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RESEARCH ARTICLE

Anti-Obesity Effect of *Dunaliella Salina*: Study on the Gastric Inhibitory Polypeptide, ROS, DNA Damage and Metabolic Gene Expression in Rats

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Abstract

This study aimed to identify the gene expression profiling of lipid related genes in animal model of obesity and the role of microalgal *Dunaliella salina* (alga) in modulating the expression of these genes during the initial period of obesity. The diet-induced obesity model was used using fifty male Wistar albino rats (weighing 150-160 g) that have obesity caused by being fed high-fat diet (HFD) for 12 weeks. These rats were randomly divided into 2 groups (10 rats each) and treated with *D. salina* extract and orlistat for 6 weeks. The anti-obesity effect of this halophilic unicellular green alga was compared to orlistat, a standard anti-obesity drug promoting weight loss as a gastric and pancreatic lipase inhibitor. Treatment with the alga and orlistat showed significant effect in reducing gastric inhibitory polypeptide (GIP). Also, the treatment of obesity-induced rats with the alga increased significantly the mRNA expression levels of lipid catabolic genes (peroxisome proliferator-activated receptor *PPAR*-α and carnitine palmitoyl transferase (CPT-1) and decreased significantly the expression levels of lipid anabolic gene (*SCD*-1) compared with obesity-induced rats. In addition, *D. salina* extract was able to decrease ROS generation and inhibit the rate of DNA damage in obese rats. The study was extended to evaluate the phytochemical composition of micro algal extract. The fatty acid methyl esters were analyzed by GLC. The extract contained considerable level of omega-3 fatty acids. The presence of palmitic (62.5%), linolenic (9%), oleic (8.6%) and linoleic (7.4%) was revealed. The percentages of saturated and unsaturated fatty acids were 67.7 and 32.1%, respectively. *D. salina* extract administration which is rich in omega-3 fatty acids has anti-obesity properties. Results suggested that *D. salina* might be available as functional alga and bioactive diet supplement for the treatment of obesity.

Keywords: *Dunaliella salina, Lipid anabolic genes, Catabolic genes, DNA damage, Gastric inhibitory polypeptide*

Introduction

The ratio of obesity and its related complications such as insulin resistance, hypertension, dyslipidemia, endothelial dysfunction, and inflammation is further increasing at a shocking rate worldwide especially in developed countries [1,2]. Gastric inhibitory polypeptide (GIP) is considered peptide hormone released in response to nutrient ingestion that potentiate glucose-stimulated insulin secretion [3]. It release from the gastrointestinal tract into circulation. Adipocyte-secreted factors affect diverse processes including energy balance and appetite, insulin sensitivity, lipid

metabolism, and inflammation [4, 5]. It is known that visceral fat store may be differentially reactive to food composition and may generate factors not yet recognized that may participate in difficulty of maintaining weight loss. The adipose gene expression profile reflects both the newly recruited adipocytes in various stages of maturity and the preexisting hypertrophic fat cells in established obesity [6,7]. The long-chain n-3 polyunsaturated fatty acids should be used preferentially when selecting food sources since they reduce the risk for many diseases including obesity [8].

A combination of bioactive compounds such as carotenoids, and omega-3 polyunsaturated fatty acids possessing different chemical characteristics can be used to prevent oxidative damage and its impact on obesity-related characteristics. Microalgae are source of such valuable molecules and have many applications in feed and food products [9].

The halophilic unicellular green alga *Dunaliella salina* with a mucus surface coat (*i.e*., with no cell wall), first discovered on the Mediterranean coast in 1838, is the most interesting cell factory for the production of different carotenoids, vitamins, and fatty acids under extreme environment conditions [10]. A wide range of abundant carotenoids (especially all-*trans*-ß-carotene and 9- or 90-*cis*-ß-carotene) are produced by microalgae that have recently received much attention as they can provide for health and pharmaceutical benefits [9, 10].

Many investigations [10-12] have demonstrated that alga extract contains abundant β-carotene and astaxanthin could ameliorate oxidative stress that can provide health benefits. This could satisfy the growing need for novel ingredient of nutriceutical, pharmaceutical, and food supplements. The main goal of the present study is to investigate the role of *D. salina* extract on obesity induced by high fat diet (HFD) through determination of different metabolic biomarkers.

Besides, the expression levels of lipid catabolic genes (*PPAR*-α and *CPT*-1) and lipid anabolic gene (*SCD*-1) in liver tissues of obesity-induced rats fed HFD were evaluated. Additionally, the therapeutic effect of microalgal extract against intracellular reactive oxygen species (ROS) generation induced DNA damage attributed by HFD was studied. To our knowledge, this is the first reported on the gene expression profiling of lipid catabolic and anabolic genes in diet-induced obese rat model and supplementation with *D. salina* in modulating the gene expression of the genes that are up-regulated during the period of obesity.

Materials and Methods

Cultivation of Microalga *D. salina* and Preparation of Extract

Cultivation of Microalga *D. salina* on Laboratory Scale

Algal species isolated from River Nile water by using BG11 media for algal isolation and purification. The organisms were grown in conical flask 5 liter containing 2 L of Bold media (Stein, J.) (Table 1), for two weeks.

Table 1: Bold's Nutrient Composition

Macro elements	Concentration
Urea	0.3g/L
K_2HPO_4	0.075g/L
$KHPO_4$	0.175g/L
$MgSO_4(7H2O)$	0.075 g/L
Na Cl	0.025 g/L
$CaCl_2$ ($2H_2O$)	0.025 g/L
Microelements	
$ZnSO_4(7H_2O)$	8.8 mg/L
$MnCl_2(4H_2O)$	0.44 mg/L
MoO_3	0.071 mg/L
$CuSO_4$ ($5H_2O$)	1.57 mg/L
H_3BO_3	11.42 mg/L

EDTA	50 mg/L
KOH	31 mg/L
Fe SO_4 ($7H_2O$)	4.98 mg/L
Co (NO_3)$2.6H_2O$	0.49 mg/L
H_2SO_4	1µl/L

Micronutrient solution (1mL) was added to the culture medium. The culture was harvested by centrifugation at 4000 rpm, dried at 50°C and then grounded into homogeneous fine powder.

The algal biomass after the growth phase was harvested and inoculated in bold media for another two weeks. In addition, the biomass was harvested and inoculated in

plastic bottles with capacity of 17 L containing 15 L of microalgal culture with continuous aeration. The culture temperature was 22±3°C.

Fluorescent light was used to supply constant light intensity ≈2500 Lux for the culture. The micronutrient solution (1mL) was added to the culture medium. The culture was harvested by centrifugation at 4000 rpm, dried at 50°C and then grounded into homogeneous fine powder.

Cultivation of Microalga *D. salina* in the Photo-bioreactor

After growing for 10 days the inoculum was transferred to a fully automated and computer controlled vertical photo-bioreactor with a capacity of 4000 L.

Preparation of Ethanolic Extract of *D. salina*

The dried biomass of *D. salina* (300 g) was soaked in ethanol (80%) and shacked on shaker (Heidolph UNIMAX 2010) for 48 hrs 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. The resulting dry extract was evaporated on a rotary vacuum evaporator to dryness. The dry extract was kept in dark and stored at -20˚C in a freeze and kept for further analysis [13].

Biological Study

Experimental Animals

Male albino rats (n=50) weighted (150±10 g), were obtained from the Animal House of the National Research Centre (NRC). Animals were quarantined and allowed to acclimate for 10 days before beginning experimentation. They were housed 10 per cage under temperature controlled environment (26-29°C) with a fixed light/dark cycle with free access to water and food. All procedures of the present study were performed according to the Ethical Committee of the NRC, Egypt, provided that the animals will not suffer at any stage of the experiment.

Induction of Obesity in Rats

Obesity was induced in rats according to the method of Adaramoye et al [13], by feeding rat's high-fat diet (cholesterol). Cholesterol was orally administrated at a dose of 30mg/0.3mL olive oil/1 kg animal five times a week for twelve consecutive weeks. Lard fat was mixed with normal diet (one kilogram of animal lard was added to 5kg of normal diet. The occurrence of obesity was determined by measuring body weight gain percentages, visceral and fecal fat percentages [14].

Doses and Routes of Administration

Obese rats received an oral dose of 2 mg/kg body weight (b.wt.) dissolved in distilled water of the anti-obesity reference drug; orlistat (12 mg/kg) for 6 weeks [15]. The rats were orally administered 150 mg/kg b.wt. of *D. salina* extract for 6 weeks [16]. Percentages change were calculated as the mean control – Mean of test / Mean of control X100 and the percentages of improvement were calculated as mean diseased – Mean of treated / Mean of control X 100.

Experimental Design

Fifty male Wistar albino rats (5 - 6 weeks old) weighing 150 .00 ± 20 g (mean ± SD, weight of rats on the day received from supplier) with adaptation period to the experimental environment were randomly divided into five groups (n = 10/group) as follows: Group 1 (1) is the group of animals fed normal diet(ND)as control group. Group (2) is the group of animals fed normal diet

and treated with 150 mg/kg b.wt. of *D. salina* for 12 consecutive weeks (ND/DS).

Group (3) is the group of animals fed HFD for 12 weeks. Group (4) is the obese rats group treated for extra 6 weeks with 150 mg/kg b.wt. of *D. salina* extract (HFD/DS). Group (5) is the obese rats group treated for extra 6 weeks with anti-obesity standard drug, orlistat (12 mg/ kg b.wt., HFD/OR). Health conditions of all rats were monitored daily and no adverse events were observed throughout the study. At the beginning of the experiment the weights of all rats were recorded at 155.00 ± 5.00 g (mean ± SD, weight of rats after 10 days of acclimatization). All experiments and biochemical analysis were conducted using 50 rats with triplicate measurements. The permission to conduct this study was according to the Ethics of National Research Centre, Egypt.

Blood Sample and Collection of Serum and Liver Tissue

Blood samples were obtained fallowing an overnight fasting state at the end of treatment (week 12) at 8 a.m. Blood samples were withdrawn from a cubital vein into sterilized tubes and immediately and centrifuged at 4000 rpm for 15 min. The separated sera were stored on ice at -80°C for further analyses [17]. All the rats were sacrificed, and liver was obtained. The hepatic tissue (0.5 g) was homogenized in 4.5 mL ice cold saline solution (10%, W/V).

Biochemical Measurements

Determination of Gastric Inhibitory Polypeptide (GIP)

Gastric inhibitory polypeptide (GIP), biomarker was measured by double -antibody radio-immunoassay [18]. Immuno-reactive GIP was measured as the antiserum recognizes the large molecular form of GIP (mol. wt. nearly 8000 Dalton), and GIP standard (mol. wt. 4.977). Apocrine standard GIP was used, while parallelism was demonstrated between standard curve and the serially diluted sample of rats' serum containing high levels of endogenous GIP. The assay sensitivity was 110 ηg/L and the inter assay coefficient of variation 4.1%, at 2.677 ηg/Land 22.5% at 138 ηg/L

Determination of Reactive Oxygen Species (ROS) Formation

Intracellular ROS generation was measured in liver tissues of male rats by a flow cytometer with an oxidation-sensitive DCFH-DA fluorescent probe, after single-cell suspension was made [19]. DCFH is oxidized to fluorescent dichlorofluoresce in by the action of cellular oxidants. DCFH-DA is a non-fluorescent compound that is freely taken up into cells. The suspension was loaded by DCFH-DA solution with a final concentration of 50 µM and was incubated for 30 min at 37ºC.

Then samples were centrifuged at 1000 rpm for 5 min (4ºC), and cells were resuspended with phosphate buffered saline (PBS, pH 7.2–7.4). The fluorescence was detected by flow cytometer (with excitation 488nm and emission 525 nm). For each treatment, 1×10^5 cells were counted, and the experiment was performed in triplicate.

Gene Expression Analysis

Isolation of Total RNA

TRIzol® Reagent (Invitrogen, Germany) was used to extract total RNA from liver tissues of male rats according to the manufacturer's instructions with minor modifications. RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip. Isolated total RNA was treated with one unit of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water and quantified photospectrometrically at 260 nm. Purity of total RNA was assessed by the 260/280 nm ratio which was between 1.8 and 2.1.

Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT), otherwise they were stored at -80°C.

Reverse Transcription (RT) Reaction

Complete Poly (A) $^+$ RNA isolated from liver tissues were reverse transcribed into cDNA in a total volume of 20 µL using Revert Aid ™ First Strand cDNA Synthesis Kit (Fermentas, Germany). An amount of total RNA (5 µg) was used with a master mix. The master mix was consisted of 50 mM $MgCl_2$,10x RT buffer (50 mMKCl; 10 mMTris-HCl; pH 8.3), 10 mM of each dNTP, 50 µM oligo-dT primer, 20 IU ribonuclease

inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 IU MuLV reverse transcriptase.

The mixture of each sample was centrifuged for 30 sec at 1000 g and transferred to the thermocycler. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with a denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for cDNA amplification through quantitative Real Time- polymerase chain reaction (qRT-PCR) [20].

Real Time- PCR (qPCR)

Step One™ Real-Time PCR System from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA USA) was used to determine the liver cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix ExTaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec.

The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers. Each experiment included a distilled water control. The sequences of specific primer of the genes used are listed in Table 3.

Table 3: Primers used for Real time- PCR (qPCR)

Genes	Primer sequence [a]	References
PPAR-α	F: 5`- CCT GAA CAT CGA GTG TCG AAT AT-3`	
	R: 5`- GGT CTT CTT CTG AAT CTT GCA GCT-3`	[21]
CPT-1	F: 5`-AAA GAT CAA TCG GAC CCT AGA CA-3`	
	R: 5`- CAG GGA GTA GCG CAT AGT CA-3`	[21]
SCD-1	F: 5'-CAT CAT TCT CAT GGT CCT GCT-3'	
	R- 5'-CCA GTC GTA CAC GTC ATT TT-3'	[21]
β-*Action*	F: 5`-GTG GGC CGC TCT AGG CAC CAA-3`	
	R: 5`-CTC TTT GAT GTC ACG CAC GAT TT-3`	[22]

[a]F: Forward primer; R: Reverse primer.

At the end of each qPCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers [23]. The relative quantification of the target to the reference was determined using the $2^{-\Delta\Delta CT}$ method as follows:

$\Delta C_{T\,(test)} = C_{T\,(target,\,test)} - C_{T\,(reference,\,test)}$,

$\Delta CT\,(calibrator) = C_{T\,(target,\,calibrator)} - C_{T\,(reference,\,calibrator)}$,

$\Delta\Delta CT = \Delta C_{T\,(Test)} - \Delta C_{T\,(calibrator)}$.

Comet Assay

Isolated liver tissues of all groups of rats were subjected to the modified single-cell gel electrophoresis or comet assay [24]. To obtain the cells, a small piece of the liver was washed with an excess of ice-cold Hank's balanced salt solution (HBSS) and minced quickly into approximately 1 mm^3 pieces while immersed in HBSS, with a pair of stainless steel scissors.

After several washings with cold phosphate-buffered saline (to remove red blood cells), the minced liver was dispersed into single cells using a pipette. In brief, the protocol for electrophoresis involved embedding of the isolated cells in agarose gel on microscopic slides and lysing them with detergent at high salt concentrations overnight (in the cold).

The cells were treated with alkali for 20 min to denature the DNA and electrophoresis under alkaline conditions (30 min) at 300 mA, 25 V.

The slides were stained with ethidium bromide and examined using a fluorescence microscope with a green filter at × 40 magnification. For each animal about 100 cells were examined to determine the percentage of cells with DNA damage that appear like comets.

The non-over lapping cells were randomly selected and were visually assigned a score on an arbitrary scale of 0–3 (*i.e.*, class 0 = no detectable DNA damage and no tail; class 1 = tail with a length less than the diameter of the nucleus; class 2 = tail with length between 1× and 2× the nuclear diameter; and class 3 = tail longer than 2× the diameter of the nucleus) based on perceived comet tail length migration and relative proportion of DNA in the nucleus [25].

Statistical Analysis

Data of biochemical analysis were expressed as mean± SD of 10 rats in each group. Statistical analysis was carried out using SPSS compute program coupled with Co-State computer program, where unshared letters were significant at $P< 0.05$. Data of ROS, gene expression and comet assays were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System followed by Scheffé-test to assess significant differences between groups. The software was used is SAS, Version 9.1 (Stat soft Inc., Tulsa, USA). The values were expressed as mean±SEM. All statements of significant were based on probability of $P< 0.05$.

Investigation of Fatty Acids Constituents

Microalgal paste was mixed with chloroform: methanol: deionized water (1:2:0.8, v/v/v) and then homogenized [11]. One part chloroform was added and the mixture was further homogenized. Then, one part deionized water was added to the homogenate giving a final ratio of 2:2:1.8 v/v/v. The mixture was re-homogenized and finally filtered to remove cell debris. The solvents were evaporated to dryness under reduced pressure at 40 °C.

Saponification of Extract

The residue was subjected to saponification by refluxing with 5% alcoholic KOH in a water bath for 2 hr [26]. After cooling, 50 mL of water was added, and the solution was extracted with ether. The organic phase was washed with water until it became alkali free and was then dried over anhydrous sodium sulphate.

Preparation of Saponifiable Matter (Fatty Acids)

The alkaline aqueous solution (soap) remained after removal of the unsaponifiable matter was rendered acidic (pH=2) with sulphuric acid (10%). The liberated free fatty acids were then exhaustively extracted with successive small portions of diethyl ether. The combined ether extracts were washed with distilled water, till the wash was neutral to litmus paper. The ether was distilled off and the residue of total fatty acids was dried over anhydrous calcium chloride overnight. It was semisolid at room temperature and brown in color represent the fatty acid fractions [26].

Preparation of Fatty Acid Methyl Esters

The fatty acids were converted to their methyl esters by refluxing with absolute methanol (50 mL) and sulphuric acid (1.5 mL) for two hrs [26]. The major part of alcohol was distilled off and the residue was diluted with distilled water and then exhaustively extracted with several portions of ether. The combined ether extracts were washed with distilled water, till the wash was free from any acidity. The solvent was distilled off and the residue was dried over anhydrous calcium chloride overnight and then kept for further investigation.

GLC of Fatty Acid Methyl Esters

The column used was a capillary column (50 m x 0.25 mm) and packed with DB-5 (5% phenyl, 95% methyl polysiloxane). The injected volume was 1 µL. The analysis was carried out at a programmed temperature. The initial temperature was 150 °C then increased at a rate of 5 °C/min and final temperature 280°C (kept for five min). The detector temperature was 280 °C. The total run time was 38 min. Identification of the fatty acid methyl esters was done by comparing of their retention times and pattern of fragmentation with those of reference compounds analyzed under the same conditions.

Results

Effect of *D. salina* on Serum GIP in Obese Rats

Table 3 demonstrated no significant differences between GIP level in control rats and normal rats-treated with *D. salina* extract. GIP level recorded significant increase (57.93%) in rats fed HFD induced obesity compared to control group. Obese rats treated with the extract showed higher marked improvement reached to 41.19 % than standard drug (31.80%).

Table 3: Effect of *D. salina* (6 weeks of treatment post induction with HFD) on serum GIP level in obese rats

Marker	Treated groups				
	Control /ND	Control /ND /DS	HFD	HFD/DS	HFD/OR
GIP (ng/L)	689.00±20.20[a]	645.70±30.25[a]	1088.12±33.00[b]	804.32±29.90[c]	869.00±24.78[c]
% change	-	-6.28	+57.93	+16.74	+26.12
% improvement	-	-	-	41.19	31.80

ND: normal diet, ND/DS: Rats feed normal diet and treated orally with *D. salina* extract. HFD/DS: Rats feed with HFD and treated orally with *D. salina* post induction. FHD/OR: Rats feed HFD and treated orally with standard drug Orlistat. Statistical analysis is carried out using SPSS computer program, combined with co-state computer program, where unshared letter is significant at $P \leq 0.05$.

Effect of *D. salina* Extract on ROS Generation

Fig.1 showed the effect of *D. salina* extract against obese rats induced by feeding on HFD induced probably intracellular ROS generation in liver tissues of male rats.

The current observations found that HFD increased significantly the intracellular ROS generationin comparison to control rats. However, only low rates of ROS generation were found in rats treated with *D. salina* extract which they were relatively similar to those in control rats.

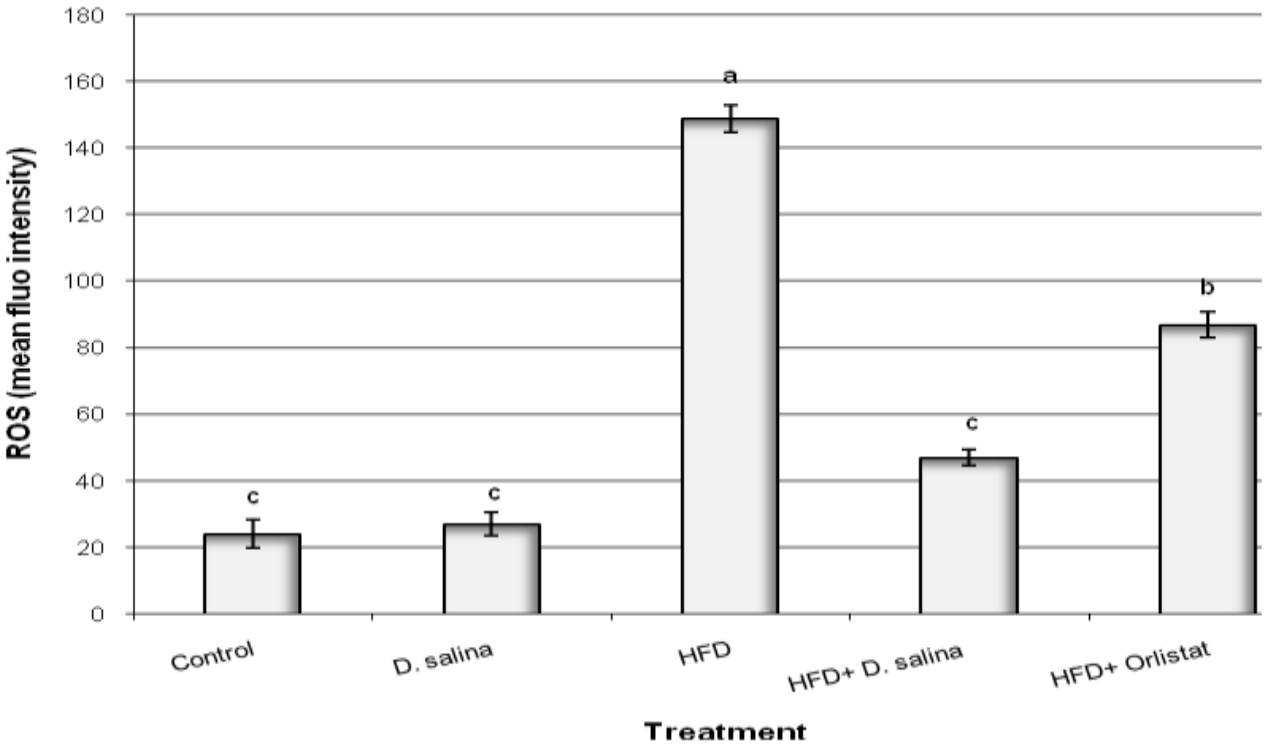

Figure 1: The intracellular ROS level alterations in liver tissues of male rats fed HFD and treated with *D. Salina* extract. Data are presented as mean ± SEM. [a,b,c] Mean values within tissue with unlike superscript letters were significantly different (P<0.05)

The extract decreased significantly the ROS generation rates induced by HFD treatment, where the rates of ROS generation in the group of rats fed HFD and treated with *D. salina* extract were 47% compared with 149% in group of rats fed only HFD (Fig. 1). Additionally, the inhibition effect of microalgal extract against ROS generation was more effective compared with orlistat drug, where the rates of ROS generation in the group of rats fed HFD and treated with *D. salina* extract were 47% compared with 87% in rats fed HFD plus orlistat drug.

Effect of *D. salina* Extract on the Gene Expression Alterations

Figs. 2-4 showed the expression levels of lipid catabolic genes (*PPAR*-α and *CPT*-1) and lipid anabolic gene (*SCD*-1) in liver tissues of male rats fed HFD alone or treated with *D. Salina* extract.

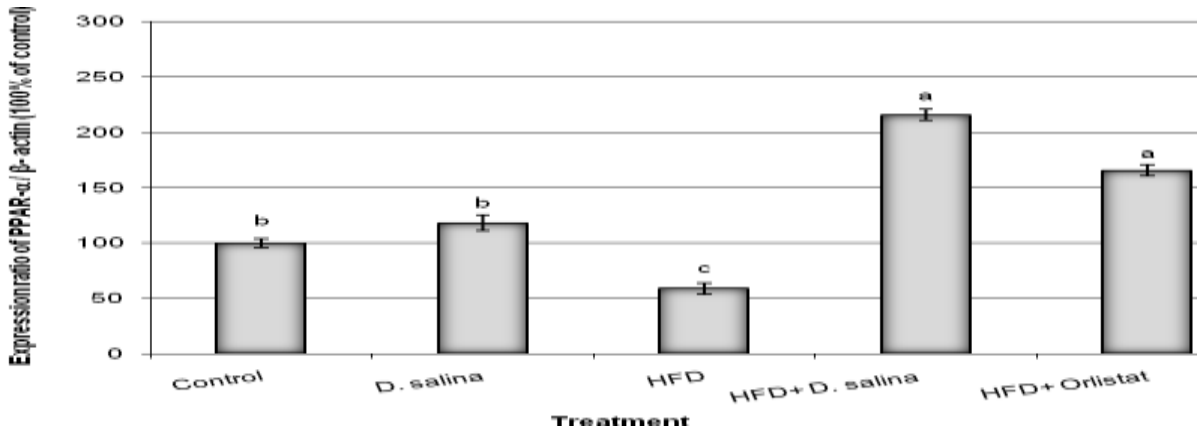

Figure 2: The alterations of *PPAR*-α gene in liver tissues collected from male rats fed high fat diet and treated with *D. Salina* extract. Data are presented as mean ± SEM. [a,b,c:] Mean values within tissue with unlike superscript letters were significantly different ([a:] *P*<0.01, [b, c,d:] *P*<0.05)

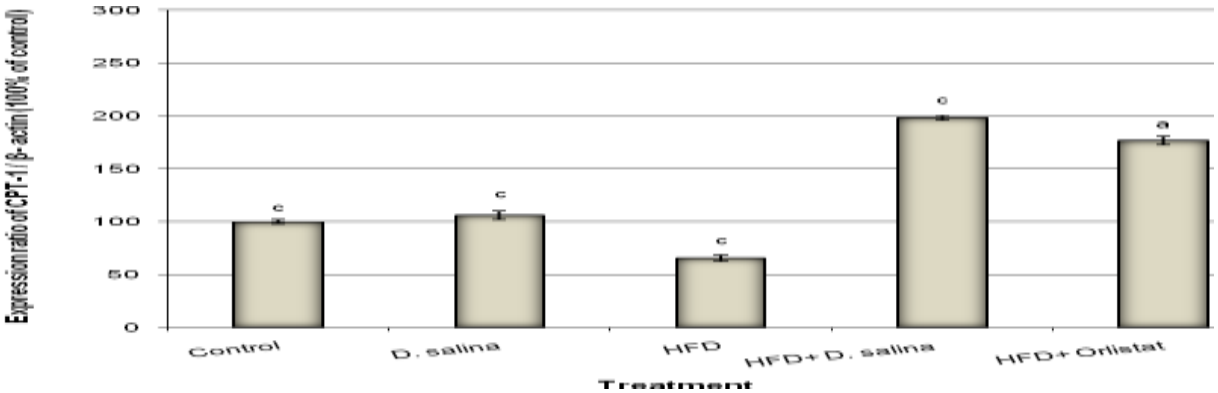

Figure 3: The alterations of CPT-1 gene in liver tissues collected from male rats fed HFD and treated with *D. Salina* extract. Data are presented as mean ± SEM. [a,b,c:] Mean values within tissue with unlike superscript letters were significantly different ([a:] *P*<0.01, [b, c:] *P*<0.05)

The obesity-induced rats which fed HFD were decreased significantly (*P*<0.01) the mRNA expression values of *PPAR*-α and CPT-1, respectively, while increased significantly (*P*<0.01) the expression levels of *SCD*-1 gene compared with control rats (Figs. 2-4). On the other hand, the expression levels of lipid catabolic genes and lipid anabolic gene in liver tissues of male rats treated with *D. salina* extract were exhibited relatively similar expression values in comparison to control rats (Figs. 2-4).

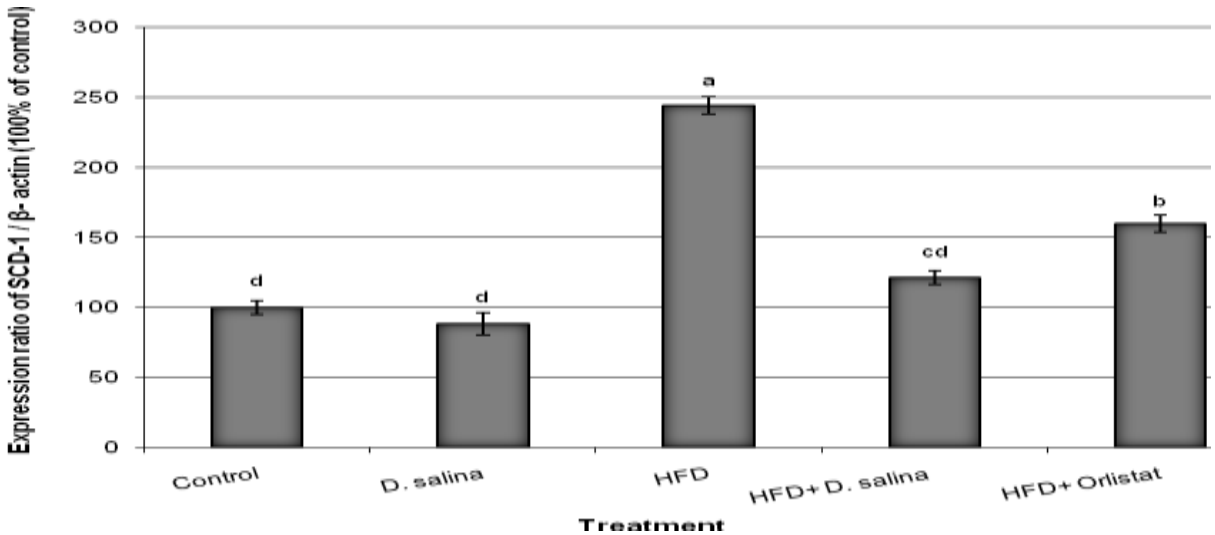

Figure 4: The alterations of *SCD*-1 gene in liver tissues collected from male rats fed HFD and treated with *D. Salina* extract. Data are presented as mean ± SEM. [a,b,c:] Mean values within tissue with unlike superscript letters were significantly different ([a:] *P*<0.01, [b,c:] *P*<0.05)

These results found that obesity-induced rats which fed HFD and treated with *D. salina* extract increased significantly (P<0.05) the mRNA expression values of *PPAR*-α and *CPT*-1, respectively, while decreased significantly (P<0.05) the expression levels of *SCD*-1 gene compared with obesity induced rats (Figs. 2-4).

Moreover, the effect of *D. salina* extract against gene expression alterations in obesity- induced rats was more effective compared with the reference drug namely orlistat.

Effect of *D. salina* Extract on the DNA Damage Assessed by Comet Assay

Table 4 shows the assessment of the DNA damage in liver tissues of male rats collected from different treated groups with HFD and/or *D. salina* extract, respectively.

The rates of DNA damage in the *D. salina* extract-treated groups of rats were relatively similar to the control group. However, the levels of DNA damage in obesity-induced rats were increased significantly (P <0.01) compared to those in control group (Table 4).

Table 4: Rate of DNA damage in liver tissues of male rats fed HFD and treated with *D. salina* extract using comet assay.

Treatment	Number of animals	No. of cells		Class¥ of comet				DNA damaged cells (%)
		Analyzed (*)	Total Comets	0	1	2	3	
Control	5	500	26	474	19	7	0	5.2±0.23^{c}
D. salina	5	500	27	473	18	9	0	5.4±0.16 ^{c}
HFD	5	500	92	408	33	38	21	18.4±2.14^{a}
HFD+ *D. salina*	5	500	41	459	16	18	7	8.2±0.31^{bc}
HFD+ Orlistat	5	500	53	447	21	19	13	10.6±0.26^{b}

¥: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus. (*): No of cells analyzed were 100 per an animal. $^{a,b,c:}$ Mean values within tissue with unlike superscript letters were significantly different ($^{a:}$ P<0.01, $^{b,c:}$ P<0.05)

The DNA damage was decreased significantly (P <0.05) in group treated with *D. salina* extract combined with HFD. Moreover, the decline in DNA damage induced by *D. salina* extract in obesity-induced rats was relatively similar to that induced by orlistat drug.

Fatty Acids Identification

The yield was 9.32% on dry weight basis for the lipids.

Fatty acids profile was determined using GLC analysis and the fatty acids identified in the algal biomass of *D. salina* are presented in Table 5. Fatty acid identification revealed the presence of palmitic (62.5%), linolenic (9%), oleic (8.6%) and linoleic (7.4%). Saturated and unsaturated fatty acids exhibited percentages of 67.7 and 32.1 %, respectively.

Table 5: Fatty acids identified in *D. salina* biomass

Fatty acids	Common name	Percentage (%)
C: 16:0	Palmitic acid	62.5
C: 16:1	Palmitoleic acid	7.1
Total, C:16		69.6
C: 18:0	Stearic acid	5.2
C: 18:1	Oleic acid	8.6
C: 18:2	Linoleic acid	7.4
C: 18:3	Linolenic acid	9.0
Total, C:18		30.2
Lipid profile		
Total, saturated fatty acids		67.7
Total, unsaturated fatty acids		32.1

Discussion

Obesity is a disease involving body weight gain. The current results demonstrated significant elevation in GIP level in obese rats induced by HFD [27, 28]. GIP is an obesity-promoting factor in HFD conditions [28]. The binding to the GIP receptor on adipocytes was directly caused the GIP to elevate the adipose tissue volume [29] and

indirectly, by enhancing deposition of fat and expansion of fat depots by elevating secretion of insulin from pancreatic ß-cells [29, 30].

The body weight and fat gain were exerted by altering the release of metabolism-regulating hormones to become more anabolic and the plasma GIP level in obese condition was increased [31, 33].GIP hypersecretion was reported to precede obesity and the HFD of healthy human subjects for 2 weeks increased the plasma GIP level [34, 35]. The gene expression profiling of lipid catabolic and lipid anabolic genes in obesity laboratory models was identified in many studies [31, 34].

This study was aimed to identify lipid metabolic gene expression in obese male rats and the therapeutic effect of microalgal extract *D. salina* in modulating the gene expression of the genes that are up-regulated during the period of obesity. Therefore, the role of *D. salina* extract on the expression levels of lipid catabolic genes (*PPAR*-α and *CPT*-1) and lipid anabolic gene (*SCD*-1) in liver tissues of obese rats fed HFD was investigated. The marine alga *Dunaliella* is wildly distributed in salt water and is known for its massive production of ß-carotene which is used commercially as food supplements in many countries [5].

Another bioactive metabolites in microlagae are the omega-3 (*ω*-3) and omega-6 (*ω*-6) polyunsaturated fatty acids which are essential type because both of them cannot be synthesized by the body itself and need to be uptaken through diets.

The important forms of omega-3 fatty acids are linolenic (which are identified in *D. salina*), eicosapentaenoic and docosahexaenoic acids. Likewise, arachidonic acid ($C_{20:4}$) and linoleic acid ($C_{18:2}$) are the most useful form of omega-6 fatty acids. The respective health effects of dietary intake of these fatty acids may differ due to genetic differences in metabolism. According to nutrition experts, a diet composed of omega-6/omega-3 in a ratio of less than 5: 1 is highly recommended [34]. Several studies demonstrated the beneficial roles of omega-3 fatty acids on animals and humans have been carried out [34-36].

These studies suggested that omega-3 fatty acids are beneficial against cancer and obesity.

Particularly, omega-3 fatty acids are stored as triglycerides in mature adipocytes (differ from other fatty acids) and improve serum lipid level. Omega-3 fatty acids can suppress adipocyte hypertrophy and reduce the lipid substances of adipose tissue [37, 38]. Our present investigated green microalga has gained much attention as potential source of bioactive compounds, including some type of polyunsaturated fatty acids.

Although the main commercial resource of omega-3 fatty acids is marine fish oil, in this study we have used marine microalga, *Dunaliella* as an alternate resource of omega-3 fatty acids. Chitranjali et al. [39] reported that treatment of peripheral blood mononuclear cells with omega-3 fatty acids significant down-regulated of anti-inflammatory related genes (IL-6, TNF-α and COX-2) as well as metalloproteinase matrix genes (MMP-2 and MMP-9).

The present study found that treatment of obesity with *D. salina* extract increased significantly the expression levels of mRNA of lipid catabolic genes (*CPT*-1 and *PPAR*-α) and the expression levels of lipid anabolic gene (*SCD*-1) were decreased significantly compared with obesity induced rats.

In agreement with our findings, Yook et al. [21] reported that dietary omega-3 fatty acids have been found to be as regulators for anabolic lipid genes suppression and promoters for catabolic lipid genes. In addition, the present study found that *D. salina* extract was able to decrease ROS generation and inhibit rate of DNA damage in obese rats.

These findings run in same line with several studies suggested that an extract that containing high omega-3 fatty acids and carotenoids content is considered as an important scavenger against generation of several free radicals including ROS [39, 41].

Microalgae such as many species of unicellular green alga *Dunaliella* sp. might be of great value in the production of various types of bioactive compounds such as n-6 /n-3polyunsaturated fatty acids and carotenoids. Thus, the protective role of the current investigated alga extract of *D. salina* could be attributed to the omega-3 fatty acids and 9-*cis*-β-carotene content which have been found to inhibit formation of micronucleus in human lymphocytes [39, 42].

Zeaxanthin and lutein are carotenoids present in a variety of green microalgae belonging to the Chlorophyta (known as chlorophytes) and they were reported with antioxidant property and prevent liver fibrosis [9, 40]. Carotenoids are effective antioxidants that can quench singlet oxygen (O_2), suppress lipid peroxidation, and prevent oxidative damage.

Many investigations have also demonstrated that various carotenoids, including lutein, astaxanthin, astaxanthin esters, zeaxanthin, all-*trans* and 9-*cis* β-carotene, and α-carotene, have good hepatoprotective, anti-inflammatory and antioxidant activities [9,10,40]. In the current study, GLC analysis revealed the presence of unique omega-3 fatty acids. These compounds were reported with potential beneficial effect for the treatment of non-alcoholic fatty liver and have been found to trigger *PPAR*-α gene [43], which is considered as a member of super-family of steroid nuclear receptors and acts as important role in lipid homeostasis regulation [4].

Furthermore, other important role of *PPAR*-αgene in lipid metabolism, which it activates genes encoding proteins coincided in lipid oxidation and transport such as ACO and CPT-1 [44-46]. Peroxiredoxin is a ubiquitously expressed protein in eukaryotic cells and is highly evolutionarily conserved from *Dunaliella*. Peroxiredoxin can protect RNA from degradation by RNase [47]. The gene expression profile of adipose tissue has been studied previously in various rodent models of established obesity [6, 7].

SCD-1 is gene encoding alipogenic enzyme coincided in the biosynthesis of triglyceride and cholesterol in liver tissues [48-50]. Where, up-regulation of hepatic *SCD*-1 enhances lipogenesis and lipid formation in liver tissues.

In the same line, the present findings revealed that mRNA expression levels of *PPAR*-α and *CPT*-1 were significantly increased and the expression of *SCD*-1 was significantly decreased with omega-3 fatty acids intake directly from foods and/or dietary supplements.

These results suggested that *D. salina* extract has hypolipidemic and hypocholestrolemic characteristics. Thus, regular administration of *D. salina* extract in obese rats significantly over expressed the mRNA levels of lipid catabolism related genes and down regulation of mRNA of lipid anabolic genes.

Conclusions

It could be conclude that both *D. salina* and orlistat can modify high fat diet-induced obesity. It seems that *D. salina* and orlistat are reversible lipase inhibitors that act by inhibiting the absorption of dietary fats. *D. salina* extract administration which is rich in omega-3 fatty acids has anti-obesity properties. On the other hand, further studies on human should be carried out to investigate its utilization as a substitute to other resources rich in omega-3 fatty acids such as fish oil for the function of controlling obesity.

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