Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 786–792

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Dunaliella salina extract effect on diabetic rats: Metabolic fingerprinting and target metabolite analysis

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ARTICLE INFO

Article history:
Received 17 October 2008
Received in revised form
23 December 2008
Accepted 23 December 2008
Available online 21 January 2009

Keywords:
Rat
Oxidative stress
Diabetes
Streptozotocin
Dunaliella salina
Metabolomics
Metabonomics

ABSTRACT

Antioxidant therapy has been proposed to improve the oxidative stress status of diabetic patients. Natural products are a source of substances such as carotenoids, with known antioxidant properties with possible benefits on diabetes. Among them, *Dunaliella salina* is a microalga with high content in carotenoids that can be extracted via an environmentally clean process such as supercritical fluid extraction with CO_2.

Five doses of *D. salina* extract with *in vitro* antioxidant properties were intragastrically administrated to adult male streptozotocin (STZ) diabetic rats. Urine fingerprints of control and diabetic rats, both with and without treatment, were obtained by capillary electrophoresis with two different modes (normal polarity and MEKC and reverse polarity and CZE). When the profiles were submitted together to pattern recognition techniques they showed the effects of *D. salina* extract on this acute and short-term treatment animal model in a rapid, simple and cost-effective way without identifying a single marker. In order to have a further biochemical knowledge of the effect, after treatment, rats were sacrificed and blood and liver glutathione, as well as plasma glucose, triglycerides, cholesterol, total protein, urea, acetoacetate, 3-hydroxybutyrate, lactate, pyruvate and urate, TBARS and urine 8-isoprostane were analysed. Vitamin E in plasma and liver was also measured. Twenty-seven parameters were individually assessed, and both univariate statistics (mean comparison after 1W-ANOVA) and multivariate data analysis were performed. *D. salina* extract induced changes showed up by the multivariate analysis. Results of the treatment from most of the parameters can be considered beneficial for diabetic animals; although an increase in hyperglycemia and 8-isoprostane excretion when STZ treated animals received the extract was observed.
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1. Introduction

Type I Diabetes is a physiopathological situation where oxidative stress is involved and can be used experimentally to evaluate the responsiveness to an antioxidant treatment [1]. In particular, the rat treated with STZ has been compared with other animal models of oxidative stress, and has recently been proposed as the most appropriate model of systemic oxidative stress for studying antioxidant therapies [2]. These therapies have demonstrated to be able to induce changes in a broad variety of experimental situations, either when treatment was administrated along more than 10 weeks [3] or just for 3 days, 2 weeks after STZ injection [4].

Traditional medicine treatments for diabetes have shown ability to decrease oxidative stress, but mainly due to a decrease in the hyperglycemia [5]. In the case of an antioxidant therapy for oxidative stress caused by diabetes, very few natural products have been found to reduce oxidative stress without reducing hyperglycemia in different studies carried out with STZ diabetic rats (*Viburnum dilatatum* [6], *Camellia sinensis* [7], *Salvia miltiorrhiza* [8] or *Citrus unshiu* [3]).

This aspect may be important if this substances are proposed as antioxidants to improve the situation in many other pathologies in which oxidative stress is involved. *Dunaliella salina* is a micro-alga from the Chlorophyceae family. *D. salina* can proliferate over a large range of salinities for massive carotenoid accumulation (400 mg/m^2/day) which makes cultivation easy and economical

Abbreviations: ANOVA, analysis of variance; BGE, background electrolyte; CE, capillary electrophoresis; EDTA, ethylene diamino tetra acetic; GSH, reduced glutathione; GSSG, oxidized glutathione; HFIP, hexafluoroisopropanol; LV, latent variable; PCA, principal component analysis; PLS-DA, partial least squares (or latent structures) discriminant analysis; STZ, streptozotocin; SDS, sodium dodecyl sulphate; TBARS, thiobarbituric acid reacting substances.

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doi:10.1016/j.jpba.2008.12.041

[9–12]. Its complex composition provides stronger antioxidant activity than the synthetic β-carotenes [13–16].

Functional foods are defined as 'foods that in addition to the nutritional and energetic requirements are able to provide a beneficial physiological action for human health' [17]. Extraction and purification using environmentally clean processes of carotenoids and other antioxidants as ingredients of functional foods has been successfully applied to microalgae [15,16,18].

Antioxidant therapy effectiveness must be demonstrated throughout the evaluation of several parameters [19], including markers of oxidative degradation such as TBARS or isoprostanes, and antioxidant defence such as glutathione, vitamin E and antioxidant capacity. In our group, after selecting a group of parameters we have been able to detect changes associated to the therapy after only five doses of antioxidants along three days, starting 11 days after STZ administration [4].

In this type of studies, statistical analysis is usually performed by one- or multiple ways analysis of variance (ANOVAs). Nowadays, it is clear that multivariate analysis offers more possibilities to simultaneously evaluate all the data from all the parameters of all the individuals.

Such an approach is commonly employed in metabolomics investigations. One of the strategies employed by the emergent science of metabolomics is metabolic fingerprinting [20]; which involves rapid, high-throughput global analysis to discriminate between samples of different biological status or origin by pattern recognition.

In a previous paper [21], working with a model mixture of antioxidants (vitamins C and E), capillary electrophoresis (CE) urine fingerprints of control and diabetic rats have shown a clear effect of an antioxidant treatment on diabetic animals not seen in controls in a rapid, simple and cost-effective way.

Hence, the present study was designed to investigate the potential nutraceutical antioxidative *in vivo* properties of a *D. salina* extract in STZ diabetic rats *vs* their controls by combining metabolic fingerprinting and target metabolite analysis and using univariate and multivariate statistical tools.

2. Materials and methods

2.1. Chemicals

Microalgae extract were processed by pressurized liquid extraction (PLE) as described by Herrero et al. [15]. Briefly, in 11 mL extraction cells 2.0 g of sample were placed, and the cell was filled with solvent to a pressure of 1500 psi. After heating-up and static extraction the cell was rinsed and purged with N_2. Different solvents and conditions were tested, and the extract which provided the highest antioxidant activity (ethanol, 160 °C, 17.5 min) was employed for the *in vivo* assay.

STZ, triolein, Tween-80, N-ethylmaleimide, sulphated β-cyclodextrin, sodium dodecyl sulphate (SDS), 2-thiobarbituric and standards of α-tocopherol (all-rac) (vitamin E), GSH, GSSG, acetoacetic, 3-hydroxybutyric, pyruvic, lactic and uric acids were obtained from Sigma (St. Louis, MO, USA) as well as castor oil. Phenyldodecane, H_3BO_3, H_3PO_4 85%, $HClO_4$ 70%, sodium tetraborate decahydrate, methanol and acetonitrile (HPLC grade) were from Merck (Darmstadt, Germany) and hexafluoroisopropanol (HFIP) from Fluka (Buchs SG, Switzerland). HCl 37%, NaCl, NaOH, EDTA-Na_2, trichloroacetic and citric acids from Panreac (Montcada i Reixac, Spain).

All the buffer solutions were prepared with water purified by a Milli-Qplus185 (Millipore, Billerica, MA, USA), and saline solution contained 0.9 g of NaCl per 100 mL H_2O. Tocol was kindly supplied by Roche (Basel, Switzerland), and 1,1,3,3-tetrametoxypropane (TMP) was purchased from Aldrich (Steinhein, Germany).

2.2. Animals and samples

Sprague–Dawley male rats, 12 ± 2 weeks of age, from the animal quarters of University San Pablo-CEU were used. Throughout the experiments the animals were kept in collective cages (less than 7 animals per cage) and controlled conditions (22 ± 2 °C and 55 ± 10% relative humidity). Animals had free access to tap water and diet (Harlan Global Diet 2014, Harlan Interfauna Ibérica, Madrid, Spain).

Rats were distributed in 4 experimental groups: animals that received an intraperitoneal dose (50 mg/kg, dissolved in 50 mM citrate, pH 4.5) of STZ and showed blood glucose levels over 200 mg/dL 4 days after treatment (diabetics, D) were sacrificed at 14 days after the STZ administration. Sex and age-matched rats that did not receive STZ were studied in parallel (controls, C). At 72, 64, 48, 40 and 24 h before sacrifice, rats of both the D and C groups received by gavage either extract of *D. salina* (150 mg) dispersed in 1 mL vehicle (200 mg castor oil and 20 mg Tween in 1 mL saline solution) or 1 mL vehicle. Animals were placed in individual metabolic cages during the last 24 h for urine collection. Tubes with 3 M HCl (50 μL for control samples and 100 μL for diabetic samples) were kept for 12 h at room temperature and pooled with the second 12 h fraction. Finally urine samples were separated in aliquots in small tubes and frozen at −80 °C until the analysis by CE.

Animals were sacrificed under anaesthesia with ketamine (75–100 mg/kg)/azepromacine (2.5 mg/ml) by cardiac puncture and blood was collected in EDTA-Na_2. Brain, liver and lumbar adipose tissue were excised, and immediately frozen in liquid nitrogen, weighed and stored at −80 °C. Blood was rapidly centrifuged to separate plasma that was stored at −20 °C until analysed. For glutathione determination, a blood aliquot was directly collected in diluted perchloric acid.

The experiments were approved by the Ethical Committee of the University San Pablo-CEU and they are in agreement with Amsterdam Treaty and Spanish legislation (RD 223/1988).

2.3. Urine fingerprinting by CE

Samples were treated and analysed as previously described [22]. Briefly, two different and complementary modes CE were employed. CE experiments were carried out on a P/ACE MDQ system (Beckman System, Palo Alto, CA, USA) equipped with diode array UV-absorbance detection (190–600 nm), a temperature-controlled (liquid cooled) capillary compartment and an autosampler. Electrophoretic data were acquired and analysed with 32 Karat software (P/ACE MDQ instrument).

CD-MEKC separations achieved with normal polarity (using background electrolyte BGE(1)) were performed in a 60 cm (total length), 50 cm (effective length) × 75 μm (internal diameter) fused silica capillary (Composite Metal Services, Hallow, Worcester, UK). BGE(1) comprised of 25 mM sodium tetraborate decahydrate, 75 mM SDS and 6.25 mM sulphated β-cyclodextrin. The pH was adjusted to pH 9.50 (apparent) with 2 M NaOH (after addition of SDS and cyclodextrin) with 2.25% v/v HFIP. The separation capillaries were maintained at 20 °C, with a 20 kV applied voltage and 10 s hydrodynamic injection.

CZE separations achieved with reversed polarity were performed using a 60 cm (total length), 50 cm (effective length) × 50 μm (internal diameter) polyacrylamide coated capillary (Beckman Coulter, Palo Alto, CA, USA). All experiments were performed at 20 °C using a separation potential of −25 kV. Samples were injected at the cathode, with 0.5 psi (3447 Pa) pressure applied for 20 s. Resolved sample components were detected at the anode. BGE(2) was prepared with 0.2 M phosphoric acid, adjusted to pH 6.10 with NaOH, and 10% (v/v) methanol was then added. The detection wavelength was 200 nm in both modes.

2.4. Target metabolite analysis

Plasma glucose, triglycerides, cholesterol and urea were analysed with commercial kits from Spinreact (Gerona, Spain). 8-Isoprostane in 24 h urine was measured with a commercial kit from Cayman Chemical (Ann Arbor, MI, USA), employing a Asys UVM340 plate reader (Asys Hitech GmbH, Eugendorf, Austria).

Thiobarbituric reacting substances (TBARS) were measured by specific fluorescence of the malondialdehyde-TBA adduct according to Viana et al. [23].

Capillary electrophoresis equipments (P/ACE 5010 and P/ACE 5500) from Beckman–Coulter (Palo Alto, CA, USA) were employed to determine glutathione as well as four short chain organic acids (acetoacetate, 3-hydroxybutyrate, lactate, and pyruvate) and urate. Glutathione (both oxidized and reduced) in liver and blood was measured according to Maeso et al. [24], and short chain organic acids and urate in plasma were determined as described elsewhere [25].

Plasma and liver α-tocopherol was determined by previously reported methods [26,27] with a HPLC instrument that consisted of a 125 Pump, a 507 autosampler, a 168 UV-DAD detector (all from Beckman–Coulter) and a Waters 470 fluorescence detector (Waters, Milford, MA, USA).

2.5. Data alignment and treatment

Baseline correction was performed with an in-house application developed in Matlab® 7.0 (Mathworks, Natick, MA, USA). Subsequently, profiles were multialigned with an in-house program developed in Matlab 7.0, using the correlation optimized warping (COW) method previously described [28]. The third step was the electropherogram normalisation followed by variable scaling, which was also performed with Matlab 7.0 application. Finally, multivariate data analysis (principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed with PLS Toolbox 4.0 (Matlab 7.0).

When working with target parameters, the multivariate data analysis selected was PLS-DA and it was done with PLS Toolbox 4.0 (Matlab 7.0). In addition, classical individual statistical analysis (one-way ANOVA and means comparison by Fisher's least significant difference) was performed with StatGraphics Centurion XVI (StatPoint Inc, Herndon, VA, USA).

3. Results and discussion

3.1. Metabolic fingerprinting of urine by CE

In a previous work [21], the capability of showing response in an acute model of non-treated STZ diabetes after a short period of diabetes (14 days) and a short treatment (5 doses) with a classic antioxidant mixture (vitamins C and E) was proved. The model was developed to be used for *in vivo* assessing the potential antioxidant properties of natural extracts, in a rapid way with less animal suffering, lower cost and lower consumption of extracts, as usually, at this point of the studies, extracts are obtained with laboratory equipment and the available amount is scanty.

The purpose of this study was to investigate the possible nutraceutical properties of a *D. salina* extract. This microalga was selected due to its high carotenoid content [11,29,30] and was submitted to an environmentally friendly extraction process with pressurized liquids [15]. The extract that showed the highest antioxidant activity *in vitro* was selected to evaluate its *in vivo* antioxidant properties in an animal model with elevated oxidative stress in which an antioxidant therapy showed effectiveness [4].

The first question was to know if there was any appreciable effect doing worthy a further biochemical study. Novel metabolic fingerprinting techniques aim rapid sample classification through high-throughput global analysis. For metabolic fingerprinting of urine, samples were consecutively measured with both CE methods (normal polarity and MEKC and reverse polarity and CZE), previously validated [22], and both profiles were added in order to have a broader picture. The set of profiles (Fig. 1) was baseline corrected, multialigned, normalized with creatinine and autoscaled, previous to multivariate analysis.

A supervised test (PLS-DA) was employed with the four groups (CV, CD, DV, and DD). Fig. 2 shows the corresponding score plot. The total variability explained by the model was 57%; the classification error was 0% in all cases and the cross-validation error was 9.6% in CV, 5% in DV, 20% in CD and 17% in DD, which makes the model quite reliable.

As could be expected, the first latent variable LV1 reveals a clear trend: looking at the complete score plot of the electrophoretic profile of the urine samples and without identifying any single peak, samples clearly separated into controls and diabetics. Moreover, controls clustered very close together, whilst diabetic animals were highly dispersed, thus showing a characteristic of diabetic non-insulin treated animals, that is, a lack of metabolic control. In addition, no outliers were found. More interestingly, latent variable LV2 clustered separately treated (upper part) and non-treated animals (lower part), showing a clear effect of the treatment, although not affecting at the diabetic status.

The main limitation of CE-UV is the biomarker identification and, till now, the compounds responsible for that classification are unknown, but once the effect of *D. salina* treatment was established a target metabolite analysis was performed to attempt a biochemical explanation of the activity. Results are summarized in Table 1 and will be discussed below.

Classical statistical analysis suffers from low number of individuals per group, and homogeneity of variances has to be assumed; moreover, non-parametrical tests need many samples for the median comparison gives real information. But in this type of *in vivo* work the number of animals must be kept at a minimum while several parameters have to be measured with the corresponding economic and personal cost.

Multivariate data analysis is particularly well suited to handle this type of data and for that reason PLS-DA (Fig. 3) was applied. One of the goals is to use data obtained from samples to build an appropriate model for classification and, if possible, determine the factors leading to differences among the samples by means of the corresponding loading plot (Fig. 4). The only previous data treatment in this case was autoscaling.

Variations along the LV1 axis in the loading plot (Fig. 4) are related to the pathology (diabetes/control), being values at right side increased in the diabetic groups, and at left side increased in the control groups. Along the LV2 axis, *Dunaliella* treatment separates variables; therefore, those variables increased when animals received the extract are in the upper part of the figure, whereas at the lower part are those variables which decreased.

Taking a first glance at the loading plot (Fig. 4), treatment could be positively evaluated, because most of the variables are located in quadrant II (13 variables) and IV (6 variables). Variables in which treatment counteracts diabetes changes are located in these quadrants; whereas quadrant I (3 variables) and quadrant III (5 variables) collect variables that correspond with parameters in which treatment enhances the effects of pathology.

Total weight and liver, brain and adipose tissue weights are parameters that multivariate analysis locates in quadrant III of the loading plot (Fig. 4, parameters #1, #2, #3 and #4), as well as relative adipose tissue weight (#6). Although as indicated in Table 1 there were not significant differences in liver weight of control

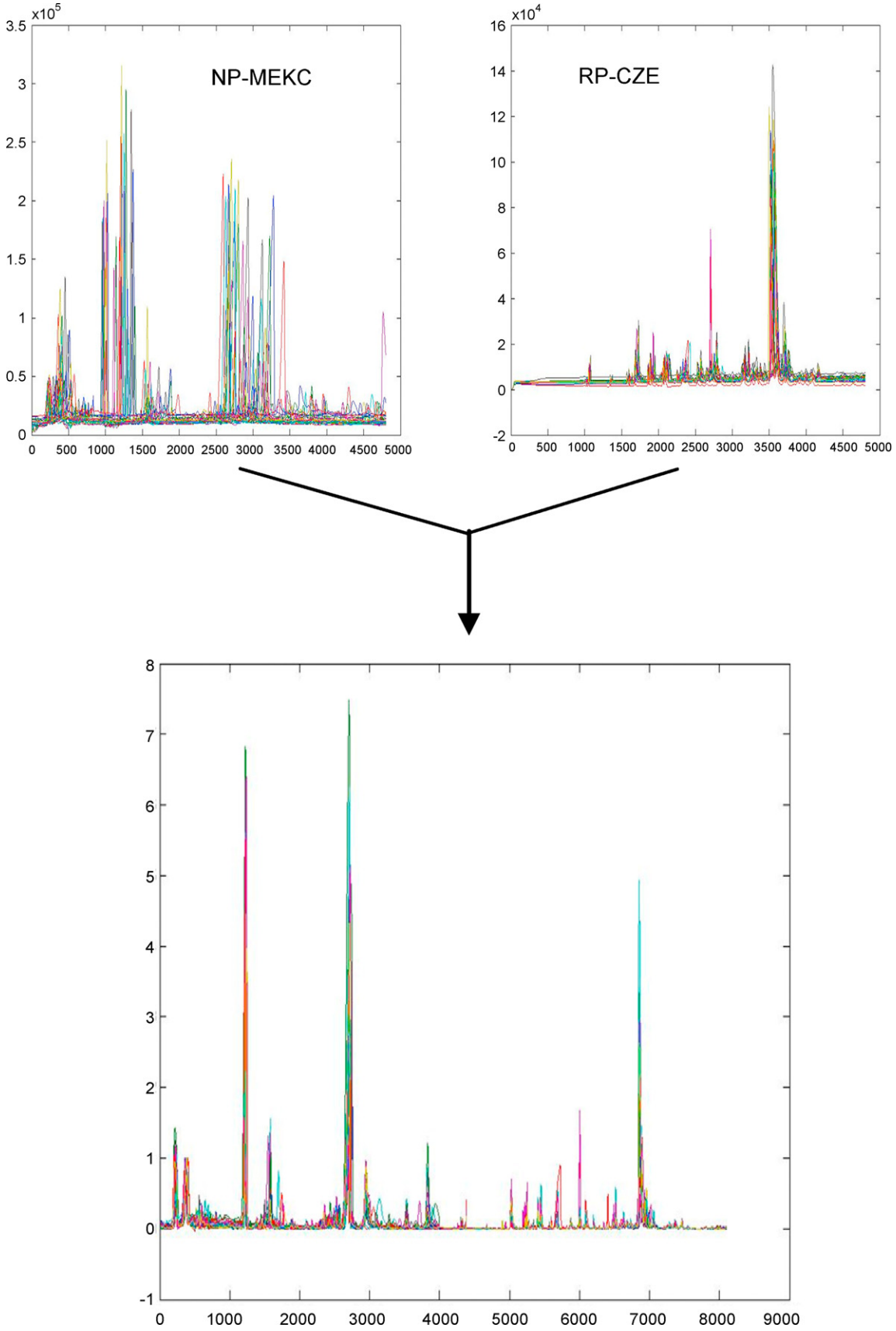

Fig. 1. 28 CE profiles, after addition of raw data from both CE methods (normal polarity and MEKC (NP-MEKC); reverse polarity and CZE (RP-CZE), baseline correction and multialignment.

Table 1
Physical, biochemical and oxidative stress parameters (mean ± standard error) in control and diabetic rats after 14 days of diabetes of control (C) and diabetic (D) rats that received either vehicle (V) or Dunaliella salina extract (D). Superscripts letters indicate homogeneity of means (homogeneous means share the same letter). Q: Quadrant in the loading plot (Fig. 4).

	Parameter	Unit (s)	Q	CV	DV	CD	DD
1	Weight	g	III	409.79 ± 14.56^{ab}	340.00 ± 14.52^{a}	449.58 ± 16.72^{b}	375.79 ± 7.02^{a}
2	Liver weight	g	III	13.96 ± 0.77	14.43 ± 1.43	14.63 ± 0.74	13.60 ± 0.43
3	Lumbar adipose tissue weight	g	III	2.87 ± 0.41^{b}	0.88 ± 0.50^{a}	3.57 ± 0.42^{b}	0.64 ± 0.08^{a}
4	Brain weight	g	III	2.06 ± 0.04	1.99 ± 0.04	2.06 ± 0.04	1.98 ± 0.03
5	Relative liver weight		II	3.40 ± 0.13%^{a}	3.96 ± 0.20%^{b}	3.25 ± 0.10%^{a}	3.62 ± 0.10%^{ab}
6	Relative LAT weight		III	0.69 ± 0.1%^{b}	0.20 ± 0.1%^{a}	0.80 ± 0.09%^{b}	0.17 ± 0.02%^{a}
7	Relative brain weight		II	0.50 ± 0.0%	0.58 ± 0.0%	0.46 ± 0.01%	0.53 ± 0.01%
8	Glucose plasma	mg/dL	II	197.3 ± 5.68^{a}	626.6 ± 73.59^{b}	185.4 ± 15.69^{a}	855.3 ± 63.30^{c}
9	Triglycerides plasma	mg/dL	II	60.8 ± 11.36^{a}	393.2 ± 94.29^{b}	44.7 ± 7.38^{a}	206.5 ± 93.69^{ab}
10	Cholesterol plasma	mg/dL	II	64.66 ± 9.62	73.10 ± 9.73	49.44 ± 3.82	66.59 ± 8.64
11	Proteins plasma	mg/dL	II	3.87 ± 0.12	4.38 ± 0.55	3.78 ± 0.12	4.66 ± ±0.63
12	Urea plasma	mg/dL	II	33.5 ± 1.32	48.2 ± 6.22	31.2 ± 2.08	45.5 ± 3.12
13	Lactate plasma	mM	II	1.90 ± 0.21	2.57 ± 0.47	2.26 ± 0.44	2.70 ± 0.30
14	Pyruvate plasma	μM	IV	20.14 ± 2.53	18.57 ± 1.97	25.20 ± 3.92	21.29 ± 3.52
15	Acetoacetate plasma	μM	II	103.22 ± 51.08	1136.88 ± 856.34	130.96 ± 30.73	1083.10 ± 629.43
16	3-Hydroxybutyrate plasma	mM	II	0.55 ± 0.11	2.23 ± 0.78	0.66 ± 0.21	1.88 ± 0.74
17	Urate plasma	μM	IV	32.15 ± 4.92	25.21 ± 12.22	56.11 ± 8.78	38.35 ± 12.59
18	TBARS plasma	μM MDA	II	1.35 ± 0.04^{a}	2.11 ± 0.14^{c}	1.39 ± 0.03^{ab}	1.69 ± 0.16^{b}
19	8-Isoprostane urine	ng/day	I	6.63 ± 1.69^{a}	14.06 ± 2.43^{ab}	7.74 ± 1.39^{a}	23.68 ± 6.83^{b}
20	Total antioxidant capacity plasma	μM trolox	IV	7.47 ± 0.81	5.42 ± 0.86	6.97 ± 0.53	8.45 ± 1.78
21	GSH + GSSG liver	μmol GS/g	IV	8.38 ± 0.44^{b}	6.41 ± 0.53^{a}	6.99 ± 0.23^{a}	7.55 ± 0.27^{ab}
22	GSSG/GSH liver		IV	1.84 ± 0.38%^{ab}	2.56 ± 0.37%^{b}	1.91 ± 0.28%^{ab}	1.15 ± 0.14%^{a}
23	GSH + GSSG blood	μM GS	I	836.57 ± 33.52	817.74 ± 20.29	682.13 ± 55.09	627.41 ± 71.29
24	GSSG/GSH blood		II	1.91 ± 0.22%	2.00 ± 0.76%	3.33 ± 1.33%	3.50 ± 1.01%
25	α-Tocopherol plasma	mg/dL	II	1.03 ± 0.10^{ab}	1.91 ± 0.45^{b}	0.83 ± 0.08^{a}	1.94 ± 0.50^{b}
26	α-Tocopherol liver	μg/g	II	20.44 ± 0.72^{ab}	39.80 ± 5.97^{c}	18.25 ± 4.21^{a}	32.62 ± 4.99^{bc}
27	α-Tocopherol/lipids plasma	μmol/mmol	IV	6.17 ± 0.93^{b}	2.48 ± 0.45^{a}	6.34 ± 0.50^{b}	3.74 ± 0.66^{a}

and diabetic rats that did not receive the extract, relative size of the liver was clearly increased in the diabetic group (due to the simultaneous reduction in body weight), and when *Dunaliella* was administered to these rats body weight slightly increased and liver size slightly decreased. Therefore, relative liver size significantly decreased and this parameter (#5) was located in quadrant II. Hepatomegaly is a classical symptom of the Mauriac syndrome, due to glycogen accumulation in liver of uncontrolled type I diabetic patients, and the best treatment for this complication is metabolic control with insulin [31]. However, it is noteworthy that with such a short treatment and without insulin this result was achieved.

Glucose concentration (parameter #8 in Table 1 and in Fig. 4) is not controlled by insulin in this model of STZ diabetic rats, but in diabetic rats that received *D. salina* extract glucose concentration was even higher. Glucose levels in plasma of these diabetic animals are highly variable, and differences found in *Dunaliella* treated group may be only related to the low number of samples. The effect of the extract in insulin-controlled diabetic animals has not been tested, and this particular aspect should be investigated more in detail if this extract should be proposed as nutraceutical ingredient. However, circulating lipids were lower when diabetic animals received the extract; although differences in cholesterol (parameter #6) were not significant, triglycerides (#5) that are higher in diabetic animals were lowered by the administration of the extract. PLE extraction procedure provided an extract with high proportion of 18:3ω3 [32], with known antihyperlipidemic properties that can be beneficial in the management of complications associated to diabetes [33].

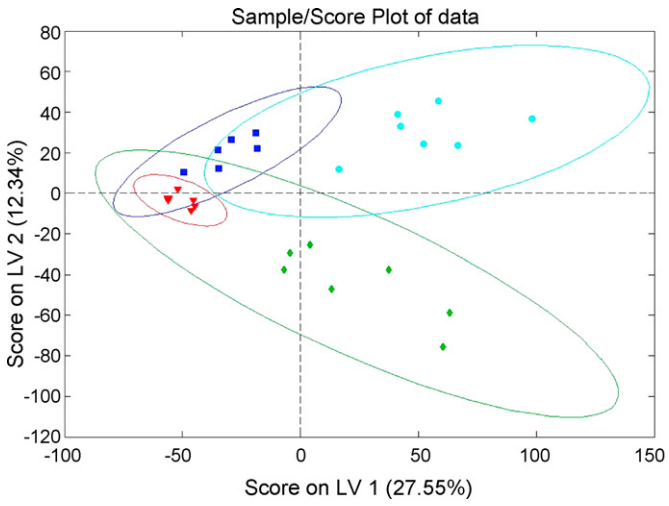

Fig. 2. PLS-DA scores plot for urine fingerprinting of control and diabetic non-treated rats (CV and DV) and control and diabetic rats after *Dunaliella* extract treatment (CD and DD).

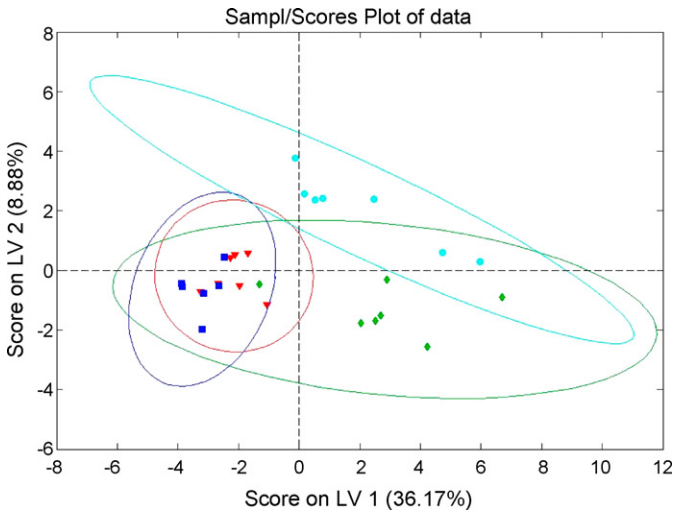

Fig. 3. PLS-DA scores plot for 27 target variables measured in the 4 experimental groups: control and diabetic non-treated rats (CV and DV) and control and diabetic rats after *Dunaliella* extract treatment (CD and DD).

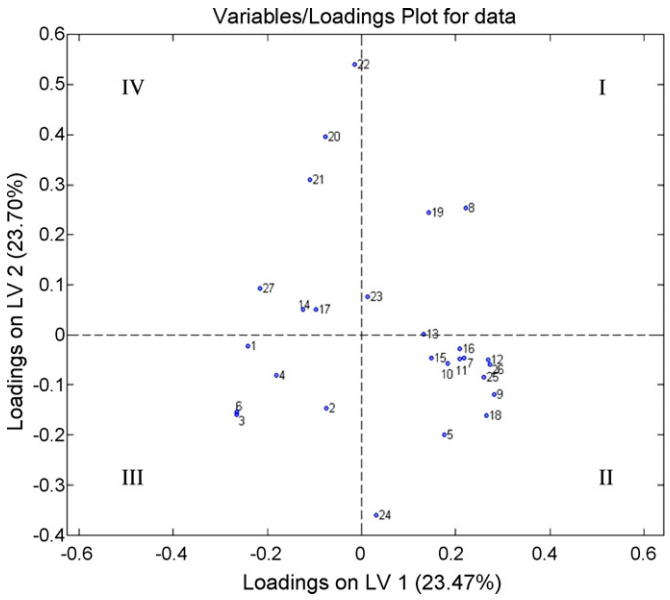

Fig. 4. Loading plot corresponding to the PLS-DA in Fig. 3.

The experiment was designed to evaluate the effect on oxidative stress, but our results indicate that some changes have been achieved in the intermediate metabolism, too, because plasma proteins (parameter #11 in Table 1 and Fig. 4) and urea (#12), as well as plasma lactate, pyruvate, acetoacetate and 3-hydroxybutyrate (#13, #14, #15 and #16, respectively) are variables located in quadrants II and IV, even though one-way ANOVA did not show significant differences. Nevertheless, it should be pointed that results are complementary and not contradictory, because none of these 6 parameters separated clearly along LV2 (the treatment associated axis).

Urate was found to be higher in control animals too, but again tendencies were not found to be significant. Uric acid is being considered one of the most abundant water-soluble antioxidants in humans, interacting with activated oxygen species and notably with the hydroxyl radical [34]. Here we have found that there was a non-significant tendency to increase plasma urate when rats received the extracts (Table 1), and this parameter (#17) was located in quadrant IV. For diabetic rats, this could be positive, because urate has been proposed as one component of the homeostatic response of the mitochondria to oxidant stress in rats [35,36].

The hereby described treatment with antioxidants reduced plasma TBARS levels (parameter #18 in Table 1 and Fig. 4, in quadrant IV), and increased (not significantly) total antioxidant capacity (parameter #20 in Table 1 and Fig. 4, in quadrant IV), similar to other antioxidant therapies [37]. Nevertheless, it must be pointed out that the antioxidant administration was performed only during three days, and that the last administration occurred 24 h before the sacrifice, whereas it was necessary 10 weeks of antioxidant treatment to observe a reduction in TBARS in other similar studies [38,39].

8-Isoprostane is a product of free radical attack to arachidonic acid [40] and can be measured as a reflection of its final degradation. In contrast with results for TBARS and Antioxidant Capacity, our findings showed an increase in total 8-isoprostane excreted in urine in 24 h in diabetic animals, that was higher when the extract was provided (Table 1). Moreover, this parameter (#19) is clearly located in quadrant I (Fig. 4), indicating a negative aspect of the administration. The amount of 8-isoprostane in 24 h urine of STZ-diabetic rats has been found higher [41,42], but antioxidant therapies decreased it [4]. As with hyperglicemia, this negative aspect must be taken into account for further studies. This increase when animals received *D. salina* may be related to the increase in the proportion in plasma of its precursor, arachidonic acid, observed in these rats in our laboratory (data not shown).

As shown in Table 1, diabetic animals that received the extract (DD group) increased total glutathione up to values comparable to those of CV group; in addition, GSSG/GSH mean of the DD group was the lowest observed among the four groups. According to these results, both of these parameters (#21 and #22) were located in quadrant IV, increasing evidences that oxidative stress was reduced in diabetic animals that received the extract.

In several studies with STZ-diabetic rats it has been found that reduced glutathione concentration is higher when they receive an antidiabetic treatment [3,43,44]. As GSSG determination is more challenging [24], results of the ratio between both forms has not been reported so often in this type of studies. The glutathione antioxidant system has been proposed to be the most important intracellular defence of an organism against free radicals, and is also known to be affected by the generalized increase in oxidative stress associated with diabetes [45]. Liver is the main contributor to plasma GSH [46], and there are many factors that may affect the synthesis in the liver and or its release. On the other hand, neither total glutathione nor glutathione ratio in blood showed significant differences among groups as shown in Table 1. Again, only trends can be observed that separate groups as a result of *Dunaliella* administration. These trends are clearly shown in the loading plot, and these parameters (#23 and #24) are clearly separated along LV2 axis (treatment), but quite close to LV1 axis origin (pathology). Differences between the results of glutathione in blood and liver stress the necessity of being cautious when these metabolites are taken into account to extrapolate conclusions about the effectiveness of a treatment.

Treatments with different extracts of algae such as *Scoparia dulcis* [47] or *Spirulina maxima* [48] have shown modulatory effects on vitamin E concentration in plasma and liver. Nevertheless, its concentration in plasma and liver is strongly related with the levels of circulating lipids [27] and for that reason, in diabetic rats there was higher concentration of α-tocopherol in plasma and liver (Table 1), but as *D. salina* extract induced a slight reduction in plasma triglycerides and cholesterol, enrichment in tocopherol of the lipoproteins occurred as the result of the treatment and this latter parameter (#27) was located in quadrant II, pointing that *D. salina* extract permits lipoproteins to be better protected against free radical damage.

In previous works, some of the compounds (9 fatty acids, 5 carotenoids and other 14 compounds) present in the extract were identified [15,32]. Among them, the main effects observed in the rats treated with extracts could be held by (i) carotenoids and vitamin E on one side, with known antioxidant properties *in vivo* and (ii) C18:18ω3 and C18:1ω9 on the other side, which have shown antihyperlipidemic properties. It must be taken into consideration, of course, that some of the identified compounds have not been reported to have any nutraceutical properties, but they cannot be ruled out. The full characterisation of the extract has not been fulfilled yet, and maybe new unidentified compounds can be responsible of these effects. Also (as with any nutraceutical) the final effect may be due to the synergistic action of the different substances.

4. Conclusions

In summary, STZ-diabetic rats respond to a moderate dose of orally administered antioxidants (750 mg *D. salina* extract) for a short period of time (3 days). This was successfully proved by pattern recognition techniques through urine fingerprinting by CE and by target metabolite analysis. Moreover, advanced statistical techniques have been successfully employed to achieve more in-depth knowledge of the results, because due to the inherent characteris-

tics of this type of studies (low amount of samples per group and high variability of data in diabetic animals), univariate analysis do not provide enough evidence about the changes.

From a practical point of view, the effect of supercritical *D. salina* extracts is not clear; whereas most of the parameters studied here showed beneficial effects, other key parameters showed the opposite change in diabetic animals as a result of the treatment. These aspects point out the need of further studies previous to the introduction of *D. salina* extracts as functional antioxidant ingredients.

Acknowledgements

The authors acknowledge MEC project AGL2005-06726-C04-03-01-02/ALI and Community of Madrid S-GEN-0247-2006 for economical support. Beatriz Baena thanks Comunidad de Madrid and Diana García-Martínez and María Vallejo to Ministry of Education and Science for their fellowships.

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