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Chemical and Therapeutic Study of *Nannochloropsis oculata* on Spleen of Streptozotocin Induced Diabetes in Rats

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ABSTRACT

Diabetes is considered as the most prevalent metabolic disease. Nannochloropsis oculata is well known by its beneficial values due to the presence of various bioactive compounds. In the present study, Streptozotocin (STZ) induced diabetes resulted in a significant (P<0.05) decrease in all hematological measurements. In addition, it caused significant (P<0.05) elevation in the Lipid Peroxidation Product (LPO) level associated with lowering Total Antioxidant Capacity (TAC) level in the spleen tissue. The N. oculata algal extract restored levels of these measurements to normalcy. The present study revealed that N. oculata contains fat soluble vitamin (β-carotene; 79.069 µg/g) and water soluble vitamins (Vitamin B3, B6 and B12; 3.811, 2.531 and 2.475 µg/g, respectively). Furthermore, it contains various micro and macro minerals (Calcium 20.90% and iron 4924 ppm) in addition to presence of phenolics, chlorophylls and carotenoids. The chromatographic analysis identified presence of flavonoids, pyrogallol (179.65 µg/g) and catachine (46.00 µg/g) in ethyl acetate extract. Also, four phenolic compounds (cinnamic acid, p-comaric acid, p-hydroxy benzoic acid and gallic acid) were isolated and identified by column chromatography. In conclusion, Nannochloropsis oculata algal extract showed scavenging activities against oxidative stress induced by STZ in spleen tissue of diabetic rats.

Keywords: Diabetes mellitus, *Nannochloropsis oculata*, Antioxidants, Phenolic, Carotenoids

INTRODUCTION

Diabetes is considered as the most prevalent metabolic disease which progresses with chronic hyperglycemias related to absolute and relative insulin deficiency. This disease occurs as a consequent to alterations in carbohydrate, protein and lipid metabolism [1]. Also, it is caused as a result of genetic factors which lead to autoimmune destruction of β-cells with adverse histopathological effects on spleen tissue that is considered as one of the most important immune organs. Million people worldwide are affected by this chronic disease [2].

Streptozotocin (STZ) is consisting of a N-methyl nitrosocarbamil-glucosamine-structured substance. It is synthesized naturally by *Streptomyces achromogenes* [3]. It induces diabetes through insulin insufficiency as a result of destruction of DNA by increasing pancreatic β-cell poly adenine diphosphate ribose synthetase activity and hence blocking pro-insulin synthesis [4].

Spleen represents a large lymphatic tissue. It plays a vital in re-circulation of the lymphocytes which exhibit ability to promptly elicit specific T or B lymphocyte mediated immune reactions [5]. It exhibited basic function in the rat, similarly as in man. It is responsible for clearance of the damaged old particles of the body itself and foreign particles from the blood. It is equipped with white and red pulps associated with a specific structure responsible for blood circulation [6]. Recently, it was postulated that diabetes decreases capacity of the immune response in addition to occurrence of atrophy of immune organs [7]. Various changes were found in spleen of diabetic rats associated with increasing rates of apoptosis in lymphocytes [8]. It was noticed that spleen size and weight increased in diabetic rats by mean of splenomegaly [9]. In addition, Ebaid et al., [5] reported that diabetes exhibited adverse pathological effects on spleen that is considered as one of the most important immune organs. The recent studies directed to search in the nature to evaluate the different natural products to assess their potential utility as immunomodulatory agents to enhance the immune responses to this chronic disease [10].

In 1980, the world health organization recommended that it is necessary to evaluate effectiveness of the natural hypoglycemic agents for conditions safe modern drugs in traditional medicine.

This leads to increasing demand for the natural drugs which are frequently considered to be less toxic and more effective than the synthetic ones [11]. Recently, microalgae are considered as an important medicinal agent, because these marine organisms are rich sources of various biologically active metabolites such as phenolics, carotenoids, chlorophyll, phycobiliproteins, amino acids, polysaccharide, vitamins, fatty acids, macro and micro elements [12,13]. So far, many of these compounds have been isolated and being developed as a new promising medicinal agent [14]. In recent years, algae have attracted universal interest in the research area as a result of its ability to produce several beneficial natural products used as anti-inflammatory, antimicrobial, antimalarial, antiproliferative, and anticancer agents [15]. Moreover, they are used as functional food ingredients or feed supplementary due to their high nutritional value in addition to their ability to grow on a large scale, even under adverse conditions, they are able to produce significant amounts of fatty acids [16].

Nannochloropsis oculata is a small green microalga genus which belongs to the class Eustigmatophyceae. It is a marine eukaryotic unicellular phytoplankton which is well known in aquaculture due to its nutritional value and potency to produce biologically active constituents and it is considered as a source of omega-3 fatty acids [17]. The active components extracted from *N. oculata* exhibited the ability to minimize production of the free radicals and enhance the antioxidant strength [18]. The beneficial effect of the algal extract refers to presence of high levels of protein, polyunsaturated fatty acids and antioxidant pigments [19]. The present study aimed to study the chemical composition of the *N. oculata* algal extract and to evaluate its efficiency against the deleterious effect of STZ induced diabetes on spleen of rats.

MATERIALS AND METHODS

Chemicals and reagents

All solvents and chemicals in the present study are of pure analytical grade; hexane, petroleum ether (40-60°C), chloroform, ethyl acetate methanol and acetone (SDFCL, India) were distilled and dehydrated before use. Cinnamic, gallic, *p*-coumaric and p-hydroxybenzoic acid (Merck, Germany) are used as authentic references. Silica gel G-60 for column chromatography, 70-230 mesh (BDH, England). Precoated TLC-sheets of silica gel with florescent indicator 254 nm (Merck, Germany).

Microalgae strain and preparation

N. oculata alga (NNO-1 UTEX Culture LB 2164) was cultivated and collected from the Algal Biotechnology Unit, National Research Centre, Dokki, and Giza, Egypt. Microalgae concentration and biochemical composition were controlled according to method described by Nuno et al. [20]. Strain was cultivated in 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. These were centrifuged at 3588 g and 20°C, and the precipitated microalgae centrifuged again at 897 g and 20°C for 10 min. Recovered biomass was freeze-dried and stored separately by species at −20°C until use.

Preparation of algal extract and determination of the median lethal dose

The fresh vegetative algal material was dried in an incubator at 50°C for 72 h and then crushed into powder in an electric blender. The algal powder was defatted by cold maceration in hexane to remove all the lipoidal matter. This was followed by subsequent extraction in ethyl acetate at room temperature then filtered. The ethyl acetate extract was dried under vacuum rotary at 45°C. The safe dose of the ethyl acetate extract was evaluated after determination the median Lethal Dose (LD_{50}). Fifty six adult albino mice weighing 20-25 g was used to study acute toxicity. 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 24 h post treatment. The LD_{50} was calculated according to the equation suggested by Paget and Barnes [21]. The therapeutic dose of algal extract was administrated orally by stomach tube at 250 mg/kg/day.

Induction of diabetes mellitus

A freshly prepared solution of STZ dissolved in 100 mM citrate buffer (pH 4.5) at a dose of 60 mg/kg b.w. and injected intraperitoneally (i.p.) in a volume of 1 ml/kg body weight to overnight fasted rats [22]. STZ injected animals exhibited massive glycosuria and hyperglycaemia within few days. Diabetes was confirmed in STZ rats by measuring the fasting blood glucose concentration. A blood glucose level of 200 mg/dl was considered as diabetic.

Animals and treatments

Healthy sixty adult male Wistar rats (weighting 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 ± 2°C.

Ethics statement

The experimental design and animal handling were performed according to the experimental protocol which was 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.

Experimental design

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

Collection of samples

At the end of the experimental period (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 to be sure for diabetes induction.

Part two: deposited into heparinized tubes for the haematological measurements. After sacrificing the animals by cervical dislocation, the spleen tissues were excised and washed in ice-cold saline then homogenized in 0.01 M Tris-HCl buffer (pH 7.4). Aliquots of these homogenate were used for measuring markers of the oxidative stress.

Haematological analysis

The heparinized blood samples were analyzed on an automatic blood analyzer (XE 2100 D; Sysmex, Kobe, Japan) to quantify Hemoglobin (HB), Red Blood Cells (RBCs), platelets, White Blood Cells (WBCs) and lymphocytes.

Markers of oxidative stress

The Lipid Peroxidation Product (LPO) was determined in the spleen tissue homogenates spectrophotometrically at wave length 535 nm using a UV-Visible spectrophotometer (Shimadzu UV-2401 pc) [23]. Furthermore, the Total Antioxidant Capacity (TAC) was measured based on capacity of the sample to inhibit production of thiobarbituric acid reactive substances from sodium benzoate under the influence of the free oxygen radicals derived from Fenton's reaction as mentioned by Koracevic et al. [24].

Statistical analysis

All data were statistically evaluated by the Statistical Package for Social Sciences (SPSS for windows, version 11.0). The results were expressed as mean ± standard error (SE). Significant difference between the groups were statistically analyzed by One-way Analysis of Variance test (ANOVA) followed by Least Significant Difference (LSD) test and confirmed by Benferoni test. A "P" value of less than 0.05 was considered to indicate statistical significance.

Histopathological examination

Spleen specimens were taken from all animals and processed to be examined using light microscopy. The tissue sections were fixed in 10% neutral buffered formalin and embedded in paraffin. The paraffin sections were then stained with hematoxylin-eosin (H&E) [25]. Spleen histopathological changes were scored according to Dommels et al. [26]. A rating score between 0 (no damage) and +++ (maximal damage) was assigned for each investigated section. Sections from at least five rats were carefully investigated.

Phytochemical study

Determination of pharmacopoeial constants and vitamins

The moisture content was determined through drying the fresh vegetative *N. oculata* at 100°C for 24 h. Subsequently, contents of the total ash, water soluble ash and acid-insoluble ash were determined in the powdered alga as described by Kirk and Sawyer [27]. Fat and water soluble vitamins were determined in the dried vegetative *N. oculata* according to method suggested by Hasan et al. [28], using High Performance Liquid Chromatographic (HPLC) system (Shimadzu-UFLC Prominence), equipped with an auto sampler (Model-SIL 20AC HT) and UV-Visible detector (Model-SPD 20A).

Determination of chlorophyll and carotenoid contents

The carotenoid content was determined in the powdered vegetative *N. oculata* according to procedures documented by Rodriguez-Amaya [29]. Wet cells, collected by centrifugation, were exhaustively extracted with acetone using a mortar and pestle. The carotenoid extract was transferred to petroleum ether in a separatory funnel by addition of water and washed free of acetone. The pigment solution was dried with anhydrous sodium sulphate and quantified spectrophotometrically [30]. The total chlorophyll content was determined spectrophotometrically at wavelengths 645 and 663 nm using formula suggested by Enwereuzoh and Onyeagoro [15]. Moreover, Contents of the total carotenoid was calculated using formula described by de Carvalho et al. [31].

Determination of macro and micro elements

Most of the micro elements present in the algal biomass are heavy metals and algae have been reported to be strongly active in heavy metals concentration. Contents of the element (phosphorus, potassium, calcium, magnesium, sodium, iron, manganese, zinc and copper) were evaluated in the dried vegetative *N. oculata* according to procedure of Norbert [32] using UNICAM solar 969 Atomic Absorption Spectrophotometer.

Isolation of pure phenolic compounds

Ten grams from algal ethyl acetate extract were chromatographed over silica gel column 150 × 4.5 cm. Elution was successively carried out by Chloroform-Methanol ($CHCl_3$-MeOH) mixtures of increasing polarities to yield 3 main fractions (I-III) which screened and purified by thin layer chromatography silica gel using $CHCl_3$-MeOH (8:2) as solvent systems. Compound (1) 35.0 mg and compound (2) 29.0 mg were isolated from fraction I (Eluted with $CHCl_3$-MeOH; 7:3), compound (3) 25.5 mg was isolated from fraction II (Eluted with $CHCl_3$-MeOH; 5:5), while compound (4) 21.5 mg was separated from fraction III (Eluted by $CHCl_3$-MeOH 4:6). The isolated compounds were identified and determined by different spectroscopic analyses using Infrared spectrophotometer Perkin-Elmer 283 (German), Mass Spectrometer Finnigan Model 3200 at 70 eV (German), nuclear magnetic resonance spectrometers (JEOL EX- 500 MHz (Japan)).

Chromatographic analysis of total phenolics and flavonoids

The total phenolic content was assayed in the ethyl acetate extract by spectrophotometric method as documented by Singleton and Rossi [33]. Briefly, 0.5 ml of the extract mixed with 2.5 ml of 10% Folin-Ciocalteu’s reagent and 2.5 ml 7.5% $NaHCO_3$. The absorbance was measured spectrophotometrically at λ_{max}=765 nm against blank. Concentration of the phenolics was calculated from calibration curve of different series of gallic acid. Content of the phenolics was expressed in terms of gallic acid equivalent (mg of gallic acid/g of extract).

The different phenolic compounds were identified by the HPLC (Shimadzu-UFLC Prominence), equipped with an auto sampler (Model-SIL 20AC HT) and UV-Visible detector (Model-SPD 20A) (Japan). The analysis was carried out using analytical column of an Eclipse XDB-C18 (150 × 4.6 µm; 5 µm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 ml/min for a total run time of 70 min and the gradient programme was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. The injection volume was 50 µl and peaks were monitored simultaneously at 280 and 320 nm for the benzoic acid and cinnamic acid derivatives, respectively. All samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with those of the standards.

Determination of antioxidant activity in the isolated compounds

Percentage of the antioxidant activity was assessed by 2,2-Diphenyl-1-Picryl-Hydrazyl-Hydrate (DPPH) free radical assay [34].

RESULTS AND DISCUSSIONS

Haematological indices and markers of oxidative stress

Blood is the most important body fluid that regulates various vital body functions through transportation of nutrients, oxygen and other metabolites to different tissues and organs then transportation of metabolic waste products away from those tissues to the excretion sites [35]. The haematological indices provide with crucial information for evaluation of vitality of the different tissues [36].

As revealed in Table 1, STZ induced diabetes resulted in a significant ($P<0.05$) decrease in all hematological measurements (HB, RBCs, platelets, WBCs and lymphocytes) with respect to control. This was in accordance with Alnahdi [37] who postulated that occurrence of anaemia and reduction of these hematological biomolecules in STZ induced diabetes have been reported due to non-enzymatic glycosylation (glycation) of the HB and RBCs membrane proteins by the excess glucose. These structural alterations may lead to impaired protein function leading to hemolysis of the blood cells and hence anemia [38]. Also, STZ caused decline in the WBCs and lymphocytes. This was in agreement with Oyedemi et al. [39], who reported that STZ suppresses the immune system by damaging WBCs. Moreover, reduction of these parameters could be linked to suppression of leukocytosis from the bone marrow which influences the immune system and phagocytic activity [40]. Administration of *N. oculata* algal extract restored levels of these measurements to normalcy by increasing their levels significantly ($P<0.05$) as compared to STZ induced diabetes. This may refer to ability of the extract to stimulate the bone marrow for over production of haematopoietic regulatory elements such as colony-stimulating factors, erythropoietin and thrombopoietin by the stromal cells and macrophages thus stimulating hematopoiesis. In addition, the algal extract increased the WBCs and lymphocytes due to ability of its active constituents to increase expression of the cellular immune cells [18].

As illustrated in Figures 1a and 1b, STZ caused elevation in the LPO associated with lowering the TAC level significantly ($P<0.05$) in spleen of diabetic rats with respect to control. This might be attributed due to the oxidative stress which occurred as a result of the diabetic toxicity starting with generation of the free radicals followed by increasing pro-inflammatory cytokines and ending by programmed cell death [5]. This can clearly explain the alterations in the spleen tissues of the diabetic rats. The effective strategy for treatment of lymphocyte-mediated diseases is concerned with regulation of lymphocytes apoptosis in the white pulps [41]. The treatment with *N. oculata* algal extract at all therapeutic modes lowered the LPO with elevation of the TAC level significantly ($P<0.05$) to normalcy with respect to STZ induced diabetic group. This might be attributed to presence of flavonoids with radical scavenging activity such as cinnamic acid, p-comaric acid, p-hydroxy benzoic acid and gallic acid.

Histopathological examination

In coincide with results of the LPO and TAC in the present study, STZ caused histopathological alterations in spleen of diabetic rats. As illustrated in Figure 2a, it was revealed that there was no histopathological alteration in spleen of control rats. Normal histological structures of the white and red pulps were noticed. In *N. oculata* algal extract treated group, there was no histopathological alteration and no deviation from control group (Figure 2b). In STZ induced diabetic group and treated with commercial insulin, there was normal histological structure of the white and red pulps with respect to control (Figure 2c). In STZ induced diabetic group, lymphoid depletion was detected with the highest severity degree in the white pulps (+++; 75-100%) associated with congestion in the red one (Figure 2d). In simultaneous-treated and post-treated groups, no histopathological alterations were detected (Figures 2e and 2f).

Although Nuno et al. [20] reported that *N. oculata* algal extract may exhibit adverse effects due to rigidity of the algal cellular structure which is relatively thick, and when freeze-dried could adversely impact the epithelium, lactic acid bacteria counts and nutrient absorption, it showed amelioration against the STZ effect which induced the peroxidation reaction and enhanced the oxidative stress in spleen of rats. This might refer to the presence of various polyphenolic compounds which were well known to be found in the microalgae at all [12].

Phytochemical study

As revealed in Table 2, the vegetative *N. oculata* is rich in high contents of moisture and different ashes. Moreover, it contains wide range of different vitamins (Table 3). As presented in Table 4, the *N. oculata* is rich in wide range of macro and micro elements. Ca is the most abundant macro element (20.90%) while iron is the most abundant micro one (4924 ppm).

The phenolic compounds were characterized and identified in the dried vegetative *N. oculata* algal extract during the current study. It was noticed that the extract contains various phenolic compounds with different quantities. Moreover, contents of the total chlorophyll and carotenoids in *N. oculata* were about 29.3199 ± 0.326 µg/ml and 14.907 ± 0.004 µg/g, respectively. Upon application of the *N. oculata* algal ethyl acetate extract on silica gel column, it was postulated that four major phenolic compounds have been isolated and identified as cinnamic acid, p-comaric acid, p-hydroxy benzoic acid and gallic acid. These phenolic compounds are well recognized by their antioxidant efficiency through single electron transfer and through hydrogen atom transfer [42]. Furthermore, presence of the different vitamins that play role in decreasing the harmful effects occurred by the action of free radicals and check degenerative disease [43]. In addition, existence of the α-tocopherol and carotenes which are considered as the most abundant fat soluble compounds [44] exhibit antioxidant activity. The α-tocopherol is capable of scavenging singlet oxygen forming the tocopheroxyl radicals that subsequently reduced back to α-tocopherol in presence of ascorbate through the ascorbate-glutathione cycle [45].

Table 1: Effect of STZ-induced diabetes and daily oral administration of *N. oculata* algal extract on hemoglobin, red blood cells, platelets, white blood cells and lymphocytes

Haematological parameters	GI	GII	GIII	GIV	GV	GVI
HB (g/dl)	13.94 ± 0.07	13.97 ± 0.07	13.91 ± 0.08	10.21^{a} ± 0.01	12.61^{ab} ± 0.06	13.55^{ab} ± 0.05
RBCs (10^6/cmm)	6.96 ± 0.05	6.89 ± 0.07	6.81 ± 0.07	5.26^{a} ± 0.04	6.31^{ab} ± 0.02	6.90^{b} ± 0.06
Platelets (10^3/cmm)	464.7 ± 2.47	464.5 ± 0.90	465.67 ± 0.82	355.83^{a} ± 5.75	460.80^{ab} ± 1.53	459.40^{ab} ± 1.52
WBCs (10^3/cmm)	9.34 ± 0.03	9.4 ± 0.01	9.41 ± 0.34	5.04^{a} ± 0.07	9.80^{ab} ± 0.02	9.37^{ab} ± 0.02
Lymph. (10^3/cmm)	4.51 ± 0.02	4.53 ± 0.03	4.54 ± 0.01	3.80^{a} ± 0.03	5.04^{ab} ± 0.02	4.49^{ab} ± 0.02

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

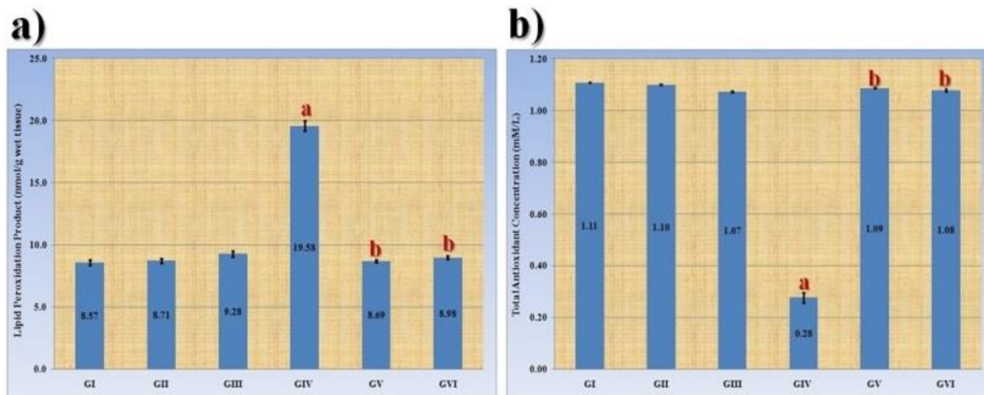

Figure 1: Effect of STZ-induced diabetes and daily oral administration of *N. oculata* algal extract on (a) Lipid peroxidation product and (b) Total antioxidant capacity level in spleen tissue. Values in center of column are expressed as mean. a shows the significance (P<0.05) in comparison to the control group and b shows the significance (P<0.05) in comparison to the STZ induced diabetic group

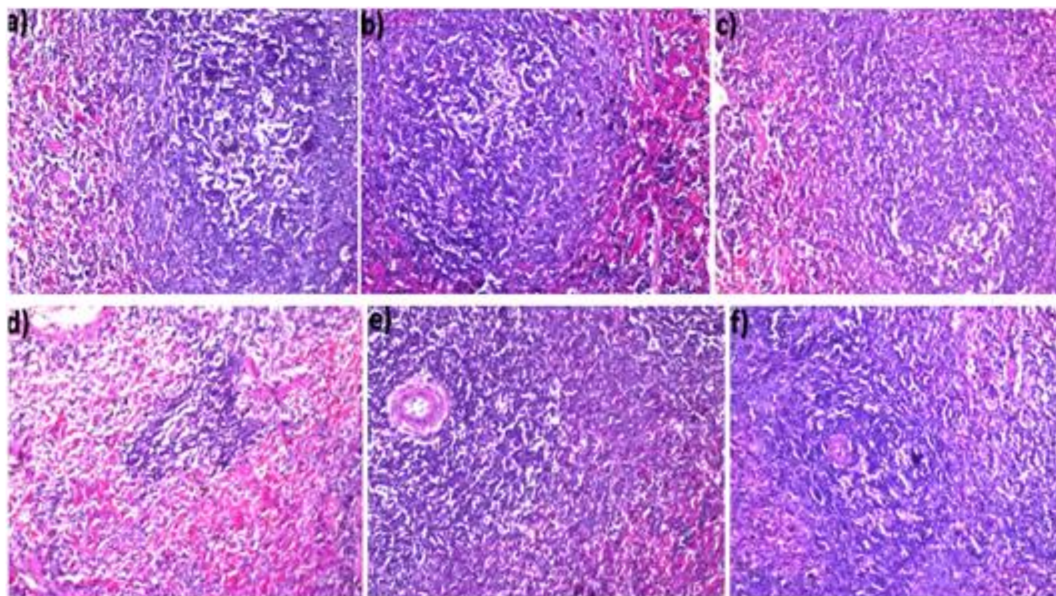

Figure 2: Spleen tissue showing (a) Control group with normal histological structure (H and E, X 40), (b) *N. oculata* algal extract treated group without deviation from control group (H and E, X 40), (c) STZ induced diabetes treated by commercial insulin with normal histological structure (H and E, X 40), (d) STZ induced diabetes with severe lymphoid depletion in the white and red pulps (H and E, X 40), (e) simultaneous-treated group with normal histological structure (H and E, X 40) and (f) Post-treated group without alterations and deviation from control group (H and E, X 40)

Table 2: Percentages of certain pharmacopoeial constants in powders of the vegetative *N. oculata*

Pharmacopoeial constants (%)			
Moisture	**Total Ash**	**Water soluble Ash**	**Acid insoluble Ash**
91.5	42.25	16.47	23.76

Table 3: Concentration of fat and water soluble vitamins in powders of the dried vegetative *N. oculata*

Fat soluble vitamins	Concentration (µg/g)	Water soluble vitamins	Concentration (µg/g)
β-carotene	79.069	B3	3.811
Vitamin A	ND	B1	ND
Vitamin D3	0.407	B6	2.531
Vitamin D2	2.744	B9	ND
Delta tocopherol (Δ-E)	ND	B2	0.290
Alpha tocopherol (α-E)	10.868	B12	2.475

ND: Not Detected concentration

Table 4: Contents of the macro and micro elements in powders of the dried vegetative *N. oculata*

Macro elements	%	Micro elements	ppm
N	4.31	Fe	4924
P	0.40	Mn	147.73
K	1.00	Zn	109.2
Ca	20.90	Cu	37.7
Mg	3.32		
Na	3.43		

Characterizations of the isolated compounds

Cinnamic acid (1): White crystalline solid soluble in ethyl alcohol, chloroform and acetone (Rf: 0.75 $CHCl_3$-MeOH 8:2 v/v). Melting point 133, yellowish brown color with $FeCl_3$ spray reagent, IR_{max} (thin film): 3352 cm^{-1} (O-H Stretch), 2955 cm^{-1} (C-H Stretch), 1731 cm^{-1} (C=O Stretch), 1535 cm^{-1} (Aromatic C=C Stretch), 1461, 1383, 1241 cm^{-1} (C–H bending), 1166, 1173 cm^{-1} (C–OH Stretch), 772 and 608 cm^{-1} (O-H bending). MS showing base peak at m/z 148 (100%) for molecular formula $C_9H_8O_2$. The characteristic fragmentations are m/z 131 (25%), 120 (33%), 103 (47%), 93 (55%), 77 (65%), 63 (58%). ^{1}H-NMR (DMSO-d6, 300 MHz): σ (ppm)=11.01 (1H, s, broad, –COOH), 7.82 (1H, d, *J*=15 Hz, C7–H), 7.60 (2H, m, C2–H, C6–H), 7.33 (3H, m, C3–H, C4–H and C5–H), 6.47 (1H, d, *J* = 15 Hz, C8–H). ^{13}C-NMR (DMSO-d6, 125 MHz): σ (ppm)=171.83 (–COOH), 148.43 (C7), 133.83 (C1), 131.00 (C4), 128.40 (C3, C5), 127.97 (C2, C6), 115.93 (C8).

p-Comaric acid (2): white crystalline solid soluble in ethyl alcohol, chloroform and acetone (Rf: 0.89 $CHCl_3$-MeOH 8:2 v/v). Melting point 210, orange color with $FeCl_3$ spray reagent, IR_{max} (thin film): 3423 cm^{-1} (O-H Stretch), 2925 cm^{-1} (C-H Stretch), 1725 cm^{-1} (C=O Stretch), 1638 cm^{-1} (Aromatic C=C Stretch), 1457, 1263 cm^{-1} (C–H bending), 1121 cm^{-1} (C–OH Stretch), 874 and 528 cm^{-1} (O-H bending). MS showing base peak at m/z 164 (100%) for molecular formula $C_9H_8O_3$. The characteristic fragmentations are m/z 147 (60%), 119 (22%), 107 (15%), 91 (20%), 65 (18%). ^{1}H-NMR (DMSO-d6, 300 MHz): σ (ppm)=12.07(1H, s, broad, –COOH), 9.12 (1H, s, broad, C4–H), 7.32 (1H, d, *J*=15 Hz, C7–H), 7.17 (2H, d, *J*=7 Hz, C2–H, C6-H), 6.91 (1H, d, J=15Hz, C8–H), 6.79 (2H, d, *J*=7 Hz, C3–H, C5-H). ^{13}C-NMR (DMSO-d6, 125 MHz): σ (ppm)=170.22 (–COOH), 145.77 (C7), 136.23 (C1), 131.69 (C4), 121.59 (C3, C5), 119.23 (C2, 6), 116.23 (C8).

p-Hydroxy benzoic acid (3): white crystalline solid soluble in ethyl alcohol, chloroform and acetone (*R*f: 0.93 $CHCl_3$-MeOH 8:2 v/v). Melting point 214, yellow color with $FeCl_3$ spray reagent, IR_{max} (thin film): 3437 cm^{-1} (O-H Stretch), 2855, 2728 cm^{-1} (C-H Stretch), 1734 cm^{-1} (C=O Stretch), 1563, 1455 cm^{-1} (Aromatic C=C Stretch), 1376, 1248 cm^{-1} (C–H bending), 1166, 1112 cm^{-1} (C–OH Stretch), 717 and 620 cm^{-1} (O-H bending). MS showing molecular ion at m/z 138 (23%) for molecular formula $C_7H_6O_3$, base peak at m/z 121. The characteristic fragmentations are m/z 111 (47%), 93 (67%), 83 (90%), 65 (75%), 53 (84%). ^{1}H-NMR (DMSO-d6, 300 MHz): σ (ppm)=12.04 (1H, s, broad, –COOH), 8.45 (1H, s, broad, C4–H), 7.13 (2H, d, J=7.7 Hz, C3–H, C5–H), 6.82 (2H, d, J=7.7 Hz, C2–H, C6–H). ^{13}C-NMR (DMSO-d6, 125 MHz): σ (ppm)=181.87(–COOH), 158.12 (C4), 145.32 (C1), 128.40 (C3, C5), 120.97 (C2, C6).

Gallic acid (4): white crystalline solid soluble in ethyl alcohol, chloroform and acetone (Rf: 0.96 $CHCl_3$-MeOH 8:2 v/v). Melting point 260, blue color with $FeCl_3$ spray reagent. IR_{max} (thin film): 3427 cm^{-1} (O-H Stretch), 2923, 2825 cm^{-1} (C-H Stretch), 1776 cm^{-1} (C=O Stretch), 1643, 1466 cm^{-1} (Aromatic C=C Stretch), 1325 cm^{-1} (C–H bending), 1157, 1121 cm^{-1} (C–OH Stretch), 874 cm^{-1} (O-H bending) cm^{-1}. MS showing base peak at m/z 170 (100%) for molecular formula $C_7H_6O_5$. The characteristic fragmentations are m/z 153 (85%), 141 (35%), 125 (20%), 93 (20%). ^{1}H-NMR (DMSO-d6, 300 MHz): σ (ppm)=11.97 (1H, s, broad, –COOH), 7.22 (2H, s, C2–H, C6–H). ^{13}C-NMR (DMSO-d6, 125 MHz): σ (ppm)=166.74 (–COOH), 144.97 (C-3 & C-5), 137.93 (C-4), 121.76 (C-1), 108.85 (C-2 & C-6).

It was noticed that the total phenolic content in the ethyl acetate *N. oculata* extract was expressed as 100 ± 0.001 mg of gallic acid/g of dried extract. As illustrated in Table 5, the HPLC analysis identified eight phenolic compounds and four flavonoids in the ethyl acetate *N. oculata* extract. It was noticed that pyrogallol (179.65 µg/g) and catachine (46.00 µg/g) were the most predominant flavonoids in the extract. The ethyl acetate *N. oculata* algal extract and the four isolated flavonoids exhibited radical scavenging activity with different percents. p-Hydroxy benzoic acid has the highest scavenging activity (81%) while cinnamic acid noticed with the lowest activity (29.4%) (Table 6).

Table 5: HPLC analysis of phenolics and flavonoids in the ethyl acetate *N. oculata* extract

Compound	Retention time (min)	Concentration (µg/g)
Pyrogallol	4.7	179.65
Gallic acid	5.7	8.17
Protocatechuic acid	9.9	2.11
p-Hydroxybenzoic acid	15.1	4.81
Catachine	18.6	46
Chlorogenic acid	20.6	0.96
Caffeic acid	21.4	0.25
p-coumaric acid	37.2	8.36
Cinnamic acid	42.8	1.47
Quercetin	43.5	0.72
Apigenin	46	6.66
Kaempferol	46.5	5.92
Chrysene	52	1.85

Table 6: The DPPH radical scavenging activity (%) of the ethyl acetate *N. oculata* extract and the isolated phenolic compounds

Samples	Abs.	Activity%
Ethyl acetate extract	0.312	72.9%
Cinnamic acid	0.814	29.4%
p-Comaric acid	0.416	64%
p-Hydroxy benzoic acid	0.214	81.4%
Gallic acid	0.443	61.5%

The current experiment postulated that the *N. oculata* algal body is rich in moisture. This might be attributed to role of moisture in dissolving the materials and carrying the nutrients for all organs in the body to enable them to make their role in a proper way [46]. The soluble fibers may undergo some metabolism in the small intestine and especially in the large intestine through bacterial enzymes, converting it to products that help in maintenance of the colonic microflora, which is useful to the digestion process. While the insoluble fiber can help in waste and toxin secretion from the body through different mechanisms [47]. The nutritional value of *N. oculata* algal extract may refer to presence of calcium (20.90%) that is important in formation of fibrinogen and subsequently fibrin and collagen [48]. In addition, presence of potassium (1%) and sodium (3.43%) which are known activators of energy potentials through nerve membrane together with calcium. Moreover, magnesium is a hormone activator in type 2 diabetes and its presence in the algae (3.32%) can be beneficial in managing this disease [49].

CONCLUSION

The study concluded that *N. oculata* algal extract exhibited scavenging activities against oxidative stress induced by STZ in spleen tissue of diabetic rats. This was attributed to presence of various active constituents such as phenolics, chlorophylls and carotenoids in addition to presence of flavonoids, pyrogallol and catachine. Furthermore, it was found that cinnamic acid, *p*-comaric acid, *p*-hydroxy benzoic acid and gallic acid were the most predominant phenolics which were isolated from *N. oculata* algal extract.

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