $15.28^{b}$± 2.24 | $9.73^{b}$± 1.24 | $5.78^{e}$± 1.21 |

a, b, c, d, e different letters indicate significant differences ($p < 0.05$) among groups.

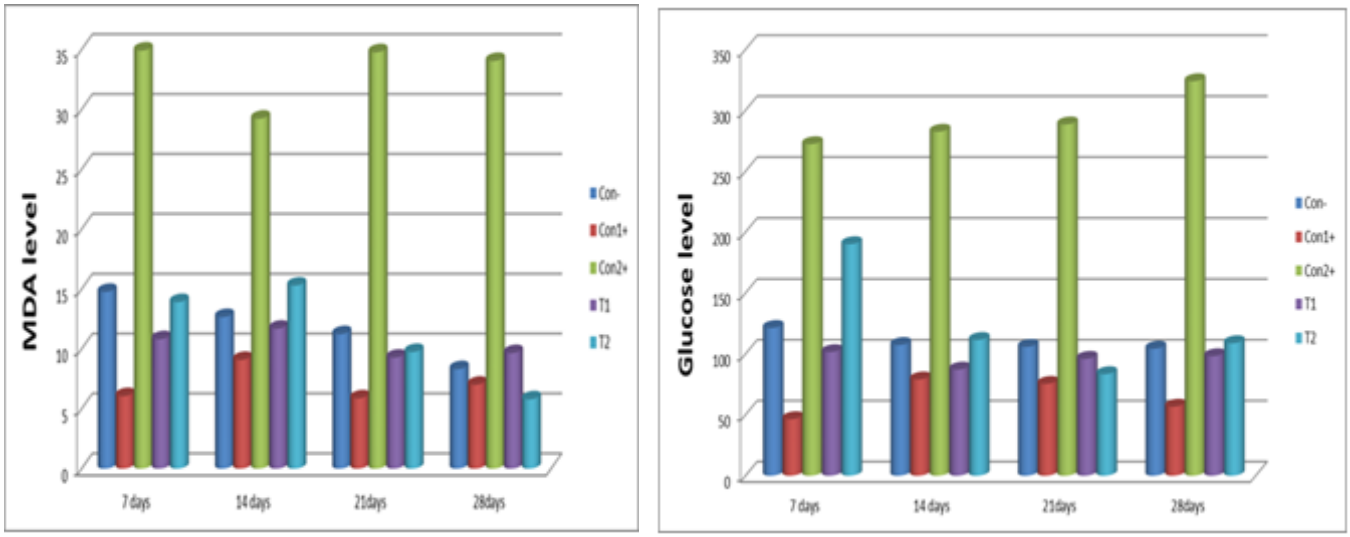


**Fig. 7 :** Effect of the algae extract on the Glucose level and MDA level in the blood serum of male laboratorymice.

compared to the negative control and the second positive and the low- and high-dose groups during the intervals of 7 and 14 days, but during a 21-day interval, this group exhibit a significant decrease compared to the negative and positive control groups and the second and high- and low-dose groups, while during the 28-day interval, the results demonstrate that there was significant decrease only with the second positive control group.

## DISCUSSION

The current study revealed that the mass spectrometry of the algal extract of *C. vulgaris* contained twenty-nine chemical compounds, three of which had the highest percentage represented by 9, 12, 15-Octadecatrienoic acid (Z, Z, Z), Hexadecanoic acid, ethyl ester and 9,12-Octadecadienoic acid ethyl ester. Other compounds had different percentages, such as Cyclohexanol, 5-methyl-2-(1-methyl ethyl) -, [1S-(1.alpha, 2.beta, 5.beta)] and Phytol and Aspidofractinine-3-methanol (2.alpha, 3.beta, 5.alpha). Manilal *et al* (2010) have clarified that the vital efficacy may be attributed to the compound with the highest percentage of the total area of diagnosed compounds identified by the GC-mass.

The induction of diabetes using alloxan in male laboratory mice had a clear role in increasing the level of blood sugar throughdamaging the function of the pancreas, as this was inferred by its high level in the blood serum throughout the experimenttime. The reason for the increase in sugar may be due to the destroying of pancreatic beta cells by alloxan, as it penetrates the cell through glucose transporters, causing damage to the pancreas, thus stopping both the secretion of insulin and the metabolism of glucose in the cells leading, of course, toits risein the blood (Inawati and Winarno, 2008). The inactivation of insulin secretion by alloxan may also be due to the oxidative stress resulted from the formation of free radicals and as an outcome of the reactionof the Thiol group (-SH) of the Glucokinase enzyme with alloxan (mostglucose phosphorylation enzymes are sensitive to alloxan). Moreover, the inhibition of Glucokinase reduces sugar oxidation and prevents the formation of ATP. Therefore, all these factors disrupt the secretion of insulin so that high blood sugar after the treatment with alloxan is a catalyst for diabetes (Kim *et al*, 2006; Tiedge *et al*, 2000). The results of some other studies have also indicated that alloxanis reduced to the toxic type (diualuric acid), which is automatically oxidized to the roots of hydroxyl radicals and Superoxide anion. These roots have a toxic effect and break down the protein of the Langerhans, lipids and nucleic acids (Majumdar *et al*, 2008). Furthermore, alloxan interferes with the effectiveness of some compounds containing the sulfhydryl (SH) group that synthesizes the enzyme glucokinase. Glucokinase is one of the liver enzymes that accelerate the conversion of glucose into glucose-6-phosphate, which is the first stage of glycolysis, and thus leads to its ineffectiveness and increaseblood sugar (Szkudelski, 2001). This detection corresponds to several studies (Sharma *et al*, 2003; Galletto *et al*, 2004; Ene *et al*, 2006; Vinuthan *et al*, 2007; Saikat *et al*, 2008).

The treatment of male laboratory mice with the ethanol extract of *C. vulgaris* caused a significant decrease in the sugar level in the high- and low-dose groups compared to the second positive control group. Perhaps the reason behind this decrease maybe because the algae extract has influenced the beta-pancreatic cells, as it worked to stimulate them to produce insulin and increase the entry of glucose into the adipocytes (Sharma and Rhyu, 2014) or phenolic compounds may have stimulated the non-affected beta cells to produce the

insulin, as beta cells are not equal in their sensitivity to alloxan, which led to a decrease in the level of sugar in the blood. Insulin performs a major function, which is to help increase the permeability of the body's cells membranesto glucose molecules, thus to increase their transport from the blood to the cells (Guyton and Hell, 2011). Moreover, this hormone stops the process of making glucose in cells by inhibiting the Pyruvate carboxylase, the Fructose 1-6 phosphatase and the Glucose 6 phosphatase responsible for this process (Murray *et al*, 2000).

The active compounds found in algae extracts have an influential response to treat diabetes, as they have worked as antioxidants caused by high sugar. Free radicals have emerged and peroxide products have accumulated in body cells. Besides, the lack of insulin secretion leads to increased free radicals through the high activity of the enzyme fatty acl-CoA that works to oxidize lipid acids and generate hydrogen peroxide $H_2O_2$ in the body (Mahadev *et al*, 2004; Lee *et al*, 2010). Algae extracts also played a prominent role in increasing the absorption of glucose without stimulating the secretion of insulin, or perhaps by disrupting the formation of sugar in the liver through Glyconeogenesis. It could be said that the extract may have disrupted the natural regulatory response to the circulating high levels of insulin, such as the release of somatostatin or glucagon (Cherng and Shih, 2005) or the 9,12,15-Octadecatrienoic acid (Z, Z, Z), which could have played an important role in increasing the sensitivity of cells to insulin, which, in turn, has led to an increase in the representation of glucose in the cells (Rajaram, 2014).

*C. vulgaris* algae extract may increase both protein glucose transporters Glucose Transporter 4 (GLUT4) in cell membranes and insulin receptors thus escalate the entry of glucose into the skeletal muscles, liver and adipose tissue. That is to say, GLUT4 is the main key to balancing glucose and transporting it from the blood to different body cells. The fact that 80% of the sugar transported to the muscles is through these proteins and it depends on the availability of small amounts of insulin, which leads to the binding of insulin to its receptors on the surface of cells, which leads to the activation of Tyrosine –kinase inside the cells. The phosphorylation process of insulin receptor substrates(IRS) takes place. These substrates are located near the plasma membrane. They stimulate the response molecules to the region, such as PI3K which occupiesa position in the transporting proteins in the plasma membrane (Mizoguchi *et al*, 2008). The results further demonstrate an increase in the level of MDA in the second positive control group, as alloxan may attack the pancreatic beta cells and produce free radicals that accumulate and become toxic and destructive. Alloxan may also lead to a disturbance in the cellular receptors of insulin, thus stopping the process of taking glucose by the cells, consequently, increase blood sugar. This increase leads to the development in the generation of free radicals through the oxidation of glucose, which attacks lipids and their compounds in the body, especially cellular membranes, and leads to oxidize and damage them as well as form dangerous products, including MDA, as a mixture is formed of the hydroperoxide of lipids during the peroxidation process. The fission of peroxyl in the lipid hydroperoxide drives to the formation of similar groups of hydroperoxides that are bound together to build the peroxyl group that allows the formation of free radicals. These free radicals can decompose again to compose the peroxide structurally linked to prostaglandins and are subject to division to produce MDA (Alhazza, 2007; Park and Giacca, 2007). Besides, the increase of free radicals and decrease of antioxidants that interfere in the attackof free radicals, take part in controlling it as well as prevent the generation of new roots can contribute to the damage of large organic molecules, expand oxidation of lipids, and develop complications of diabetes (Devasagayam *et al*, 2004). The current study revealed a significant decrease in the level of MDA in the treatment groups; this may be because the algae extract contained effective compounds that acted as defensive mechanisms against oxygen breakdown. One of these natural compounds is the phenolic compounds, which are very effective as antioxidants, as well as vitamins C and E, which areamong the most influentialsecondary metabolites. They may act as hydrogen precursors, thus they interact with active free radicals, such as the negative superoxide radicalO-2, the hydroxyl OH radical, and the hydrogen peroxide $H_2O_2$, making them more stable and terminatetheir reaction (Zakaria, 2011).

Various types of algae produce multisulfuric sugar compounds that have antioxidant activity (Vijayabaskar *et al*, 2012; Michalak and Chojnacka, 2015). Perhaps the algae extract has acted to increase the effectiveness and activity of antioxidants in serum and body cells, especially Superoxide dismutase, Glutathione peroxidase, and Catalase, thus raising the concentration of antioxidants in the body, inhibiting the lipid peroxidation and the preventing Malondialdehyde MDA (Vuppalapati *et al*, 2016). Alternatively, flavonoids extracted from the algae may have removed the free radicals (ROS, RNS), especially active oxygen classes, thus reducing the oxidative stress and expanding and activating antioxidant concentrations (Jerez-Martel *et al*, 2017). Furthermore,

the study algae contained various components, which were diagnosed by GC-mass technology, including unsaturated lipid acids, particularly Hexadecanoic acid, ethyl ester, which acts as a strong antioxidant and immunostimulatory, as well as Oleic acid, which lessens levels of saturated lipid in the body, thus reducing oxidative stress as well aslipid oxidation and its products from MDA. On the other hand, the algae contain vitamins, especially vitamin E, and because of this it haveacted to remove free radicals, prevent protein oxidation and loss, reduce antioxidant consumption and inhibit lipid oxidation by breaking the propagation chain (Solomons *et al*, 2012; Rezq *et al*, 2010)

The ethanol extract of *C. vulgaris* algae contains chemically effective compounds that have a biological act diagnosed by GC-mass. From the above-mentioned results, it is clear that there was an important role for the extract in reducing the levels of both the glucose andMalondialdehyde (MDA) in the blood serum of diabetes-induced male laboratory mice. The extract also had a strong ability to inhibit or suppress oxidative stress, as it contained antioxidants represented by the compounds diagnosed in the current study.

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