Oral administration of *Nitraria retusa* ethanolic extract enhances hepatic lipid metabolism in *db/db* mice model ‘BKS.Cg-Dock7^m+/+ *Lepr*^{db/J}’ through the modulation of lipogenesis-lipolysis balance

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Abstract

The medicinal plants can be used in the prevention or treatment of many diseases. Several studies concerning the potential of bioactive components in plants and food products and their link to obesity and related metabolic disorders, have been gaining big interest. Diabetes is a serious metabolic syndrome. Searching for alternative natural bioactive molecules is considered main strategy to manage diabetes through weight management. In the present study, an edible halophyte *Nitraria retusa* was selected and *in vivo* experiment was conducted using *db/db* model mice. We orally administrated its ethanol extract (NRE) to BKS.Cg-*Dock7*^m/+ *Lepr*^{db}/J mice model for a period of 4 weeks. The effect was evaluated on the body weight and adiposity changes and on the biochemical parameters of *db/db* NRE-treated mice. The molecular mechanism underlying the anti-obesity effect was investigated by testing the gene expression related to hepatic lipid metabolism. NRE was found to significantly suppress increases in body and fat mass weight, decreases triglycerides and LDL-cholesterol levels and enhances gene expression related to lipid homeostasis in liver showing anti-obesity actions. Our findings, indicate that NRE possesses potential anti-obesity effects in BKS.Cg-*Dock7*^m/+ *Lepr*^{db}/J model mice and may relieve obesity-related symptoms including hyperlipidemia through modulating the lipolysis-lipogenesis balance.

Keywords: *Nitraria retusa*, BKS.Cg-*Dock7*^m/+ *Lepr*^{db}/J mice, lipid metabolism, diabetes, liver.

Introduction

Prevention and treatment of obesity and obesity-related diseases are an important deal for health systems. The main target of many *in vivo* studies and clinical trials, is to reduce fat mass and overweight prevalence, and related health problems over the world (Ranjbar et al., 2013). Obesity and being overweight are problems of growing importance, being an essential risk factor for type-2 diabetes, when accompanied with other known complications. Therefore, it has been an important therapeutic goal to reduce the risk of type-2 diabetes through weight management (Kang and Oh., 2012). In order to improve our knowledge concerning the pathological mechanisms of human obesity disease, good animal models are very helpful. In this context,

61 several mice models of obesity are used in the research of obesity-related human health
62 symptoms and metabolic disorders mainly diabetes. *Db/db* mice model BKS.Cg-*Dock7^m*^{+/+}
63 *Lepr^{db}*/J are mice homozygous for the diabetes spontaneous mutation (*Lepr^{db}*) which become
64 obese at approximately three to four weeks of age. Homozygous mutant mice are polyphagic,
65 polydipsic, and polyuric (Lutz and Woods., 2012). At one month of age, *db/db* mice are larger
66 (obese) presenting increased fat deposition in the inguinal and axillary regions when compared to
67 control (heterozygous) littermates (Lee et al., 1996). Different methods have been used to reduce
68 body weight and its complications for many years. Herbal medicines have been in consideration
69 as a current alternative to reduce body weight gain and fat mass. For example, *Nigella sativa*,
70 *Camellia sinensis*, green tea, and black Chinese tea were found to possess effective anti-obesity
71 actions resulting in a potential management of other health disorders related to obesity, mainly
72 cardiovascular diseases and diabetes (Ranjbar et al., 2013). The mechanisms whereby bioactives
73 molecules affect body weight and body composition may include modifications in appetite
74 control, enhancement of hepatic lipid metabolism and decreased food intake (Rainsa et al., 2011).
75 The most common complementary and alternative medicine modalities are based in food
76 supplements and herbal therapies. In this regard, many crude extracts of plants and their isolated
77 bioactive compounds were shown to be effective in prevention and treatment of obesity by either
78 inducing weight loss or preventing weight gain (Ranjbar et al., 2013). Halophytes, salt-tolerant
79 species, are considered a potent source of various chemical secondary components with a big
80 economic interest (Ksouri et al., 2012). Compared to the glycophytic species, halophytes contain
81 much higher amounts of secondary metabolites or even restricted to halophytic species which
82 could be used in various application fields such as pharmacognosy and functional foods
83 (Boughalleb and Denden., 2011). The biological function of bioactive-compounds might be

single, or combined with others present in whole plant extracts mixtures leading to synergistic effect and depending on its chemical composition and efficacy (Buhmann and Papenbrock, 2013). Until present, several potential applications of halophytes are still in the research and development phase (Atia et al., 2011). The genus *Nitraria* is an edible halophyte shrub well adapted to arid climate. Species of *Nitraria* are found in desert as well as in salty regions. *Nitraria retusa* (Forssk.) is known in Tunisia as “Ghardaq”. Fruits of *Nitraria* species have been used as nutritional food and traditional herb for the treatment of hypertension and abnormal menstruation (Hadj Salem et al., 2011). The leaves serve as supplement for tea and are used as poultice. The ashes of this species have the ability to remove fluids of infected wounds. Fresh leaves of *Nitraria retusa* decoction is used in Morocco in case of poisoning, upset stomach, ulcers, gastritis, colitis and colonic abdominal pain (Boubaker et al., 2010).

Prevention and controlling weight gain effect of *Nitraria retusa* ethanolic extract (NRE) was investigated in our previous study using high fat diet-induced obesity experiment in C57B6J/L mice. Nonetheless, it is unknown whether NRE is effective in reducing weight gain in transgenic *db/db* mice with spontaneous diabetes, or in the enhancement of lipid metabolism in the liver and generally is effective in treating or ameliorating obesity in this monogenic model of mice. Our findings, which focus on both safety and efficacy of *in vivo* NRE oral administration, could be useful for further pharmaceutical or industrial applications to find a mixture of NRE components with higher efficacy of the anti-obesity agents.

2. Materials and Methods

2.1. Plant sampling

The halophytic species *Nitraria retusa* was selected in this study. Shoots collect was conducted in August 2010 from salt flat “Sabkha Elkelbia” located at N 35 48 44, E 10 09 06 of Kairouan. This

locality is characterized by a semi-arid climate with less rainfall < 200 mm/year and higher salinity mean (20g/l). The collected samples were rinsed with distilled water, kept in laboratory temperature; oven dried at 60°C and then ground finely using a ball mill type “Dangoumeau”. The plant powder obtained was stored at room temperature for different analysis.

2.2. The preparation of *Nitraria retusa* extract for animal experiment

Seventy percent ethanol extraction of *Nitraria retusa* powder sample was conducted with 10% (w/v). The ethanol extract was kept in dark at room temperature for 2 weeks, with shaking at least once a day. After centrifugation of the mixture, liquid fraction was then collected, filtered through 0.45µm filter (MILLIPORE, U.S.A), and concentrated using SpeedVac (SCRUM Inc., Japan). Finally it was dissolved in MilliQ water depending on the determined dose selected for oral administration.

2.3. Chemical analyses of the general composition of *Nitraria retusa* extract using reverse phase- high performance liquid chromatography (RP-HPLC)

During this experiment, the same extract of *Nitraria retusa* used previously was kept. Before injection into the HPLC system, extract was passed through a 0.45 µm nylon filter. The separation of selected phenolic compounds was carried out using HPLC system (consisting of a vacuum degasser, an autosampler, and a binary pump with a maximum pressure of 600 bar; Agilent 1260, Agilent technologies, Germany) equipped with a reversed phase C₁₈ analytical column of 4.6 x 100 mm and 3.5µm particle size (Zorbax Eclipse XDB C₁₈). Column temperature was maintained at 25°C. The injected sample volume was 2 µl and the flow-rate of mobile phase was 0.4 mL/min .Mobile phase B was milli-Q water consisted of 0.1% formic acid and mobile

phase A was acetonitrile. The optimized chromatographic condition was revealed as follows: 10% A, 90% B (0 min); 20% A, 80% B (5 min); 30% A, 70% B (10 min); 50% A, 50% B (15 min); 70% A, 30% B (20 min); 90% A, 10% B (25 min); 50% A, 50% B (30 min); 10% A, 90% B (35 min). UV absorption spectra were recorded online during the HPLC analysis. The DAD detector was set to a scanning range of 200-400 nm. Peak identification was obtained comparing the retention time and the UV spectra of *Nitraria retusa* phenolics chromatogram with those of available standards (Guyot et al., 1998). Quantification was performed by reporting the measured integration area in the calibration equation of the corresponding standard.

2. 4. Animals and experimental design

Five-week-old, male BKS.Cg-*Dock7^m*+/+ *Lepr^{db/J}* (*db/db*) mice and their litter mates (*db/+*) were purchased from Charles River and maintained under a light cycle (12h light/dark), fed with a diet contained 3.13 Kcal/g with 10.5% calories from fat, 25% from protein and 64.5 % from carbohydrates purchased from PMI Nutrition International . The animals were allowed 1 week to acclimatize themselves to the housing conditions before the beginning of the experiment. Then mice were randomly divided into 4 groups, 2 groups; *db/db* mice control and *db/db* mice NRE; were orally administrated with a daily dose of 0 and 50 mg/kg of body weight (BW) respectively. Their two litter mates groups; *db/+* (lean mice) control and *db/+* (lean mice) NRE were also orally administrated with the same doses as described above. Food consumption and weight gain were measured daily and weekly at regular times. At the end of experimental period (4 weeks), mice were sacrificed; organs were removed after collecting blood for analysis, rinsed with a physiological saline solution and immediately stored at -80°C. All procedures were performed in accordance with the Ethics Animal Care and Use Committee of the University of Tsukuba, Japan.

2.5. Measurements of body and white adipose tissue weights

Body weight was measured once a day during the feeding period. As much fat tissue as possible was collected from the visceral and white adipose tissue (WAT) including the retroperitoneal, perirenal, perigonadal and mesenteric adipose tissues. The total white adipose tissue mass was weighed, and then, stored until analysis for further experiments.

2.6. Measurements of glucose, triglyceride and cholesterol levels

After 4 weeks of treatment, blood samples were collected and centrifuged at 3,000 rpm for 15 min at 4°C, and serum glucose, triglyceride (TG), total cholesterol (TCHO), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels were measured according to the kit manufacturer's instruction.

2. 7. Isolation of total RNA from liver and real-time-PCR analysis

- Total RNA extraction:

Liver samples were prepared (50mg) and homogenized by Isogen solution using a Polytron homogenizer. Then total RNA was purified using the ISOGEN kit (Nippon GeneCo. Ltd., Japan) following the manufacturer's instructions.

- cDNA synthesis:

Total RNA was quantified using Thermo scientific Nanodrop 2000 (USA), and the reverse transcription reactions were performed using the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad/CA, USA) using 1 µg of total RNA. Briefly, RNA was denatured by incubation at 65°C for 5 min, with 1 µL oligo (dT) primers, and chilled at 4°C. Then SuperScript

III reverse transcriptase was added and the reaction mix was then incubated at 42°C for 60 min, then 10 min at 70°C (Han and Isoda, 2009).

- Real-time PCR:

The expression of Peroxisome proliferator-activated receptor gamma (PPAR- γ 1), Peroxisome proliferator-activated receptor alpha (PPAR- α), Lipoprotein lipase (LPL), Fatty acid synthase (FAS), Acetyl-CoA –Carboxylase 1 (ACC1), Sterol regulatory element-binding protein 1-c (SREBP-1-c) and Carnitine palmitoyl-transferase 1 (CPT-1); were determined by real-time PCR using Beta –actin as housekeeping gene. Primers and TaqMan probes used for these experiments were purchased from Applied Bio-systems. Primers were inventoried gene expression assays. TaqMan real-time PCR amplification reactions were performed in a 20 μ l reaction mixtures containing: 10 μ l of TaqMan Universal PCR Master Mix UNG (2X), 9 μ l of template c-DNA (100 ng μ l⁻¹) and 1 μ l of the corresponding primer/probe mix, using an AB 7500 fast real-time system (Applied Bio-systems). For the amplification, the following cycling conditions were applied: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95 °C / 1 min at 60°C.

3. Statistical analysis

All experiments were repeated at least three times. Data were expressed as the mean $\pm$ SD. Differences between control and treatments were assessed by Student's unpaired t-test. P values below 0.05, 0.01, and 0.001 were considered significant.

4. Results

4.1. Chemical analyses of the general composition of *Nitraria retusa* extract using reverse phase- high performance liquid chromatography (RP-HPLC)

Based on the HPLC analysis, the chromatographic profile of *Nitraria retusa* extract acquired at 254 nm was presented in **Fig.1**. Three main components were identified; the quercetin was eluted at retention time of 17.696 min, the isorhamnetin-3-O-glucoside was at 22.460 min, and the isorhamnetin-3-O-rutinoside was at 25.682 min. According to quantification measurements, the concentrations in sample ($\mu\text{g/ml}$) of quercetin, isorhamnetin-3-O-glucoside and isorhamnetin-3-O-rutinoside were: 22.34 $\mu\text{g/ml}$, 331.42 $\mu\text{g/ml}$, and 24.91 $\mu\text{g/ml}$, respectively (**Table.1**). The retention time of standards were presented in **Fig.2**.

Figure.1. Chromatographic profile of *Nitraria retusa* extract acquired at 254 nm.

Identified peaks are: 1; quercetin, 2; isorhamnetin-3-O-glucoside, 3; isorhamnetin -3-O-rutinoside.

Figure.2. Retention time and Chromatographic profile of standards acquired at 254 nm. Retention times of quercetin, isorhamnetin-3-O-glucoside and isorhamnetin-3-O-rutinoside were 17.889, 22.479, 25.626; respectively.

Table.1. Identification and quantification of three phenolic compounds of *Nitraria retusa* extract

Peak	Retention time (min)	Area	Identification	Quantification (µg/mL)
1	17.696	7.8	Quercetin	22.343
2	22.460	92.4	Isorhamnetin-3-O glucoside	331.420
3	25.682	20.2	Isorhamnetin-3-O rutinoside	24.915

4.2. NRE administration reduces body weight gain as well as adipose tissue weight in *db/db* obese mice model BKS.Cg-*Dock7^m*^{+/+} *Lepr^{db}*/J without affecting appetite

As shown in **Fig.3** and **Table.2.**, the body weight and body weight gain of the control *db/db* increased significantly, compared to NRE *db/db* over 4 weeks treatment period in BKS.Cg-*Dock7^m*^{+/+} *Lepr^{db}*/J mice. Moreover, final body weight was significantly lower in the NRE *db/db* (34.83 ± 1.75 g) group compared to control *db/db* group (42.16 ± 2.56 g) (**Table.2**) ($p < 0.05$), without affecting food intake, which was around 3g/day/mice for all different experimental groups (**Table.3**).

Fig. 3 Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on weight gain of BKS.Cg-*Dock7^m*^{+/+} *Lepr^{db}*/J mice.

Mice were divided to 4 groups: 2 groups; *db/db* mice control and *db/db* mice NRE; were orally administrated with a daily dose of 0 and 50 mg/kg of body weight (BW) respectively for 4 weeks. Their two litter mates groups; *db⁺* (lean mice) control and *db⁺* (lean mice) NRE were also orally administrated with a daily dose of 0 and 50 mg/kg of body weight (BW) respectively for 4 weeks. Body weight was daily measured at regular time. Data represent mean $\pm$ SD, $n=8$, * $p < 0.05$ compared to *db/db* mice control group.

In the current study, NRE treatment was demonstrated not only to decrease final body weight, but also cumulative body weight (**Table.2**) and adipose tissue weight leading to an improvement of adiposity. In fact 50 mg/kg NRE treatment in *db/db* mice decreased adipose tissue weight from 5.41 ± 0.8 g to 2.83 ± 0.7 in *db/db* control group g (**Fig.4**). However, NRE treatment did affect neither the body weight nor the adiposity in lean mice group.

Table 2 .Body Weight gain in *db/db* mice control, *db/db* mice NRE, lean mice control and lean mice NRE groups after 4 weeks

Groups	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
<i>db/db</i> mice control	31.00 ± 1.00	42.16 ± 2.56	11.16 ± 1.56
<i>db/db</i> mice NRE	29.00 ± 1.50	$34.83 \pm 1.75 *$	$5.83 \pm 0.25 *$
Lean mice control	$22.50 \pm 1.22^{**}$	$23.60 \pm 1.71^{**}$	$1.10 \pm 0.49^{**}$
Lean mice NRE	$22.20 \pm 0.75^{**}$	$23.00 \pm 1.32^{**}$	$0.80 \pm 0.57^{**}$

Data represent the mean $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, when compared to the *db/db* mice control group (Student's unpaired t-Test). No significant difference between lean mice control and lean mice NRE.

db/db mice control: BKS.Cg-*Dock7*^{m+/+} *Lepr*^{db/J} (*db/db*) mice control group. *db/db* mice NRE: BKS.Cg-*Dock7*^{m+/+} *Lepr*^{db/J} (*db/db*) mice treated with ethanolic extract of *Nitraria retusa* (50 mg/kg body weight) group. Lean mice control: BKS.Cg-*Dock7*^{m+/+} *Lepr*^{db/J} (*db*+) mice control group. Lean mice NRE: BKS.Cg-*Dock7*^{m+/+} *Lepr*^{db/J} (*db*+) mice treated with ethanolic extract of *Nitraria retusa* (50 mg/kg body weight) group.

Table 3 .Food intake (g/week) and (g/day) in in *db/db* mice control, *db/db* mice NRE, lean mice control and lean mice NRE groups after 4 weeks

Total food intake		
Groups	g/week	g/day
<i>db/db</i> mice control	42.77 ± 7.59	6.10 ± 0.65
<i>db/db</i> mice NRE	38.57 ± 5.10	5.50 ± 0.50
Lean mice control	25.17 ± 3.80	3.59 ± 0.69
Lean mice NRE	25.87 ± 4.75	3.69 ± 0.85

There was no significant difference between the total food intake of the *db/db* mice NRE group and the *db/db* mice control group (Student's unpaired t-Test). *db/db* mice control: BKS.Cg-*Dock7^m+/+ Lepr^{db/J}* (*db/db*) mice control group. *db/db* mice NRE: BKS.Cg-*Dock7^m+/+ Lepr^{db/J}* (*db/db*) mice treated with ethanolic extract of *Nitraria retusa* (50 mg/kg body weight) group. Lean mice control: BKS.Cg-*Dock7^m+/+ Lepr^{db/J}* (*db*+) mice control group. Lean mice NRE: BKS.Cg-*Dock7^m+/+ Lepr^{db/J}* (*db*+) mice treated with ethanolic extract of *Nitraria retusa* (50 mg/kg body weight) group.

Fig. 4 Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on adipose tissue weight of BKS.Cg-*Dock7^m+/+ Lepr^{db/J}* mice.

Data of *db/db* mice are shown on (A) and data of lean mice results are shown in (B). At the end of experiment, adipose tissue, for all groups, was weighed. Data represent mean ± SD, n=8, * p<0.05, compared to *db/db* mice control group (Student's unpaired t-Test).

4.3. NRE administration ameliorates lipid serum profile in *db/db* obese mice model

BKS.Cg-Dock7^m+/+ *Lepr*^{db/J}

Serum glucose and lipid levels (triglycerides, total cholesterol, HDL-cholesterol (HDL-c), LDL-cholesterol (LDL-c)) of all mice groups were analyzed. Results are summarized in **Fig.5**. An increase in triglyceride levels, particularly when accompanied by decreased high-density lipoprotein (HDL) levels, has been shown to be a surrogate marker for insulin resistance, a strong predisposing condition for Type 2 Diabetes. Thus, the *in vivo* effects of *Nitraria retusa* extracts, on cholesterol levels (total, low-density lipoprotein (LDL) cholesterol, and HDL cholesterol) was assayed. As shown in **Fig.5A1** and **5C1**, oral administration of a *Nitraria retusa* extract did not reduce levels of total cholesterol or levels of HDL cholesterol (the “good cholesterol”). However, as shown in **Fig.5B1**, oral administration of a *Nitraria retusa* extract significantly reduced LDL cholesterol (the “bad cholesterol”) levels in diabetic mice model by 50 % approximately, compared to the control.

Regarding triglycerides and glucose measurement results, as shown in Figures **5.D1** and **5.E1**, triglycerides level was slightly decreased from 23.75 ± 3.80 in *db/db* mice control to 15.00 ± 2.89 in *db/db* NRE-treated mice, while glucose level was not significantly affected by NRE administration for 30 days of treatment. This is likely may due to the duration of the disclosed experiments. Taken together, these data indicate that *in vivo* administration of a *Nitraria retusa* extract is effective in reducing diabetic hypercholesterolemia.

A1

A2

B1

B2

C1

C2

Fig.5 Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on Blood constituents; Total cholesterol (A; A1, A2), LDL-cholesterol (B; B1, B2), HDL-cholesterol (C; C1, C2), Triglycerides (D; D1, D2) and Glucose (E; E1, E2); of BKS.Cg-*Dock7^m+/+ Lepr^{db}/J* mice.

At the end of experiment, blood samples were collected and analyzed. Data represent mean $\pm$ SD, n=8, * p<0.05, compared to *db/db* mice control group (Student's unpaired t-Test).

4.5. Effect of NRE administration on genes expression related to hepatic lipid metabolism which modulate lipolysis-lipogenesis balance in diabetic mice

Understanding the molecular mechanism that undergoes dyslipidemia should facilitate improved strategies for serum lipid management. In this respect, the effect of NRE administration in BKS.Cg-*Dock7*^m+/+ *Lepr*^{db/J} mice on hepatic lipid metabolism biomarkers at translational level was investigated, following 4 weeks experimental study.

To assay the *in vivo* effects of *Nitraria retusa* extract on these genes which regulate lipid homeostasis, *Nitraria retusa* extract was administrated orally and daily to mice during 30 days and expression of said genes in liver of all groups of mice were determined by real-time PCR. As shown in Figures 6 to 8, oral administration of *Nitraria retusa* extract significantly over-expressed expression of PPAR- γ , PPAR- α , ACC1, FAS, SREBP-1c, LPL as well as CPT1 genes ; in diabetic mice models. These results were concordant with weight loss, adiposity and biochemical metabolites investigation. The current data indicates that *in vivo* administration of NRE could be an effective plant preparation in enhancing liver lipid metabolism which treats or ameliorates obesity.

A1

A2

B1

B2

Fig.6. Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on genes regulating lipid metabolism in liver of BKS.Cg-Dock7m+/+ *Leprdb/J* mice.

At the end of experiment, liver samples were collected and analyzed. Peroxisome proliferator activated receptor alpha; *PPAR-α*; (A; A1, A2) and Peroxisome proliferator activated receptor gamma; *PPAR-γ1*; (B; B1, B2). Real time PCR was conducted and result was represented as mRNA expression fold change compared to the *db/db* mice control group and lean mice control group. Bars represent mean $\pm$ SD, n=3, * p<0.05, ** p<0.001 compared to *db/db* mice control group (Student's unpaired t-Test).

Fig.7. Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on genes regulating lipid metabolism in liver of BKS.Cg-*Dock7m*^{+/+} *Leprdb/J* mice.

At the end of experiment, liver samples were collected and analyzed. Fatty acid synthase; *FAS*; (A; A1, A2), and Acetyl-CoA-Carboxylase; *ACCI*; (B; B1, B2). Real time PCR was conducted and result was represented as mRNA expression fold change compared to the *db/db* mice control group and lean mice control group. Bars represent mean $\pm$ SD, n=3, * p<0.05, ** p<0.001 compared to *db/db* mice control group (Student's unpaired t-Test).

A1

A2

B1

B2

C1

C2

Fig.8. Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on genes regulating lipid metabolism in liver of BKS.Cg-*Dock7m*^{+/+} *Leprdb/J* mice.

At the end of experiment, liver samples were collected and analyzed. Sterol regulatory element-binding protein-1-c: *SREBP-1c*; (A; A1, A2), Lipoprotein Lipase: *LPL*; (B; B1, B2) and Carnitine palmitoyltransferase I; *CPT1*; (C; C1, C2). Real time PCR was conducted and result was represented as mRNA expression fold change compared to the *db/db* mice control group and lean mice control group. Bars represent mean $\pm$ SD, n=3, * p<0.05, ** p<0.001 compared to *db/db* mice control group (Student's unpaired t-Test).

4.5. Discussion

Our previous anti-obesity study was conducted in mice fed with high-fat diet-induced obesity and daily administrated with *Nitraria retusa* extracts (NRE) at final doses of 50 mg/kg BW and 100 mg/kg BW for a period of 4 weeks. Main results revealed that oral administration of these doses was effective to control the weight gain and reduce significantly the cumulative body weight and fat mass compared to mice fed only with high-fat diet (HFD) (Zar kalai et al., 2013). In this current study our objective was to test whether the NRE could also attenuate the metabolic complications related to spontaneous obesity, using a *Lepr^{db}/Lepr^{db}* mice which is a model of obesity and type-2-diabetes that is deficient in leptin receptor activity due to a mutation in leptin receptor (*db*) gene. Therefore, the anti-obesity effects of NRE were first investigated by evaluating body weight, feed intake and white adipose tissue weight. At the end of treatment, measurements of lipid and glucose concentrations in serum, as well as mRNA expressions of genes related to lipogenesis-lypolysis balance in liver of scarified mice were conducted. We used NRE at a dose of 50 mg/kg BW, which was a minimal effective dose in our previous study on HFD-induced obesity experiment (Zar kalai et al., 2013), to evaluate its oral administration effect on *db/db* mice model used in the present study. Thus, supplementation with NRE significantly reduced body weight and white adipose tissue mass, without affecting feed intake. However, in many previous studies, some medicinal plants possessed anti-obesity actions through several

mechanisms either by preventing weight gain or promoting weight loss, accompanying with appetite suppression (Sahib et al., 2012). In other hand, it was reported that the anti-obesity activity could be ameliorated by the synergistic effect of a combination of some components present in a plant extract resulting in the enhancement of the herbal medicine's therapeutic activity (Rayalam et al., 2011). Although not bound by theory, the *Nitraria retusa* extract disclosed herein likely has superior anti-obesity activity which could be due to synergy brought by the combination of many compounds. *Nitraria retusa* extract disclosed herein is likely rich in naturally present flavonoids especially glycosides ones such as; Isorhamnetin-3-*O*-glucoside, Isorhamnetin-3-*O*-rutoside, and Isorhamnetin-3-*O*-robinobioside, quercetin and others, that could work all in synergy (Hadj Salem et al., 2011; Hussein et al., 2009; Boubaker et al., 2012). The whole extract mixture of NRE as described in the chemical analysis results section done by RP-HPLC analysis, and confirmed by other chemical investigations studies (Hadj Salem et al., 2011; Hussein et al., 2009); could also significantly reduced LDL cholesterol (the "bad cholesterol") and triglycerides levels in diabetic mice model (**Fig.5 B1 and D1**). In this respect, previous studies reported that the increased of plasma total cholesterol and LDL cholesterol levels are caused by diabetic dyslipidemia (Dokken, 2008). NRE treated *db/db* mice group had significantly lower levels of plasma LDL cholesterol when compared to control group (**Fig.5 B1**). Thus, NRE treatment might reduce the risk of diabetic hypercholesterolemia in *db/db* mice. Furthermore, treated *db/db* mice group revealed a lower level of triglycerides compared to control group (**Fig.5 D1**) which reflect the enhancement of the lipid metabolism in liver. Liver occupies a central position in the metabolic homeostasis; it plays an essential role in oxidizing triglycerides to produce energy not only to meet its own needs, but also to cover those of other tissues (Nguyen et al., 2008). Eventually, in obesity the accumulation of lipids into hepatocytes

518 leads to many metabolic complications due mainly to the alteration of the expression of many
519 genes (*PPAR-γ1* *PPAR-α*, *FAS*, *LPL*, *SREBP-1c*, and *CPT-1*) related to lipid and cholesterol
520 metabolism. Therefore, evaluating the effect of NRE exposure on the said genes regulating the
521 hepatic lipid metabolism in *db/db* mice used in this work was conducted. Generally, hepatic lipid
522 metabolism is controlled by a balance between lipogenesis and lipolysis processes. This balance
523 is maintained by determined level of the encoded genes expressions of lipogenic and lipolytic
524 enzymes. *PPAR-γ* and *PPAR-α* (ligand-activated transcription factors and members of the
525 nuclear hormone receptor superfamily) are the main regulators of energy and lipid homeostasis
526 (Varga et al., 2011). They act as a master regulator of fatty acid oxidation and lipid storage,
527 respectively (Abbott, 2009; Ribet and Langin, 2010). Our findings revealed that the genes
528 expressions of *PPAR-γ* and *PPAR-α* were overexpressed in *db/db* treated mice compared to the
529 control group (**Fig.6 A1 and B1**), which probably reflect the modulation of serum lipid profile.
530 Our results were concordant with those reported in previous clinical studies describing that the
531 activation of *PPAR-α* enhanced fatty acid oxidation in liver and decreased the levels of
532 circulating and cellular lipids in obese diabetic patients (Kim and Kawada, 2012). The mRNA
533 expression of *FAS* and *ACCI* genes encoding for two important lipogenic enzymes in liver; fatty
534 acid synthase and acetyl-CoA-carboxylase, respectively, were overexpressed by NRE
535 administration (**Fig.7A1 and B1**). The level of these enzymes involved in lipogenesis, were
536 reported to be maintained and controlled by the *SREBP-1c* (Sterol Regulatory Element-Binding
537 Protein-1-c), the main regulator of hepatic cholesterol homeostasis (Deng and Elam, 2012). Its
538 hepatic overexpression in diabetic mice was reported to induce lipogenic enzymes leading to a
539 marked decrease in hyperglycemia (Becard et al., 2001), which is in turn positively correlated
540 with our results shown in (**Fig. 5D1, 6A1, 6B1 and 7A1**). The level of gene expression encoding

541 another important lipogenic enzyme, Lipoprotein lipase (*LPL*), playing a central role in fat
542 mobilization and regulating the plasma triglycerides and cholesterol levels, was also
543 overexpressed in *db/db* mice due to the NRE oral administration. Its overexpression was reported
544 to prevent hypertriglyceridemia and hypercholesterolemia especially LDL levels (Yamamoto et
545 al., 1995). These described results strongly confirm our findings related to the expression of
546 hepatic *LPL* gene due to NRE exposure. In order to maintain the balance of lipid metabolism in
547 liver between fat storage, regulated by lipogenic pathway and fat burning, regulated by lipolytic
548 pathway. The mitochondrial fatty acid oxidation which is controlled by *PPAR- α* plays a crucial
549 function. Carnitine palmitoyltransferase- 1 (*CPT1*) is one of main target enzymes of *PPAR- α* ,
550 contribute the transport of activated long-chain fatty acids from the cytosol to the mitochondrial
551 matrix, where β -oxidation is occurring (Koo et al., 2008). The lack of carnitine will slow
552 oxidation of fatty acids and fuel and increase in serum lipid level (Park et al., 2008). NRE oral
553 administration in the present study up-regulate *CPT1* gene expression (**Fig.7C1**), which clearly
554 affect the plasma lipid levels and reduce finally the body weight in *db/db* mice. However, normal
555 mice or lean mice NRE-treated groups were not affected by NRE administration either in body
556 weight or in serum lipid profile correlated with hepatic genes expression described in all results.
557 These findings could probably reflect the safety of the combined effect of NRE components as
558 well as to the difference in leptin signaling pathway role in the normal mice. In this study, main
559 results showed that NRE administration in *db/db* mice significantly increased gene expression of
560 hepatic lipolytic enzymes such as *CPT1* (**Fig.7C1**) as well as the transcriptional activity of
561 *PPAR- α* (**Fig.5A1**), the key role in the induction of fatty acid oxidation enzymes. In other side,
562 NRE administration significantly increased lipogenic enzymes expression such as *ACC* and *FAS*
563 (**Fig. 6A1 and B1**) resulting finally in a modulation of lipid metabolism. NRE compounds were

reported for their strong antioxidant activity *in vitro* as well as *in vivo* (Boubaker et al., 2012; Yokozawa et al., 2002). Therefore, the enhancement of hepatic lipid metabolism could be attributed also, to the improvement of the antioxidant system following treatment with the NRE mixture. This in turn increases the expressions of *FAS*, *SREBP-1c* genes that may be compromised by the presence of reactive oxygen species within the fat cells, preventing further lipid storage. (Furukawa and Shimomura, 2004). However as shown in our results, *FAS* and *SREBP-1c* were overexpressed in liver of *db/db* mice treated with NRE which might reflect the enhancement of the hepatic antioxidant status. Taken together all results, NRE could attenuate the development of metabolic disorders in transgenic diabetic mice due to its enhancement of fatty acid oxidation process through regulation of lipid metabolism.

4.6. Conclusion

The treatment of obesity is difficult and challenging. A large number of scientific works indicates that variety crude extracts and isolated components from plants can promote body weight loss and prevent metabolic syndrome-related diseases. Indeed, the effects of NRE on *db/db* obese mice model BKS.Cg-*Dock7^m*^{+/+} *Lepr^{db}*/J indicate that *Nitraria retusa* extract mixture possesses potential anti-obesity effects and may relieve obesity-related symptoms comprising hyperlipidemia through modulating the lipolysis (β -oxidation) – lipogenesis balance. Thus NRE could be one of possible ways to control obesity and to prevent or reduce the risk of getting obesity-related metabolic disorders.

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References

- Abbott, B.D., 2009. Review of the expression of peroxisome proliferator-activated receptors alpha (PPAR α), beta (PPAR β), and gamma (PPAR γ) in rodent and human development. *Reprod. Toxicol.* 7, 246–257. DOI: 10.1016/j.reprotox.2008.10.001.
- Adams, L.A., Angulo, P., Lindor, K.D., 2005. Nonalcoholic fatty liver disease. *Can. Med. Assoc. J.* 172, 899–905. DOI: 10.1503/cmaj.045232.
- Alvarez, A.F., Alvarez, M.S., Gonzalez, R., Cucarella, C., Muntane, J., Casado, M., 2011. Human SREBP1c Expression in Liver Is Directly Regulated by Peroxisome Proliferator-activated Receptor (PPAR α). *J. Biol. Chem.* 286, 21466–21477. DOI: 10.1210/me.2003-0151.
- Atia, A., Barhoumi, Z., Rabhi, M., Abdelly, C., Smaoui, A., 2011. Environmental eco-physiology and economical potential of the halophyte *Crithmum maritimum* L. (Apiaceae). *J. Med. Plants. Res.* 5, 3564–3571. <http://www.academicjournals.org/JMPR> .
- Becard, D., Hainault, I., Marniche, D.A., Coussot, L.B., Pascal, F., Fougelle, F., 2001. Adenovirus-Mediated Overexpression of Sterol Regulatory Element Binding Protein-1c Mimics Insulin Effects on Hepatic Gene Expression and Glucose Homeostasis in Diabetic Mice. *DIABETES.* 50, 2425–2430. DOI:10.2337/diabetes.50.11.2425.
- Boubaker, J., Skandrani, I., Bouhlel, I., Ben sghaier, M., Neffati, A., Ghedira, K., Ghediraa, LG., 2010. Mutagenic, antimutagenic and antioxidant potency of leaf extracts from *Nitraria retusa*. *J. Food. Chem. Toxicol.* 48, 2283–2290. DOI: 10.1016/j.fct.2010.05.061.

Boughalleb, F., Denden, M., 2011. Physiological and biochemical changes of two halophytes, *Nitraria retusa* (Forssk.) and *Atriplex halimus* (L.) under increasing salinity. Agric. J. 6, 327–339. DOI: 10.3923/aj.2011.327.339.

Boubaker, J., Ben Sghaier, M., Skandrani, I., Ghedira, L., Chekir-Ghedira, L., 2012. Isorhamnetin 3-*O*-robinobioside from *Nitraria retusa* leaves enhance antioxidant and antigenotoxic activity in human chronic myelogenous leukemia cell line K562. BMC Complement. Altern. Med.12, 1–9. DOI: 10.1186/1472-6882-12-135.

Browning, J.D., Horton, J.D., 2004. Molecular mediators of hepatic steatosis and liver injury. J. Clin. Invest. 114, 147–152. DOI: 10.1172/JCI22422.

Browning, J.D., Szczepaniak, L.S., Dobbins, R., Nuremberg, P., Horton, J.D., Cohen, J.C., Grundy, S.M., Hobbs, H.H., 2004. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. J. Hepatol. 40, 1387–1395. DOI: 10.1002/hep.20466.

Buhmann, A., Papenbrock, J., 2013. An economic point of view of secondary compounds in halophytes. Funct. Plant. Bio.40, 952–967. DOI: 10.1071/FP12342.

Deng, X., Elam, M.B., 2012. FoxO1 Inhibits Sterol Regulatory Element Binding Protein-1c gene expression via the transcription factors Sp1 and SREBP-1c. J. Biol. Chem. 287, 20132–20143. DOI: 10.1042/BJ20020430.

Dokken, B.B., 2008. The Pathophysiology of Cardiovascular Disease and Diabetes: Beyond Blood Pressure and Lipids. Diabetes. Spectr. 21, 160–165. DOI:10.2337/diaspect.21.3.160.

Foufelle, F., Ferre, P., 2002. New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. Biochem. J. 366, 377–391. DOI:10.1042/BJ20020430.

Furukawa, S., Shimomura, I., 2004. Increased oxidative stress in obesity and its impact on metabolic syndrome. J. Clin. Invest. 114, 1752–1761. DOI:10.1172/JCI21625.

Guyot, S., Marnet, N., Laraba, D., Sanoner, P., Drilleau, J.F., 1998. Reversed-Phase HPLC following Thiolytic for Quantitative Estimation and Characterization of the Four Main Classes of Phenolic Compounds in Different Tissue Zones of a French Cider Apple Variety (*Malus domestica* Var. Kermesmerrien). J. Food. Agric. Chem. 46, 1698–1705. DOI: 10.1021/jf970832p.

Hadj Salem, J.H., Chevalot, I., Schiavo, C.H., Paris, C., Fick, M., Humeau, C., 2011. Biological activities of flavonoids from *Nitraria retusa* (Forssk.) Asch and their acylated derivatives. J. Food. Chem. 124, 486–494. DOI: 10.1016/j.foodchem.2010.06.059.

Hamaguchi, M., Ida, K., 2005. The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. Ann. Intern. Med. 143, 722–728. DOI: 10.7326/0003-4819-143-10-200511150-00009.

Han, J., Isoda, H., 2009. Capsaicin Induced the Upregulation of Transcriptional and Translational Expression of Glycolytic Enzymes Related to Energy Metabolism in Human Intestinal Epithelial Cells. J. Agric. Food. Chem. 23, 11148–11153. DOI:10.1007/978-90-481-3892-0_46.

Hussein, S.R., Kwashty, S.A., Tantawy, M.E., Saleh, N.A.M., 2009. Chemosystematic studies of *Nitraria retusa* and selected taxa of zygophyllaceae in Egypt. Plant. Syst. Evol. 277, 251–264. DOI: 10.1007/s00606-008-0108-x.

Kang, Y.R., Oh, H.G., 2012. Anti-obesity and anti-diabetic effects of Yerba Mate (*Ilex paraguariensis*) in C57BL/6J mice fed a high-fat diet. Lab. Anim. Res. 28, 23–29. DOI: 10.5625/lar.2012.28.1.23.

Kim, Y.I., Kawada, T., 2012. Potent PPAR α Activator Derived from Tomato Juice, 13-oxo-9,11-Octadecadienoic Acid, Decreases Plasma and Hepatic Triglyceride in Obese Diabetic Mice. PloS One. 7, 1–8. DOI: 10.1371/journal.pone.0031317.

Koo, B.S., Seong, S.H., Kwon, D.Y., Sohn, H.S., Cha, Y.S., 2008. Fermented kochujang supplement shows anti-obesity effects by controlling lipid metabolism in C57BL/6J mice fed high fat diet. Food. Sci. Biotechnol. 17, 336–342. <http://www.dbpia.co.kr/Article/1563518>.

Lee, G.H., Proenca, R., Montez, J.M., Carroll, K.M., Darvishzadeh, J.G., Lee, J.I., Friedman, J.M., 1996. Abnormal splicing of the leptin receptor in diabetic mice. Nature. 379, 632–635. DOI: 10.1038/379632a0.

Lutz, T.A., Woods, S.C., 1996. Overview of Animal Models of Obesity. Curr. Protoc. Pharmacol. 58, pp. 1–18. DOI: 10.1002/0471141755.ph0561s58.

Moro, C., Basile, G., 2000. Obesity and medicinal plants. Fitoterapia. 71, 73–82. DOI: 10.1016/S0367-326X(00)00177-5.

Nguyen, P., Leray, V., Diez, M., Serisier, S., Le Bloc'h, J., Siliart, B., Dumon, H., 2008. Liver lipid metabolism. J. Anim. Physiol. Anim. Nutr. 92, 272–283. DOI: 10.1111/j.1439-0396.2007.00752.x.

Park, S.H., Park, T.S., Cha, Y.S., 2008. Grape seed extract (*Vitis vinifera*) partially reverses high fat diet-induced obesity in C57BL/6J mice. NRP. 2, 227–233. DOI: 10.4162/nrp.2008.2.4.227.

Rainsa, T.M., Agarwal, S., Mak, K.C., 2011. Antiobesity effects of green tea catechins: a mechanistic review. J. Nutr. Biochem. 22, 1–7. DOI: 10.1016/j.jnutbio.2010.06.006.

Ranganathan, G., Kern, P.A., 2006. The lipogenic enzymes DGAT1, FAS, and LPL in adipose tissue: effects of obesity, insulin resistance, and TZD treatment. J. Lipid. Res. 47, 2444–2450. DOI: 10.1194/jlr.M600248-JLR200.

Ranjbar, S.H., Jouyandeh, Z., Abdollahi M., 2013. A systematic review of anti-obesity medicinal plants - an update. J. Diabetes. Metab. Disord. 12, 28. DOI:10.1186/2251-6581-12-28.

Rayalam, S., Della-Fera, MA., Baile, C.A., 2011. Synergism between resveratrol and other phytochemicals: Implications for obesity and osteoporosis. *Mol. Nutr. Food Res.* 55, 1–9. DOI: 10.1002/mnfr.201000616.

Reddy, J.K., Rao, M.S., 2006. Lipid metabolism and liver inflammation.II. Fatty liver disease and fatty acid oxidation. *Am. J. Physiol. Gastrointest. Liver. Physiol.* 290, 852–858. DOI: 10.1152/ajpgi.00521.2005.

Reddy, J.K., Hashimoto, T., 2001. Peroxisomal β -oxidation and peroxisome proliferator-activated receptor: an adaptive metabolic system. *Annu. Rev. Nutr.* 21, 193–230. DOI: 10.1146/annurev.nutr.21.1.193.

Ribet, C., Langin, D., 2010. Peroxisome Proliferator-Activated Receptor- α , control of Lipid and Glucose Metabolism in Human White Adipocytes. *Endocrinology.* 151, 123–133. DOI: 10.2337/diab.46.8.1319.

Sahib, N.G., Saari, N., Ismail, A., Khatib, A., Mahomoodally, F., Abdul Hamid, A., 2012. Plants' Metabolites as Potential Antiobesity Agents. *Scientific.World.Journal.* 2012, 1–8. DOI: 10.1100/2012/436039.

Varga, T., Czimmerer, Z., Nagy, L., 2011. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochim. Biophys. Acta.* 1812, 1007–1022. DOI: 10.1016/j.bbadis.2011.02.014.

Yamamoto, K., Shimano, H., Shimada, M., Kawamura, M., Gotoda, T., Harada, K., ichiohsuga, J., Yazaki, Y., Yamada, N., 1995. Overexpression of apolipoprotein E prevents development of diabetic hyperlipidemia in transgenic mice. *Diabetes*, 44, 580–585. DOI: 10.2337/diab.44.5.580.

Zar Kalai, F., Han,J., Ksouri, R., El Omri, A., Abdelly,C., Isoda, H., 2013. Antiobesity Effects of an Edible Halophyte *Nitraria retusa* Forssk in 3T3-L1 Preadipocyte Differentiation and in

740 C57B6J/L Mice Fed a High Fat Diet-Induced Obesity. J. Evid. Based Complementary. Altern.
741 Med. 2013, 1–11. DOI: 10.1155/2013/368658.