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Evaluation of the Pancreatoprotective Effect of *Nannochloropsis oculata* Extract against Streptozotocin-Induced Diabetes in Rats

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ABSTRACT

Diabetes mellitus is a chronic degenerative disease. In the developed countries, it is considered as the third leading cause of death following heart and cancer diseases. The most effective algal extract was prepared from *Nannochloropsis oculata* to treat diabetes-induced by Streptozotocin (STZ). In the present study, STZ showed a significant (P < 0.05) increase in glucose and C-reactive protein (CRP) levels associated with a decline in insulin level. This was parallel to histopathological alterations in the pancreas of diabetic rats. The *N. oculata* algal extract lowered glucose and CRP levels significant (P < 0.05) with restoring the insulin level to normalcy. The electrophoretic protein and lipoprotein patterns showed that STZ caused the disappearance of several normal bands. The α -amylase and α -esterase (EST) were expressed severely and represented as one thick band in the diabetic group. Also, STZ caused qualitative alterations in the arrangement of the bands of β -EST pattern. The algal extract decreased the quantity of α -amylase and α -esterase (CAT) and peroxidase (POX) patterns associated with the existence of abnormal bands. The algal extract restored the normal bands with hiding the abnormal ones. STZ induced cleavage of the genomic DNA leading to the existence of several unique bands. The algal extract maintained the integrity of the genomic DNA. The study concluded that algal extract showed an ameliorative effect against diabetes induced by STZ at biochemical, histopathological and molecular levels in the pancreas of rats.

INTRODUCTION

Diabetes mellitus is a chronic degenerative disease. It is considered as one of the most common endocrine disorders. It is a serious complex and a multifarious group of metabolic syndromes that disturbs the metabolism of carbohydrates, fats, and proteins (Kar *et al.*, 1999). It is characterized by elevation of glucose level by mean of hyperglycemia which subsequently leads to hypercholesterolemia, hypertriglyceridemia and cardiovascular diseases. Moreover, it leads to renal failure, blindness or diabetic cataract worldwide (Prasad *et al.*, 2009). It is caused as a result of underproduction of pancreatic insulin and/or lowered sensitivity of the tissue to insulin by mean of insulin resistance (Kumar *et al.*, 2015). According to the worldwide survey reported by World Health Organization (WHO), it was found that diabetes affects about 10% of the population and more than 220 million people worldwide suffer from this disease (Kim *et al.*, 2006). As documented by Guariguata *et al.* (2011), it was emphasized that diabetes is considered as a major cause of morbidity and mortality. Moreover, in the developed countries, it is arranged as the third

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leading cause of death following heart and cancer diseases.

Streptozotocin (STZ) is a naturally occurring nitrosourea product of Streptomyces achromogenes. It exerts a selectively direct necrotic effect on β cells and insulin-producing pancreatic endocrine cells causing hyperglycemia within 48-72 h when it is injected intraperitoneally (Kumar et al., 2015). Although STZ is less toxic than alloxan during the experimental induction of diabetes mellitus, it induces various side effects represented by liver and kidney injury with depression of bone marrow (Brenna et al., 2003). Proteins are the most important organic constituents in tissues because of all the enzymes and hormones which regulate all biological activities are consisting of proteins. In addition, it is well known that proteins exhibit a pivotal role in the tissue through activating the compensatory mechanisms under toxic stress (Venkataramana Sandhya et al., 2006). For this reason, assessment of protein and enzymes activities can be considered as an effective tool for revealing the physiological status of tissues (Manoj, 1999). Diet, exercise, oral hypoglycemic drugs, and insulin therapy belong to the therapeutic strategy for diabetes treatment. Currently, there are several available drugs suitable to decrease hyperglycemia in diabetic patients (Mutalik et al., 2003). The beneficial effects of standard hypoglycemic drugs are well documented during the recent studies which postulated that activity of the standard medications is still not effective against the natural progression of the disease. Although insulin is considered as an effective hyperglycemic therapy, it exhibits shortcomings due to its ineffectiveness on oral administration and fatal hypoglycemia in the event of excess dosage (Kasiviswanath et al., 2005). Moreover, biguanides and sulphonylurea belong to the oral antidiabetic agents which are available with insulin for diabetes treatment but associated with adverse side effects (Patil et al., 2012). As a result of the undesirable side effects of these drugs, it is necessary to search in the nature for new drug/compound for overcoming problems of this disorder without side effects (Noor et al., 2008). For all these reasons, the WHO recommended that it is necessary to evaluate the effectiveness of the natural hypoglycemic agents of plants origin for conditions safe modern drugs in traditional medicine (WHO, 1980). This leads to increasing demand for the natural drugs which frequently exhibit higher affectivity and lowest toxicity than the synthetic ones (Loew and Kaszkin, 2002).

Marine organisms are rich sources of biologically active metabolites. So far, many of these primary and secondary metabolites have been isolated and being developed as new pharmaceutical products (Schwartsmann et al., 2001; Selvendran, 2013). It was emphasized recently that algae have attracted global attention due to its potential to produce lots of valuable natural products (Enwereuzoh and Onyeagoro, 2014). Nannochloropsis oculata is a small green microalga genus which belongs to the class Eustigmatophyceae. It is well known marine eukaryotic unicellular phytoplankton in aquaculture by its nutritional value and potency to produce highly valuable compounds (Scholz et al., 2014; Hamidi et al., 2014; Kent et al., 2015). The active components extracted from N. oculata exhibited the ability to reduce the generation of the free radicals associated with increasing capacity of the antioxidant system (Yanuhar et al., 2011; Selvendran, 2013). These algal components in N. oculata may aid in regulating glucose, lipids, and lipoproteins during the metabolic pathways in the endocrine tissues of diabetic rats. Also,

they may contribute to the maintenance of the intestinal tissues (Nuno *et al.*, 2013). Moreover, it was documented recently that the sterol content in *N. oculata* might provide a promising antiinflammatory and anti-cancer role (Sanjeewa *et al.*, 2016). The purpose of this study was to appraise the potential use of the most effective microalgae *N. oculata* extract in the treatment of diabetes mellitus induced by STZ using a rat model.

MATERIALS AND METHODS

All other chemicals and reagents used were of analytical grade and of the highest purity. Acrylamide, Bis-acrylamide, Ammonium persulfate (APS), N,N,N,N-Tetramethylethylnediamine (TEMED), Coomassie Brilliant Blue G-250 (CBBR-250) and Sudan Black B (SBB) were procured from Sigma-Aldrich. Hematoxylin and Eosin stains were obtained from SRL, India and the chemicals used for in-gel esterase staining including α - and β -naphthyl acetate, Fast Blue RR was purchased from Qualigens Fine Chemicals, India. Streptozotocin (STZ) was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Benzidine and 2-Thio-barbituric Acid (TBA) were purchased from Sigma Chemicals Company (London, UK). The PCR kit and primers were purchased from Promega.

Microalgae strain and preparation

Nannochloropsis oculata alga (NNO-1 UTEX Culture LB 2164) was cultivated and collected from the Algal Biotechnology Unit, Biological and Agricultural Research Division, National Research Centre, Dokki, Giza, Egypt. Microalgae concentration and biochemical composition were controlled according to the method described by Nuno *et al.* (2013). The strain was cultivated under conditions consisting of the f/2 medium at 21°C, 30 ppm NaCl, pH 8.2, and under 2×75 W fluorescent lights. Samples were collected on the 6th day and centrifuged at 3588 g and 20°C. The precipitated microalgae were centrifuged again for 10 min at 897 g and 20°C. Recovered biomass was freeze-dried and stored separately by species at -20° C until use.

Total polyphenols and antioxidant activity

The concentration of the total polyphenols (Singleton and Rossi, 1965) and total reducing power (Oyaizu, 1986) were estimated in different algal extracts to select the most suitable extract. Percentage of the antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH) free radical assay (Brand-Williams *et al.*, 1995). Moreover, the cytotoxic activity was determined based on the method suggested by Mosmann (1983) on human hepatocellular carcinoma cell line (HepG2) purchased from CSIR-National Chemical Laboratory, Pune, India.

Administration of the N. oculata algal extract

The crude algal material of the most effective extract was dried by incubation at 50°C for 72 h and then crushed into powder in an electric blender. Fifty-six adult albino mice of weight 20-25 g were used to evaluate the median lethal dose (LD_{50}) and hence the therapeutic dose of the algal extract. It was divided into 7 groups each of 8 mice. The groups were treated orally with rising doses of 500, 1000, 2000, 3000, 4000, 5000 and 6000 mg/kg body weight (b.w.) of algal extract. Mortality was recorded after 24 hrs of the extract treatment. The LD₅₀ was calculated using equation

suggested by Paget and Barnes (1964). The therapeutic dose of the algal extract was administrated orally by stomach tube at 250 mg/Kg/day.

Animals and treatments

Healthy sixty adult male Wistar rats (weighing 170–200 g) were housed in ten per cage. The animals were provided with water *ad libitum* and standard food and maintained under normal environmental conditions at $25 \pm 2^{\circ}$ C.

Ethics statement

The experimental design and animal handling were carried out based on the experimental protocol approved by Institutional Animal Ethics Committee of National Research Centre, Dokki, Giza, Egypt (No: 471/2016) and were conducted in accordance with guidelines as per "Guide for the care and use of laboratory animal" and with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals.

Induction of diabetes mellitus

A fresh STZ solution was prepared at a dose of 60 mg/ kg b.w. by dissolving STZ in 100 mM citrate buffer (pH 4.5) and injected intraperitoneally (i.p.) in a volume of 1 ml/kg b.w. to rats after fasting overnight (Archana *et al.*, 2001). STZ injected animals exhibited massive glycosuria and hyperglycemia within few days. Diabetes was confirmed in STZ injected rats by measuring the fasting blood glucose concentration. Rats were considered as diabetic when a blood glucose level reaches 200 mg/dl.

Experimental design

The rats were randomly divided into six groups. Group I (termed as a control group): Rats were received distilled water for 21 days and fed with a normal diet as ad libitum. Group II (termed as N. oculata algal extract treated group): Rats were treated with aqueous algal extract at a dose of 250 mg/Kg for 21 days and fed with normal diet. Group III (termed as diabetic rats treated with commercial insulin): Rats were injected i.p. with STZ at a dose of 60 mg/kg b.w. and then treated with insulin (Insulatard human insulin purchased from Novo Nordisk A/S, DK-2880 Bagsvaerd, Denmark) at a dose of 4 to 8U followed by 1 to 2 U daily to obtain euglycemia for 7 days. Group IV (termed as a diabetic group): Rats were injected with a single dose of STZ i.p. and then diabetic rats were sacrificed after one week of STZ injection. Group V (termed as a Simultaneously treated group): Rats injected with STZ i.p. and administrated algal extract in a parallel manner for 21 days. Group VI (termed as a post-treated group): Rats injected with STZ i.p. for 7 days then treated with algal extract for 21 days.

Collection of samples

At end of the experiment (i.e., on 21st day), the animals were anesthetized through slight exposure to diethyl ether. The blood samples were drawn from retro-orbital plexus and divided into two parts, part one: deposited in NaF glucose vacuum tubes (Becton Dickinson, New York, NJ) for plasma separation during glucose estimation. Part two of the blood samples: was allowed for clotting at room temperature and then centrifuged at 3000 rpm for 15 minutes; the serum was separated and kept in clean stoppered vials at -20° C until the biochemical assay. After sacrificing the animals by cervical dislocation, the pancreas tissues were excised and washed in ice-cold saline then homogenized in 0.01 M Tris buffer (pH 7.4). Aliquots of this homogenate were used for measuring lipid peroxidation product (LPO) and for the different electrophoretic assays.

Biochemical analysis

Glucose, Serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), Urea, Creatinine, Total Protein, Cholesterol, Triglycerides, HDL-cholesterol, LDL-cholesterol, Creatine Phosphate Kinase (CPK) and Lactate Dehydrogenase (LDH) were measured in all experimental animals by using commercially available kits purchased from Spectrum Diagnostics Egyptian Company for Biotechnology (Cairo, Egypt). Level of plasma insulin was determined with sandwich enzyme-linked immunosorbent assay kit (ELISA, Boehringer Mannheim, Mannheim, Germany). Serum C-reactive protein (CRP) level was assayed using a solid phase ELISA that uses affinity purified anti-rat CRP antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidize (HRP) conjugated anti-rat CRP antibodies for detection. The LPO product was determined in the pancreatic tissue homogenates by spectrophotometrical technique at wavelength 535 nm using a UV-vise spectrophotometer (Shimadzu UV-2401 pc) based on the method suggested by Ohkawa et al. (1979).

Statistical analysis

The analyses were expressed as mean \pm standard error (SE) in the tables and figures. Statistical comparisons were carried out by one-way analysis of variance (one-way ANOVA) followed by least significant difference (LSD) test and confirmed by Bonferroni test. A "*P*" value of less than 0.05 indicates the statistically significant difference.

Histopathological examination

After sacrifice, small portions were autopsied from the splenic lobes of the pancreas were taken from different groups and immediately preserved in 10% neutral buffered formalin solution for 24 hr. The tissue specimens were dehydrated in serial dilutions of alcohol solutions, cleared in xylene and embedded in paraffin then sectioned at 4 μ m. Subsequently, the tissue sections were collected on glass slides and deparaffinized then stained by hematoxylin and eosin (H&E) stain for histological examination according to the method described by Banchroft *et al.* (1996). The histopathological changes were scored as mean of at least five rats and assigned between 0 (no damage) and +++ (maximal damage) for each investigated section according to the method suggested by Dommels *et al.* (2007).

Electrophoretic patterns

The tissues were washed and ground then homogenized in water-soluble extraction buffer. The homogenates were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatants containing water-soluble proteins were transferred to new tubes. Samples of each group were pooled together and used as one sample. Total protein concentration was determined in all pooled samples using bovine serum albumin as standard according to Bradford (1976). Quantities of protein should be equal in all wells and must be about 70 μ g protein.

The native proteins were separated through Polyacrylamide Gel Electrophoresis (PAGE) according to the methods described by Hames, (1990) and modified by Darwesh *et al.* (2015) who documented that samples, gels, and running buffers were lacking sodium dodecyl sulfate. The native bands were stained by Coomassie Brilliant Blue G-250 for visualizing protein bands. Furthermore, the native gel was stained for lipids with Sudan Black B (SBB) by mean of isoelectrophoresis (Subramaniam and Chaubal, 1990). The native gel was stained for electrophoretic catalase (CAT) and peroxidase (POX) patterns according to the method suggested by Siciliano and Shaw (1976) and Rescigno *et al.* (1997), respectively. It was processed for localization of in-gel α -amylase activity by the method suggested by Rammesmayer and Praznik (1992) by incubation with a solution containing soluble starch followed by staining with iodine solution. Moreover, the native gel was incubated in reaction mixture containing α , β -naphthyl acetate (5.58 × 10⁻³ mM, pH 7.5) as substrates along with dye coupler Fast Blue RR based on the method suggested by Baker and Manwell (1977) and modified recently by Ahmad *et al.* (2012) for studying localization of in-gel α - and β -esterase (EST) activities.

Electrophoretic localization of in-gel enzyme activity

Table 1: Polyphenol concentration, Iron reducing power, antioxidant and anticancer activities in different N. oculata algal extracts.

	Solvent	Polyphenol (mg gallic acid/100 gm)	Reducing power (μg/mL)	Antioxidant activity (%)	IC50 (µg/mL)
Vegetative dried	Hot water	0.23 ± 0.00	3.08 ± 0.35	5.20	16.19
	70% EtOH	0.21 ± 0.00	3.79 ± 0.43	6.10	36.19
Stress dried	Hot water	0.30 ± 0.00	3.13 ± 0.35	0.10	31.17
	70% EtOH	0.10 ± 0.00	1.89 ± 0.20	3.62	34.92
Vegetative fresh	Hot water	0.15 ± 0.00	0.71 ± 0.08	0.65	27.64
	Cold water	0.28 ± 0.00	3.84 ± 0.44	3.61	20.00
Stress fresh	Hot water	0.49 ± 0.01	1.41 ± 0.15	2.87	27.37
	Cold water	0.20 ± 0.00	0.97 ± 0.10	2.35	39.3
Ethyl acetate extract		$0.70\pm0.01*$	$4.94\pm0.45^{\boldsymbol{*}}$	12.72*	14.29*

*: The most effective extract with the highest polyphenol concentration, reducing power, antioxidant and anticancer activity.

Genomic DNA pattern

The genomic DNA was extracted from pancreatic tissues using the method described by Barker et al. (2004). It was amplified with 5 random primers of different nucleotide sequences (OPA-04 (5'-AATCGGGCTG-3'), OPA-05 (5'-AGGGGTCTTG-3'), (5'-GAAACGGGTG-3'), **OPA-07 OPA-10** (5'-GTGATCGCAG-3') and OPA-11 (5'-CAATCGCCGT-3')) during the polymerase chain reaction (PCR) based on method suggested by Rapley (1998). The amplification process was performed using thermal cycler (Progeny 30, Techno, Cambridge Ltd. Dux ford Cambridge, UK) through the thermal program initial denaturation at 94°C for 5 min. followed by 40 cycles of DNA amplification. Each cycle was consisting of denaturation at 94°C for 2 min. then annealing at 40°C for 2 min. and extension at 72°C for 7 min. After separation of the amplified fragments on 2% agarose gel, they visualized on a UV transilluminator then photographed by Gel Documentation System.

Data analysis

The polyacrylamide and agarose gel plates were photographed and analyzed using Quantity One software (Version 4.6.2). The relative mobility (Rf), band percent (B %) and band quantity (Qty) of the electrophoretically separated bands were determined. Furthermore, percent of the similarity index (SI %) was calculated to compare all treated groups to control group according to equation suggested by Nei and Li (1979).

RESULTS

As illustrated in Table 1, it was found that the most effective algal extract noticed with high concentrations of the total polyphenols, total reducing power and free radical scavenging activity 0.70 \pm 0.01 mg gallic acid/100 gm, 4.94 \pm 0.45 µg/ml and 12.72%, respectively. Furthermore, the lowest cell viability % of cancer cells was noticed with this effective extract which exhibited IC₅₀ 14.29 µg/mL.

Biochemical measurements

As reported in Table 2, it was noticed that STZ caused significant (P < 0.05) elevation in circulating glucose and CRP level and significant (P < 0.05) decrease in insulin level with respect to control. With respect to STZ induced diabetic group, the *N. oculata* algal extract decreased glucose and CRP levels in parallel to an elevation of insulin level significantly (P < 0.05). As compared to control, STZ caused significant (P < 0.05) elevation in ALT, AST and ALP levels. The *N. oculata* algal extract decreased activities of these enzymes significantly (P < 0.05).

As shown in Table 3, significant (P < 0.05) elevation was observed in urea and creatinine levels associated with significant

(P < 0.05) decrease in TP level of STZ induced diabetic rats. The *N. oculata* algal extract lowered levels of these parameters significantly (P < 0.05) with restoring TP level to a normal value when compared to STZ induced diabetic group.

Table 2: Effect of STZ-induced diabetes and daily oral administration of N. oculata algal extract on levels of glucose, insulin, C-reactive protein (CRP) and liver enzymes.

	GI	GII	GIII	GIV	GV	GVI
Glucose (mg.dl-1)	114.7 ± 1.08	117.90 ± 0.81	118.00 ± 0.87	$407.67^{\mathtt{a}}\pm4.13$	$227.50^{ab}\pm \ 1.40$	$126.40^{ab}\pm1.08$
Insulin (µg/L)	10.50 ± 0.03	10.41 ± 0.03	10.40 ± 0.04	$4.51^{\rm a}\pm 0.04$	$7.84^{\rm ab}\pm0.06$	$8.84^{ab}\ \pm 0.02$
CRP (ng.ml ⁻¹)	2.73 ± 0.03	2.75 ± 0.03	2.84 ± 0.02	$12.47^{\mathtt{a}}\pm0.03$	$3.54^{\rm ab}\pm0.04$	$2.90^{\text{ab}}\pm0.05$
ALT (U.L-1)	30.10 ± 0.43	31.00 ± 0.37	31.00 ± 0.24	$90.17^{\mathtt{a}}\pm1.08$	$43.10^{ab}\pm0.53$	$33.60^{\text{ab}}\pm0.50$
AST (U.L-1)	29.10 ± 0.62	30.30 ± 0.40	30.44 ± 0.53	$170.17^{\rm a}\pm0.79$	$42.60^{ab}\pm0.40$	$34.80^{\text{ab}}\pm0.25$
ALP (U.L ⁻¹)	77.70 ± 0.58	77.40 ± 0.45	79.33 ± 0.33	$234.00^{\mathtt{a}}\pm1.51$	$126.40^{\mathrm{ab}}\pm0.87$	$84.00^{\text{ab}}\pm0.58$

a: Values compared to control group (GI); b: Values compared to STZ induced diabetic group (GIV) (significant $P \le 0.05$).

Group I: Control group, Group II: N. oculata algal extract treated group, Group III: diabetic rats treated with commercial insulin, Group IV: diabetic group, Group V: Simultaneously treated group, Group VI: Post-treated group.

Table 3: Effect of STZ-induced diabetes and daily oral administration of N. oculata algal extract on kidney functions.

	GI	GII	GIII	GIV	GV	GVI
Urea (mg.dl ⁻¹)	42.40 ± 0.45	41.70 ± 0.37	42.33 ± 0.33	$74.50^{\mathtt{a}}\pm0.72$	$57.60^{ab}\pm0.31$	$46.10^{\mathrm{ab}}\pm0.48$
Creat. (mg.dl ⁻¹)	0.73 ± 0.00	0.74 ± 0.01	0.74 ± 0.00	$0.94^{\rm a}\pm 0.01$	$0.73^{\rm b}\pm0.00$	$0.73^{\rm b}\pm0.01$
TP (g.dl ⁻¹)	6.19 ± 0.01	6.20 ± 0.01	$\boldsymbol{6.16} \pm \boldsymbol{0.03}$	$4.24^{\rm a}\pm0.03$	$5.28^{ab}\pm0.01$	$6.28^{ab}\pm0.02$

a: Values compared to control group (GI); b: Values compared to STZ induced diabetic group (GIV) (significant P < 0.05).

Group I: Control group, Group II: N. oculata algal extract treated group, Group III: diabetic rats treated with commercial insulin, Group IV: diabetic group, Group V: Simultaneously treated group, Group VI: Post-treated group.

Table 4: Effect of STZ-induced diabetes and dail	v oral administration of <i>N. oculata</i> algal	extract on lipid profile and heart enzymes.

	GI	GII	GIII	GIV	GV	GVI
Cholest. (mg.dl-1)	83.30 ± 0.42	84.00 ± 0.47	85.78 ± 0.60	$134.83^{\rm a}\pm0.83$	$104.50^{ab}\pm2.21$	$89.30^{ab}\pm0.67$
T.Gs (mg.dl ⁻¹)	101.80 ± 1.53	101.70 ± 1.78	101.89 ± 2.04	$371.33^{\mathtt{a}}\pm2.26$	$217.70^{ab}\pm0.72$	$119.60^{ab}\pm1.45$
LDL-c (mg.dl ⁻¹)	29.47 ± 0.28	29.77 ± 0.43	30.42 ± 0.47	$61.08^{\mathtt{a}}\pm0.36$	$36.04^{\mathrm{a}}\pm0.15$	$30.17^{\text{b}}\pm0.13$
HDL-c (mg.dl ⁻¹)	34.90 ± 0.50	34.20 ± 0.53	33.56 ± 0.29	$16.30^{\mathtt{a}}\pm0.10$	$33.00^{\text{b}}\pm0.45$	$34.50^{\rm b}\pm0.52$
CPK (U.L-1)	62.81 ± 0.36	62.91 ± 0.28	62.60 ± 0.38	$227.17^{\mathrm{a}}\pm2.27$	$125.40^{ab}\pm1.27$	$63.24^{\rm b}\pm0.33$
LDH (U.L ⁻¹)	86.10 ± 0.50	88.10 ± 0.50	88.22 ± 0.68	$227.33^{a}\pm2.32$	$128.30^{ab}\pm0.82$	$94.50^{ab}\pm0.45$

a: Values compared to control group (GI); b: Values compared to STZ induced diabetic group (GIV) (significant P < 0.05).

Group I: Control group, Group II: N. oculata algal extract treated group, Group III: diabetic rats treated with commercial insulin, Group IV: diabetic group, Group V: Simultaneously treated group, Group VI: Post-treated group.

As revealed in Table 4, levels of total cholest., T.Gs, and LDL-c elevated significantly (P < 0.05) associated with significant (P < 0.05) decline in HDL-c in STZ induced diabetic group with respect to control. The treatment with *N. oculata* algal extract lowered levels of the elevated measurements with increasing the in HDL-c level significantly (P < 0.05) as compared to STZ induced diabetic group. Moreover, it was noticed that STZ caused significant (P < 0.05) increase in CPK and LDH levels as compared to control. The *N. oculata* algal extract decreased activities of these enzymes significantly (P < 0.05) with respect to STZ induced diabetic group.

While, as illustrated in Figure 1, STZ caused significant (P < 0.05) elevation in LPO level in pancreas tissue as compared to control. The *N. oculata* algal extract lowered the LPO level significantly (P < 0.05) when compared to STZ induced diabetic group.

Histopathological examination

As illustrated in Figure 2a, it was revealed that there was no histopathological alteration in the pancreas of control rats. Normal histological structure of the islands of Langerhans cells (S) as the endocrine portion as well as the acini (a) with the duct system as exocrine one was noticed. In *N. oculata* algal extract treated group, there was no histopathological alteration and no deviation from the control group (Figure 2b). While in group III there was the normal histological structure of islands of the Langerhans cells (S) associated with mild dilatation in the duct system (pd) (Figure 2c). In contrast, in group IV (diabetic group), atrophy was detected in the islands of Langerhans cells (S) in a

diffuse manner (high degree of severity +++; 75-100%) associated with congestion in the stromal blood vessels (V) (Figure 2d). Atrophy was only detected in the islands of Langerhans cells (S) in a diffuse manner (mild degree of severity +; 25–50%) in group V (simult-treated group) (Figure 2e). While, in group VI (post-treated group), no histopathological alterations were detected in the islands of Langerhans cells (S) (Figure 2f).



Fig. 1: Effect of STZ-induced diabetes and daily oral administration of *N. oculata* algal extract on LPO product in pancreas tissue. Group I: Control group, Group II: *N. oculata* algal extract treated group, Group II: diabetic rats treated with commercial insulin, Group IV: diabetic group, Group V: Simultaneously treated group, Group VI: Post-treated group.



Fig. 2: Pancreas tissue showing a) control group (GI) with normal histological structure (H&E, X 40), b) *N. oculata* algal extract treated group (GII) without deviation from control group (H&E, X 40), c) STZ induced diabetes treated by commercial insulin (GIII) with normal histological structure and mild dilatation in the duct system (pd) (H&E, X 40), d) STZ induced diabetes (GIV) with severe histological alterations (H&E, X 40), e) simult-treated group (GV) with atrophy was only in islands of Langerhans cells (S) (H&E, X 40) and f) post-treated group (GVI) without alterations and deviation from control group (H&E, X 40).

Electrophoretic patterns

As compared to the corresponding control (Figure 3a),

several protein bands disappeared in STZ induced diabetes with the appearance of one characteristic band (Rf 0.46, Int 2565.71 and

Qty 10.86). Moreover, the lowest SI value was noticed with the STZ induced diabetic group (22%). Also, it could be observed that STZ decreased bands number in the lipoprotein pattern without the existence of further bands (Figure 3b). The lowest SI value was observed with the STZ induced diabetic group (61.54%) and

this was constant with results of the protein pattern. The treatment with N. *oculata* algal extract restored all the normal bands and hence the SI values reached the highest value in the STZ induced diabetic group treated with N. *oculata* algal extract.



Fig. 3: Electrophoretic patterns showing the curative effect of *N. oculata* algal extract against streptozotocin-induced diabetes on a) native protein and b) lipoprotein in pancreas tissue of rats. Group I: Control group, Group II: *N. oculata* algal extract treated group, Group III: diabetic rats treated with commercial insulin, Group IV: diabetic group, Group V: Simultaneously treated group, Group VI: Post-treated group.

Electrophoretic localization of in-gel enzyme activity

As illustrated in Figure 4a, α -amylase was represented by only one thick band in STZ induced diabetic group (Rf 0.06, Int 426.3 and Qty 2.86). Also, in the STZ induced diabetic group treated with N. oculata algal extract, the enzyme was represented by the only band but with lower intensity and quantity (Rf 0.05, Int 186.97 and Qty 1.21) with respect to the STZ induced diabetic group. In addition, a-EST (Figure 4b) was represented by one thick band in STZ induced diabetic group (Rf 0.06, Int 399.24 and Qty 1.93). Furthermore, the treatment with N. oculata algal extract decreased the band intensity and quantity (Rf 0.04, Int 208.64 and Qty 0.94). On the other hand, STZ injection caused alterations in the β -EST pattern (Figure 4c) represented by shifting the first band (EST-1) to be noticed with different data (Rf 0.04, Int 1873.50 and Qty 9.87) as compared to control. Moreover, the lowest SI value was noticed with the diabetic group (50%). In the diabetic group treated with N. oculata algal extract, one abnormal band was noticed (Rf 0.04, Int 1026.33 and Qty 5.31) in addition to the normal bands. Moreover, the SI value in this group (SI 80) was higher than that in the diabetic group.

With respect to the corresponding control (Figure 5a), several CAT bands disappeared in STZ induced diabetes with the appearance of 2 bands (Rf 0.03, Int 545.65 and Qty 1.50; Rf 0.05, Int 1344.69 and Qty 3.69, respectively). Moreover, only one abnormal band was identified (Rf 0.02, Int 1107.42 and Qty 2.90) in STZ induced diabetic group treated with *N. oculata* algal extract. The SI value in STZ induced diabetic group treated with *N. oculata* algal extract. Also, it could be observed that STZ

decreased bands number in the POX pattern (Fig. 5b) with the existence of 2 abnormal bands (Rf 0.02, Int 1147.69 and Qty 3.54; Rf 0.63, Int 1430.87 and Qty 4.42, respectively). Furthermore, only one abnormal band was identified (Rf 0.01, Int 1308.45 and Qty 3.90) in STZ induced diabetic group treated with *N. oculata* algal extract. The lowest SI value was observed with the STZ induced diabetic group (28.57%) and this was constant with results of the CAT pattern.

Genomic DNA fragmentation

As shown in Figure 6a, STZ induced cleavage of DNA into 4 oligonucleosome length fragments (Rf 0.16, 0.51, 0.76 and 0.92; B % 15.38, 26.24, 25.37 and 33.01; Qty 8.42, 14.36, 13.89 and 18.07, respectively). Based on result of the DNA fragmentation, the amplicon with the primer OPA-04 (Figure 6b) showed that STZ caused qualitative alterations appeared through disappearance of all normal bands with existence of 3 characteristic ones (Rf 0.17, 0.35 and 0.48; B% 38.01, 35.62 and 26.37; Qty 0.91, 0.85 and 0.63, respectively). As revealed in Figure 6c, the amplicon with the primer OPA-07 showed that STZ caused the disappearance of one normal band with the existence of 3 characteristic bands (Rf 0.10, 0.20 and 0.31; B% 14.66, 26.87 and 26.87; 1.09, 1.99 and 1.99, respectively). As illustrated in Figs. 6d &e, the amplicon showed that STZ exhibited the same mutagenic effect with the primers OPA-10 and OPA-11. These disturbances were represented qualitatively by disappearance of one normal band with existence of 2 characteristic ones at Rf 0.21 and 0.36 (B% 29.28 and 30.56; Qty 11.95 and 12.47, respectively) with OPA-10 and at Rf 0.23 and 0.39 (B % 16.01 and 25.98 ; Qty

0.34 and 0.55, respectively) with the primer OPA-11. Moreover, STZ caused alterations with the primer OPA-12 (Figure 6f) represented by disappearance of all normal bands with existence of 5 characteristic ones (Rf 0.18, 0.36, 0.49, 0.54 and 0.78; B% 10.60, 32.89, 12.89, 17.22 and 26.71; Qty 0.35, 1.10, 0.42, 0.57

and 0.89, respectively). The lowest SI Value was noticed with all the primers in STZ induced diabetic rats. The genomic DNA pattern remains integrated into the STZ induced diabetic group treated with *N. oculata* algal extract. The SI value reached the highest value in all *N. oculata* algal extract treated group.



Fig. 4: Electrophoretic isozymes showing the curative effect of *N. oculata* algal extract against streptozotocin-induced diabetes on a) α-amylase, b) α-EST and c) β-EST in pancreas tissue of rats. Group I: Control group, Group II: *N. oculata* algal extract treated group, Group III: diabetic rats treated with commercial insulin, Group IV: diabetic group, Group V: Simultaneously treated group, Group VI: Post-treated group.



Fig. 5: Electrophoretic isozymes showing the curative effect of *N. oculata* algal extract against streptozotocin-induced diabetes on a) CAT pattern and b) POX pattern in pancreas tissue of rats. Group I: Control group, Group II: *N. oculata* algal extract treated group, Group III: diabetic rats treated with commercial insulin, Group IV: diabetic group, Group V: Simultaneously treated group, Group VI: Post-treated group.



Fig. 6: Genomic DNA pattern showing the curative effect of *N. oculata* algal extract against streptozotocin-induced diabetes in pancreas tissue of rats a) DNA fragmentation, b) amplicon with random primer OPA-04, c) with primer OPA-07, d) with primer OPA-10, e) with primer OPA-11 and f) with the primer OPA-12. Group I: Control group, Group II: *N. oculata* algal extract treated group, Group III: diabetic rats treated with commercial insulin, Group IV: diabetic group, Group V: Simultaneously treated group, Group VI: Post-treated group.

DISCUSSION

The STZ is a broad-spectrum antibiotic. It induced diabetes through the destruction of β -cells of islets of Langerhans cells and insulin-producing pancreatic endocrine cells selectively. Subsequently, the blood glucose level elevated as a result of an insufficient release of insulin (Bailey and Flatt, 1986). The major disadvantage of STZ was represented by liver and kidney injury (Brenna et al., 2003). Because of the undesired sideeffects of synthetic antidiabetic drugs, it was necessary to search in the nature to suggest easily accessible natural products with hypoglycemic activity through insulinogenic effect (insulinreleasing stimulatory effect) by activation of the β cells (Mahfouz, 2011). In the present study, STZ induced hyperglycemia. This was in accordance with Najafian et al. (2010). This might be attributed to destruction of the pancreatic β cells and hence lowering level of insulin (Mythili et al., 2004). The CRP is the most effective and stable inflammatory marker (Ridker, 2003). Its level elevated in the circulation as a result of insulin deficiency (Jain et al., 2007). It has been attributed that the hypoglycemic effect of N. oculata algal extract may refer to its ability to restore integrity and function of pancreatic cells. This leads to elevation of insulin output associated with inhibition of the intestinal absorption of glucose (Elder, 2004). Hyperglycemia is related to various defects in metabolic pathways. It is associated with the generation of the free radicals which can exhaust antioxidant defenses thus leading to the oxidative damage to membranes and enhanced susceptibility to LPO (Giugliano *et al.*, 1996). Serum enzymes such as ALT, AST, and ALP are used to evaluate hepatic disorders. In the present study, STZ caused an increase in activity of these enzymes. This may refer to the membrane permeability that was altered due to active or cholestatic hepatic injuries (Zhang *et al.*, 2010). Alteration of the membrane permeability causes derangement in the transport of metabolites leading to leakage of these enzymes (Mehana *et al.*, 2012). Restoration of the transaminases to their normal levels after treatment with algal extract indicates a revival of the insulin secretion. This is consequently associated with the decline of glucose level and inhibition of LPO (Iweala and Oludare, 2011).

Elevated urea and creatinine levels are considered as significant markers of renal dysfunction from diabetic hyperglycemia (Almdal and Vilstrup, 1988). Our results indicated that levels of the urea and creatinine elevated in STZ induced diabetic rats. This was consistent with Ronco *et al.* (2010) who suggested that the abnormally high levels of urea and creatinine were related to the impaired kidney function. Moreover, it was demonstrated that there was a positive correlation between hyperglycemia and the development of nephropathy (Ceriello *et al.*, 2000). In 2008, Punithavathi *et al.* postulated that elevation of these parameters may be associated with the metabolic abnormalities in gluconeogenesis and greater protein catabolism in diabetic rats. Level of TP decreased in STZ induced diabetes. These findings were supported by Bhargavi *et al.* (2015) who reported that the reduction in TP might refer to the stimulated conversion of glycogenic amino acids to carbon dioxide and water. The *N. oculata* algal extract prevented negative impact on kidney function in STZ induced diabetes. This may be attributable to the presence of the polysaccharides and fatty acids which may aid in regulating nitrogen compounds in endocrine metabolism (Nuno *et al.*, 2013).

It is well known that the lipid profile appears to be a vital factor in the development of atherosclerosis (Chattopadhyay and Bandyopadhyay, 2005). During the present study, STZ induced diabetes exhibited dyslipidemia through an elevation in total cholesterol, T.Gs, LDL-c and hence decline in HDL-c. This was in accordance with many previous studies (Yadav et al., 2008). This might be attributed to several biochemical mechanisms represented by activation of the hormone-sensitive lipase which facilitate mobilization of fatty acids from T.Gs in adipocytes (Cullen et al., 1999) and/or activation of the lipoprotein lipase which facilitates hydrolysis of T.Gs in endothelial cells (Kondo et al., 2007). The N. oculata algal extract exerted its role in lowering cholesterol and T.Gs levels due to the presence of polysaccharides which play role in reductions in the intestinal absorption (Niewold et al., 2012). Moreover, the algal extract decreased the LDL-c and increased HDL-c. This may refer to the presence of the eicosapentaenoic and docosahexaenoic fatty acids which aid in reducing LDL-c and hence inducing HDL-c (Komprda, 2012). The activity of the serum CPK used as a sensitive indicator in the early stage of myocardial ischemia, while LDH is roughly proportional to extent of the myocardial tissue injury (Chatterjea and Shinde, 2002).

In the present experiment, STZ injection resulted in a significant increase in CPK and LDH activities. These findings were supported by Alnahdi (2012) who reported that levels of these enzymes in STZ-induced diabetic rats. This may be due to the infarcted myocardium occurred as a result of STZ metabolism (Hamm *et al.*, 1992). Restoration of these enzymes to their normalcy after treatment with the *N. oculata* algal extract may be due to presence polyphenols and the natural antioxidants which preserve the integrity of the tissue. In the current study, STZ caused elevation of the effect of the ROS which generated as a result of depletion of these enzymes at persistently elevated glucose level (Maritim *et al.*, 2003). Subsequently, this leads to an increase in peroxidation reaction (Koo *et al.*, 2001).

The *N. oculata* algal extract scavenging activities against oxidative stress and hence lowered the LPO level. Furthermore, the algal extract exhibited the reducing power which may serve as a significant reflection of the antioxidant activity (Oktay *et al.*, 2003). Subsequently, the compounds exhibit the power to reduce the oxidized intermediates during the peroxidation processes. Therefore, they can act as primary and secondary antioxidants (Chanda and Dave, 2009). STZ has been shown to induce production of the free radicals which cause tissue injury. The pancreas is especially susceptible to be damaged by STZ due to its ability to induce the generation of the free radicals which attack tissue and hence cause tissue injury (Basha and Subramanian, 2011). Based on recent studies carried out by El-Feky *et al.* (2017), this *N. oculata* algal extract is rich in various phenolics, chlorophylls, and carotenoids. Moreover, the chromatographic analysis identified presence of flavonoids, pyrogallol, and catechine in addition to the presence of four phenolic compounds (cinnamic acid, p-coumaric acid, p-hydroxybenzoic acid and gallic acid).

In the current study, STZ caused histopathological alterations in the pancreatic tissue. This was in accordance with Higdon *et al.* (2001) who reported that STZ caused inflammatory changes in pancreatic islets of diabetic rats. Consequently, this leads to atrophy in islands of the Langerhans cells associated with vacuolation of islet cells. This might be due to the destruction of β -cells and hence decrease in a number of the pancreatic islets. The *N. oculata* algal extract may exhibit antioxidant activity and protect the pancreas from the free radicals attack due to the scavenging properties and the ability to decrease the production of the free radicals (Selvendran, 2013).

The current study revealed that the alterations in the native electrophoretic protein and lipoprotein patterns in the pancreas of STZ induced diabetic rats may refer to depletion of the antioxidant defenses and accumulation of products of the oxidative stress. This leads to damage to these biological macromolecules (Blasiak et al., 2004). Also, these electrophoretic alterations may occur as a result of nonenzymatic glycation of the native proteins through reaction of sugar with the protein portions (Kennedy and Baynes, 1984). Moreover, the glycation process causes a decrease in the biological role of the chaperone which is responsible for the protein folding (Hook and Harding, 2002). The activity of the antioxidant enzymes is tissue dependent and varies from tissue to tissue. The changes in the activity of the antioxidants in diabetic rats might be attributable to alterations in the metabolic pathways or degeneration of the protein contents as a result of attacking the free radicals (Ramanathan et al., 1999). During the present study, the STZ caused alterations in the different electrophoretic isoenzymes. This might refer to the glycation process which inhibits enzyme activity (Hook and Harding, 1998). Furthermore, Al-Enazi (2014) supported the findings that STZ caused alterations in the electrophoretic CAT and POX patterns due to glycation of these enzymes. The α -amylase catalyzes the degradation of the dietary carbohydrates to oligosaccharides and disaccharides through hydrolysis of α -(1,4)-D-glycosidic linkages of starch and other glucose polymers. It has been looked at as potential targets for diabetes (Najafian *et al.*, 2011). The α -amylase was represented by only one thick band in STZ induced diabetes. This may refer to its effective role in glucose hydrolysis. Moreover, it may refer to changes in amylase protein level in parallel to changes in amylase mRNA level (Kim et al., 1990) or due to the abnormal efficiency of the acinar pancreatic cells to biosynthesize this enzyme (Kamarýt *et al.*, 1993). During our experiment, it was noticed that the α -EST was represented by only one band in STZ induced diabetic rats. Also, STZ caused abnormalities in number and arrangement of bands of the β -EST pattern. This may refer to the binding of the esterase to sugar or sugar phosphate during the glycation process. This leads to the protein cross-linking, aggregation or fragmentation and hence enzyme alterations (Yan and Harding,

2005). The *N. oculata* algal extract minimized the alterations induced by STZ injection in the different electrophoretic patterns. This may refer to the presence of various active phytoconstituents with antioxidant efficiencies, such as carotenoids, polyunsaturated fatty acids and polysaccharides (Holtin *et al.*, 2009). In addition, the presence of the phenolic compounds is considered as part of a complex defense mechanism against a wide range of stressors and thus accumulates in response to these adverse factors (Dixon and Palva, 1995). The anti-glycating may refer to the presence of ellagic acid (Muthenna *et al.*, 2012), flavonoids, tannic acid and gallic acid (Obrenovich *et al.*, 2010). Walter *et al.* (2010) emphasized that these polyphenols exhibit the anti-glycating activity through other mechanisms irrespective of glycation inhibition.

During the current experiment, STZ enhanced the DNA fragmentation in pancreas tissue. This was in agreement with Morgan *et al.* (1994) and supported by Kroncke *et al.* (1995) who documented that STZ is a donor of the nitric oxide which has been found to bring about the destruction of the pancreatic islet cells. This active molecule contributes to STZ-induced DNA damage and exhibited cytotoxicity due to the formation of peroxynitrite as a result of the interaction of nitric oxide with superoxide anion. The *N. oculata* algal extract exhibited protection against DNA injuries associated with oxidative stress. This may refer to the presence of the bioactive peptides in addition to the natural effective antioxidants (Kim *et al.*, 2012).

CONCLUSIONS

In this study, it was observed that the *N. oculata* algal extract restored all the biochemical measurements changed in STZ induced diabetes to normalcy. Furthermore, it lowered the LPO level and hence minimized the histopathological alterations occurred in pancreas tissue as a result of STZ effect. It decreased the qualitative mutagenicity in electrophoretic protein, lipoprotein patterns and isoenzymes (α -amylase, α -, β -EST, CAT, and POX) in pancreas tissue through hiding the abnormal bands and restoring the normal ones. Also, it lowered the quantitative mutation through decreasing quantity of the bands which expressed severely in diabetic rats. Moreover, it maintained the integrity of the genomic DNA which cleaved by STZ giving several unique bands.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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