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Research Article

POTENTIAL ROLE OF *HAEMATOCOCCUS PLUVIALIS* AGAINST DIABETES INDUCED OXIDATIVE STRESS AND INFLAMMATION IN RATS

FAROUK K EL-BAZ[1]*, HANAN F ALY[2], WAGDY KB KHALIL[3], GAMILA H ALI[4], NAGLAA A HAFIZ[3], SAFAA A SAAD[1]

[1]Department of Plant Biochemistry, National Research Centre, Dokki, Giza, P.O. 12622, Egypt. [2]Department of Therapeutic Chemistry, National Research Centre, Dokki, Giza, P.O. 12622, Egypt. [3]Department of Cell Biology, National Research Centre, Dokki, Giza, P.O. 12622, Egypt. [4]Department of Water Pollution Research, National Research Centre, Dokki, Giza, P.O. 12622, Egypt. Email: fa_elbaz@hotmail.com

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ABSTRACT

Objective: The aim of this study is to investigate the impact of *Haematococcus pluvialis* extract against oxidative stress and inflammatory cytokines induced by hyperglycemia in diabetic rats.

Methods: Oxidative stress; lipid peroxide (as presented by Malondialdehyde; MDA) and nitric oxide (NO), beside total antioxidant capacity, enzymatic and non-enzymatic antioxidants including reduced glutathione, glutathione peroxidase, and glutathione reductase were evaluated. The inflammatory cytokines; tumor necrosis factor-alpha and interleukin-1 beta were also investigated in rats' serum. Several analyses including expression of antioxidant enzyme related genes, reactive oxygen species (ROS) formation and DNA adducts were performed.

Results: The results showed that diabetes mellitus induced-rats exhibited increase in oxidative stress biomarkers and inflammatory cytokines, lower expression levels of the antioxidant enzyme genes; superoxide dismutase and glutathione S-transferase than those in control rats. In addition, diabetic rats exhibited significantly higher levels of ROS generation and 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation. In contrary, supplementation of diabetic rats with *H. pluvialis* extract improved the negative effect of the hyperglycemia on antioxidant enzymes, the gene expression of antioxidant enzymes, and ROS generation as well as 8-OHdG formation.

Conclusion: *H. pluvialis* extract decreased the oxidative stress, enhanced antioxidant status and inflammatory cytokines induced by hyperglycemia in diabetic rats. The effect of *H. pluvialis* extract involved in the increase of expression levels of antioxidant enzyme genes; decreased the levels of ROS generation and 8-OHdG formation which may be attributed to the presence of astaxanthin in *H. pluvialis* extract.

Keywords: *Haematococcus pluvialis*, Hyperglycemia, Diabetes mellitus, Oxidative stress, Inflammatory cytokines, DNA adducts.

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INTRODUCTION

Several scientific reports have been indicated that reactive oxygen species (ROS) were participated in the occurrence of diabetes mellitus (DM) and consequently with the health complications coinciding with this disease [1]. By the increase of the markers of oxidative stress such as 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is considered as a marker of the oxidized and damaged DNA molecules, in the serum of the diabetic patients promotes the DM disease into the complicated case [2-4]. There are several reactions that are responsible to produce the ROS molecules in diabetic patients in which the most important one of them is the reaction of the nonenzymatic through glycation. This reaction is existed in the mitochondria, proteins, and protein kinase activated by nicotinamide adenine dinucleotide phosphate oxidase in several kinds of cells such as endothelial and smooth muscle cells.

It has been found that, along with the previous sources of ROS production is advanced glycation end-products (AGEs), namely end products of advanced glycosylation, which are found in the β-cells and kept under high levels of glucose. Under the conditions of ROS production by AGEs, it was found that ROS production decreased the transcription of the insulin gene and induced the apoptosis of b-cells [5]. This mechanism suggests that the increase of the glucose levels in the blood of the diabetic patients, which subsequently induces the oxidative stress may be considered as important reason for the toxicity produced from hyperglycemia. Thus, using antioxidative products by the diabetic patients might decrease or prevent the toxicity of hyperglycemia.

On the other hand, cytokines are a group of pharmacologically active, low molecular weight polypeptides that possess autocrine, paracrine and juxtacrine effects with characteristic features [6]. These molecules cluster into several classes (i.e., interleukins [ILs], tumor necrosis factors [TNFs], interferons, colony-stimulating factors, transforming growth factors and chemokines), which are relevant humoral mediators in a highly complex, coordinated network regulating inflammatory immune responses with the participation of different cytokine-associated signaling pathways. In addition, they exert important pleiotropic actions such as cardinal effectors of injury [7]. Cytokines are produced by a wide variety of cells in the body, playing an important role in many physiological responses that have a therapeutic potential [8]. In type 2 diabetes, chronic hyperglycemia encourages an inflammatory state, where cytokines increase may result in the destruction of the pancreatic β-cells and malfunction of the endocrine pancreas [9]. There are no data regarding the protective effect of *Haematococcus pluvialis* extract against oxidative stress in diabetic rats. On the other hand, there are several studies have been conducted on the protective effect of the active ingredient of *H. pluvialis* namely astaxanthin (ASTA). There are numerous studies investigated the pharmacological effect of ASTA on the animals models and found different protective impacts. Its effect varied from protection against gastric inflammation [10] and hepatoxicity [11] induced by *Helicobacter pylori* and carbon tetrachloride, respectively. In addition, it has been found that ASTA exhibited enhancer role for immune function [12,13].

Therefore, the main objective of this study was to study the impact of *H. pluvialis* extract against oxidative stress and inflammatory cytokines induced by hyperglycemia in diabetic rats.

MATERIALS AND METHODS

Chemicals

All chemicals in this study are of analytical grade, products of Sigma, Merck and Aldrich. STZ was purchased from Sigma-Aldrich, India. All kits were the products of Biosystems (Alcobendas, Madrid, Spain), Sigma Chemical Company (St. Louis, MO, USA), and Biodiagnostic Company (Cairo, Egypt). TRIzol reagent was bought from Invitrogen (Germany). The reverse transcription and polymerase chain reaction (PCR) kits were obtained from Fermentas (USA). SYBR Green Mix was purchased from Stratagene (USA).

Materials

Cultivation of H. pluvialis

H. pluvialis (strain No. CCAP 34/7) was isolated by spreading 0.1 ml of water samples collected from Nile River phytoplankton using BG11 media for algal isolation [14] into petri dishes containing 1.5% agar for solidification. Then, single colonies of algae were re-cultivated in the specified liquid media as nonaxenic batch cultures (50 ml) at 25±2°C and 24 hr with continuous white fluorescent lamp intensity ≈2500 Lux. Cultivation was carried out on an open pond with a capacity of 70 L containing 55 L of growth media. After cultivation, the biomass was initially separated from the water by gravitational settling, then further concentrated by centrifugation [15] and dried at 40°C.

Ethanolic extract preparation of *H. pluvialis*

About 100 g of *H. pluvialis* powder was soaked in ethanol (80%) and shacked on shaker (Heidolph UNIMAX 2010) for 48 hr at 150 rpm. The extract was filtered using a Buchner funnel and Whatman No. 4 filter paper, and the algal residue was re-extracted with the addition of fresh ethanol for another two times. Combined filtrates were concentrated using Rotary evaporator (Heidolph-Germany) at 40°C under vacuum to dryness. The dry resulting extract was stored at −20°C in a freeze and kept for further analysis [16].

EXPERIMENT

Animals

About 50 male Wistar albino rats (180-200 g) procured from Central Animal House, National Research Centre (NRC) were used. Animals were acclimatized to the laboratory conditions at room temperature before the experimentation. Animals were kept under standard conditions of a 12 hr light/dark cycle with food and water in plastic cages with soft bedding. All the experiments were carried out between 9.00 and 15.00 hr. The protocol was approved by the NRC Ethics Committee Guidelines (approval no: 0111457) for the use and care of animals.

Drug and treatment schedule

Induction of diabetes model: For the evaluation of streptozotocin (STZ) diabetic effect, type 2 diabetes was induced by intraperitoneally injection of a single dose of STZ (45 mg/kg b.wt.) dissolved in 0.01 M citrate buffer immediately before use [17]. After STZ injection, rats had free access to food, water and were given 5% glucose solution to drink overnight to encounter hypoglycemic shock [18]. Rats were checked daily for the presence of glycosuria. Rats were considered to be diabetic if glycosuria was present for 3 consecutive days [19]. Three days after STZ injection, fasting blood samples were obtained and blood sugar was determined (≥300 mg/dl). The antidiabetic glibenclamide (daonil) reference drug was orally administrated at a dose of 10 mg/kg b.wt. daily for 30 days [20].

Design

A total of 50 rats were divided randomly into 5 groups (10 rats each), as follows:

Group 1: Considered as normal, healthy control rats.
Group 2: Considered as normal rats treated with *H. pluvialis* ethanolic extract (150 mg/kg b.wt.) [21].
Group 3: Considered as diabetic group.

Hyperglycemic rats were used for the experiment and classified as follows:

Groups 4: Considered as diabetic rats orally administered with *H. pluvialis* ethanolic extract (150 mg/kg b.wt.).
Groups 5: Considered as diabetic rats orally administered antidiabetic glibenclamide reference drug (10 mg/kg b.wt.) daily for 30 days.

Collection of blood, organs, and tissue samples

Rats were fasted overnight (12-14 hrs), anesthetized by diethyl ether and blood collected by puncture of the sublingual vein in clean and dry test tube, left 10 minutes to clot and centrifuged at 3000 rpm for serum. The separated serum was used for biochemical analysis of total antioxidant capacity (TAC), TNF-alpha (TNF-α), and IL-1 beta (IL-1β). Liver was removed immediately, homogenized in 5-10 volumes of appropriate medium using electrical homogenizer, centrifuged at 3000 rpm for 15 minutes, the supernatants (10%) were collected and placed in Eppendorff tubes then stored at −80°C and quantified for the determination of oxidative stress markers; MDA, NO as well as nonenzymatic antioxidant; reduced glutathione (GSH), antioxidant enzyme glutathione peroxidase (GPx) and glutathione reductase (GR) spectrophotometrically.

Biochemical determinations

GPx activity

GPx activity was assayed according to the method of Paglia and Valentine [22], using NADPH-coupled reduction of GSSG cata-lyzed by GR which can be measured at 340 nm.

GSH

Brain GSH was measured colorimetrically according to the method of Beutler *et al.* [23]. This method is based on determination of the relatively stable yellow color when 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) is added to sulfhydryl compounds which can be measured at 503 nm.

Lipid peroxide (MDA)

MDA was measured by the thiobarbituric reactive species assay, which measures the production of MDA that reacts with thiobarbituric acid [24].

Nitric oxide (NO)

NO level was assayed in liver tissue by the spectrophotometric method according to Berkels *et al.* [25]. NO level was assayed by the spectrophotometric method. Promega's griess reagent system is based on the chemical reaction between sulfanilamide and N-1-naphthylethylenediamine dihydrochloride under acidic condition (phosphoric acid) to give colored azo-compound which can be measured at 520–550 nm.

TAC

TAC was assayed according to the method of Koracevic *et al.* [26]. The method is based on determination of the ability to eliminate added hydrogen peroxide. The remaining H 2O2 is determined colorimetrically by an enzymatic reaction converting 3,5-dichloro-2-hydroxyl benzenesulfonate to a colored product that is measured at 532 nm.

GR activity

The activity of GR was determined spectrophotometrically where the decrease in absorbance at 340 nm was red according to the method of Zanetti [27].

Inflammatory cytokines; TNF-α and IL-1β

Serum inflammatory cytokines were performed by ELISA; a sandwich enzyme immunoassay.

Gene expression analysis

Extraction of total RNA and complementary DNA (cDNA) synthesis

Liver tissues of male rats were used to extract the total RNA using TRIzol® Reagent (Invitrogen, Germany) Kit. The isolation method was carried out according to the manufacturer's instructions of the above Kit. To synthesize the cDNA isolated RNA from liver tissues was reverse transcribed into cDNA [28,29].

Quantitative real time-PCR (qRT-PCR)

A StepOne RT-PCR System (Applied Biosystem, USA) was used to assess the copy of the cDNA of male rats to detect the expression values of the tested genes. To perform the PCR reaction, a volume of 25 ml of reaction mixtures was prepared containing 12.5 ml of SYBR® green (TaKaRa, Biotech. Co. Ltd.), 0.5 ml of 0.2 mM forward and reverse primers, 6.5 ml DNA-RNA free water, and 2.5 ml of the synthesized cDNA. The sequences of specific primer of the genes used are listed in Table 1. The relative quantification of the target genes to the reference (β-Actin) was determined using the 2−ΔΔCT method.

Determination of ROS formation

Intracellular ROS generation was measured in pancreatic tissue by a flow cytometer with an oxidation-sensitive dichlorodihydrofluorescein diacetate fluorescent probe, after single-cell suspensions were made [32].

Determination of 8-OHdG by high-performance liquid chromatography (HPLC)

DNA was extracted from rat pancreatic tissue by homogenization in buffer containing 1% sodium dodecyl sulfate, 10 mM Tris, 1 mM EDTA (pH 7.4) and an overnight incubation in 0.5 mg/ml proteinase K at 55°C. Homogenates were incubated with RNase (0.1 mg/ml) at 50°C for 10 minutes and extracted with chloroform/isoamyl alcohol. The extracts were mixed with 3 M sodium acetate and two volumes of 100% ethanol to precipitate DNA at −20°C. The samples were washed twice with 70% ethanol, air-dried for 15 minutes and dissolved in 100 µl of 10 mM Tris/1 mM EDTA (pH 7.4) [15]. DNA was then digested and the adduct 8-OHdG was measured with HPLC equipped with a Coul Array system (Model 5600). Analytes were detected on two coulometric array modules, each containing four electrochemical sensors attached in series which allowed identification targets based on reduction potential. The UV detection was set to 260 nm. The HPLC was controlled and the data acquired and analyzed using CoulArray software. The mobile phase was composed of 50 mM sodium acetate 5% methanol at pH 5.2. Electrochemical detector potentials for 8-OHdG and 2-deoxy guanesine (2-dG) were 120/230/280/420/600/750/840/900 mV and the flow rate was 1 ml/minutes [33].

Table 1: Primer sequences used for qPCR

Gene	Primer sequence (5′–3′)	References
SOD-1	F: TAGCAGGACAGCAGATGAGT R: GCAGAAGGCAAGCGGTGAAC	Sadi *et al.* [30]
GST-Mu	F: AGAAGCAGAAGCCAGAGTTC R: GGGGTGAGGTTGAGGAGATG	
β-actin	F: GTG GGC CGC TCT AGG CAC CAA R: CTC TTT GAT GTC ACG CAC GAT TTC	El-Baz *et al.* [31]

F: Forward primer; R: Reverse primer; PI3K: Phosphati-dylinositol-3 kinase, Akt: Serine/threonine protein kinase B, qPCR: Quantitative-polymerase chain reaction

Statistical analysis

Data were analyzed by one-way analysis of variance using the Statistical Package for the Social Sciences program, version 11 followed by least significant difference to compare significance between groups. In addition, co-state computer program was applied, where unshared letters are significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Effect of the *H. pluvialis* extract on oxidative stress biomarkers in different therapeutic groups

Table 2 declared insignificant change in oxidative stress biomarkers, MDA and NO levels in normal rats treated with *H. pluvialis* ethanolic extract while, significant increase in MDA and NO levels in STZ-induced rats with percentages 373.68% and 158.48%, respectively. The treatment of hyperglycemic rats with ethanolic extract of *H. pluvialis* showed marked amelioration in MDA and NO levels with percentages of improvement 294.74% and 127.14%, respectively, compared to standard drug which recorded improvement percentages reached to 315.78% and 129.24%, respectively.

Effect of the *H. pluvialis* extract on TAC, enzymatic, nonenzymatic antioxidant in different therapeutic groups

Regarding to TAC, enzymatic and nonenzymatic antioxidant (Table 3), insignificant change was recorded in TAC, GSH, GR levels in normal rats administered ethanolic extract of *H. pluvialis*, while a significant increase in GPx (17.36%), was detected as compared to normal untreated rats. On the other hand, STZ-induced diabetic rats showed a significant reduction in TAC, GSH levels, GPx and GR activities with percentages 65.31, 44.52, 53.15 and 67.46%, respectively, as compared to normal control rats. Marked amelioration in all detected parameters on treated diabetic rats with ethanolic extract of *H. pluvialis* recorded 52.19, 52.43, 37.37 and 61.23%, respectively, for TAC, GSH levels, GPx and GR activities.

Table 2: Effect of *H. pluvialis* extract on oxidative stress biomarkers in different therapeutic groups

Groups	Biomarkers				
	Control	Control+*H.pluvialis*	Hyperglycemia	Hyperglycemia+*H.pluvialis*	Hyperglycemia+standard drug
MDA (umol/l)	0.19±0.01[a]	0.17±0.03[a]	0.90±0.01[b]	0.34±0.05[c]	0.30±0.04[c]
% change	-	10.53	373.68	78.95	57.89
% of improvement	-	-	-	294.74	315.78
NO (µmol/l)	6.19±0.60[a]	5.22±0.95[a]	16.00±1.22[b]	8.13±0.52[e]	8.00±0.92[e]
% change	-	15.67	158.48	31.34	29.24
% of improvement	-	-	-	127.14	129.24

Data are expressed as mean±SD. Statistical analysis is carried out using SPSS computer program (one-way ANOVA) coupled with co-state computer program, where unshared letters are significant at $p \leq 0.05$. SD: Standard Deviation, ANOVA: Analysis of variance, *H. pluvialis*: *Haematococcus pluvialis*, NO: Nitric oxide. Calculations:

$$\%\ \text{Change to control} = \frac{\text{Mean of control} - \text{Mean of treated}}{\text{Mean of control}} \times 100$$

$$\%\ \text{of improvement} = \frac{\text{Mean of disease} - \text{Mean of treated}}{\text{Mean of control}} \times 100$$

The results of this study demonstrated that *H. pluvialis* can promote the glycemic regulation, reduced oxidative stress, attenuate antioxidant biomarkers, improve inflammatory cytokines of diabetic rats and consequently improve the immune reaction against hyperglycemia induced oxidative stress. In a good agreement with the present results, several studies clearly indicated significant increase in oxidative stress biomarkers while low level of antioxidant biomarkers in type 2 DM [9,34-36]. They explained this perturbations in antioxidant system on the basis of, high blood sugar levels elicited high levels of ROS which caused depletion of antioxidant pool, GSH, GPx, and GR in liver rats indicates the damage of the second line of antioxidant defense system. This probably further exacerbates oxidative damage via adverse effect on critical GSH-related processes [37]. Reduced antioxidant status as a result of increased ROS production in experimental STZ induced rats has been reported previously by Ceriello [38] who declared that, hyperglycemia causes impairment of the antioxidant defense system as well as low antioxidant concentration that may lead to oxidative stress. In addition, the drastic depletion of liver GSH in this study may be due to high cytotoxic effect of H_2O_2 in endothelial cells as a result of inhibition of GR [37].

Effect of the *H. pluvialis* extract on the inflammatory cytokines in different therapeutic groups

TNF-α and IL-1β exhibited insignificant change in normal rats treated with ethanolic extract of *H. pluvialis* as compared to untreated rats (Table 4). A significant increase in TNF-α and IL-1β levels (50.62 and 42.23%) were recorded in diabetic rats as compared to normal rats. Treatment of diabetic rats with *H. pluvialis* extract showed noticeable improvement in TNF-α (47.24%) and IL-1β (44.29%) levels comparing to standard which revealed 40.43% and 49.92%, respectively.

The current results demonstrated elevated levels of TNF-α and IL-1β in diabetic rats. In accordance with the present finding El-Baz *et al.* [9] declared that, chronic hyperglycemia initiated an inflammatory state where cytokines levels are elevated which may result in the destruction of the pancreatic β-cells and malfunction of the endocrine pancreas. TNF-α is an inflammatory adipocytokine that has a major effect in the progress of insulin resistance [39]. Furthermore, IL-1β has an inverse relation with low insulin level [39]. The present results are in concomitant with the results of Al-Dahr and Jiffri [40] who found an

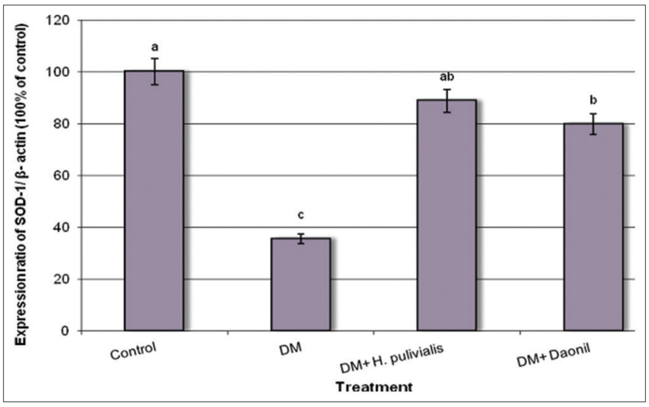

Fig. 1: Expression levels of superoxide dismutase-1 gene in liver tissues of diabetes mellitus-induced rats treated with *Haematococcus pluvialis* extract. Data are presented as mean±standard error of mean. a,b,cFollowed by different superscripts are significantly different (p≤0.05)

Table 3: Effect of *H. pluvialis* extract on TAC, enzymatic and nonenzymatic antioxidant in different therapeutic groups

Groups	Biomarkers				
	Control	Control+*H.pluvialis*	Hyperglycemia	Hyperglycemia+*H.pluvialis*	Hyperglycemia+Standard drug
TAC (mmol/mg protein)	15.54±1.21[a]	16.90±0.91[a]	5.39±0.25[b]	13.50±1.51[a]	10.50±1.21[c]
% Change	-	8.04	65.31	13.13	32.43
% of improvement	-	-	-	52.19	32.88
GSH (U/mg protein)	36.05±213[a]	37.00±3.45[a]	20.00±1.00[b]	38.90±2.23[a]	28.99±2.29[c]
% change	-	2.64	44.52	7.90	19.58
% of improvement	-	-	-	52.43	24.93
GPX (U/mg protein)	1.90±0.01[a]	2.23±0.01[c]	0.89±0.05[b]	1.60±0.1[c]	1.20±0.09[e]
% change	-	17.36	53.16	37.37	36.84
% of improvement	-	-	-	37.37	16.31
GR (U/mg protein)	13.00±2.20[a]	13.65±1.00[a]	4.23±0.55[b]	12.19±0.56[a]	9.10±0.20[c]
% change	-	5.00	67.46	6.23	30.00
% of improvement	-	-	-	61.23	37.46

Data are expressed as mean±SD. Statistical analysis is carried out using SPSS computer program (one-way ANOVA) coupled with co-state computer program, where unshared letters are significant at p≤0.05. SD: Standard deviation, ANOVA: Analysis of variance, *H. pluvialis*: *Haematococcus pluvialis*, TAC: Total antioxidant capacity, GSH: Reduced glutathione, GPx: Glutathione peroxidase, GR: Glutathione reductase

Table 4: Effect of *H. pluvialis* extract on proinflammatory TNF-α and IL-1β in different therapeutic groups

Groups	Biomarkers				
	Control	Control+*H.pluvialis*	Hyperglycemia	Hyperglycemia+*H.pluvialis*	Hyperglycemia+standard drug
TNF-α (ng/l)	26.12±3.10[a]				
25.00±1.92[a]	39.34±3.49[b]	27.00±3.52[a]	28.78±1.20[a]		
% change	-	4.29	50.62	3.37	10.18
% of improvement	-	-	-	47.24	40.43
IL-1β (Pg/l)	3370.70±78.10[a]	3389.00±98.45[a]	4794.00±560.60[b]	3301.00±57.89[a]	3111.33±100.70[a]
% change	-	0.5	42.23	2.06	7.69
% of improvement	-	-	-	44.29	49.92

Data are expressed as mean±SD. Statistical analysis is carried out using SPSS computer program (one-way ANOVA) coupled with co-state computer program, where unshared letters are significant at p≤0.05. SD: Standard deviation, ANOVA: Analysis of variance, *H. pluvialis*: *Haematococcus pluvialis*, TNF-α: Tumor necrosis factors-α, IL-1β: Interleukin-1 beta increment in TNF-α level in all diabetic patients. In addition, Mojtaba *et al*. [39] found elevation in IL-1β level in type II diabetic patients which was higher than the nondiabetic counterparts. The authors backed this elevation in IL-1β to β-cell dysfunction.

Effect of the *H. pluvialis* extract on the expression of genes encoding antioxidant enzymes

The results of the gene expression analysis using quantitative real-time RT-PCR are summarized in Figs. 1 and 2. The genes encoding superoxide dismutase (SOD-1) and glutathione S-transferase (GST)-Mu were determined in liver tissues of DM rats after before or after treatment with *H. pluvialis* extract. The results found that, DM-rats revealed very lower expression levels of SOD-1 and GST-Mu genes than those in control rats, where the differences were highly significant ($p\leq0.05$). In contrary, treatment of male DM-rats with *H. pluvialis* extract increased the expression levels of SOD-1 and GST-Mu genes compared with those in DM-rats without treatment, where the differences were also highly significant ($p\leq0.05$). In addition, treatment of male DM-rats with the reference drug for diabetes, namely glibenclamide, increased significantly the expression levels of SOD-1 and GST-Mu genes compared with those in DM-rats without treatment.

Effect of *H. pluvialis* extract on the ROS generation as oxidative stress indicator

The impact of *H. pluvialis* extract as preventive effect on the intracellular ROS generation is summarized in Fig. 3. The results indicated that the generation of ROS in DM-rats was significantly higher than those in control rats. However, the generation of ROS in DM-rats treated with *H. pluvialis* extract decreased significantly the ROS generation compared with those in DM-rats without treatment. Moreover, treatment of male DM-rats with glibenclamide decreased significantly the ROS generation compared with those in DM-rats without treatment.

Effect of *H. pluvialis* extract on the apoptosis rate

The determination of the 8-OHdG generation in pancreatic tissues of male DM-rats' genome before or after *H. pluvialis* extract treatment as an indicator for oxidative stress induced DNA damage is summarized in Fig. 4.

The present results found that the ratio of 8-OHdG/2-dG generation increased significantly in DM-rats in comparison to that of the control group. In contrast, the ratio of 8-OHdG/2-dG generation decreased significantly following *H. pluvialis* extract treatment compared with those in DM-rats. Furthermore, treatment of male DM-rats with glibenclamide decreased significantly the ratio of 8-OHdG/2-dG generation compared with those in DM-rats without treatment.

A numerous studies have been suggested that hyperglycemia increased the formation of ROS and reduced the antioxidant enzyme activity through glycation of these proteins (binding the protein molecules with the sugar molecules) [38]. In agreement with these findings, the present results found that DM-rats exhibited lower expression levels of the antioxidant enzyme genes, SOD-1 and GST-Mu, than those in control rats. In an explanation for these results, the low expression of the genes encoding antioxidant enzymes in the diabetic animal tissues is attributed to the attack by the ROS molecules to the diabetic tissues [41,42]. On the other hand, the current results revealed that, diabetic rats exhibited significantly higher levels of ROS generation and 8-OHdG formation. In the same line, several studies reported that increase the levels of glucose in diabetic rats was coincided with increase the levels of 8-OHdG [5,43].

It has been found that the increase of the antioxidants levels is acting to delay or prevent the disorders of the pancreatic β-cells in diabetic rats which caused by increase the glucose concentrations [43,44]. Therefore, one of the potentially therapeutic tools in diabetes treatment is using antioxidant supplementation which could improve the control of this disease. This study showed that treatment of diabetic rats with *H. pluvialis* extract increased the levels of the TAC, antioxidant

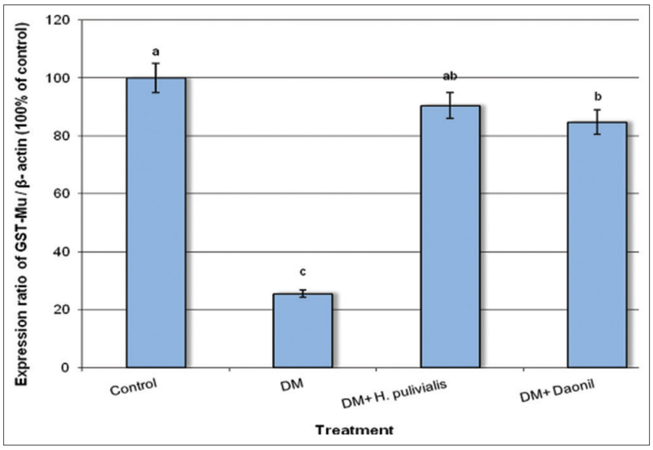

Fig. 2: Expression levels of glutathione S-transferase-Mu gene in liver tissues of diabetes mellitus-induced rats treated with *Haematococcus pluvialis* extract. Data are presented as mean±standard error of mean.[a,b,c]Followed by different superscripts are significantly different ($p\leq0.05$)

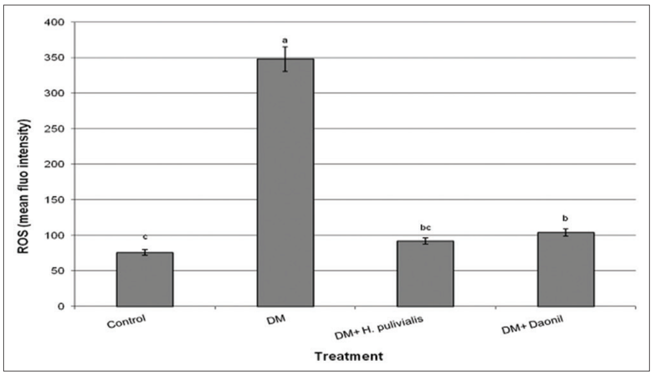

Fig. 3: The changes of intracellular reactive oxygen species levels in pancreatic tissues of diabetes mellitus-rats supplemented with *Haematococcus pluvialis* extract. Data are presented as mean±standard error of mean.[a,b,c]Followed by different superscripts are significantly different ($p\leq0.05$)

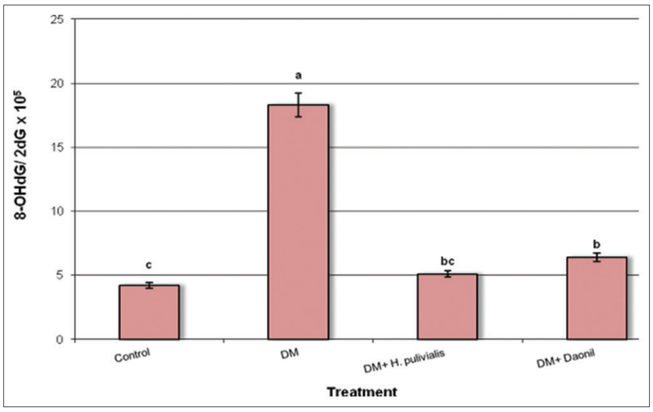

Fig. 4: Generation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in pancreatic tissues of diabetes mellitus-rats supplemented with *Haematococcus pluvialis* extract. DNA damage was expressed as the ratio of oxidized DNA base (8-OHdG) to nonoxidized base in pancreatic tissue DNA. [a,b,c]Followed by different superscripts are significantly different ($p\leq0.05$)

biomarkers, antioxidant enzyme genes and decreased the levels of oxidative stress biomarkers, inflammatory cytokines, ROS generation and 8-OHdG formation compared with those in diabetic rats without *H. pluvialis* extract treatment.

The results of the current work were in the same line with Ojo *et al.* [45] and El-Baz *et al.* [9] who found the amelioration in TNF-α IL-1β levels post-treatment of STZ induced rats with *H. pluvialis* may be attributed to the fact that, antioxidants have the ability to suppress inflammatory markers like TNF-α and IL-1β. Furthermore, Uchiyama *et al.* [46] reported that treatment of diabetic mice with the active ingredient of *H. pluvialis* extracts namely ASTA improved the tolerance against diabetes which coincided with increase the levels of the antioxidants and decrease the ROS formation. They also found that the improving action of ASTA in diabetic mice was associated with decrease the levels of glucose concentration as the main reason in diabetes toxicity. Moreover, they found at 120 minutes, ASTA treatment decreased the levels of glucose ($p<0.001$) and increased the levels of insulin levels ($p<0.001$) in diabetic mice compared with diabetic mice without ASTA treatment. Moreover, treatment of diabetic mice with ASTA improved the lesions in diabetic tissues.

CONCLUSION

The findings of this study suggested that *H. pluvialis* extract supplementation was able to decline the oxidative stress, enhanced antioxidant status and improved inflammatory cytokines induced by hyperglycemia in diabetic rats. The therapeutic action of *H. pluvialis* also involved in the increase of expression levels of antioxidant enzyme genes, decrease the levels of ROS generation and 8-OHdG formation which may be attributed to the presence of ASTA in *H. pluvialis* extract.

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