Anti-Obesity Effects of Tunisian Halophyte *Nitraria retusa* (Forsk.) Asch. Through Modulation of Lipid Metabolism

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Anti-Obesity Effects of Tunisian Halophyte *Nitraria retusa* (Forsk.) Asch. Through Modulation of Lipid Metabolism

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ABREVIATIONS

ACC1: Acetyl-CoA Carboxylase

AI: *Atriplex infalata*

$AlCl_3$ $6H_2O$: Aluminum chloride hexahydrate solution

AS: *Arthrophytum scoparium*

BW: Body weight

c-DNA: Complementary DNA

CE: Catechin equivalents

CEBP- α: CCAAT/enhancer-binding protein alpha

CHFD: Control High-fat diet fed group

CND: Control Normal diet fed group

CPT1: Carnitine palmitoyl-transferase I

DAD: Diode array detector

DMEM: Dulbecco's modified Eagle's medium

DNA: Deoxyribonucleic acid

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

***Db/db* mice**: Diabetic mice

EC_{50} (µg/mL): Effective concentration, at which the absorbance was 0.5

FAS: Fatty acid synthase

FBS: Fetal bovine serum

Fe^{3+}: Denoted Iron (III)

$FeCl_3$: Iron (III) chloride anhydrous

FRAP: Ferric reducing antioxidant power

GAE: Gallic acid equivalents

HCl: Hydrochloric acid,

HDL: High-density lipoprotein

HDL-c: High-density lipoprotein cholesterol

HFD: High-fat diet

HFD+PC: High-fat diet + positive control (Naringenin 10 mg/kg body weight)

HFD+NR50: High-fat diet + *Nitraria retusa* 50 mg/kg body weight

HFD+NR100: High-fat diet+ *Nitraria retusa* 100 mg/kg body weight

HPLC: High performance liquid chromatography

IC_{50} (µg/mL): Inhibiting concentration of 50% of the synthetic radical DPPH

Iso: Isorhamnetin

$K_3Fe(CN)_6$: Potassium ferricyanide (III)

LDL: Low-density lipoprotein

LDL-c: Low-density lipoprotein cholesterol

LPL: Lipoprotein lipase

ME: *Mesembryanthemum edule*

mg CE g/ DW: Milligram catechin equivalent per gram dry weight

mg GAE g/ DW: Milligram gallic acid equivalent per gram dry weight

mRNA: Messenger Ribonucleic acid

MTT: 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide

Na_2CO_3: Sodium carbonate anhydrous

$NaNO_2$: Sodium nitrite

NaOH: Sodium hydroxide

ND: normal diet

NR: *Nitraria retusa*

NRE: *Nitraria retusa* ethanolic extract

PCR: The polymerase chain reaction

PPAR-α: Peroxisome proliferator-activated receptor alpha

PPAR-γ: Peroxisome proliferator-activated receptor gamma

RP: Reverse phase

RP-HPLC: Reverse phase high performanceliquid chromatography

RS: *Rantherium sueavelons*

SDS: Sodium dodecyl sulfate

SREBP-1c: Sterol Regulatory Element-Binding Protein-1-c

TAA: Total antioxidant activity

TCA: Trichloroacetic acid

TCHO: Total cholesterol

TEAC: Trolox equivalent antioxidant capacity

TG: *Tamarix gallica* or triglycerides

TMA: Total monomeric anthocyanins

TNF-α: Tumor necrosis-factor-alpha

TP: Total phenolics

UV-vis: Ultra violet visible

WAT: White adipose tissue

WHO: World health organization

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CHAPTER 1

General introduction

1.1. Definition of obesity

According to WHO, obesity can be defined as an excess of body fat relative to lean body mass. A human subject is considered obese if they have a body mass index (BMI) of 30 and above. However, term of obesity is not limiting and depends on cultural and social definitions (Font et al., 2010). By 2005, obesity had affected 400 million adult, and since 1997, WHO has cited obesity as a global epidemic (Kazemipoor et al., 2012). At the cell biological level, obesity is characterized by an increase in the number (Hyperplasia) and size (Hypertrophy) of adipocytes. A lean adult has adult 35 billion adipocytes, each containing about 0.4 to 0.6 µg of triglycerides; an extremely obese adult can have four times as many adipocytes (125 billion), each containing twice as much lipid (0.8-1.2 µg of triglyceride) (Hirsch et al., 1970). Many reports defined obesity as a medical and physical condition that results from excessive storage of fat in the body, and, characterized by the accumulation of fat to the extent that it may induce side effects on health, leading to reduced life expectancy and/or increased health problems (Kazemipoor et al., 2012; Haslam and James, 2005). Therefore, over consumption of calories and decreased energetic activity could be the major cause of obesity. Losing weight became a big interest to have major health benefits for overweight people and also enhances life expectancy in people having obesity-related disorders (Rayalam et al., 2008). The main treatment for obesity consists of dieting and physical exercise (Lau et al., 2007). Diet programs may produce weight loss over the short term (Sacks et al., 2009), but keeping this weight loss is often difficult requiring a lower and enduring food energy diet. Therefore managing obesity and maintaining optimum body weight through an alternative therapy could help and be a vital part to manage life style disorders and related metabolic syndrome disease.

1.2. Obesity and adipogenesis

Obesity is produced due to an imbalance between energy intake and energy expenditure that finally leads to the uncontrolled growth of fat cells called adipocytes (Song et al., 2013). Adipocytes accumulate lipids through adipogenesis in adipose tissue, and adipogenesis is the process by which undifferentiated precursor cells (fibroblast-like-pre-adipocytes) differentiate into fat storage cells or mature cells called adipocytes (Fruhbeck et al., 2001). The conversion from undifferentiated fibroblast-like pre-adipocytes into complete adipocytes constitutes the adipocyte life cycle (Rayalam et al., 2008). Thus, a dramatic increase in the rate of obesity is a result of an excessive accumulation in white adipose tissue. Numerous studies have demonstrated that adipocyte differentiation and the amount of fat accumulation as well as adipocyte dysfunction are strongly associated with development of obesity, which is a major risk factor for many disorders including diabetes, hypertension, and cardio-vascular disease (Lee et al., 2009).

1.3. Obesity and related-metabolic disorders

1.3.1. Western-style diet (High-fat diet) and metabolic syndrome diseases

Generally, life styles are associated to increasing occurrences of overweight and risks of obesity related-diseases. The etiology of obesity is complex, dietary factors, particularly the feeding of a high-fat (HF) diet is supposed to be one of the central factors for its development (Woods et al., 2003). To date, progresses in the understanding of energy metabolism have revealed many of the regulatory systems involved in the homeostasis of body weight. Consuming high fat diets regularly may lead to an increasing of fat storage (Astrup, 1993; Woods et al., 2003). Therefore,

epidemiological studies have recognized a positive correlation between mean dietary fat intake and the incidence of obesity and its related health disorders and risk factors (Lichtenstein, 1998). The incidence of metabolic syndrome rises with the severity of obesity and touches 50% in morbidly obese youngsters (Lee, 2013). Obesity is considered as the most crucial predisposing factor for insulin resistance, resulting in high levels of glycemia and insulinemia which finally induce the development of diabetes (Shulman, 2000). In addition metabolic syndrome may lead to the chronic inflammatory state and metabolic disorders that predisposes on the other health problems mainly related to cardiovascular diseases, such as, hypertension, lipoprotein abnormalities and atherosclerosis (Wahba and Mak, 2007). Consequently, the increased prevalence of obesity and obesity-associated complications continues to be a foremost global health concern. This is due mainly to the cause of obesity that is multifactorial.

1.4. Proposed mechanisms for prevention and treatment of obesity (*in vitro* and *in vivo* models):

Several reports have outlined the mechanisms of proposed anti-obesity including decreased energy intake and increased energy expenditure (*in vivo* studies), decreased pre-adipocyte differentiation and proliferation, decreased lipogenesis and increased lipolysis, and fat oxidation (*in vitro* and *in vivo* studies) (Manchini et al., 2001).

1.4.1. Obesity studies using *in vitro* models:

-3T3-L1 cell line

Many models and techniques are being used in order to evaluate and understand adipocyte biology. 3T3-L1 is a pre-adipose and not cancer cell line, which was generated from clonal expansion of murine Swiss 3T3 cells and contains only a single cell type. This cell line is one of

the best-characterized and reliable models for studying the conversion of pre-adipocytes into adipocytes (Zhou and Zhu, 2009). Reports have outlined the mechanisms of proposed ant-obesity. Among them, pre-adipocytes play a key role in increased fat mass through increased number and size of adipocytes leading to the differentiation into mature adipocytes. Thus, both suppression of pre-adipocyte differentiation and decrease in cell viability of pre-adipocytes as well as adipocytes are potential means to manage and treat obesity (Wang and Jones, 2004; Lii et al., 2011). Moreover, many research studies used 3T3-L1 cells to identify key molecular markers and related pathways of pre-adipocyte differentiation. The cell differentiation is a multi-stage process involving synchronized regulation of specific genes and proteins expression linked with each phase of adipogenesis (Ntambi and Young-Cheul, 2000). Adipogenic transcriptions factors such as of the CCAAT/enhacer binding protein-beta (*C/EBP-β*), nuclear receptor peroxisome proliferator-activated receptor gamma (*PPAR-γ*), and CCAAT/enhacer binding protein-alpha (*CEBP-α*) play a key role in the complex transcriptional cascade that occurs during adipogeneis (Cristancho and Lazar, 2011). Therefore, understanding the mechanism through which a determined compound or mixture of compounds affect the differentiation to adipocytes would be one of the approaches which lead to reduce or prevent the onset and progression of obesity.

-Chronological changes and transcriptional cascade during adipocyte differentiation

Adipocyte differentiation is known to be mediated by cell cycle and differentiation factors and controlled by diverse signaling events which could inhibit or stimulate the adipogenesis process. This process involves two main phases the recruitment and proliferation of adipocyte precursor cells called pre-adipocytes, followed by their differentiation into mature fat cells. The maturation step is characterized by the alteration of cell shape, growth arrest and clonal expansion. Consequently, a sequence of specific changes in gene expression related to lipid storage occurs

(Gregoire, 2001). Interaction among the members of *C/EBP* and *PPAR* families play a crucial role during adipogenesis process. *CCAAt*/enhancer binding protein-beta (*CEBP-β*) is expressed immediately after the induction of differentiation, and then *PPAR-γ* and *CCAAt*/enhancer binding protein-alpha (*CEBP-α*) act synergistically to maintain their high levels and promote adipogenesis (Rayalam et al., 2007). They activate in coordinated functions, the expression of their target genes; adipocyte-specific genes; such as adipocyte fatty acid-binding protein (*aP2*), lipoprotein lipase (*LPL*), leptin, adiponectin, glucose transporter-4 and fatty acid synthase (*FAS*). This cooperative regulation leads to morphological changes and fat accumulation within cells (Takahashi et al., 2009).

1.4.2. Obesity studies using *in vivo* models:

Obesity is associated with several health problems, including diabetes, cardiovascular disease, respiratory failure, muscle weakness and cancer (Kanasaki and Koya, 2011). To better understand the pathological mechanisms of human disease, good animal models are essential. Thus, many rodent models of obesity are used in the research of obesity-related human health conditions and diseases such as diabetes and cancer.

1.4.2.1. Monogenic mice obesity models:

In rodents, a number of autosomal recessive gene mutations that result in the obese phenotype have been described, including *ob/ob* and *db/db* mice (Zhang et al., 1994; Tartaglia et al., 1995). In 1949, researchers from the Jackson laboratory discovered obese mice. The *db/db* mouse was identified initially in 1966 by researchers in the Jackson Laboratory as an obese mouse (Kanazaki and Koya, 2011). It was also reported that, the products of the ob and db genes represent a hormone-receptor pair (leptin, produced and released into the plasma by adipocytes,

and its receptor, present in hypothalamic nuclei, respectively) that acts as a component of a negative feedback system involved in the regulation of fat mass (Lin et al., 2000). Impaired leptin signaling in the hypothalamus of *ob/ob* mice (unable to produce leptin) and *db/db* mice (unable to respond to leptin) leads to persistent hyperphagia, and obesity. Other metabolic abnormalities could occur such as hyperlipidemia, insulin resistance, decreased energy expenditure and diabetes (Chen et al., 1996; Pelleymounter et al., 1995).

-Monogenic mice model used in this study: *db/db* mice

Strain Name: BKS.Cg-*Dock7m* +/+ *Lepr* ^{db}/J: Congenic; Mutant Strain.

Appearance

-Leprdb: black, fat

Related Genotype: a/a + *Leprdb*/+ *Lepr*^{db}

-Dock7m Leprdb: black, lean

Related Genotype: a/a Dock7m +/+ *Lepr*^{db}

-Dock7m: misty (grey), lean

Related Genotype: a/a *Dock7m* +/*Dock7m* +

Mice homozygous for the diabetes spontaneous mutation (*Lepr*^{db}) become identifiably obese around three to four weeks of age. Elevations of plasma insulin begin at 10 to 14 days of age and of blood sugar at four to eight weeks. Homozygous mutant mice are polyphagic, polydipsic, and polyuric (http://www.renasci.co.uk/experimental/obesity/dietary-induced-obese-animals).

At one month of age, *db/db* mice are larger (obese) when compared to control (heterozygous) littermates, and *db/db* mice present increased fat deposition in the inguinal and axillary regions. They also develop frank hyperglycemia by 8 weeks of age (Lee et al., 1996). Consequently these mice are widely used as a model for the study of obesity and type-2- diabetes.

1.4.2.2. Diet-induced obese mice model:

Dietary-induced obese (DIO) mice and rats are well-established animal models of obesity. A high-fat diet (HFD) is often utilized in obesity research as a non leptin-deficient model. There are mouse strain-specific differences in responses to the HFD (West et al., 1992). C57BL/6J mice are the most widely used for HFD-induced obesity because they display aberrations similar to human metabolic syndrome when fed the HFD (Kanasaki and Koya, 2011). There are major differences among sub-strains in response to the HFD. For example, while C57BL/6J mice exhibit HFD-induced obesity, hyperinsulinemia, and insulin resistances that nearly parallel the progression of human disease; C57BL/KsJ mice exhibit a weak phenotype (Kanasaki and Koya, 2011).

-Diet-induced obese mice model used in this study

-Strain Name: C57B6J/L

Common Names: C57 Black; B6; B6J; Black 6;

Appearance: black; Related Genotype: a/a

C57BL/6J is the most widely used inbred strain and the first to have its genome sequenced. Even though this strain is refractory to many tumors, it is a permissive background for maximal expression of most mutations (Buettner et al., 2007). They are predisposed to diet-induced obesity, type 2-diabetes, and atherosclerosis (Buettner et al., 2007). C57BL/6J mice fed a high-fat diet develop obesity, mild to moderate hyperglycemia, and hyperinsulinemia. Obesity is induced in male C57BL/6J mice by allowing them unlimited access to high fat diet supplying either 45% or 60% energy (JAX® Diet-induced Obesity (DIO) Models). Studies in dietary-induced obese mice are popular as an initial screen as they require fewer compounds than

dietary-induced obese rats and are easily to perform as mice are given their food as a single-source (Kanasaki and Koya, 2011). In conclusion, using such high-fat diet-induced obesity mice models could be one of alternatives to fight against human obesity.

1.5. Role of traditional medicinal plants and their bioactive compounds in the management and treatment of obesity: an overview

Medicinal plants and plant extracts represent the ancient and most prevalent form of medication. The use of natural therapies for weight loss has enlarged, based on consistency, safety, and cost compared with artificial drugs or surgical procedures, which may have limitations (Calixto, 2000). A large group of literature indicates that considerable progress has been made relating to our knowledge of bioactive components in plant foods and their links to obesity. Therefore, a sum of studies have been carried out to investigate the anti-obesity effects of polyphenols like apigenin and luteolin (Han et al., 2003), kaempferol (Yu et al., 2006), genistein and diadzein (Kim et al., 2006; Dang and Lowik, 2004), grape seed proanthocyanidin extract (GSPE) (Preuss et al., 2000), and epigallocatechin gallate (EGCG) (Wolfram et al., 2006). In other hand, certain animal studies reported the efficiency of some known herbs and vegetables lowering overweight and related disorders caused by obesity. The most known are, *Foeniculum vulgare* miller (Fennel), Camellia sinesis (Tea), Trigonella foenum graecum (Fenugreek) and *Ceratonia siliqua* (Locust bean) which act in different ways (Kazemipoor et al., 2012). In same respect and based on a number of *in vivo* studies regarding the efficacy of anti-obesity medicinal plant preparations, they may act by stimulating thermogenesis, reducing lipogenesis, increasing lipolysis through inducing β-oxidation (Okuda et al., 2001), suppressing appetite, and decreasing lipid absorption (Kazemipoor et al., 2012). Other medicinal plants have been repoted for their

effect to inhibit adipocyte differentiation and fat cell formation (Van heerden and Gordonii, 2008).

1.6. Potential of halophytes as medicinal plants

Plants having the ability to grow and complete their life cycle in habitats with a high salt content are called salt plants or halophytes. Halophytes have particular characteristics allowing them to evade and/or resist and tolerate salinity in arid regions as well as in humid regions (Abdelly et al., 2011). Eco-physiological studies on halophytes in arid lands shows that these plants are endowed with particular mechanisms and adaptations which enable them to survive, grow and reproduce, or even survive without net growth under the extreme conditions (severe drought and salinity) prevailing in arid zones (Khan et al., 2006).

Salt resistance can include either salt tolerance strategy or salt avoidance strategy:

-Salt tolerance involves physiological and biochemical adaptations for maintaining protoplasmic viability as cells accumulate electrolytes.

-Salt avoidance implicates structural with physiological adaptations to reduce salt concentrations of the cells or physiological excretion by root membranes.

Halophytes are frequently classified as excretive or (exclusive) and succulents (inclusive):

-Excretive halophytes possess gland cells capable of secreting excess salts from plant organs.

-Succulents have high water content within large vacuoles to minimize salt toxicity (Foyer and Noctor, 2005).

According to Ben Hamed et al., (2013) halophytes are distinguished from glycophytes mainly by their growth response to salinity. Comparative studies between halophytes and glycophytes have shown that halophytes are better equipped with the mechanisms of cross-stress tolerance and are constitutively prepared for stress. Halophytes existing in the extreme environments have to deal with numerous changes in salinity level. These species are known for their ability to endure and quench toxic Reactive Oxygen Species (ROS), since they are equipped with a powerful antioxidant system (Ksouri et al., 2009). At present, an increasing interest is granted to these species because of their high content in bioactive compounds (primary and secondary metabolites) such as vitamins, sterols, essential oils (terpenes), polysaccharides, glycosides, and phenolic compounds (Ksouri et al., 2011). In Tunisia, there is a large diversity of halophytic plants of multiple interests including therapeutic practices, economic and agro-alimentary applications.

1.7. Plant material used in anti-obesity study:

In current study, six halophytes samples were selected, based on the screening results as well as on the scientific reports related to their biological activities and their chemical composition.

1.7.1. Botanical description and geographical distribution:

-***Nitraria retusa*** (Forssk) is a salt shrub or bush in the Nitrariaceae family. It is known locally as Al-Ghardaq and described as a perennial and spiny plant. The plant grows to 2.5 m tall, although it is usually less than 1 m in height. It has tiny, white to green, fragrant flowers, and small edible red fruits (Hadj Salem et al., 2011). The plant is native to desert areas and saline soils of northern Africa, where it grows in primary succession on barren sand dunes (Suleiman et al., 2008).

Order: Sapindales

Family: Nitrariaceae

Genus: *Nitraria*

Species: *retusa*

- ***Tamarix gallica*** (L.), or French Tamarisk, is a deciduous, herbaceous, shrub or small tree reaching up to about 5 meters high. It is native in Saudi Arabia and the Sinai Peninsula, and very common around the Mediterranean region (Lawrence, 1985). *Tamarix gallica* is a tree or shrub halophyte widespread in coastal regions and desert, relatively long-living plant that can tolerate a wide range of environmental conditions and resist abiotic stresses such as salt, high luminosity, and drought stresses (Saïdana et al., 2007).

Order: Caryophyllales

Family: Tamaricaceae

Genus: *Tamarix*

Species: *gallica*

-***Mesembryanthemum edule*** (L.) or (*Carpobrotus edulis*), is succulent plant adapted to arid conditions and characterized by fleshy water-storing tissues that act as water reservoirs, having a capsular fruit containing edible pulp (Watson, 2007). It is an invasive species adapted to many regions throughout the world, notably Australia, California and the Mediterranean having similar climates (Van Der Watt and Pretorius, 2001). On the Mediterranean coast, *Messembryanthemum edule* has extent rapidly and many parts of the coastline are fully covered by this invasive species.

Order: Caryophyllales

Family: Aizoaceae

Genus: *Mesembryanthemum*

Species: *edule*

-***Atriplex inflata*** or Atriplex lampifer (Muell.)

Atriplex is a plant genus of 250-300 species, known by the common names of saltbush distributed closely worldwide from subtropical to temperate and to subarctic regions (Mavrodiev et al., 2010). The species in genus *Atriplex* are annual or perennial herbs, subshrubs, or shrubs (Welsh, 2003). *Atriplex inflata* is saltbush, found around salt flats and low salinized area. It is an annual Chenopodiaceae species that reaches a height of about 50 cm. It has simple leaves that are alternate. It mostly exist in saline habitats, namely in arid regions. This species is introduced in many regions in the world for its great forage value (Atia et al., 2011).

Order: Caryophyllales

Family: Chenopodiaceae

Genus: *Atriplex*

Species: *inflata*

-***Arthrophytum scoparium*** or *Hammada scoparia* (POMEL)

Arthrophytum scoparium or *Hammada scoparia* belonging to Chenopodiaceae family, is a small, highly branched halophytic shrub that are widely distributed in south-east Spain, North Africa, and parts of Iran, Turkey, Iraq and Syria (Irano-Turanian region) (A guide to medicinal plants in North Africa, 2005). This plant grows wild in dry habitats of the Mediterranean region and the Near East (Maire, 1962; Ben Salah et al., 2002).

Order Caryophyllales

Family Amaranthaceae

Genus: *Arthrophytum*

Species: *Scoparium*

-Rhanterium suaveolens

Rantherium suaveolens (Desf), endemic North African species locally known as Arfej, is a perennial weedy shrub widely distributed in the northern Sahara in the extreme south of Tunisia (Issaoui et al., 1996).

Order: Asterales

Family: Asteraceae

Genus: *Rhanterium*

Species: *suaveolens*

1.7.2. Traditional uses, biological activities and chemical composition:

***-Nitraria retusa* Forssk. Asch.**

This plant is used in traditional medicine in Tunisia and Egypt. The dry leaves are used in decoction as a substitute to tea and to make cataplasms. Ashes of this plant have the anti-inflamatory property treating the infected wounds. Its fleshy red fruits are eaten by human beings and birds; its wood is used as fuel by the local inhabitants (Hadj Salem et al., 2011). In a previous chemo-systematic investigation of leaves and young stems of *N. retusa*, Halim et al, (1995), isolated and fully characterized six flavonol glycosides: isorhamnetin-3-*O*-4-rhamgalactosyl-robinobioside, isorhamnetin-3-*O*-robinobioside, isorhamnetin-3-*O*-rutinoside, isorhamnetin-3-*O*-galactoside, isorhamnetin-3-*O*-glucoside, and, free isorhamnetin. These bioactives molecules isolated from *Nitraria retusa* have been reported to promote apoptosis in

human myelogenous erythroleukemia cells (Boubaker et al., 2012), and to exert anti-oxidant and anti-mutagenic activities (Boubaker et al., 2010).

-*Tamarix gallica* L.

Tamarix spp. was employed in traditional medicine as astringent, aperitif, diuretic (Saïdana et al., 2007), active against leucoderma, spleen trouble and eye diseases (Sharma and Parmar, 1998). Younos et al. (2005) indicate the importance of this genus in some old civilizations and the actual uses of the different parts (leaves, flowers, and galls) of *Tamarix* species in the traditional Asian therapy (anti-inflammatory, anti-diarrheic, cicatrizing agent, and antiseptic) in relation to their chemical composition, mainly constituted by polyphenols. The extract of *Tamarix gallica* is very rich in acid compounds such as; Gallic, sinapic, chlorogenic, syringic, vanillic, *p*-coumaric, and trans-cinnamic acids. As well as in flavonoids: (+)-catechin, isoquercetin, quercetin, apigenin, amentoflavone, and flavones and Tannins. The presence of these compounds exhibit a wide spectrum of medicinal properties, such as; anti-allergic, anti-inflammatory, cardio-protective and vasodilatory effects (Ksouri et al., 2009).

-*Mesembryanthemum edule* L.

Leaves of *Mesembryanthemum edule* are edible as are its fruit. Reports have shown that genus *Mesembryanthemum* has traditionally been used as food for the treatment of diabetes and liver diseases and (Bouftira, et al., 2009). It possesses biological activities, such as the anti-oxidant and anti-viral activity of *Mesembryanthemum edule* L. and *Mesembryanthemum crystallinum* L. (Bouftira, et al., 2009, Falleh, et al., 2011). Rutin, neohesperidin, hyperoside, catechin and ferulic acid were identified in *C.edulis*, and contribute to the antibacterial and antioxidant properties. It

also contains procyanidins and propelargonidins (Van der Watt and Pretorius, 2001; Falleh et al., 2009).

-*Atriplex inflata* Muell.

Atriplex species have been used for their good medicinal values. *Atriplex inflata* Muell extracts were revealed to be active against Herpes simplex viral infection (Mohammed et al., 2012). Many of these species has been investigated for their chemical constituents such as, terpenoids, flavonoids and saponins. *Atriplex inflata*, was reported to contain; Flavonol aglycones: Kaempferol, quercetin, isorhamnetin, patuletin, spinacetin and tricin, and Flavonoid sulphates (Mohammed et al., 2012).

- *Arthrophytum scoparium* Pomel

Arthrophytum scoparium is used in traditional medicine to treat eye disorders, and as a snuff powder (Boukef, 1986). *A scoparium* has been reported to have hepatoprotective, antioxidant, anticancer, antiplasmodial and larvicidal activity (Bourogaa et al., 2011; Bourogaa et al., 2013). Phenolic acids such as, coumaric acid, cinnamic acid, caffeoylquinic acid, were identified in *A. scoparium* as well as glycoside flavonoids such as; Isorhamnetin 3-*O*-*β*-D-xylopyranosyl Isorhamnetin-3-*O*-robinobioside and isorhamnetin-galactose-xylose (Bourogaa et al., 2011).

- *Rantherium suaveolens* Desf.

Rantherium species have been used by the local population in the production of cheese and in folk medicine as an antidiuretic (Hamia et al., 2013). Five kinds of ceramides was isolated from the aerial parts of *Rantherium suaveolens* and characterized by spectroscopic and chemical methods (Oueslati et al., 2004). Ceramides are key sphingolipid metabolites, having clinical

potential as therapeutics. They play the role as second messengers for different cellular processes mainly cell cycle arrest, differentiation, senescence, apoptosis. Moreover, it has been previously reported the isolation of polyacetyleneic alcohols, terpenoids, and coumarins from extracts of *R. suaveolens* (Oueslati et al., 2006).

1.8. Objectives and outlines of dissertation

Obesity is caused by the accumulation of lipid through adipogenesis in adipose tissue, and adipogenesis is the process by which undifferentiated precursor cells differentiate into fat storage cells. Excess size and number of adipocytes are known to be a hallmark of obesity; therefore, the inhibition of adipocyte differentiation is one of the strategies for the treatment of obesity. This current study attempted to investigate the beneficial effects of halophytes extracts on obesity *in vitro* using 3T3-L1 cell line model and based on the ability of these cells to differentiate from pre-adipocytes to mature adipocytes. Adipocyte differentiation is a multifaceted process involving synchronized expression of specific genes associated with each phase of adipogenesis. Thus, dysregulation of transcription factors (*CEBP-α*, *PPAR-γ*) and functional impairment of adipocyte differentiation might be appropriate targets for preventive intervention against obesity. Therefore, the effect of the most effective halophytes samples on this process through genes expression was evaluated. Maintaining high levels of *PPAR-γ* and *CEBP-α*, stimulates differentiation, so their regulation would be a good alternative or target for inhibition of differentiation. As artificial drugs fail to provide preferred effects and with side effects involved, the utilization of traditional and alternative medicines is fast gaining interest. Medicinal plants are believed to possess potential anti-obesity agents that can act through several mechanisms either by preventing weight gain or promoting weight loss amongst them and this may be an

excellent alternative strategy for developing future effective, safe anti-obesity medications or functional foods (Kang and Park, 2012).

In this context our aims were:

1- Investigation of an edible or medicinal halophyte extract (selected after screening) effect whether could prevent or control weight gain in C57B6J/L mice fed daily with high fat diet induced obesity.
2- Test the efficiency of this extract on transgenic *db/db* mice in order to evaluate the treatment effect of obesity-related diseases such as diabetes.

The outlines of this dissertation are summarized in the following flow chart (**Fig. 1. 1.**).

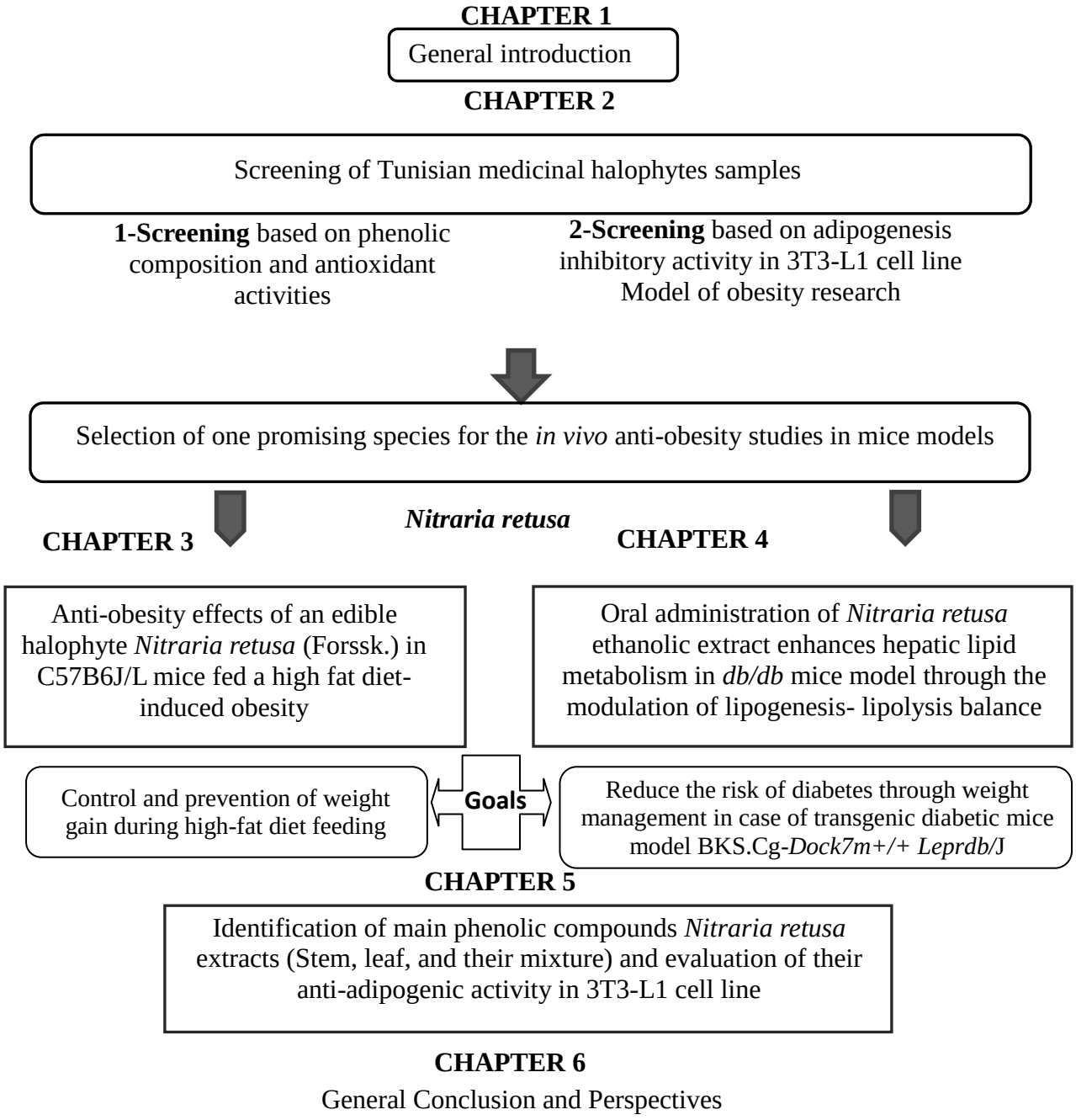

Fig. 1. 1. Flow chart of dissertation's outlines.

CHAPTER 2

Screening of medicinal halophytes based on phenolic composition, antioxidant activities and anti-adipogenic activity in 3T3-L1 cell line

2.1. Introduction

The uses of traditional medicine; during last decade, have expanded globally and are gaining popularity. The plant kingdom has become a target for the search by multinational drug companies and research institutes for new drugs and biologically active lead compounds (Li and Vederas, 2009). The world health organization (WHO) reported in 2002 that the herbal medicine serve the health need of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries (Soltan et al., 2009). Some antioxidant compounds are extracted from easy sources, such as agricultural and horticultural crops, or medicinal plants. Among those, halophytes are naturally salt-tolerant plants that may be potentially useful for economical applications as new sources of natural antioxidants in dietary food (Ksouri et al., 2009). These plants have ethno-pharmacological data indicating their utilization in folk medicine. Thus, the role of these medicinal species in the prevention or treatment of diseases has been largely attributed to their antioxidant properties associated with a wide range of bioactive molecules (Ivanova et al., 2005). The clinical importance of herbal drugs and polyphenols for treatment of diseases such as obesity and its related complications, has received considerable attention (Dave et al., 2012). The WHO defines obesity as an abnormal excessive fat accumulation detrimental to human health. Obesity has also defined as an increased adipose tissue mass, which is the result of an enlargement in fat cells and/or an increase in their number (Dave et al., 2012). These therapies have been variably efficacious on adipocyte differentiation and lipid accumulation in adipocytes. A number dietary inhibitors of adipose differentiation have been identified, including isorhamnetin (Lee et al., 2009), quercetin, resveratrol (Yang et al., 2008), naringenin (Harmon and Patel, 2001). This family of phenolic compounds are potent scavengers of free radicals and potentially useful in the prevention of cancer, arteriosclerosis and

also have been associated with several health promoting activities such as decreasing blood sugar levels and reducing body weight (Ksouri et al., 2011). The main objectives of this work were (*i*) to explore the inter-specific variability of three medicinal halophytes collected in same biotope (salt flat) on the basis of their phenolic contents and antioxidant activities, (*ii*) to screen other halophytes samples collected in different biotopes (coastal regions, salt flats, south regions) on the basis of their phenolic contents and antioxidant activities, (*iii*) to select the most promising species based on obtained results as well as on scientific reports, in order to test their efficiency on inhibiting pre-adipocyte differentiation and lipid accumulation in 3T3-L1 cells as a model in obesity research. The final goal was to focus on the ability of some selected medicinal and edible halophytes to reduce or alleviate obesity and related-complications *in vitro* or *in vivo* models.

2.2. Materials and Methods

2.2.1. Plant sampling of three halophytes collected in same biotope

Three halophytic species (*Limonium densiflorum*, *Plantago crassifolia* and *Suaeda maritima*) were screened in this study. Shoots were collected in August 2009 from "Sidi El Hani" a salt flat located at 30 kilometers southwest of Sousse and 20 kilometers east of Kairouan in Tunisia, African continent. This locality is characterized by a semi-arid climate with less rainfall < 200 mm/year and higher salinity mean (20 g/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 under refrigeration at 4° C for different analysis.

2.2.2. Preparation of plant extracts

Extracts were obtained by magnetic stirring of 3 g dry powder in 80% aqueous methanol (v/v) for 30 min. Then were kept for 24 h at 4°C, filtered through a Whatman No. 4, filter paper, and evaporated under vacuum. Then, they were stored at 4°C until analysis.

2.2.3. Plant sampling of halophytes from different locations

Arial parts of halophyte samples were collected during August 2010 from different type of biotopes. *Nitraria retusa*, *Tamarix gallica* and *Atriplex inflata*, were collected from a salt flat called "Sabkha El kelbia" (Kairouan, Tunisia) with a semi-arid climate. *Mesembryanthemum edule* sample was collected from the coastal region "Soliman" (Nabeul, Tunisia) with sub-humid climate. *Rantherium suaveolens* and *Arthrophytum scoparium* samples were collected from the south region of Tunisia (Gabes) with a Saharan climate. 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 further experiments.

2.2.4 Extraction methods

Seventy percent ethanol extraction of halophytes samples was conducted with 10% (w/v). The ethanol extracts were kept in the dark at room temperature for 2 weeks, with shaking at least once a day. The liquid fraction was then collected, filtered through 0.22 μm filter (MILLIPORE, U.S.A.), and concentrated using SpeedVac (SCRUM Inc., Japan). The dried residue was re-dissolved in seventy percent ethanol or milliQ by vortexing and stored at -80°C for further experiments.

2.2.5. Colorimetric quantification of phenolics

2.2.5.1 Determination of total polyphenol content

Colorimetric quantification of total polyphenols was determined, as described by Dewanto et al. (2002). An aliquot of 125 µL of diluted extract were added to 500 µL of distilled water and 125 µL of the Folin-Ciocalteu reagent. The mixture was shaken, before adding 1250 µL Na_2CO_3 (7%), adjusting with distilled water to a final volume of 3 mL, and mixed thoroughly. After incubation for 90 min at 23 °C in the dark, the absorbance versus prepared blank was read at 760 nm. A standard curve of gallic acid was used. Total phenolic content of organs was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g DW) through the calibration curve with gallic acid, ranging from 0 to 400 µg/mL. All samples were analyzed in triplicate.

2.2.5.2 Estimation of total flavonoid content

Total flavonoid content was measured by a colorimetric assay developed by Dewanto et al. (2002). An aliquot of suitable diluted samples of standard solution of (+)-catechin was added to $NaNO_2$ and mixed for 6 min, before adding 0.15 mL of a freshly prepared $AlCl_3$ (10 g/100 mL). After 5 min, 0.5 mL of 1 mol/ L NaOH solution was added. The final volume was adjusted to 2.5 mL with distilled water and thoroughly mixed. Absorbance was measured at 510 nm. Total flavonoid content was expressed as mg catechin per gram of DW (mg CE/g DW), through the calibration curve of (+)-catechin, ranging from 0 to 400 µg/mL. All samples were analyzed in triplicate.

2.2.5.3. Determination of condensed tannin content

The analysis of condensed tannins was carried out to the method of Sun et al. (1998). To 50 µL of appropriately diluted sample, 3 mL of 4% methanol vanillin solution and 1.5 mL of concentrated hydrochloric acid were added. The mixture was allowed to stand for 15 min, and the absorption was measured at 500 nm. Total condensed tannins amount was expressed as mg (+)-Catechin g^{-1} DW. The calibration curve range of catechin was established between 0 and 400 µg/mL. All samples were analyzed in triplicate.

2.2.6. Assessment of antioxidant activities

2.2.6.1. Evaluation of total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH (Prieto et al., 1999). An aliquot (0.1 mL) of plant extract was combined to 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a thermal block at 95°C for 90 min. After, the mixture cooled to room temperature. The absorbance of each sample was measured at 695 nm against a blank. The total antioxidant activity was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DW). The calibration curve range was 0–500 µg/mL. All samples were analyzed in triplicate.

2.2.6.2. Scavenging ability on DPPH radical

Scavenging ability of organ extracts was measured according to Hanato et al. (1988). One milliliter of the extract at known concentrations was added to 0.25 mL of a $DPPH^{\cdot}$ methanolic solution. The mixture was shaken vigorously and left standing at room temperature for 30 min in

the dark. The absorbance of the resulting solution was then measured at 517 nm and corresponded to the ability of extracts to reduce the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine. The antiradical activity was expressed as IC_{50} (µg/mL), the extract dose required to cause a 50% inhibition. A lower IC_{50} value corresponds to a higher antioxidant activity of plant extract. The ability to scavenge the DPPH radical was calculated using the following equation (1):

$$DPPH^{\cdot} \text{ scavenging effect (\%)} = [(A0-A1)/A0]*100 \quad (1)$$

Where, A0 and A1 are the absorbance values of the control and of the sample at 30 min, respectively. All samples were analyzed in triplicate.

2.2.6.3. Iron reducing power

The capacity of plant extracts to reduce Fe^{3+} was assessed by the method of Oyaizu (1986). Methanol extract (1 mL) was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide, and the mixture was incubated at 50 °C for 20 min. After that, 2.5 mL of 10% trichloroacetic acid were added, and the mixture was centrifuged at 650×*g* for 10 min. The upper layer fraction (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of ferric chloride and thoroughly mixed. The absorbance was measured at 700 nm and ascorbic acid was used as a positive control. A higher absorbance indicates a higher reducing power. EC_{50} value (mg/mL) is the effective concentration giving an absorbance of 0.5 for reducing power and was obtained from linear regression analysis.

2.2.7. Cell culture

Murine 3T3-L1 preadipocytes (Riken Tsukuba japan) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin

(5000 μg/mL)-streptomycin (5000 IU/mL) in 75-cm^2 tissue culture flasks. Medium was changed every 3 days and cell passage was carried out at 80% confluence at one on two ratio using 0.25% trypsin (1 mM EDTA). 3T3-L1 cells were cultured in a humidified incubator at 37° C and 5% CO_2.

2.2.8. Treatment of halophyte extracts on 3T3-L1 cells, pre-adipocytes differentiation and oil-Red-O staining procedures

3T3-L1 pre-adipocytes were seeded into 96-well plates at 1.0 x 10^4 cells/well, and cultured for additional two days until full confluence. Two days later (Day 0), cells were incubated with a differentiation cocktail (MDI) containing 1/10 insulin solution, 1/10 dexamethasone solution and 1/10 3-isobutyl-1-methylxanthine solution in standard culture medium for 3 days followed by additional 48 h with standard culture medium containing insulin alone, which will be changed every 2 days until full differentiation of pre-adipocytes into adipocytes. To investigate the effect of halophyte extracts; *Nitraria retusa* (NR), *Tamarix gallica* (TG), *Atriplex inflata* (AI), *Mesembryanthemum edule* (ME), *Arthrophytum scoparium* (AS) and *Rantherium sueavelons* (RS); on adipogenesis in 3T3-L1; 25, 50, 100, 200 and 400 μg/mL doses of each extract were added to the differentiation-induction and differentiation-maintenance media. The staining procedure was conducted according to the adipogensis assay kit (Cayman chemical company). The absorbance was read at 490 nm with a 96-well plate reader. The lipid droplet content was reported as percentage of control cells, and isorhamnetin-treated cells were used as positive control.

2.2.9. Cell proliferation assay (MTT assay)

Cell proliferation was investigated by MTT (3-(4-5-Dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay. 3T3-L1 cells were seeded in 96-well plates at 1×10^5 cells/mL. After incubation for 24 h, 48 h and 72 h (Pre-adipocytes) and 7 days (adipocytes) halophytes samples, (NR, TG, AI, ME, AS and RS), diluted in medium was added at final concentrations of 25, 50, 100, 200, 400 µg/mL. MTT was added after treatment for 24, 48, 72 h and 7 days, and the resulting formazan was completely dissolved by 100 µL of 10% sodium dodecyl sulfate (SDS) for 24 h. The absorbance was determined at 570 nm in a multi-detection microplate reader (Power-scan HT, Dainippon Pharmaceutical, NJ, USA). Absorbance caused by the ability of the sample to reduce MTT or by its color, was corrected using plates as blanks, prepared in the same conditions in the absence of cells.

2.2.10. RNA isolation from 3T3-L1 adipocytes and real-time-PCR analysis

Liver samples were prepared (50 mg) and homogenized with a Polytron homogenizer. Then total RNA was purified using the ISOGEN kit (Nippon GeneCo. Ltd., Japan) following the manufacturer's instructions. 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). The expression of Peroxisome proliferator-activated receptor gamma (*PPAR-γ1*), *CCAT*/enhancer binding protein alpha (*CEBP-α*), Lipoprotein lipase (*LPL)*, Fatty acid synthase (*FAS*); 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 µL reaction mixtures containing: 10 µL of TaqMan Universal PCR Master Mix UNG (2X), 9 µL of template c-DNA (100 ng μL^{-1}) and 1 µL 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.

2.2.11. Statistical analysis

All experiments were repeated at least three times. Data were expressed as the mean ± 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.

2.3. Results

2.3.1. Inter-specific variability of three medicinal halophytes collected in same biotope (Salt flat) on the basis of their phenolic contents and antioxidant activities.

Based on the absorbance values after reaction with Folin–Ciocalteu reagent, results of the colorimetric analysis are given in **Table 2. 1**. The amount of total polyphenol was species-dependent. *Limonium densiflorum* displayed the highest polyphenol content, flavonoids content as well as the condensed tannin content; 66.80± 1.83, 18.74± 0.64 and 42.43± 1.52, respectively. These results reveal the richness of *L. densiflorum* in phenolics compared to the other 2 species, despite their presence in the same biotope and undergo almost the same environmental conditions (semi-arid climate).Concerning the antioxidant capacity evaluated by 3 complementary tests (TAA, DPPH, Fe reducing power), *L. densiflorum* was also the most

efficient species as compared to the 2 other halophytes (**Table 2. 2.**). The superiority of *L. densiflorum* may be related to the higher phenolic content and quality of this species.

2.3.2. Inter-specific variability of six halophytes collected in different biotopes (coastal region, salt flats and south regions on the basis of their phenolic contents and antioxidant activities.

Results of phenolic contents and antioxidant activities of six halophytes collected in different biotopes are summarized in **Table 2. 3.** and **Table 2. 4**. It was clearly demonstrated that the environmental factors and climate changes affected the variability of halophytes on the basis of their phenolic contents and antioxidant activities. These results revealed also a positive correlation between the polyphenols amounts and the antioxidant activities. However it was also reported in many studies that the biological activities depend not only on the quantity of bioactive components but also in many cases depend on the quality or the chemical structure of these metabolites.

2.3.3. Effect of halophyte samples on cell differentiation and lipid droplet formation in 3T3-L1 cells

Adipogenesis assay was performed to investigate the effect of halophyte samples on the adipocyte differentiation and on the lipid droplets accumulation in 3T3-L1 cells using Oil red O staining. Differentiated 3T3-L1 cells were treated every two days with different halophyte extracts at various concentration and with 25 μM isorhamnetin (as a positive control), for 7 days. Based on Oil-red-O content quantification, results showed that *Nitraria retusa* sample (NR) treatments at 25, 50, 100, 200 and 400 μg/mL, in 3T3-L1 cells could inhibit the lipid droplet accumulation compared to untreated cells, in dose dependent manner (**Fig. 2. 1. A.**). The

triglyceride accumulation significantly decreased to 76.60 ± 7.30 %, 69.08 ± 8.08 %, 62.90 ± 4.80 %, 46.80 ± 7.50 %, and 42.70 ± 2.10% respectively (**Fig. 2. 1. A.**). NRE treatments at doses of 200 and 400 µg/mL, showed a similar strong effect as isorhamnetin (25 µM). Moreover, we noticed that NRE treatment in 3T3-L1 was accompanied by modulation of cell hypertrophy rather than cell hyperplasia as indicated in microscopic observation. Thus NRE treatment might induce the cell differentiation into smaller adipocytes compared to untreated cells. Another sample showed a very interesting data in increasing the lipid accumulation in dose dependent manner, is *Tamarix gallica*. Its ethanolic extract (TG) exhibit a strong anti-adipogenic activity leading to highly significant reduction in lipid accumulation within cells expressed by a low Oil-Red content as shown in **Fig. 2. 1. B.**. Regarding the rest of samples, results showed that not all these samples are effective in reducing the lipid content within cells. Only some determined high concentrations of certain samples were shown to inhibit lipid droplets accumulation within 3T3-L1 cells. In addition, results of *Arthrophytum scoparium* (AS), *Rantherium suavealens* (RS), *Mesembryanthemum edule* (ME) and *Atriple inflata* (AI) halophytes samples did not show dose-dependent effect **(Fig. 2. 2.)**. Therefore, it is necessary to correlate the obtained results with the MTT assay results in next section.

2.3.4. Effect of halophyte samples on cell proliferation and viability

Observation of the cell morphological changes of 3T3-L1 adipocytes after treatment with different doses of *Nitraria retusa* extract (NR), revealed a decrease in cell size especially in cells treated with high concentrations without affecting cell viability of differentiated 3T3-L1 adipocytes after 7 days (**Fig. 2. 3. A.**). The higher doses of NR (100, 200 and 400 µg/mL), slightly decrease cell viability of pre-adipocytes after 48 h of treatments (**Fig. 2. 5. A.**) .However, *Tamarix gallica* extract (TG) treatment was shown to inhibit cell proliferation in dose dependent

manner in both differentiated adipocytes (**Fig. 2. 3. B.**) and pre-adipocytes (**Fig. 2. 5. B.**). Thus, NR and TG extracts act, finally, with different ways but have a similar effect; which is the reduction of lipid accumulation within cells. Concenring other samples of halophytes; AS, RS, ME and AI, their ethanolic extracts did not significantly affect the cell viability and cell proliferation of 3T3-L1 cells as shown in **Fig. 2. 6.** and **Fig. 2. 7.**, respectively.

2.3.5. Cell viability and cell number by Flow cytometry (Via-Count)

To understand whether the proliferation inhibition effect was due to cell death or growth inhibition, we determined the cell number and cell viability after treatment with either *Nitraria retusa* extract or *Tamarix gallica* extract. Results showed that both extracts treatments didn't affect the cell viability compared to the control (**Fig. 2. 9. B.** and **C.**). However the cell number was decreased by around 20% after *Tamarix gallica* extract treatments (50 µg/mL and 100 µg/mL), and was increased by around 50% and 100% respectively for 200 µg/mL and 400 µg/mL of *Nitraria retusa* extract (**Fig. 2. 9. E.** and **F.**). The positive control used for this study (Isorhamnetin at 25 µM) increase little cell viability (**Fig. 2. 9. A.)** and cell number (**Fig. 2. 9. D.**).

2.3.6. Effect of NR and TG treatments on the expressions of genes related to 3T3-L1 differentiation and lipid accumulation

Gene expressions showing the effect of *Nitraria retusa* extract (NR) and *Tamarix gallica* extract (TG) on the genes associated with fat differentiation and lipogenesis were analyzed by real-Time PCR. The *PPAR-γ* mRNA expression and *CEBP-α* mRNA expression were significantly induced by NR treatments (200, 400 µg/mL). Their target genes *FAS*, *LPL* and *SREBP-1c* were also up-regulated for both concentrations (200 and 400 µg/mL) (**Fig. 2. 10.** and **Fig. 2. 11.**). These data

could be related to the increasing of cell number caused by these two doses of NRE which lead to the induction of differentiation into smaller adipocytes. However, TGE treatments showed different effects especially for *PPAR-γ* which was down-regulated by the high dose (100µg/mL) causing the down-regulation of their target genes *FAS* and *LPL* (**Fig. 2. 10.** and **Fig. 2. 11.**). *CEBP-α*, a gene with anti-mitotic and anti-proliferative effect, its mRNA expression was highly overexpressed by TG treatment which could explain or reflect the growth inhibition of 3T3-L1 cells leading to the inhibition of cell differentiation and lipid accumulation.

2.4. Discussion

The inter-specific variability illustrated by phenolic amount showed the importance of endogenous factors on the distribution of phenolics. Indeed, the work of Ksouri et al., (2008), on some halophytic species, has shown the involvement of intrinsic factors on the biosynthesis of these molecules. In addition, Djeridane et al., (2006) indicate that the secondary metabolites are more variable in plants of different species (inter-specific variability) than in plants of the same species (intra-specific variability). The superiority of *L. densiflorum* on antioxidant activities may be related to the higher phenolic content and quality of this species. In fact, Ozgen et al., (2008) displayed a considerable variation in phenolics correlated to antioxidant properties of pomegranate cultivars from Turkey, with significant positive correlations (ranged from 0.82 to 0.96) between antioxidant capacity (FRAP and TEAC), total phenolic (TP) and total anthocyanin (TMA). Our data demonstrated a great inter-specific variability on antioxidant capacities which was most probably, determined by genetic factors as proposed by others studies (Reynertson et al, 2008; Sousa et al., 2006). Evaluation of phenolic contents and antioxidant activities of different halophytes species collected from different locations with different environmental and climatic factors, showed a very big variability dependent on the species and probably their

mechanisms of salt or drought tolerance. These results revealed also a positive correlation between the polyphenols amounts and the antioxidant activities. However it was also reported in many studies that the biological activities depending not only on the quantity of bioactive components but also in many cases depend on the quality or the chemical structure of these metabolites (Balasundram et al., 2006). In other hand, the anti-adipogenic activity was variable and dependent on species and probably correlated with their richness on polyphenols either on the quantity or on the quality. Two different species (NR and TG) showed a strong activity on inhibiting the 3T3-L1 cell differentiation and lipid accumulation by regulating either size or number of cells. Excess size and number of adipocytes are known to be a hallmark of obesity; therefore regulation of the malfunction of the cellular metabolism, and the impairment of adipocyte differentiation might be appropriate targets for preventive interventions against obesity. In our study, *Nitraria retusa* extract could reduce the lipid accumulation in dose dependent manner by modulation of cell size or hypertrophy of adipocytes as well as cell number or hyperplasia; consequently it induced the differentiation with smaller lipid droplets compared to the control. In same regard, it has been reported that the increased number of small adipocytes and the decreased number of large adipocytes in white adipose tissues of troglitazone-treated obese rats appear to be an important mechanism by which increased expression levels of tumor-necrosis-factor-α (TNF-α) and higher levels of plasma lipids are normalized, leading to alleviation of insulin resistance (Okuno et al., 1998). In the other side, *Tamarix gallica* extract was demonstrated that could inhibit lipid formation through the inhibition of cell proliferation and cell growth by decreasing the cell number without affecting the cell viability. The differentiation of 3T3-L1 pre-adipocytes is regulated particularly by the action of *PPAR* and *CEBP* families. It was reported that their under-expression reduce the maturation of 3T3-L1

adipocytes (Rosen et al., 2002). *PPAR- γ* and *CEBP- α* are known to play a synergistic role in fat cell function and adipocyte differentiation of pre-adipocytes. Their cooperative functions lead to maintain high levels of *PPAR-γ* and *CEBP-α* expressions which stimulate consequently 3T3-L1 differentiation (Cristancho et al., 2011), so their down-regulation would be a good alternative or target for inhibition of differentiation. However, high gene expression level of *PPAR-γ* has been reported to be related to the induction of 3T3-L1 differentiation in small adipocytes by increasing their number via troglitazone action which in turn alleviates the insulin resistance in obese zucker rats (Okuno et al., 1998). This reported study is concordant with our findings concerning *Nitraria retusa* extract. In the other hand, *Tamarix gallica* extract was shown to inhibit cell differentiation due to the under-expression of adipocyte differentiation-related genes (**Fig. 2. 10.** and **Fig. 2. 11.).** It was shown that many natural products beneficially affect adipocytes during specific stages of development (Fukumitsu et al., 2008; Rayalam et al., 2008; Chen et al., 2009). Treatment with berberine elevated expression levels of the DNA binding proteins interacting with *CEBP-α* and inhibitded adipocyte differentiation (Hu et al., 2009). In 3T3-L1 cells, luteolin exhibited anti-adipogenic effects by inhibiting the transactivation of *PPAR-γ* (Park et al., 2009). In other animal studies, green tea seed oil used as supplement, suppressed expression of 3T3-L1 differentiation-related *PPAR-γ* and *CEBP-α* in adipose tissue (Kim et al., 2008). *SREBP-1c*, a transcription factor that controls fatty acid synthase, is additional regulator of adipogenesis in parallel with *PPAR-γ* and *CEBP-α* (Drira et al., 2011). In this regard, and, in many studies many compounds have been described as inhibiting adipocyte differentiation via *SREBP-1c* regulation (Kim et al., 2010). In current study , *Tamarix gallica* extract inhibit probably the growth of 3T3-L1 cells from the first two days after differentiation induction, leading to an inhibition in cell proliferation and later differentiation thus a strong reduction in lipid accumulation within cells

which was very clear in final day of full differentiation and conformed by cell number and cell viability assays. In same context, it was reported that oleuropein could inhibit differentiation during early stages, which runs parallel to clonal expansion; it inhibited DMI induced clonal expansion and delayed the cell cycle progression (Drira et al., 2011). Several studies revealed that arresting or delaying the cell cycle of 3T3-L1 cells during the first two days of differentiation decreased cell number and inhibited the differentiation rate to adipocytes (Lee et al., 2009).

2.5. Conclusion

Taken together all results, we concluded that *Tamarix gallica* extract mixture played a role in decreased pre-adipocytes proliferation for the possible anti-obesity via growth inhibition of pre-adipocytes in early stages of differentiation. In addition, this current study showed that *Nitraria retusa* extract mixture could be a potent anti-obesity agent via alleviation of lipid accumulation by increasing the number of small adipocytes leading probably to an alleviation of insulin resisitance within adipose tissue.

Table 2. 1. Variability of total polyphenol, flavonoid, and condensed tannin contents of three halophytes collected in same biotope.

Shoot sample	**Total polyphenol content (mgGAE.g^{-1}DW)**	**Total Flavonoid content (mgCE.g^{-1}DW)**	**Condensed tannin content (mgCE.g^{-1}DW)**
Limonium densiflorum	66.80± 1.83	18.74± 0.64	42.43± 1.52
Plantago crassiflolia	9.70± 0.29	6.42± 0.04	0.30± 0.05
Sueada maritima	5.93± 0.15	5.25± 0.15	0.27± 0.05

Table 2. 2. Evaluation of Antioxidant activities of three halophytes collected in same biotope.

Shoot sample	**Total antioxidant activity (mgGAE.g^{-1}DW)**	**DPPH test (IC_{50} µg.mL^{-1})**	**Fe-reducing power (EC_{50} µg.mL^{-1})**
Limonium densiflorum	98.30± 4.18	5.97± 0.64	90.00± 0.57
Plantago crassiflolia	13.50± 0.17	57.67± 2.51	1700.00± 26.45
Sueada maritima	14.31± 0.10	121.00± 6.55	3050.00± 36.05

Table 2. 3. Variability of total polyphenol, flavonoid, and condensed tannin contents of six halophytes collected from different biotopes.

Plant species	Total polyphenol content ($mgGAE.g^{-1}DW$)	Total Flavonoid content ($mgCE.g^{-1}DW$)	Condensed tannin content ($mgCE.g^{-1}DW$)
Tamarix gallica	152.80± 3.21	43.40± 1.21	123.44 ± 2.61
Nitraria retusa	42.70± 1.12	22.60± 0.91	21.87± 1.21
Mesembryanthemum edule	73.10± 0.72	45.00± 0.82	7.50± 0.07
Atriplex inflata	5.32± 0.07	2.90± 0.03	3.50± 0.02
Arthrophytum scoparium	6.80± 0.09	2.62± 0.02	3.23± 0.01
Rantherium suaveolens	58.92± 1.32	21.75± 1.11	38.82± 2.13

Table 2. 4. Evaluation of Antioxidant activities of six halophytes collected from different biotopes.

Plant species	Total antioxidant activity ($mgGAE.g^{-1}DW$)	DPPH test (IC_{50} $\mu g.mL^{-1}$)	Fe-reducing power (EC_{50} $\mu g.mL^{-1}$)
Tamarix gallica	123.70± 2.52	2.50± 0.05	82.00 ± 2.61
Nitraria retusa	45.82± 1.71	22.50± 0.02	160.00± 5.20
Mesembryanthemum edule	82.15± 2.21	21.36± 2.00	110.00± 4.31
Atriplex inflata	10.28± 0.50	97.23± 3.21	2150.00± 17.21
Arthrophytum scoparium	17.22± 0.72	109.15± 4.10	1020.00± 15.10
Rantherium suaveolens	32.19± 1.23	8.52± 0.56	180.00± 5.10

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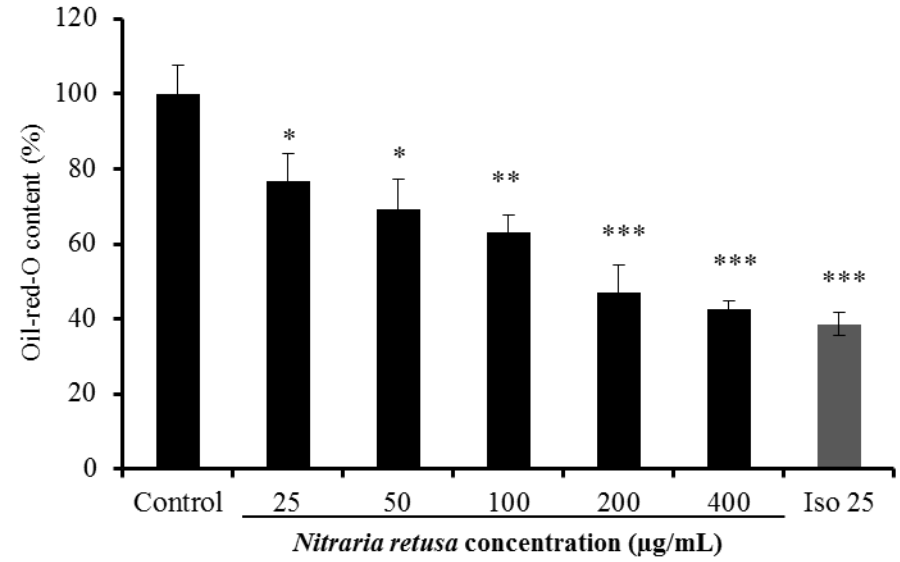

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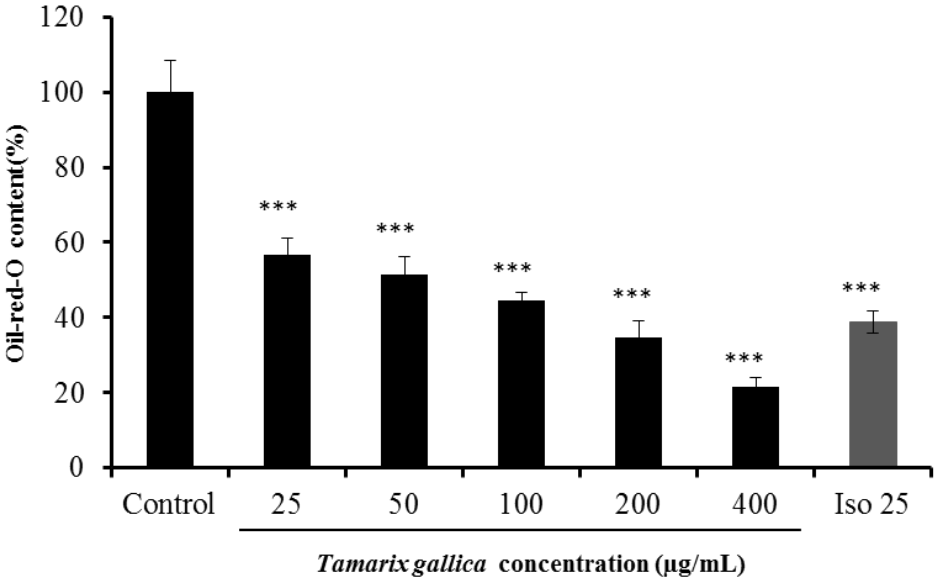


Fig. 2. 1. Effects of halophytes extracts: NR (A) and TG (B), using different concentrations (25, 50, 100, 200 and 400 µg/mL), on lipid droplet content in 3T3-L1 cells.

Fat droplets in adipocytes differentiated for 7 days with or without sample extracts and isorhamnetin at 25 µM dose (the positive control) treatments were stained with oil Red-O dye and examined used a light microscope. Relative Oil-Red-O absorbance was measured at 490 nm. Lipid droplet accumulation in treated cells was expressed as a percentage of control (untreated cells). Bars represent mean ± SD, n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control.

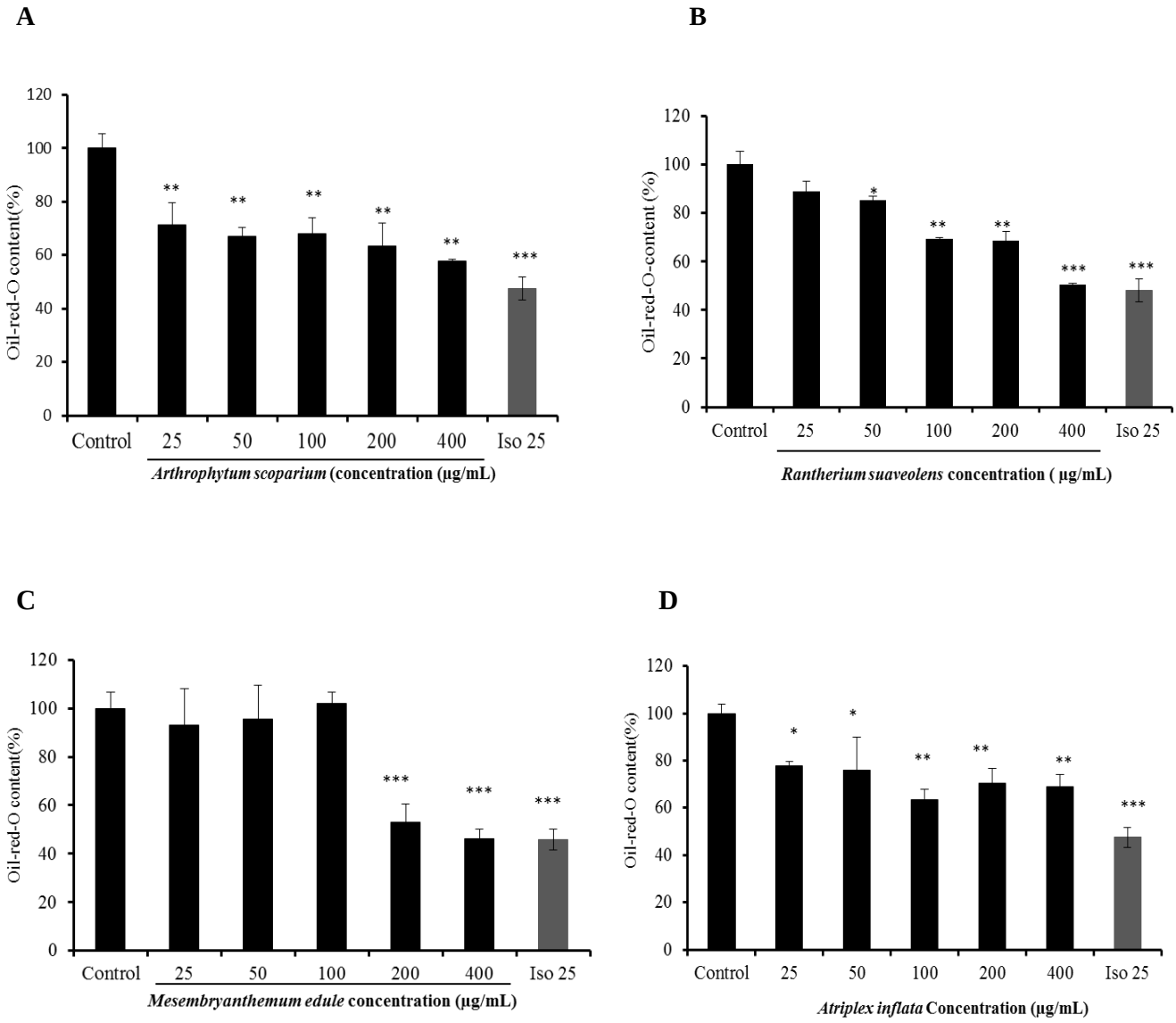


Fig. 2. 2. Effects of halophytes extracts: AS (A), RS (B), ME(C) and AI (D) using different concentrations (25, 50, 100, 200 and 400 µg/mL), on lipid droplet content in 3T3-L1 cells.
Fat droplets in adipocytes differentiated for 7days with or without sample extracts and isorhamnetin at 25 µM dose (the positive control) treatments were stained with oil Red-O dye and examined used a light microscope. Relative Oil-Red-O absorbance was measured at 490 nm. Lipid droplet accumulation in treated cells was expressed as a percentage of control (untreated cells). Bars represent mean ± SD, n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control.

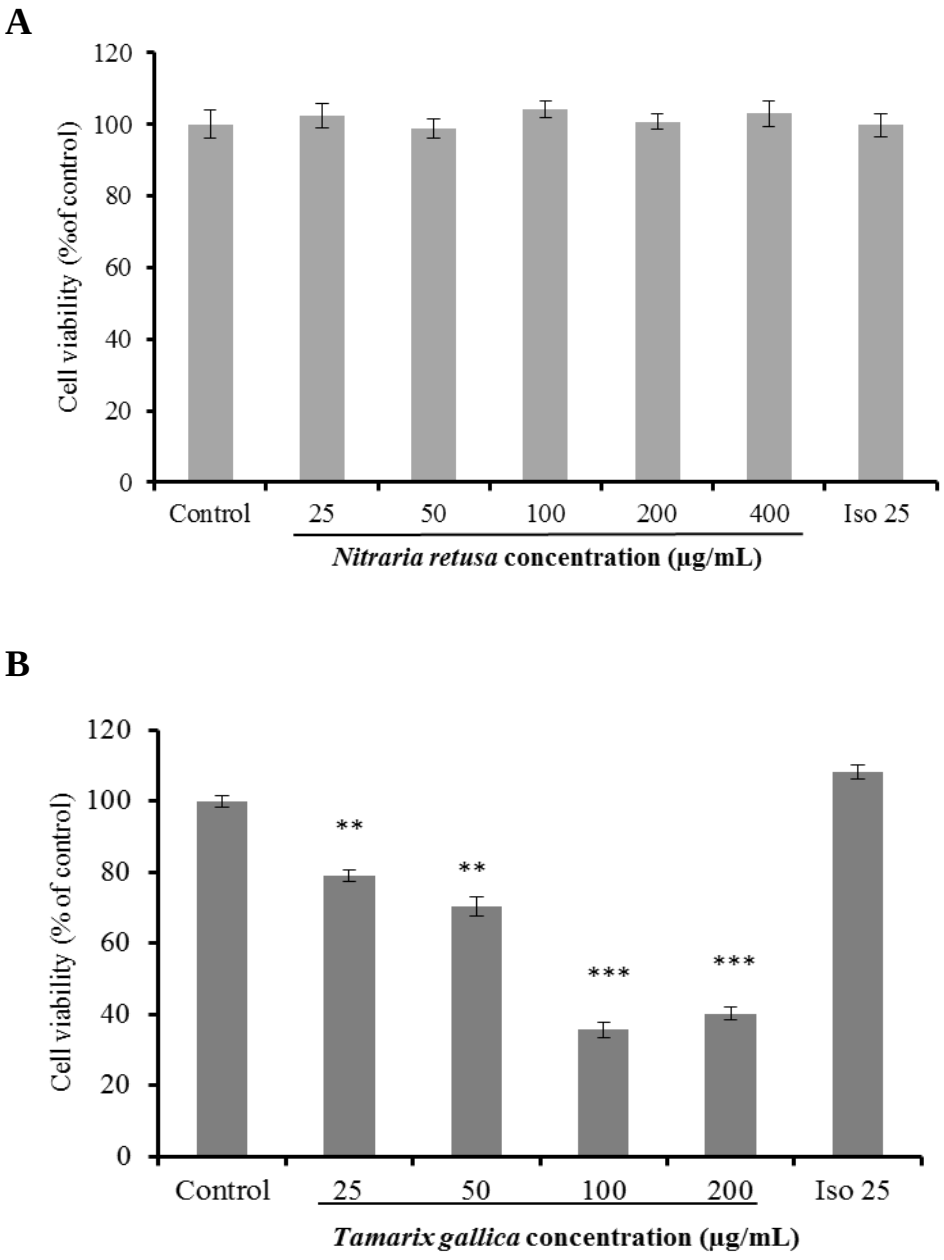
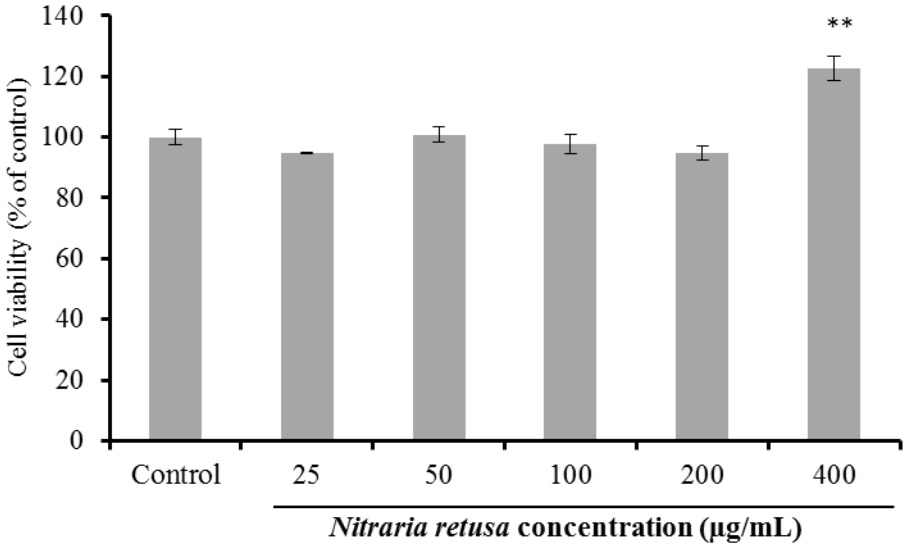


Fig. 2. 3. Effects of halophytes extracts: NR (A) and TG (B) using different concentrations (25, 50, 100, 200 and 400 µg/mL) and isorhamnetin at 25 µM dose (the positive control), on 3T3-L1 pre-adipocytes viability after cell differentiation (7 days treatment). Bars represent mean ± SD, n=3, ** $p<0.01$, *** $p< 0.001$ vs. control.

A



B

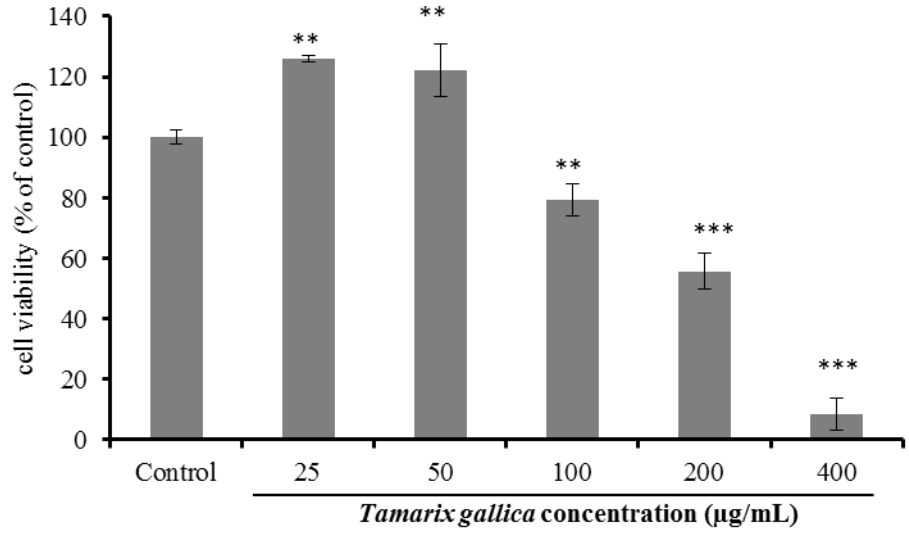
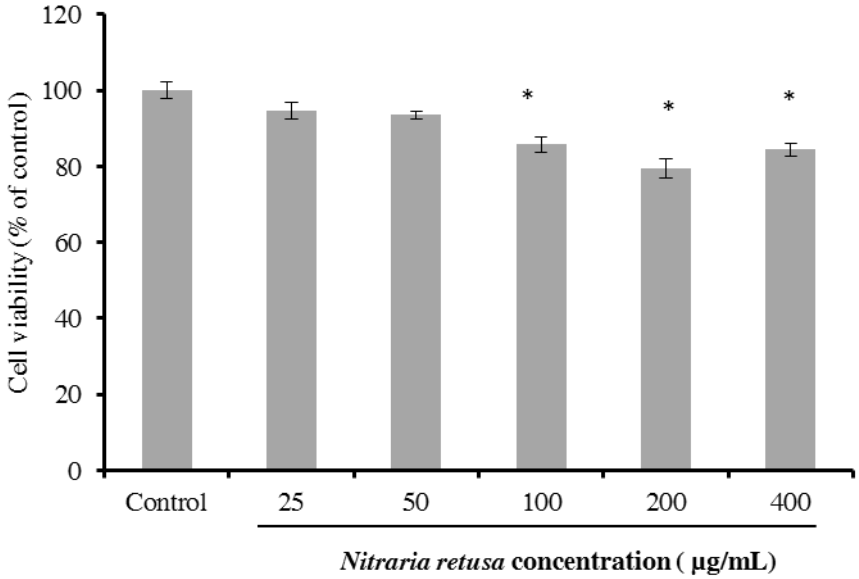


Fig. 2. 4. Effects of halophytes extracts: NR (A) and TG (B) using different concentrations (25, 50, 100, 200 and 400 µg/mL), on 3T3-L1pre-adipocytes proliferation (24 hours treatment). Bars represent mean ± SD, n=3, ** $p<0.01$, *** $p<0.001$ vs. control.

A



B

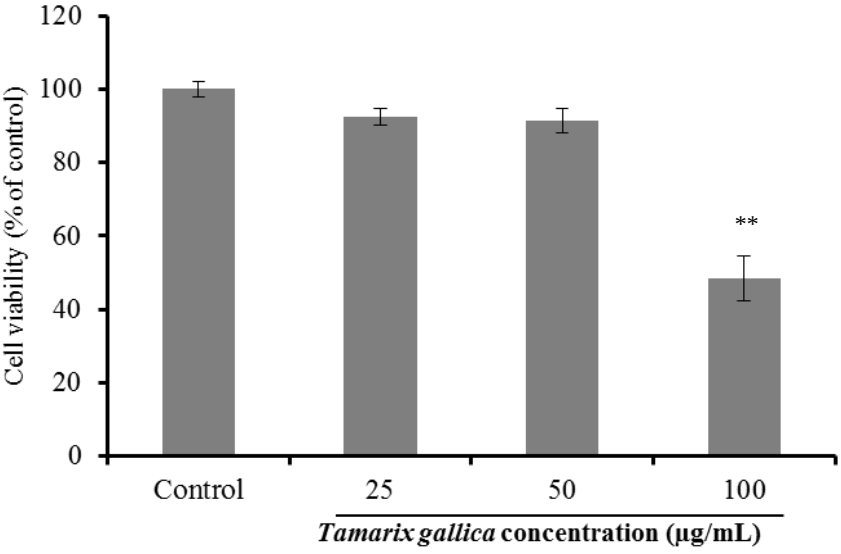


Fig. 2. 5. Effects of halophytes extracts: NR (A) and TG (B) using different concentrations (25, 50, 100, 200 and 400 µg/mL), on 3T3-L1 pre-adipocytes proliferation (48 hours treatment). Bars represent mean ± SD, n=3, * $p<0.05$, ** $p<0.01$ vs. control.

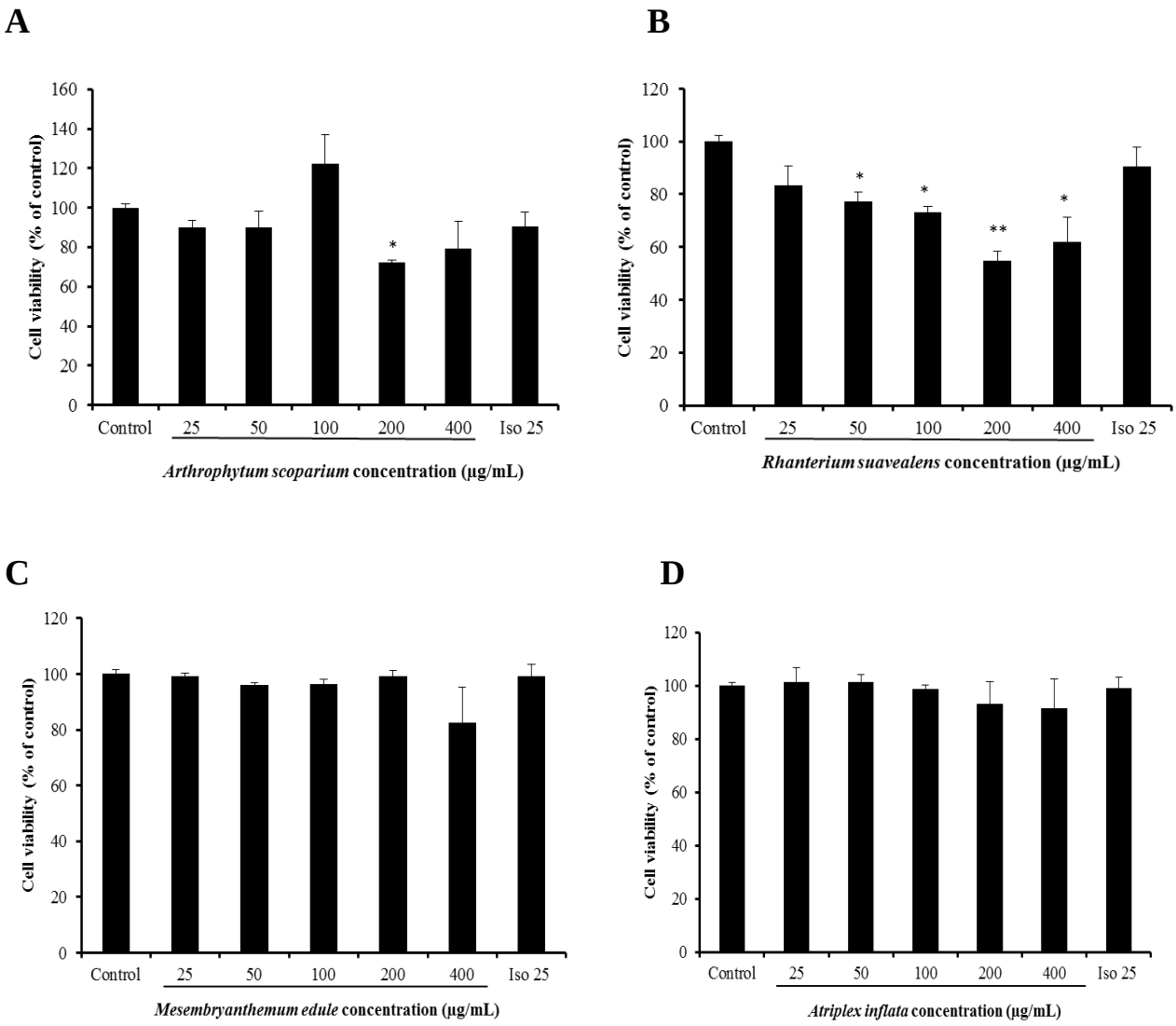


Fig. 2. 6. Effects of halophytes extracts: AS (A), RS (B), ME(C) and AI (D) using different concentrations (25, 50, 100, 200 and 400 µg/mL) isorhamnetin at 25 µM dose (the positive control), on 3T3-L1 pre-adipocytes viability after cell differentiation (7 days treatment). Bars represent mean ± SD, n=3, * $p<0.05$, ** $p<0.01$ vs. control.

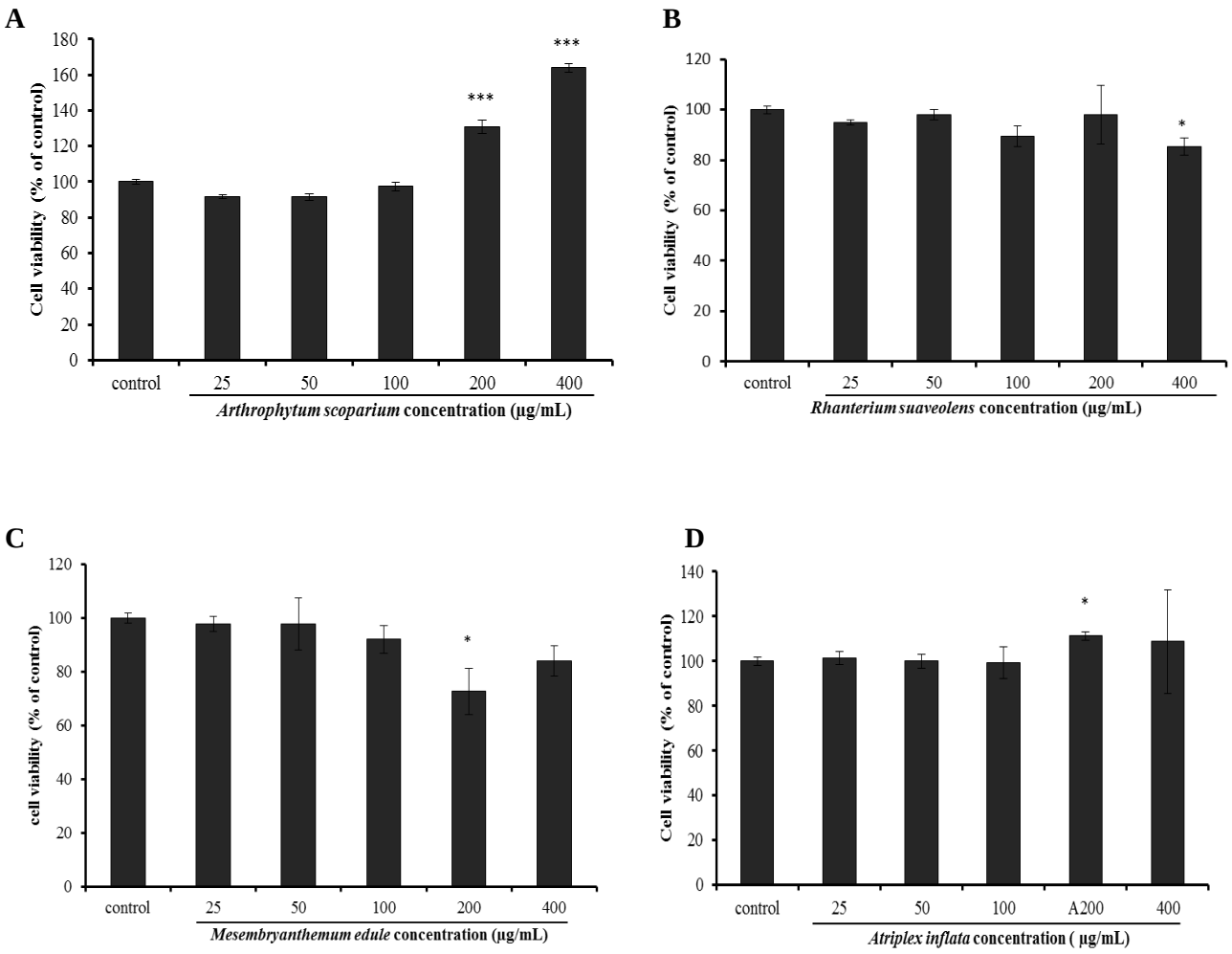


Fig. 2. 7. Effects of halophytes extracts: AS (A), RS (B), ME(C) and AI (D) using different concentrations (25, 50, 100, 200 and 400 µg/mL), on 3T3-L1 pre-adipocytes proliferation (24 hours treatment). Bars represent mean ± SD, n=3, * $p<0.05$, *** $p<0.001$ vs. control.

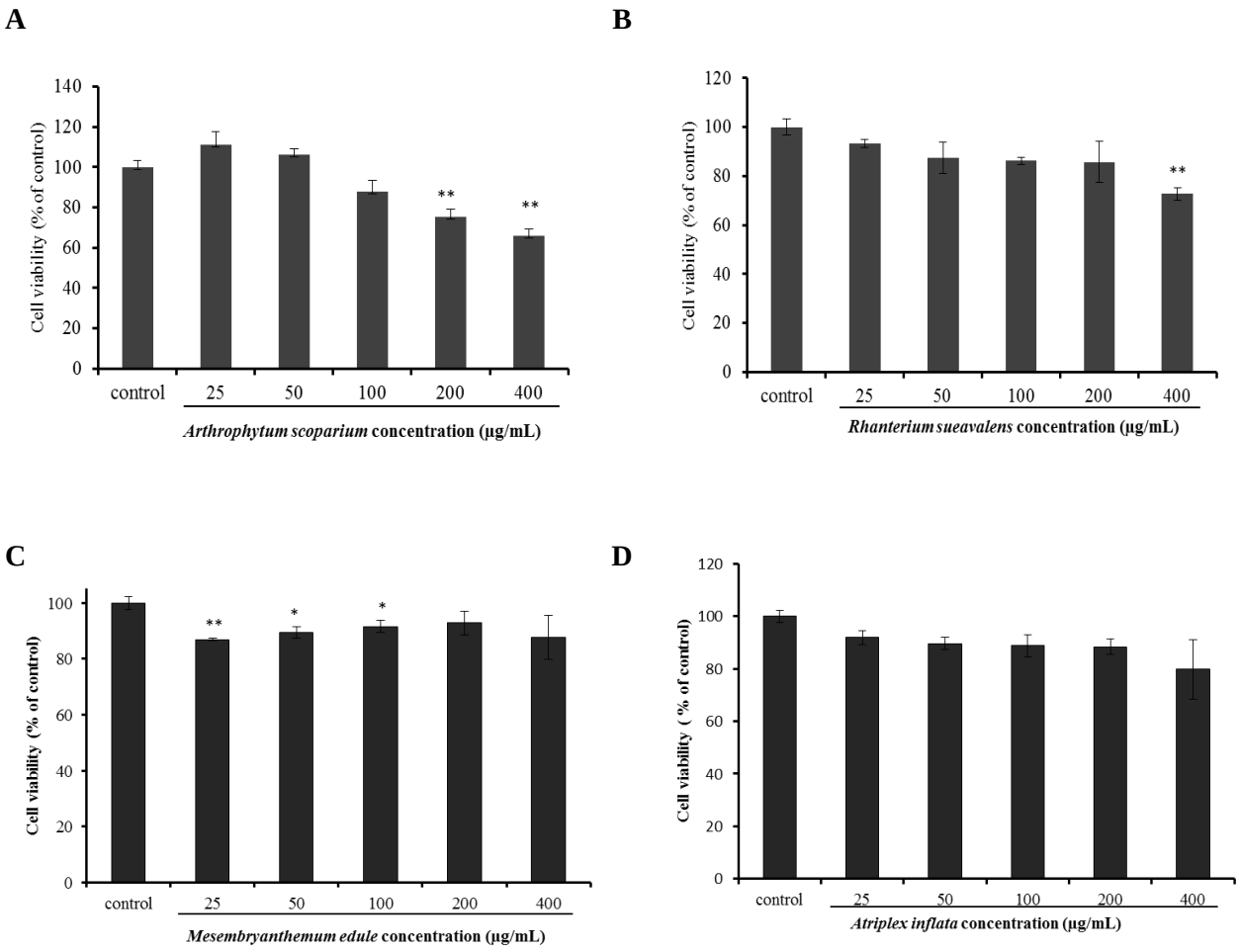


Fig. 2. 8. Effects of halophytes extracts: AS (A), RS (B), ME(C) and AI (D) using different concentrations (25, 50, 100, 200 and 400 µg/mL), on 3T3-L1pre-adipocytes proliferation (48 hours treatment). Bars represent mean ± SD, n=3, * $p<0.05$, ** $p<0.01$ vs. control.

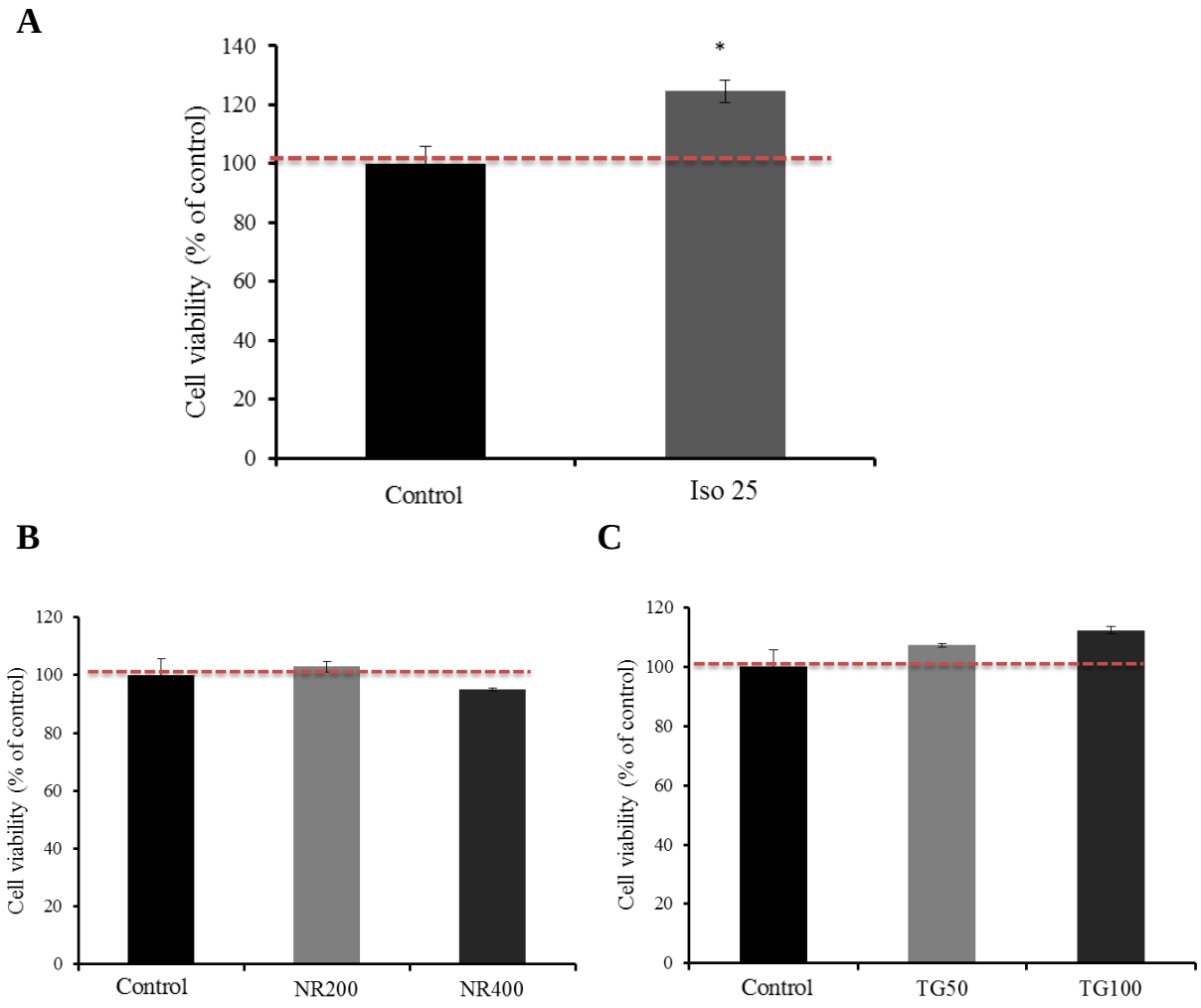


Fig. 2. 9. Effect of Isorhamnetin (Iso), *Nitraria retusa* extract (NR) and *Tamarix gallica* extract (TG) on the cell number and viability of 3T3-L1 cells. (A), (B), (C): Percentage of viability in 3T3-L1 cells treated with Iso, NR and TG, repectively, to 7 days. (D), (E), (F): Relative number of viable cells after treatment with Iso, NR and TG, repectively, after cell differentiation up to 7 days 3T3-L1 cells were treated at a final concentration of 25 µM of isorhamnetin; 50, 100 µg/mL of TG and 200, 400 µg/mL of NR . Cell number and cell viability were measured by flow cytometry (Guava ViaCount). Bars represent mean ± SD, n=3, * $p<0.05$ vs. control.

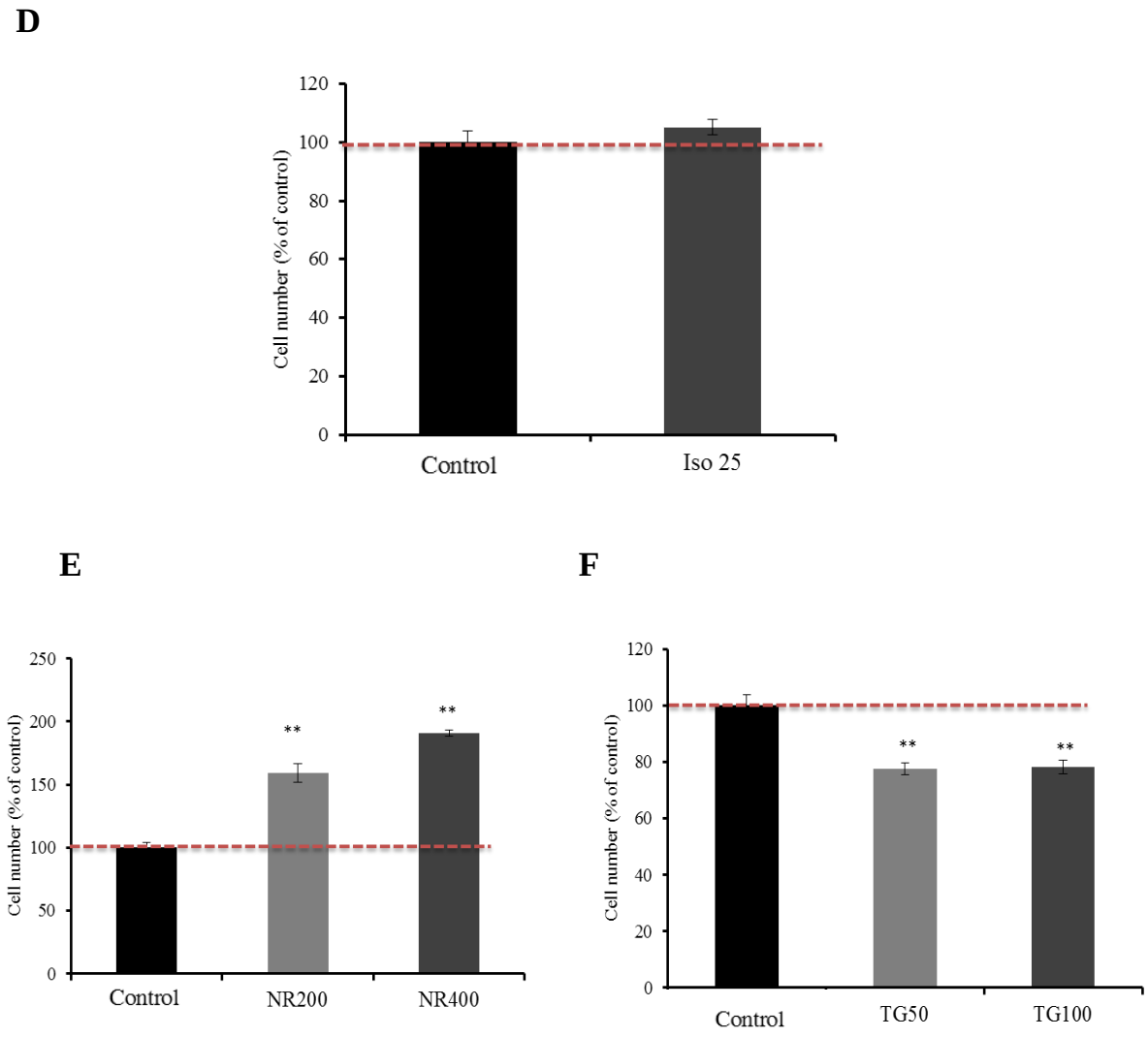


Fig. 2. 9. Effect of Isorhamnetin (Iso), *Nitraria retusa* extract (NR) and *Tamarix gallica* extract (TG) on the cell number and viability of 3T3-L1 cells. (A), (B), (C): Percentage of viability in 3T3-L1 cells treated with Iso, NR and TG, repectively, to 7 days. (D), (E), (F): Relative number of viable cells after treatment with Iso, NR and TG, repectively, after cell differentiation up to 7 days 3T3-L1 cells were treated at a final concentration of 25 µM of isorhamnetin; 50, 100 µg/mL of TG and 200, 400 µg/mL of NR . Cell number and cell viability were measured by flow cytometry (Guava ViaCount). Bars represent mean ± SD, n=3, ** $p<0.01$ vs. control.

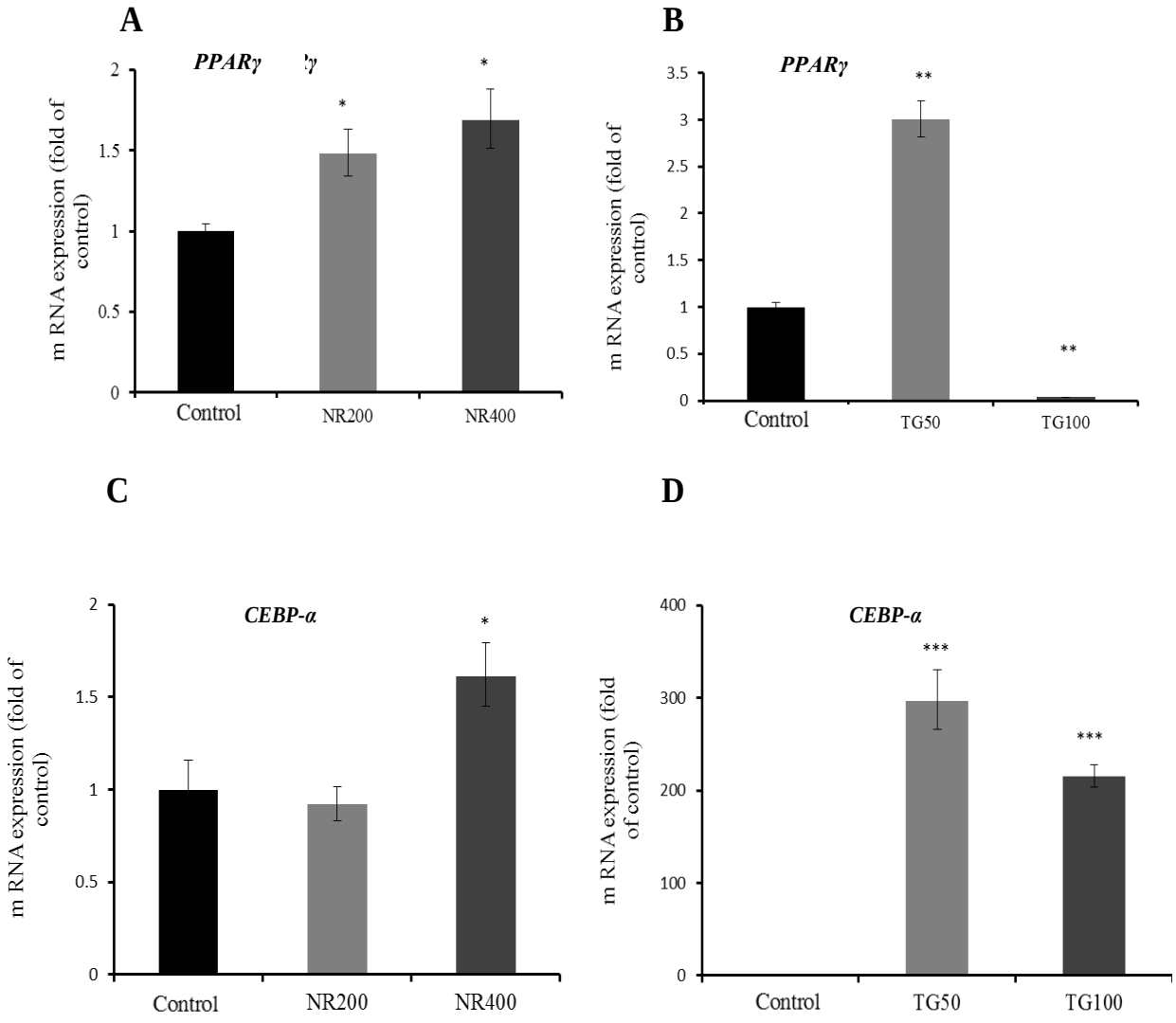


Fig. 2. 10. Effect of *Nitraria retusa* extract (NR) and *Tamarix gallica* (TG) treatments on the expressions of genes related to 3T3-L1 differentiation and lipid accumulation after 7 days of incubation. (A) Effect of NR (200, 400 µg/mL) on mRNA of *PPARγ* expression in 3T3-L1 adipocytes. (B) Effect of TG (50, 100 µg/mL) on mRNA of *PPARγ* expression in 3T3-L1 adipocytes. (C) Effect of NR (200, 400 µg/mL) on mRNA of *CEBP-α* expression in 3T3-L1 adipocytes. (D) Effect of TG (50, 100 µg/mL) on mRNA of *CEBP-α* expression in 3T3-L1 adipocytes. Bars represent mean ± SD, n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control.

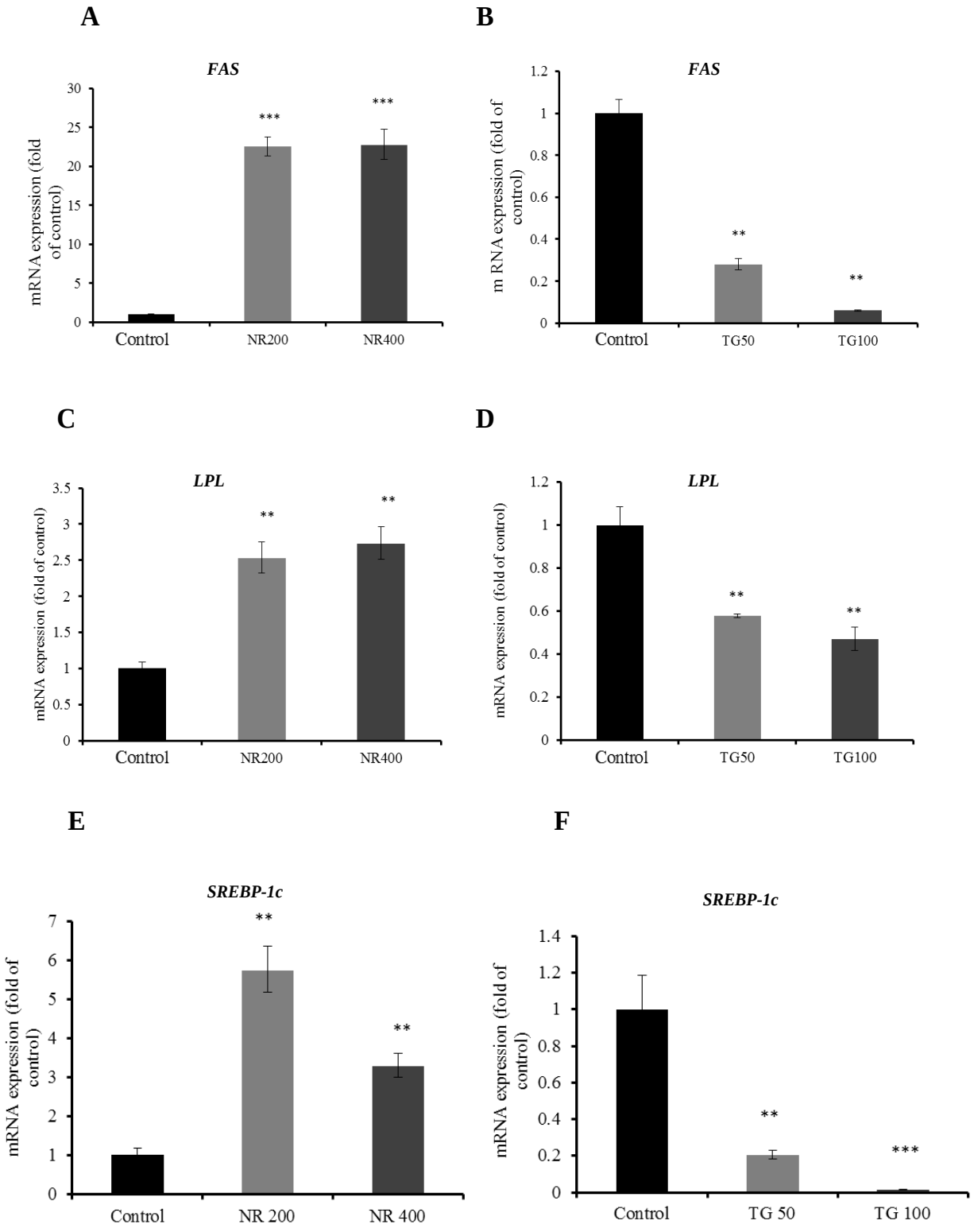

A
FAS
mRNA expression (fold of control)
30
25
20
15
10
5
0
***
***
Control
NR200
NR400
B
FAS
m RNA expression (fold of control)
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Control
TG50
TG100
C
LPL
mRNA expression (fold of control)
3.5
3
2.5
2
1.5
1
0.5
0
**
**
Control
NR200
NR400
D
LPL
mRNA expression (fold of control)
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Control
TG50
TG100
E
SREBP-1c
mRNA expression (fold of control)
7
6
5
4
3
2
1
0
**
**
Control
NR 200
NR 400
F
SREBP-1c
mRNA expression (fold of control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
***
Control
TG 50
TG 100

Fig. 2. 11. Effect of *Nitraria retusa* extract (NR) and *Tamarix gallica* (TG) treatments on the expressions of genes related to 3T3-L1 differentiation and lipid accumulation after 7 days of incubation. (A) Effect of NR (200, 400 µg/mL) on mRNA of *FAS* expression in 3T3-L1 adipocytes. (B) Effect of TG (50, 100 µg/mL) on mRNA of *FAS* expression in 3T3-L1 adipocytes. (C) Effect of NR (200, 400 µg/mL) on mRNA of *LPL* expression in 3T3-L1 adipocytes. (D) Effect of TG (50, 100 µg/mL) on mRNA of *LPL* expression in 3T3-L1 adipocytes. (E) Effect of NR (200, 400 µg/mL) on mRNA of *SREBP-1c* expression in 3T3-L1 adipocytes. (F) Effect of TG (50, 100 µg/mL) on mRNA of *SREBP-1c* expression in 3T3-L1 adipocytes.Bars represent mean ± SD, n=3, ** $p<0.01$, *** $p<0.001$ vs. control.

CHAPTER 3

Anti-obesity effects of an edible halophyte *Nitraria retusa* (Forsk.) in C57B6J/L mice fed a high fat diet-induced obesity

3.1. Introduction

Obesity is rapidly growing epidemic worldwide, presenting an increase in the risk of morbidity and mortality in many countries across the world (WHO, 2012). It has become an increasingly prevalent public health problem and represents the complex interaction of genetic, developmental, behavioral, and environmental influences (Goran, 2000). Moreover, obesity is fundamentally a problem of energy balance in that self-evidently it can develop when energy intake exceeds energy expenditure, resulting in fat accumulation and excessive adipose tissue mass. Adipose tissue, in addition to its function as the major storage depot for triglycerides, is an active endocrine tissue sensing metabolic signals and secreting hormones called adipocytokines that affect whole-body energy homeostasis (Dridi et al., 2009). Since it is an endocrine organ, it has a fundamental role in metabolism and homeostasis regulation and where numerous chemical messengers called adipokines are released for better communication. The production and secretion of an excess or insufficient amount of adipokines may provide a molecular link between increased adiposity and the development of diabetes mellitus, metabolic syndrome and cardiovascular diseases (Hidekuni et al., 2008). The main metabolic fuels of the body are glucose, fatty acids and ketone bodies. In the metabolic homeostasis of the body as a whole, the liver occupies a central position. Indeed, besides building up glycogen in its own cells, the liver plays an essential role in the synthesis of adipose tissue triglycerides, by producing very- low – density lipoproteins. Furthermore, the liver furnishes oxidizable substrates, not only to meet its own needs, but also to cover those of other tissues (Van Den Berghe, 1991). Besides, it has been reported that the liver plays an important role in modulating western diet-associated metabolic disorders. High-fat diets significantly alter the expression of many genes related to lipid, cholesterol and oxido-reductive metabolism (Radonjic et al., 2009). Nowadays, diets high in fats

tend to promote obesity; hence inhibition of digestion and absorption of dietary fats is a biological remedy in treating obesity (Gooda et al., 2012). As synthetic drugs fail to give desired effects and with side effects involved, the utilization of traditional and alternative medicines is fast gaining acceptance. Medicinal plants are believed to harbor potential anti-obesity agents that can act through various mechanisms either by preventing weight gain or promoting weight loss amongst them and this may be on excellent alternative strategy for developing future effective, safe anti-obesity drugs (Kamiya et al., 2012). Halophytes are a salt-tolerant species from salt and arid regions and desert that can tolerate a wide range of environmental conditions and resist to abiotic stresses such as salt, high temperature and luminosity, and drought stresses (Ksouri et al., 2009). In fact, able to withstand and quench these severe environmental stresses, halophytes are equipped with powerful antioxidant systems constitute mainly on phenolic compounds so called "stress metabolites". These plants have ethno-pharmacological data indicating their utilization in folk medicine. Thus, the role of these medicinal species in the prevention or treatment of diseases has been largely attributed to their antioxidant properties associated with a wide range of bioactive molecules (Ivanova et al., 2005). *Nitraria retusa* is one of the native perennial halophyte species that belong to the botanical family Nitrariaceae. It is distributed in North Africa and restricted to Algeria and Tunisia. In Tunisia, it is widespread in central and south parts. This salt-tolerant and drought resistant shrub grows along shallow and hummocks on saline grounds near the coastal areas and produces fleshy red fruits from which a tasty and refreshing juice may be extracted. *Nitraria retusa* is known in Tunisia as "Ghardaq". The sweet drupes are edible and for the treatment of hypertension. Leaves infusion and decoction are used as tea or cataplasm for their anti-inflammatory properties (Le Floc, 1952). In previous chemosystematic investigation, the flavonoids contained in *Nitraria retusa* leaves were studied;

six isorhamnetin glycosides (isorhamnetin-3-*O*-robinobioside, isorhamnetin-3-*O*-rutinoside, isorhamnetin-3-*O*-galactoside, isorhamnetin-3-*O*-glucoside, isorhamnetin 3-xylosylrobinobioside and isorhamnetin- 3-*O*-4-Rhamgalactosylrobinobioside) and free isorhamnetin were identified (El-Alali et al., 2012). These bioactives molecules isolated from *Nitraria retusa* have been reported to promote apoptosis in human myelogenous erythroleukemia cells (Boubaker et al., 2012), and to exert antioxidant and anti-mutagenic activities (Boubaker et al., 2010). To the best of our knowledge, this is the first time to report the effect of *Nitraria retusa* ethanol extract (NRE) reducing body weight in mice fed with high-fat diet in correlation with lipid metabolism in liver.

3.2. Materials and Methods

3.2.1. Plant sampling

Nitraria retusa shoots were collected during August 2010 from the salt flat "Sabkha El kelbia" located at N 35 48 44, E 10 09 06 (Kairouan, Tunisia). This locality is characterized by a semi-arid climate with less rainfall < 200 mm/year and higher soil salinity (20 g/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 further experiments.

3.2.2. Extraction methods

Seventy percent ethanol extraction *Nitraria retusa* sample was conducted with 10% (w/v). The ethanol extract was kept in the dark at room temperature for 2 weeks, with shaking at least once a day. The liquid fraction was then collected, filtered through 0.22 μm filter (MILLIPORE, U.S.A.), and concentrated using SpeedVac (SCRUM Inc., Japan). The dried residue was re-

dissolved in seventy percent ethanol or milliQ by vortexing and stored at -80°C for further experiments.

3.2.3. Animals and experimental design

Four-weeks-old, male C57B6/JL mice, were purchased from Charles River (Japan) and were maintained under a light cycle (12 h light/dark), fed with a high fat diet (HFD) or a normal diet (ND), purchased from Oriental Yeast Company (Japan) and according to the composition described in **Table 3. 1**.. After 1 week acclimatization, mice were divided randomly to 5 groups with 8 individuals for each group; Control normal diet group (CND) fed with normal diet and orally administrated with water as vehicle, control high-fat diet group fed with HFD and orally administrated with water as vehicle (CHFD), High fat diet group fed with HFD and orally administrated with naringenin as positive control well known for its activity *in vivo* assays, especially by amelioration of hepatic steatosis and attenuation of dislypedimia without affecting caloric absorption in fat-fed C57B6J/L mice (Mulvihill et al., 2009) at a dose of 10 mg/kg of body weight (HFD+PC), high-fat diet group fed with HFD and orally administrated with *Nitraria retusa* extract at 50 mg/kg of body weight (HFD+NR50) and high-fat diet group fed with HFD and orally administrated with NRE at 100 mg/kg of body weight (HFD+NR100). Body weight and food intakes were measured daily at regular intervals during the feeding period (28 days). Following 4 weeks treatment, animals were sacrificed. Blood samples were collected, liver and fat tissue were dissected, weighed and kept at -80°C until use. All procedures were performed in accordance with the Ethics Animal Care and Use Committee of the University of Tsukuba, Japan.

3.2.4. Biochemical analysis of blood serum

Collected blood samples were centrifuged at 2,000×*g* for 15 min at 4°C. Then several metabolites like: 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. The cytokine tumor necrosis factor-alpha (TNF-α) level in serum was also analyzed using the Enzyme Linked-Immuno-Sorbent Assay (ELISA) (Invitrogen Ms TNF-α kit) according to manufacturer's instructions.

3.2.5. RNA isolation from liver and real-time-PCR analysis

Fifty mg liver samples were homogenized using Polytron® PT 1200 E homogenizer (Switzerland). Then total RNA was purified using the ISOGEN kit (Nippon GeneCo. Ltd., Japan) following the manufacturer's instructions. Total RNA was quantified and quality-checked 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). The gene expression of Peroxisome Proliferator-Activated Receptor Gamma 1 (*PPAR-γ 1*), Peroxisome Proliferator-Activated Receptor Alpha (*PPAR-α*), Lipoprotein Lipase (*LPL*), Fatty Acid Synthase (*FAS*), Acetyl-CoA Carboxylase 1 (*ACC1*) and Carnitine palmitoyl transferase I (*CPT1*) were determined by real-time PCR, normalized to beta-actin and reported as fold of control. Primers and TaqMan probes used for these experiments were purchased from Applied Biosystems. Primers were inventoried gene expression assays. TaqMan real-time PCR amplification reactions were performed in a 20 μL reaction mixtures containing: 10 μL of

TaqMan Universal PCR Master Mix UNG (2X), 9 µL of template cDNA (100 ng /µL) and 1 µL of the corresponding primer/probe mix, using an AB 7500 fast real-time system (Applied Biosystems). 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.

3.2.6. Statistical analysis

All experiments were repeated at least three times. Data were expressed as the mean ± SD or the mean ± SEM. 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.

3.3. Results

3.3.1. Anti-obesity effects of NRE in HFD-induced C57B6J/L obese mice

As shown in **Fig. 3. 1.**, the HFD increased body weight gain significantly compared to ND over 4 weeks treatment period in C57B6J/L mice. Moreover, final body weight was significantly lower in the HFD+NR50 (24.60 ±0.50 g) and HFD+NR100 (24.40 ± 0.70 g) groups compared to CHFD group (27.62 ± 0.50 g) (**Table 3. 2.**) ($p<0.05$), without affecting food intake, which was around 3g/day/mice for all different experimental groups (**Table 3. 3.**). It is well known that, body weight and fat stores are determined by the net excess or deficit of food intake over energy expenditure. In the current study, NRE treatment was demonstrated not only to decrease cumulative body weight gain, but also adipose tissue weight and improve adiposity index. In fact 50 mg/kg BW and 100 mg/kg BW NRE treatment in HFD mice decreased adipose tissue weight from 1.80 ± 0.20 g in vehicle group to 1.10 ± 0.21 g and 1.20 ± 0.16 g, respectively (**Fig. 3. 2.**).

3.3.2. Effect of NRE administration on glucose, triglycerides, cholesterol and TNF-α in HFD-induced C57B6/JL obese mice

Serum glucose and lipid levels (triglycerides, total cholesterol, HDL-cholesterol (HDL-c), LDL-cholesterol (LDL-c)) and also the cytokine TNF-α level of all mice groups were analyzed. Results are summarized in **Table 3. 4.**. The HFD-fed mice showed significant high levels of serum glucose (200.00 ± 4.10 mg/dL), triglycerides (22.00 ± 1.63 mg/dL) and higher level of HDL-c (80.00 ± 1.55 mg/dL) when compared to those fed with normal diet (CND group). As shown in **Table 3. 4.**, 50 mg/kg and 100 mg/kg NRE and naringenin (positive control) treatments significantly decreased glucose levels in blood serum of HFD-fed mice to reach 168.00 ± 2.69 mg/dL, 153.00 ± 9.41 mg/dL, and 146.00 ± 1.63 mg/dL, respectively. Triglycerides levels were also significantly decreased to 10.00 ± 1.63 mg/dL and 14.00 ± 1.63 mg/dL in 50 mg/kg and 100 mg/kg NRE treated HFD-mice, respectively. However, naringenin treatment did not affect their levels. NRE administration for 4 weeks did not affect the total cholesterol in HFD-induced obese mice blood serum. However, it significantly increased HDL-c fraction (the good cholesterol) from 80.00 ± 1.55 mg/dL in vehicle group to 98.00 ± 4.10 mg/dL and 92.00 ± 4.10 mg/dL respectively for HFD+NR50 and HFD+100 groups ($p<0.05$). This cholesterol fraction in large numbers correlates with better health outcomes since transport cholesterol back to the liver for excretion. On the other hand, only 100 mg/kg NRE administration, significantly, decreased LDL-c (the bad cholesterol) from 16.50 ± 2.03 mg/dL for vehicle group to 12.00 mg/dL ($p<0.05$).

3.3.3. Effect of NRE treatment on hepatic lipid metabolism gene expression in HFD-induced C57B6/JL obese mice

High serum lipid level mainly triglycerides and cholesterol are a hallmark of many metabolic syndrome diseases such as type 2 diabetes. Understanding the molecular mechanism that

undergoes dyslipidemia should facilitate improved strategies for serum lipid management. In this respect, the effect of NRE administration in HFD-induced mice on hepatic lipid metabolism biomarkers at translational level was investigated, following 4 weeks experimental study. In fact, 50 mg/kg BW and 100 mg /kg BW NRE treatments significantly increased the gene expression of the hepatic *PPAR- γ1* (**Fig. 3. 3. a.**) to 6 and 4 folds, respectively. While, only 50 mg/kg BW NRE dose improved *PPAR-α* gene expression (**Fig. 3. 3. b.**) by 2 folds. Moreover, NRE treatments significantly modulated the lipogenic enzyme genes *ACC1* and *FAS*. In fact, *FAS* gene expression was decreased by half at 100 mg/kg BW dose (**Fig. 3. 4. a.**), and *ACC1* gene expression was increased by 2.5 folds at 50 mg/kg BW dose (**Fig. 3. 4. b.**). Additionally, 50 mg/kg NRE treatment in HFD-induced obese C57BL/6J mice significantly increased *CPT1* (**Fig. 3. 5.**) and *LPL* (**Fig. 3. 6.**) genes expression to reach 6 and 30 folds respectively. These results were concordant with weight loss, adiposity index 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 and preventing obesity.

3.4. Discussion

In the current study, NRE administration in HFD-induced obese C57BL/6J mice for 4 consecutive weeks, significantly reduced body weight gain and adipose tissue accumulation without affecting food intake. Furthermore, these effects were in concordance with a significant improvement of glucose and lipid metabolism in blood serum and the expression profiles of genes related to beta-oxidation, lipolysis and lipogenesis in the liver. These findings demonstrated that NRE suppresses obesity in HFD-induced obese mice. It is well known that obesity is caused by imbalanced homeostasis between low energy expenditure and increased energy intake and accumulation (Galgani and Ravussin, 2010). Excess energy is mainly stored as

triglycerides in adipose tissue which increase the visceral adipose tissue mass through adipocyte hypertrophy and hyperplasia (Hajer et al., 2008). Several strategies are proposed to reduce or suppress obesity, among them dietary supplements and natural products (Chandrasekaran et al., 2012). In fact, herbal and botanical preparations are gaining a lot of interest either to substitute chemical drugs or to be combined with them. In this respect, *Nitraria retusa*, an edible halophyte plant growing wild in Tunisia, could be a potent candidate. Previous phytochemical studies from our research and others demonstrated that *Nitraria retusa* has a strong anti-oxidant and free radical scavenging properties due mainly to its high contents in polyophenols and flavonoids (Hadj Salem et al., 2011). The HPLC analysis showed the presence of several alkaloids like 5, 7-dihydroxy-3-deoxy-vasiciene I, 7-hydroxy-3-deoxy1-vasiciene II and O-acetylnitraraine I (El-Alali et al., 2012). The phenolic profile showed mainly, high contents of isorhamnetin aglycone and glycosides (Sameh et al., 2009) and other flavonoids like apigenin, quercetin, kaempherol and luteolin (Pandey and Rizvi, 2009). The high content in flavonoids and their possible synergetic effect may explain in part the anti-obesity effect of NRE, since these compounds were individually demonstrated to have high potential to prevent metabolic syndrome diseases (Hsu and Yen, 2006), and their mixture showed synergetic anti-obesity effect (Yang et al., 2008; Aguirre et al., 2011; De Santi et al., 2000; Liang et al., 2003). The investigation of biochemical markers like glucose, total triglycerides, total cholesterol, LDL, HDL, and TNF α in mice blood serum, demonstrated that NRE significantly improved these parameters except for TNFα and overall data showed higher activity than naringenin, commonly used as positive control. In general, the accumulation of triglycerides in the liver is due to an imbalance between the availability of hepatic triglycerides for export and the exporting capacity of the liver via very low-density lipoproteins (VLDLs) (Tirosh et al., 2008). Furthermore, in the liver cells an

increase in glucose exerts, both directly and indirectly, a series of effects which result in the orientation of its metabolism towards glycogen synthesis, glycolysis and formation of fatty acids (Van Den Berghe, 1991). On the one hand a first direct effect of glucose is to stimulate its hepatic uptake which could be ameliorated with NRE activity on the lowering effect of the serum glucose level. On the other hand, an increase in triglycerides levels, particularly when accompanied by a decrease in high-density lipoprotein (HDL) levels, has been shown to be a surrogate marker of insulin resistance, a strong predisposing condition for type-2-diabetes (Mulvihill et al., 2009). Lipids and carbohydrates metabolism in liver is controlled by several genes. In this respect, the investigations of NRE treatment in HFD-induced obese mice on hepatic genes related to beta-oxidation, lipolysis and lipogenesis, showed a significant improvement of their expression when compared to vehicle group or naringenin treatment leading to a control effect of weight gain and fat mass. However, naringenin was reported to ameliorate hepatic steatosis and attenuate dyslipidemia, without affecting caloric absorption (Hajer et al., 2008), with an improvement of hepatic fatty-acid oxidation through *PPAR-α* coactivator 1 alpha. This study is very concordant with our current results revealing that naringenin could regulate some gene expressions related to lipid metabolism (**Fig. 3. 3.**, **Fig. 3. 4.** and **Fig. 3. 5.**) and ameliorate the serum lipid profile (**Table 3. 4.**) of mice fed a high fat diet but without any suppression effect on body weight gain (**Fig. 3. 1.** and **Table 3. 2.**). Fatty acid metabolism in the liver involves three main pathways; catabolism by *β*-oxidation, synthesis, from acetyl CoA and esterification into triglycerides. Herein in our study, NRE administration in HFD- fed mice significantly over-expressed *PPAR-α* by 2 folds increase and promoted fatty acid *β*-oxidation via overexpression of *CPT1* gene, by 6 folds. In fact, *PPAR-α* are homogenous group of genes that participate in lipid catabolism such as fatty acid uptake through membrane, fatty

acid binding in cells, fatty acid oxidation and lipoprotein assembly and transport (Hihi et al., 2002). *PPAR-γ1* are known to influence the storage of fatty acids in the adipose tissue (Maglich et al., 2009), but its mRNA expression is detected at lower level in liver. This in turn could be one of other factors (period of high-fat diet feeding, etc…) affecting its expression in liver of mice fed with HFD compared to those fed with ND (**Fig. 3. 3. a.**). *PPAR-α* activation is known to mediate the expression of genes promoting fatty acid *β*-oxidation mainly *CPT1* gene which is the encoding gene of carnitine palmitoyltransferase system which is a critical and essential step in the *β*-oxidation of long chain fatty acids (Flanagan et al., 2010). Such cascade of molecular events will lead finally to lowering the circulating fatty acids and triglycerides-rich-lipoproteins (Wakil et al., 2009). Furthermore, NRE administration in HFD-fed mice significantly increased *ACC1* gene expression encoding for the lipogenic enzyme Acetyl-CoA Carboxylase which is an attractive target for therapeutic in the control of obesity (Yamamoto et al., 1995). However, NRE administration slightly decreased *FAS* gene expression encoding for the fatty acid synthase at 50 mg/kg dose. Such effect demonstrates that NRE administration might not negatively affect the fatty acid metabolism since there was an enhancement of *CPT1* expression in the liver of NRE-treated mice. In our case, gene expressions of lipogenic enzymes were analyzed after an experimental period of 4 weeks. It was already known that, *FAS* gene expression could be influenced by the period of high-fat diet feeding. Actually, there is a difference on its expression depending on the short term or long term of feeding. Actually, in 1 week and 2 weeks FAS gene expression could increase but in long term feeding, an adaptation to excess fat accumulation will occur and *β*-oxidation rate will increase. Thus, *FAS* expression will decrease or return to normal level as control (Nishikawa et al., 2012). Concerning the gene expression of *LPL* that encodes the enzyme responsible for the hydrolysis of triglycerides in lipoproteins, and its effect on the

plasma cholesterol level. Results showed that the oral administration of NRE in mice fed with high fat diet had a highly significant overexpression of *LPL* more than 30 fold. In this regard, it has been demonstrated that *LPL* overexpression prevents the development of diet-induced hypertriglyceridemia and hypercholesterolemia and decreases VLDL and LDL fractions levels in LPL transgenic mice after cholesterol loading (Wang and Eckel, 2009). These findings suggest that *LPL* plays an important role in determining cholesterol levels. Furthermore, it has been also highlighted that free fatty acids uptake into adipocytes is also facilitated by the extracellular expression and activity of lipoprotein lipase (Wang and Eckel, 2009). *LPL* activity changes dramatically in various tissues in response to energy requirements and its systemic overexpression results in increases in whole body insulin sensitivity (Wang and Eckel, 2009).

Our study demonstrated that NRE treatment in HFD fed mice significantly ameliorated the hepatic gene profile expression involved in lipid homeostasis. In this respect, several herbal preparations cited in the literature showed similar effects. Flavonoids like isorhamnetin (Lee et al., 2010), naringenin (Yoshida et al., 2010) and quercetin (Ahn et al., 2008), individually or mixed showed significant reduction in obesity and type-2-diabetes incidence.

3.5. Conclusion

Taken together, our results demonstrated that NRE treatment *in vivo* levels; exerts anti-obesity action through lowering glucose and triglycerides, and the enhancement of the lipid metabolism in liver due to the increasing of serum HDL-cholesterol, the decreasing of LDL-cholesterol modulating the gene expression related to lipid metabolism. This effect may be due to the improvement of the antioxidant status within hepatic cells by the strong antioxidant activities of many phenolic components present in NRE especially flavonoids such as isorhamnetin aglycones and glycosides. Thus, the identification of possible active compounds and

standardization of NRE may provide an opportunity to develop a novel class of anti-obesity supplement or functional food. Further investigations will be needed in order to evaluate NRE anti-obesity bio-active molecules efficacy and their bio-availability.

Table 3. 1. Composition of experimental diets.

Ingredient	**Normal diet (ND) (%)**	**High fat diet (HFD) (%)**
Casein	18.5	25.6
L-cystine	0.28	0.36
Maltodextrin	29.01	6.01
α-Corn starch	9.67	16.00
Sucrose	2.00	5.50
Soybean oil	25.00	2.00
Lard	2.00	33.00
Cellulose	6.61	6.61
Mineral mix AIN 93G	3.50	3.50
Calcium carbonate	0.18	0.18
Vitamin mix AIN93	1.00	1.00
Choline bitartrate	0.25	0.25
Total	100	100

Table 3. 2. Body weight in ND, HFD, HFD+PC, HFD+NR50 and HFD+NR100 groups for 4 weeks.

	CND	CHFD	HFD+PC	HFD+NR50	HFD+NR100
			Body weight (g)		
Initial	19.12 ± 0.40	19.18 ± 0.30	18.93 ± 0.60	19.40 ± 0.40	19.60 ± 0.40
Final	23.25± 0.40*	27.62 ± 0.50	26.64 ± 0.20	24.60 ±0.50*	24.40 ±0.70*

Data represent the mean ± SEM, * $p<0.05$, when compared to the CHFD group (n=8 per group)

CND: Control Normal diet fed group

CHFD: Control High-fat diet fed group

HFD+PC: High-fat diet + positive control (Naringenin 10 mg/kg body weight)

HFD+NR50: High-fat diet + *Nitraria retusa* 50 mg/kg body weight

HFD+NR100: High-fat diet+ *Nitraria retusa* 100 mg/kg body weight

Table 3. 3. Food intake (g/day) and Food efficiency ratio (FER) in CND, CHFD, HFD+PC, HFD+NR50 and HFD+NR100 groups for 4 weeks

	CND	**CHFD**	**HFD+PC**	**HFD+NR50**	**HFD+NR100**
Food intake (g)	3.25 ± 0.10	3.12 ± 0.10	3.00 ±0.10	3.25 ± 0.20	3.06 ± 0.10
Food efficiency ratio (FER)	1.26 ± 0.07	2.70 ± 0.10	2.42 ± 0.20	1.60 ±0.07***	1.56 ± 0.10***

Data represent the mean ± SEM, ***p<0.001, when compared to the CHFD group (n=8 per group)

CND: Control Normal diet fed group

CHFD: Control High-fat diet fed group

HFD+PC: High-fat diet + positive control (Naringenin 10 mg/kg body weight)

HFD+NR50: High-fat diet + *Nitraria retusa* 50 mg/kg body weight

HFD+NR100: High-fat diet+ *Nitraria retusa* 100 mg/kg body weight

Table 3. 4. Blood constituents in CND, CHFD, HFD+PC, HFD+NR50 and HFD+NR100 groups after 4 weeks.

	CND	**CHFD**	**HFD+PC**	**HFD+NR50**	**HFD+NR100**
Serum total cholesterol (mg/dL)	134.00 ± 4.65	174.00 ± 8.64	154.00 ± 4.10	182.00 ± 1.55	170.00 ± 5.59
Serum HDL-cholesterol (mg/dL)	66.00 ± 4.65	80.00 ± 1.55	64.00 ± 1.55**	98.00 ± 4.10*	92.00 ± 4.10
Serum LDL-cholesterol (mg/dL)	9.00 ± 1.22	16.50 ± 2.03	6.00 ± 0.00**	18.00 ± 0.00	12.00 ± 0.00*
Serum triglycerides (mg/dL)	16.00 ± 3.11	22.00 ± 1.63	22.00 ± 1.63	10.00 ± 1.63*	14.00 ± 1.63*
Serum glucose (mg/dL)	102.00 ± 14.97	200.00 ± 4.10	146.00 ±1.63**	168.00 ± 2.69**	153.00 ± 9.41*
Serum TNF α (pg/ml)	63.20 ± 1.40	61.08 ± 0.58	61.31 ± 0.58	64.43 ± 0.56	65.72 ± 1.22

Data represent the mean ± SEM, * $p<0.05$, ** $p<0.01$, when compared to the CHFD group (n =5 per group)

CND: Control Normal diet fed group

CHFD: Control High-fat diet fed group

HFD+PC: High-fat diet + positive control (Naringenin 10 mg/kg body weight)

HFD+NR50: High-fat diet + *Nitraria retusa* 50 mg/kg body weight

HFD+NR100: High-fat diet+ *Nitraria retusa* 100 mg/kg body weight

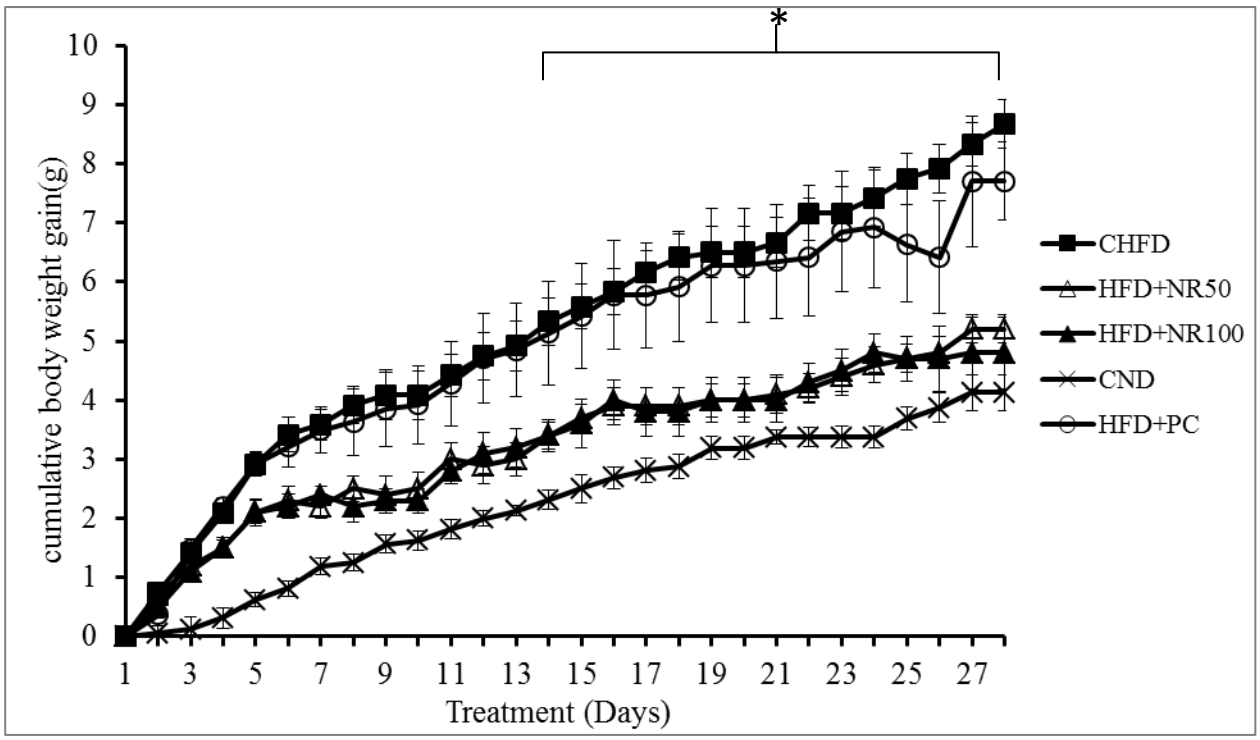


Fig. 3. 1. Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on cumulative body weight gain of mice fed with high-fat diet.

Mice were fed normal diet (CND), High-fat diet (CHFD), high-fat diet supplemented with positive control Naringenin 10 mg/kg body weight (HFD+PC),), high-fat diet supplemented with *Nitraria retusa* 50 mg/kg body weight (HFD+NR50) or high-fat diet supplemented with *Nitraria retusa* 100 mg/kg body weight (HFD+NR100) for 4 weeks. Body weight was daily measured at regular time. Data represent mean ± SEM, n=8, * $p<0.05$, significant between HFD+NR50 or HFD+NR100 and CHFD.

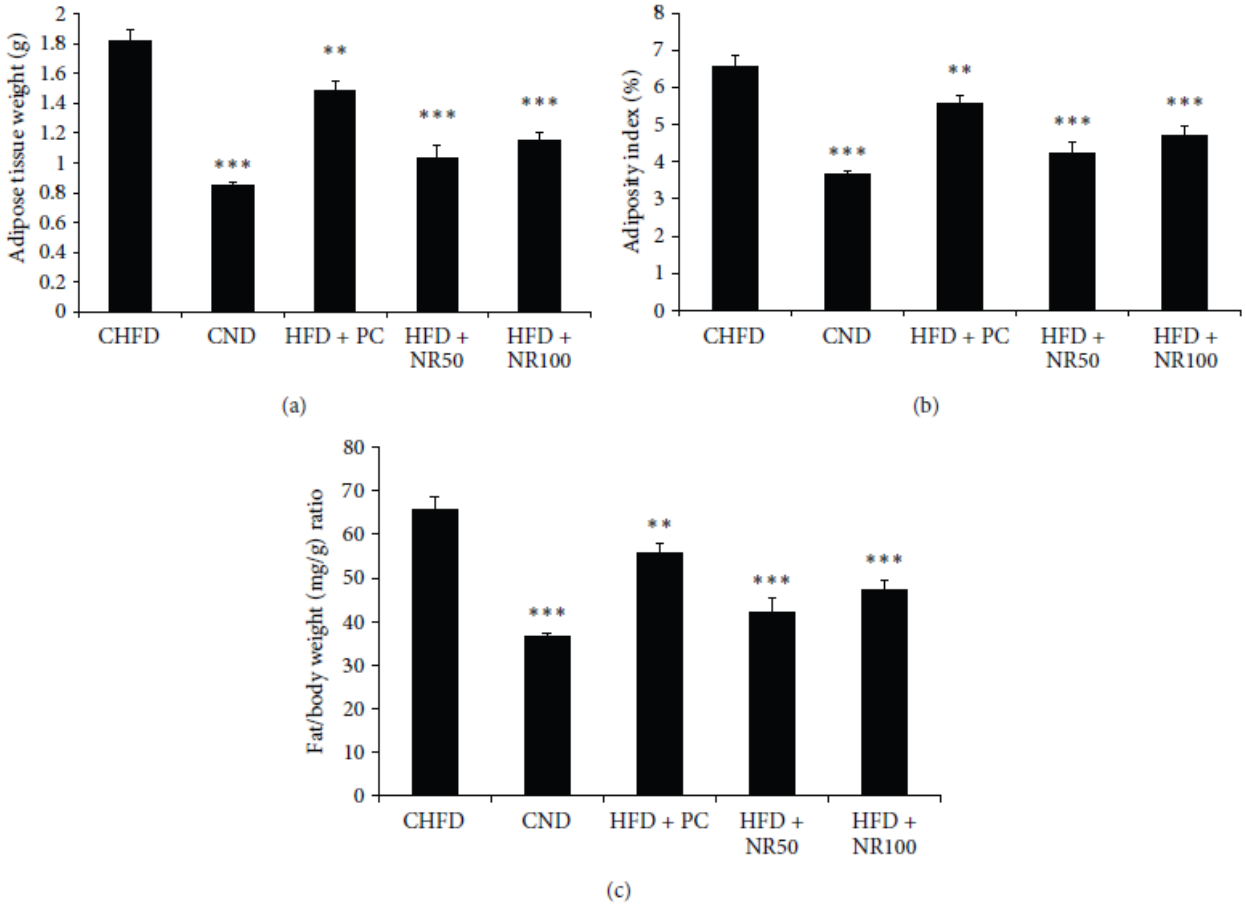


Fig. 3. 2. Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on adipose tissue weight (a), adiposity index (b) and Fat/body weight (mg/g) ratio (c) of mice fed with high-fat diet.

Mice were fed normal diet (CND), High-fat diet (CHFD), high-fat diet supplemented with positive control Naringenin 10 mg/kg body weight (HFD+PC), high-fat diet supplemented with *Nitraria retusa* 50 mg/kg body weight (HFD+NR50) or high-fat diet supplemented with *Nitraria retusa* 100 mg/kg body weight (HFD+NR100) for 4 weeks. At the end of experiment, adipose tissue, for all groups, was weighed. Data represent mean ± SEM, n=8, ** $p<0.01$, *** $p<0.001$ compared to the high-fat diet group.

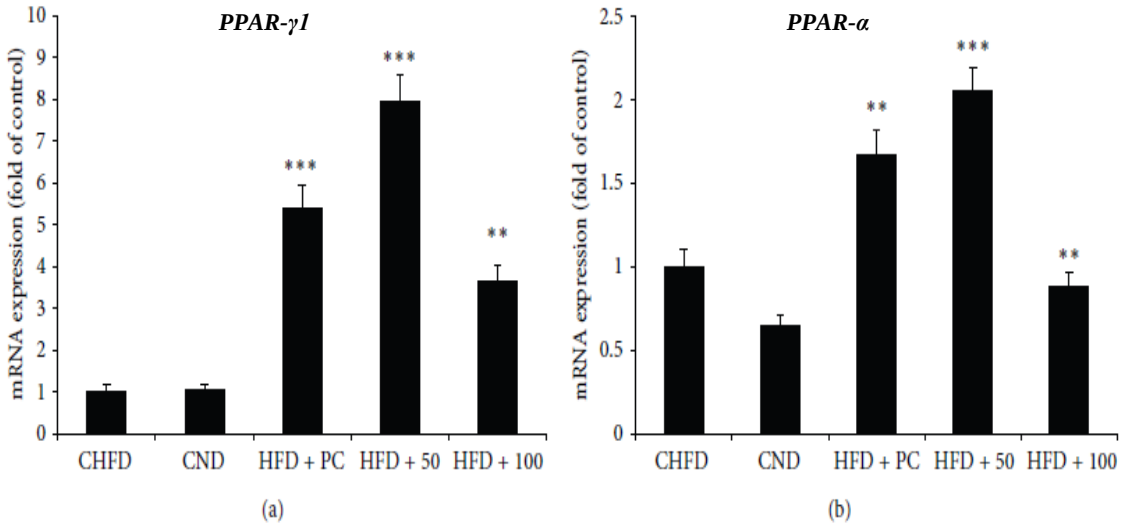


Fig. 3. 3. Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on genes regulating lipid metabolism in liver of mice fed with high-fat diet. Peroxisome proliferator activated receptor gamma (*PPAR-γ1*) (a) and Peroxisome proliferator activated receptor alpha (*PPAR-α*) (b). Real time PCR was conducted and result was expressed as m RNA expression fold change compared to the control high-fat diet HFD. Bars represent mean ± SD. ** $p<0.01$, *** $p<0.001$, compared to the high-fat diet group.

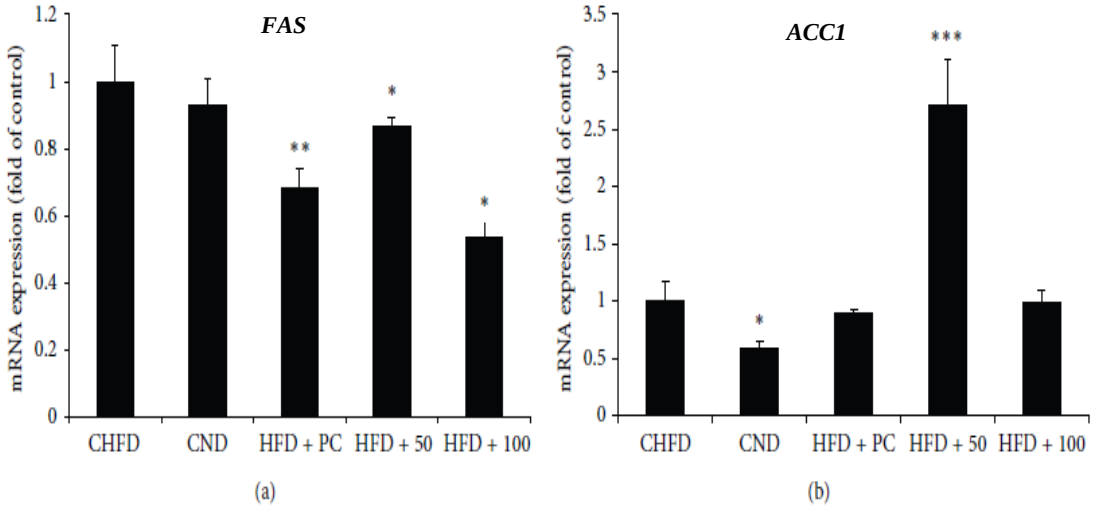


Fig. 3. 4. Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on genes regulating lipid metabolism in liver of mice fed with high-fat diet. Lipogenic enzymes: Fatty acid synthase (*FAS*) (a), and Acetyl-CoA Carboxylase1 (*ACC1*) (b). Real time PCR was conducted and result was expressed as m RNA expression fold change compared to the control high-fat diet HFD. Bars represent mean ± SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to the high-fat diet group.

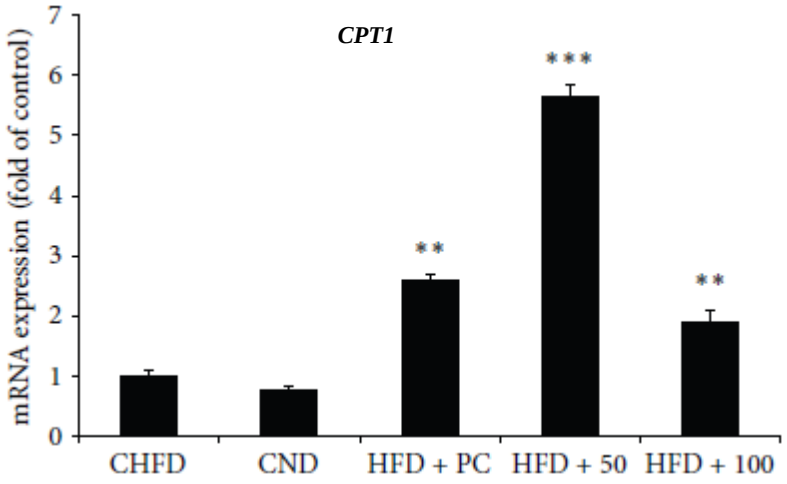


Fig. 3. 5. Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on genes regulating lipid metabolism in liver of mice fed with high-fat diet. Carnitine palmitoyltransferase I (*CPT1*) essential step in the beta-oxidation of long chain fatty acids. Real time PCR was conducted and result was expressed as m RNA expression fold change compared to the control high-fat diet HFD. Bars represent mean ± SD. ** $p<0.01$, *** $p<0.001$, compared to the high-fat diet group.

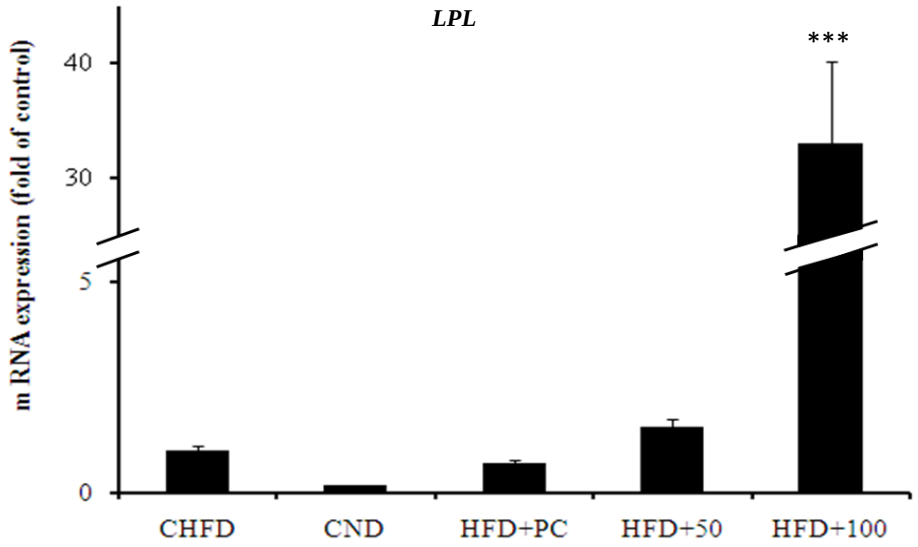


Fig. 3. 6. Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on genes regulating lipid metabolism in liver of mice fed with high-fat diet. Lipoprotein lipase (*LPL*): enzyme responsible for the hydrolysis of triglycerides in lipoproteins. Real time PCR was conducted and result was expressed as mRNA expression fold change compared to the control high-fat diet HFD. Bars represent mean ± SD. *** $p<0.001$ compared to the high-fat diet group.

CHAPTER 4

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

4.1. 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 prevealence, 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 (Yumuk et al., 2014). In order to improve our knowledge concerning the pathological mechanisms of human obesity disease, good animal models are very helpful. In this context, several mice models of obesity are used in the research of obesity-related human health symptoms and metabolic disorders mainly diabetes. The *db/db* mice model BKS.Cg-*Dock7*^{m}+/+ *Lepr*$^{db/}$J are mice homozygous for the diabetes spontaneous mutation (*Lepr*^{db}) which become obese at approximately three to four weeks of age. Homozygous mutant mice are polyphagic, polydipsic, and polyuric (Lutz and Woods, 2012). At one month of age, *db/db* mice are larger (obese) presenting increased fat deposition in the inguinal and axillary regions when compared to control (heterozygous) littermates (Lee et al., 1996). Different methods have been used to reduce body weight and its complications for many years. Herbal medicines have been in consideration as a current alternative to reduce body weight gain and fat mass, for example, *Nigella sativa, Camellia synensis*, green tea, and black Chinese tea were found to possess effective anti-obesity actions resulting in a potential management of other health disorders related to obesity, mainly cardiovascular diseases and diabetes (Ranjbar et al., 2013). The mechanisms whereby bioactives molecules affect body weight and body composition may include modifications in appetite control, enhancement of hepatic lipid metabolism and decreased food intake (Rainsa et al.,

2011). The most common complementary and alternative medicine modalities are based in food supplements and herbal therapies. In this regard, many crude extracts of plants and their isolated bioactive compounds were shown to be effective in prevention and treatment of obesity by either inducing weight loss or preventing weight gain (Ranjbar et al., 2013). Halophytes, salt-tolerant species, are considered a potent source of various chemical secondary components with a big economic interest (Ksouri et al., 2011). Compared to the glycophytic species, halophytes contain much higher amounts of secondary metabolites or even restricted to halophytic species which could be used in various application fields such as pharmacognosy and functional foods (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.

4.2. Materials and Methods

4.2.1. 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.

4.2.2. 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, filtred through 0.45 μm 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.

4.2.3. 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 -80ºC. All procedures were performed in accordance with the Ethics Animal Care and Use Committee of the University of Tsukuba, Japan.

4.2.4. Measurements of body and fat weights

Body weight was measured once a day during the feeding period. Internal organs were dissected, weighed and then, stored until they were analyzed for further experiments.

4.2.5. Measurements of glucose, triglyceride and cholesterol levels

After 4 weeks of treatment, blood samples were collected and centrifuged at 2,000×*g* for 15 min at 4ºC, and serum glucose, triglycerides (TG), total cholesterol (TCHO), high-density lipoprotein

(HDL) and low-density lipoprotein (LDL) levels were measured according to the kit manufacturer's instruction.

4.2.6. RNA isolation from organs liver and real-time-PCR analysis

Liver samples were prepared (50 mg) and homogenized with a Polytron homogenizer. Then total RNA was purified using the ISOGEN kit (Nippon GeneCo. Ltd., Japan) following the manufacturer's instructions. 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). The expression of Peroxisome proliferator-activated receptor gamma (*PPAR-γ1*), 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 µL reaction mixtures containing: 10 µl of TaqMan Universal PCR Master Mix UNG (2X), 9 µL of template c-DNA (100 ng μL^{-1}) and 1 µL 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.

4.2.7. Statistical analysis

All experiments were repeated at least three times. Data were expressed as the mean ± SD or the mean ± SEM. 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.

4.3. Results

4.3.1. 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. 4. 1.,** the control *db/db* increased body weight gain 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 4. 1.)** ($p<0.05$), without affecting food intake, which was around 6g/day/mice for all different experimental groups **(Table 4. 2.)**. In the current study, NRE treatment was demonstrated not only to decrease final body weight, but also cumulative body weight (**Table 4. 1.**) 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 **(Fig. 4. 2.).** However, NRE treatment did affect neither the body weight nor the adiposity in lean mice group.

4.3.2. 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. 4. 3**. 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. 4. 3. A1.** and **C1.**, 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. 4. 3. B1.**, 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 shown in **Fig. 4. 3. D1.** and **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 4 weeks 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.

4.3.3. Effect of NRE administration on genes expression related to hepatic lipid metabolism which modulate lipolysis-lipogenesis balance in *db/db* obese mice model

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 transcriptional level was investigated, following 30 days experimental study. Peroxisomes Proliferator-Activated Receptor (*PPAR-α*) and *PPAR- γ1*, acts as a master regulator of fatty acid oxidation and lipid storage, respectively. Sterol Regulatory Element Binding Protein-1c (*SREBP-1c*) is a transcription factor that controls genes involved in cholesterol uptake and biosynthesis. *FAS* and

ACC1 are two main lipogenic enzymes and are strongly correlated with insulin sensitivity. *CPT1* is the encoding gene of carnitine palmitoyl-transferase system which is an essential step in the *β*-oxidation of long chain fatty acids. *LPL* gene encodes the enzyme responsible for the hydrolysis of triglycerides in lipoproteins. To assay the *in vivo* effects of *Nitraria retusa* extracts on these genes which regulate lipid homeostasis, *Nitraria retusa* extracts were administrated orally and daily to mice during 4 weeks and expression of said genes in liver of all groups of mice were determined by real-time PCR. As shown in **Fig. 4. 4.** to **4. 6.**, 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.

4.4. Discussion

Our previous *in vivo* anti-obesity study (done in chapter 3), was conducted in mice fed with high-fat diet-induced obesity and daily administrated with *Nitraria retusa* extracts at final doses (50 mg/kg BW and 100 mg/kg BW) for a period of 4 weeks. Main results revealed that oral administration of this extract 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 investigated by evaluating body weight gain and feed intake, white adipose tissue weight. Then,

by measuring lipid and glucose concentrations in serum, as well as mRNA expressions of genes related to lipogenesis-lypolysis balance in liver of transgenic *db/db* obese mice model BKS.Cg-*Dock7*^{m}+/+ *Lepr*$^{db/}$J for a treatment period of 4 weeks. 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 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* extracts disclosed herein likely have superior anti-obesity activity which could be due to synergy brought by the combination of compounds included in an extract. *Nitraria retusa* extract disclosed herein is likely rich in naturally present flavonoids especially glycosides ones such as; Isorhamnetin-3-*O*-glucoside, Isorhamnetin-3-*O*-rutinoside, 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 could also significantly reduced LDL cholesterol (the "bad cholesterol") and triglycerides levels in diabetic mice model **(Fig. 4. 3. 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. 4. 3. B1.).** Thus, NRE treatment

might reduce the risk of diabetic hypercholesterolemia. Furthermore, in obesity and diabetes, the amounts of hepatic triglycerides available for export are increased which leads to an imbalance between the availability of hepatic triglycerides for export and the exporting capacity of the liver (Nguyen et al., 2008). However, treated *db/db* mice group revealed a lower level of triglycerides compared to control group (**Fig. 4. 3. 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 leads to many metabolic complications due mainly to the alteration of the expression of many genes (*PPAR-γ*, *PPAR-α*, *FAS*, *LPL*, *SREBP-1c*, and *CPT-1*) related to lipid and cholesterol metabolism. Therefore, evaluating the effect of NRE exposure on the said genes regulating the hepatic lipid metabolism in mice model used was conducted. Generally, hepatic lipid metabolism is controlled by a balance between lipogenesis and lipolysis processes. This balance is maintained by determined level of the encoded genes expressions of lipogenic and lipolytic enzymes. Peroxisome Proliferator-Activated Receptors (*PPAR*s) are ligand-activated transcription factors and members of the nuclear hormone receptor superfamily, which regulate energy homeostasis, inflammation, proliferation and differentiation (Varga et al., 2011). *PPAR-γ* promotes adipocyte differentiation and lipid storage, while *PPAR-α* play a main role in the regulation of fatty acid oxidation (Abbott, 2009), especially in tissues with high lipid catabolic capacities, such as the liver (Ribet and Langin, 2010). Our findings revealed that the genes expressions of these two later main transcription factors were overexpressed in *db/db* treated mice compared to the control group (**Fig. 4. 4. A1.** and **B1.**), which probably reflect the effect on serum lipid profile. The results were concordant with those reported in previous clinical studies

describing that the activation of PPARα enhanced fatty acid oxidation in liver and decreased the levels of circulating and cellular lipids in obese diabetic patients (Kim and Kawada, 2012). The genes *FAS* and *ACC1* involved in lipogenic pathway and regulating by *PPAR-γ* were also overexpressed (**Fig. 4. 5. A1. and B1.**) in *db/db* mice by NRE administration. The level of these enzymes involved in lipogenesis, were reported to be maintained and controlled by the *SREBP-1c*, the main regulator of hepatic cholesterol homeostasis (Deng and Elam, 2012). Its hepatic overexpression in diabetic mice was reported to induce lipogenic enzymes leading to a marked decrease in hyperglycemia (Becard et al., 2001), which is in turn positively correlated with our results shown in (**Fig. 4. 3. D1.**, **4. 5. A1.**, **4. 5. B1.** and **4. 6. A1.**). Moreover, it has been known that high *FAS* gene expression was correlated with insulin sensitivity in normal conditions conversely to *db/db* mice which have a low *FAS* gene expression caused by especially hyperphagia and insulin resistance (Lan et al., 2003). NRE administration could enhance the expression level of *FAS* gene in only *db/db* mice having the metabolic complications described above. The level of gene expression encoding another important lipogenic enzyme, lipoprotein lipase (*LPL*), playing a central role in fat mobilization and regulating the plasma triglycerides and cholesterol levels, was also overexpressed in *db/db* mice due to the NRE oral administration. Its overexpression was reported to prevent hypertriglyceridemia and hypercholesterolemia especially LDL levels (Yamamoto et al., 1995). These described results strongly confirm our findings related to the expression of hepatic *LPL* gene due to NRE exposure. In order to maintain the balance of lipid metabolism in liver between fat storage (lipogenic pathway) and fat burning, (lipolytic pathway), the mitochondrial fatty acid oxidation which is controlled by *PPAR-α* plays a crucial function. Carnitine palmitoyltransferase- 1 (*CPT-1*) is one of main target enzymes of *PPAR-α*, contribute the transport of activated long-chain fatty acids from the cytosol to the

mitochondrial matrix, where *β*-oxidation is occurring (Koo et al., 2008). The lack of carnitine will slow oxidation of fatty acids and fuel and increase in serum lipid level (Park et al., 2008). NRE oral administration in the present study up-regulates *CPT1* gene expression (**Fig. 4. 6. C1.**), which could in turn clearly affects the plasma lipid levels and reduce finally the body weight gain in *db/db* mice. However, normal mice or lean mice NRE-treated groups were not affected by NRE administration either in body weight gain or in serum lipid profile correlated with hepatic genes expression described in all results. These findings could probably reflect the safety of the combined effect of NRE components as well as the difference in leptin signaling role between *db/db* mice and normal mice. In normal conditions, leptin signaling was reported to be an important mediator of feeding behavior, energy homeostasis as we as another crucial roles such as in immune and reproductive functions (Osborn et al., 2003). In leptin receptor deficient (*db/db*) mouse model used in this study, deficiency in leptin signaling causes a dysregulation in energy balance leading to obesity characterized by hyperphagia and inslun resitance caused by decreased energy expenditure. These metabolic complications in turn were alleviated by NRE administration in control (*db/db*) mice resulting in a decreasing on body weight gain on these mice (**Fig. 4. 1.** and **Table 4. 1.**). However, the same effect was not observed on lean normal mice since they don't represent a leptin resistance and leptin acts normally as a feedback signal reflecting the nutritional status leading to a maintained energy homeostasis (Ahima et al., 1996). In current study, main results showed that NRE administration in *db/db* mice significantly increased hepatic lipolytic enzymes expression such as *CPT1* (**Fig. 4. 6. C1.**) and the transcriptional activity of *PPAR-α* (**Fig. 4. 4. A1.**), having the key role in the induction of fatty acid oxidation enzymes. In other side, NRE administration significantly increased lipogenic enzymes expression such as *FAS* and *ACC1* (**Fig. 4. 5. A1.** 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 due to the efficiency of NRE mixture, which in turn increases genes expression (*FAS* and *SREBP-1c*) that can be damaged by reactive oxygen species production within fat cells, that may prevent 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 and whole the lipid metabolism.

4.5. 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-lipogenesis balance. Thus NRE could be one of possible ways to control obesity and to prevent or reduce the risk of getting various obesity-related diseases.

Table 4. 1. Body weight gain in *db/db* mice control, *db/db* mice NRE, lean mice control and lean mice NRE groups after 30 days.

Groups	Body weight (g)		
	Initial	Final	Cumulative body weight (g)
db/db mice control	31.00 ± 1.00	42.16 ± 2.56	11.16 ±1.56
db/db mice NRE	29.00 ± 1.50	34.83 ± 1.75 *	5.83 ± 0.25 *
Lean mice control	22.50 ± 1.22	23.60 ± 1.71 *	1.10 ± 0.49 *
Lean mice NRE	22.20 ± 0.75	23.00 ±1.32 *	0.80 ± 0.57 *

Data represent the mean ± SD, * $p<0.05$, when compared to the *db/db* mice control group.

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 4. 2. 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 30 days.

Groups	Total food intake	
	g/week	g/day
db/db mice control	42.77 ± 7.59	6.10 ± 0.65
db/db 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

Data represent the mean ± SD, * $p<0.05$, when compared to the *db/db* mice control group.

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.

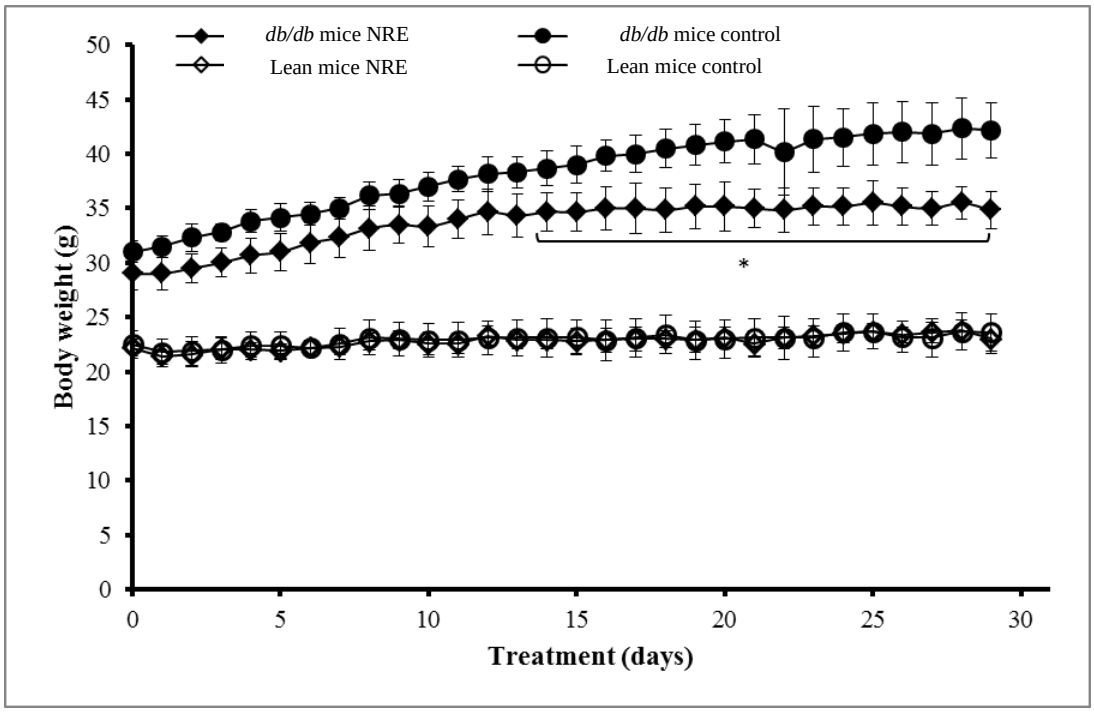
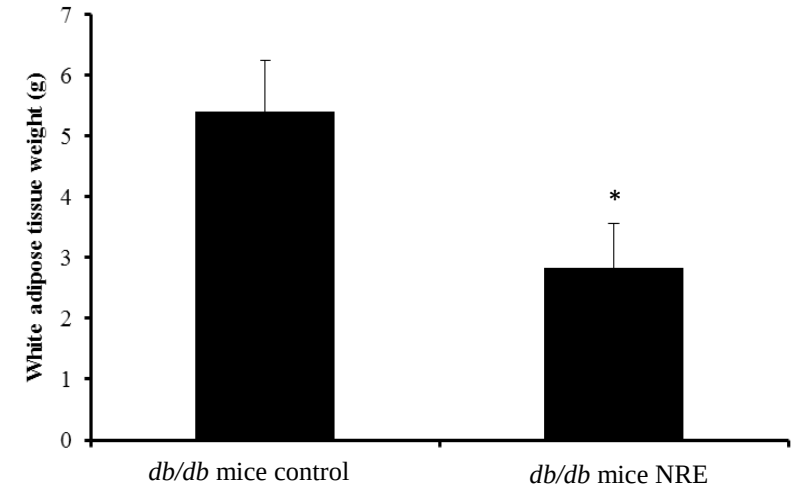


Fig. 4. 1. Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on weight gain of BKS.Cg-*Dock7*^{m}+/+ *Lepr*$^{db/}$J mice. Mice were divided to 4 groups: 2 groups; *db/db* mice control and *db/db* mice NRE; were orally administrated with a daily dose of 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 30 days. Body weight was daily measured at regular time. Data represent mean ± SD, n=8, * $p<0.05$ compared to *db/db* mice control group.

A



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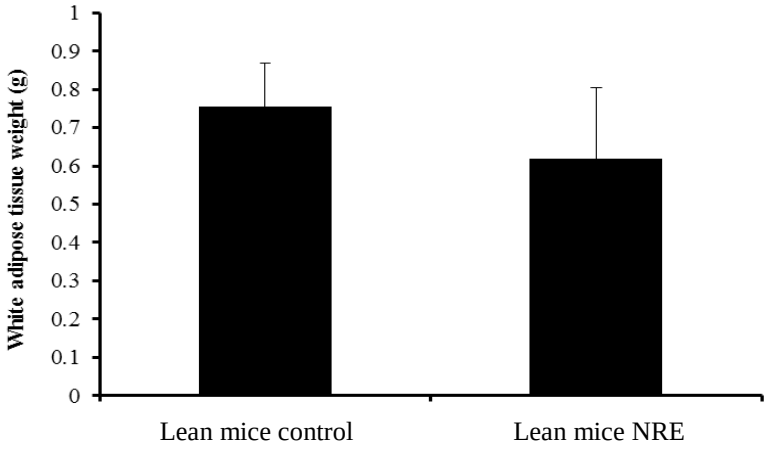

Fig. 4. 2. Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on adipose tissue weight of BKS.Cg-*Dock7*^{m}+/+ *Lepr*$^{db/}$J mice; Mice were divided to 4 groups: 2 groups; *db/db* mice control and *db/db* mice NRE; were orally administrated with a daily 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 30 days. 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 ± SEM, n=8, * $p<0.05$, compared to *db/db* mice control group.

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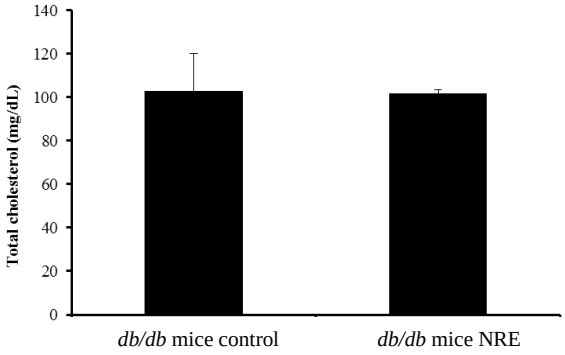

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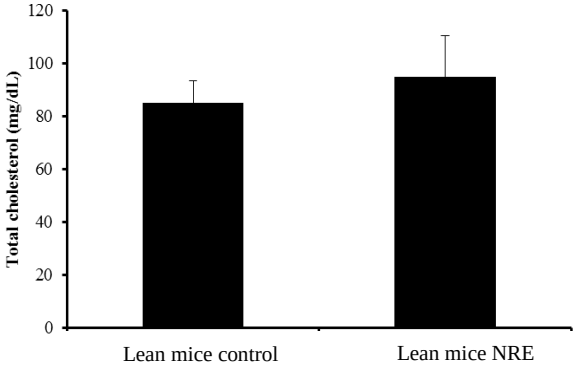

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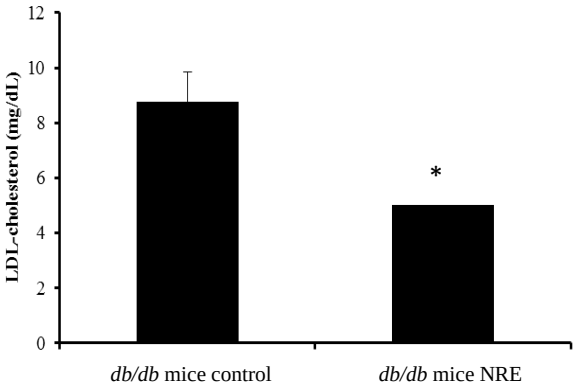

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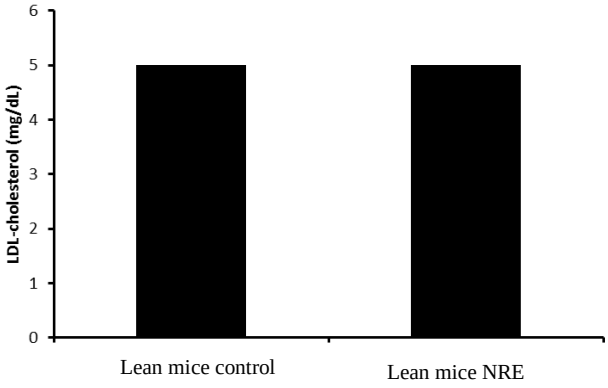

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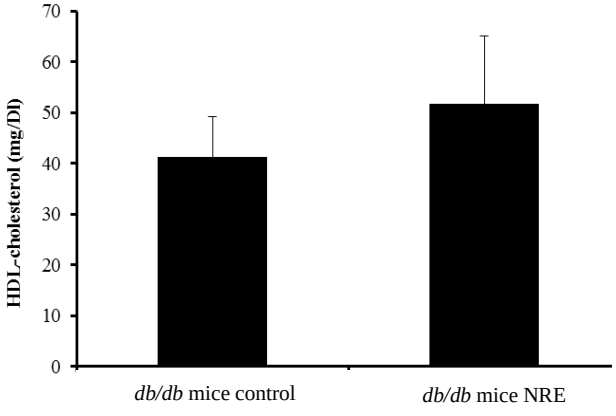

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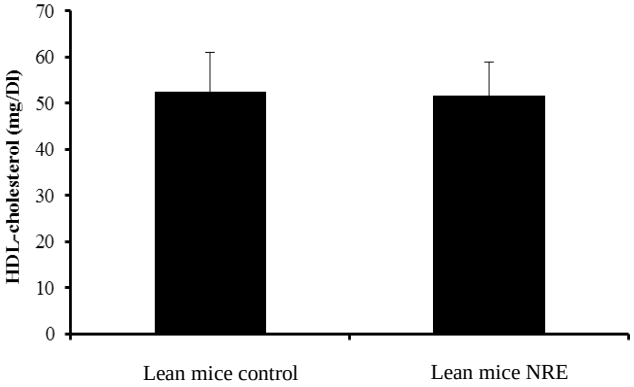

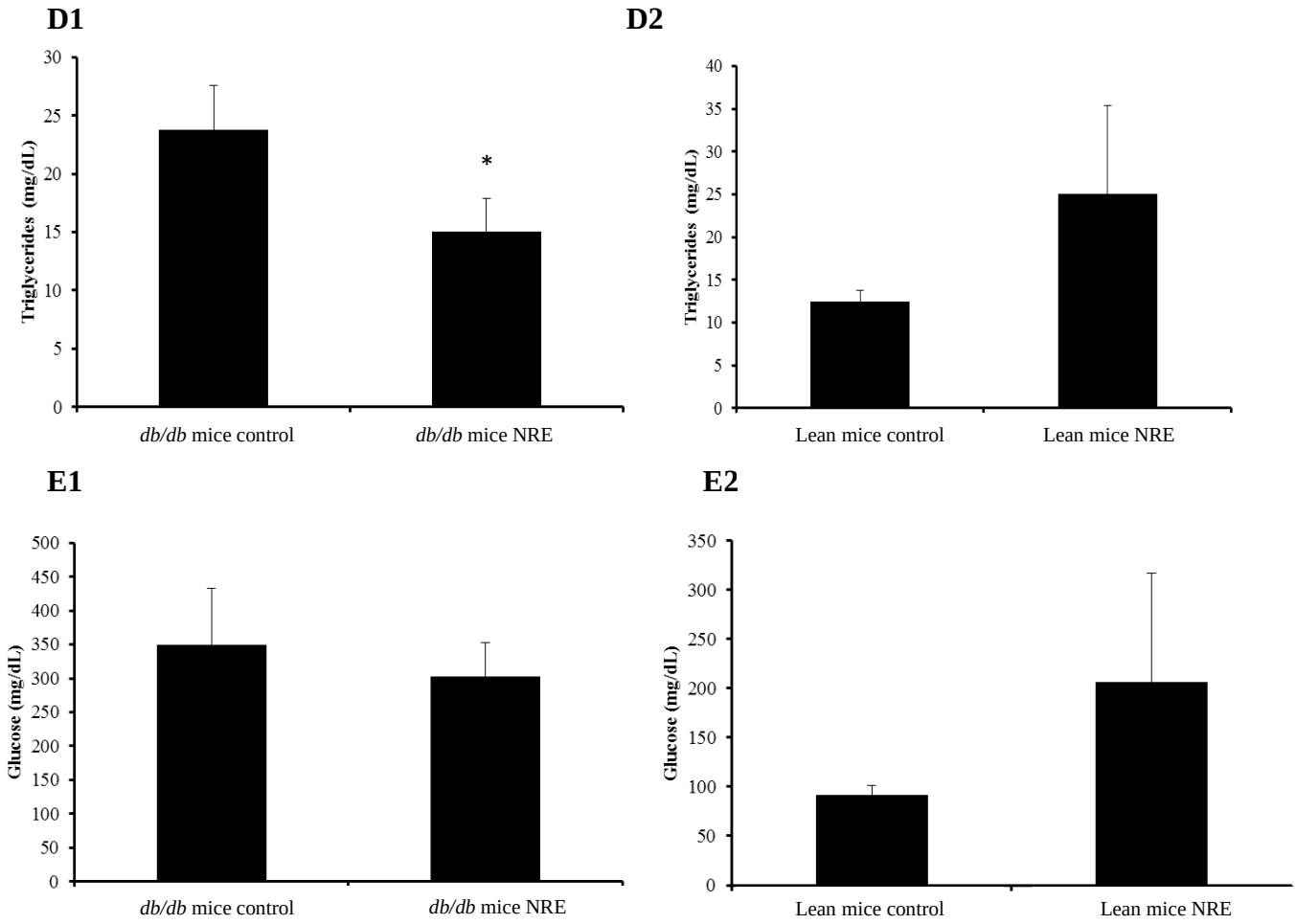

Fig. 4. 3. Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on blood constituents; Total cholesterol (A; A1, A2), LDL-cholesterol (B; B1, B2), HDL-cholesterol (C; C1, C2), Triglycerides (D; D1, D2) and Glucose (E; E1, E2); of BKS.Cg-*Dock7*^{m}+/+ *Lepr*$^{db/}$J mice. 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. At the end of experiment, blood samples were collected and analyzed. Data represent mean ± SEM, n=8, * $p<0.05$, compared to *db/db* mice control group.

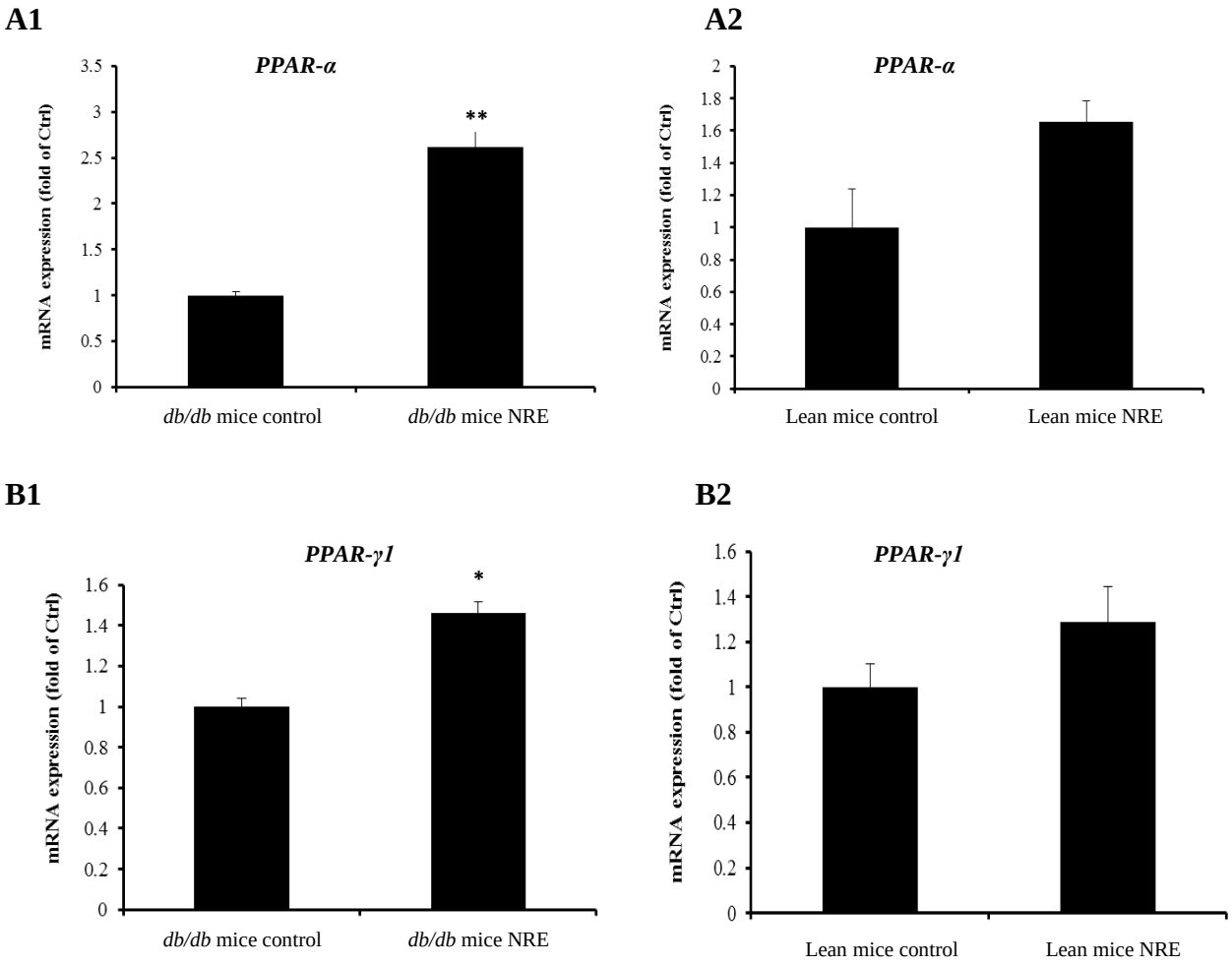

Fig. 4. 4. Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on genes regulating lipid metabolism in liver of BKS.Cg-*Dock7*^{m}+/+ *Lepr*$^{db/}$J mice. Mice were divided to 4 groups: 2 groups; *db/db* mice control and *db/db* mice NRE; were orally administrated 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. 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 m RNA expression fold change compared to the *db/db* mice control group and lean mice control group. Bars represent mean ± SD, n=3,* $p<0.05$, ** $p<0.01$ compared to *db/db* mice control group

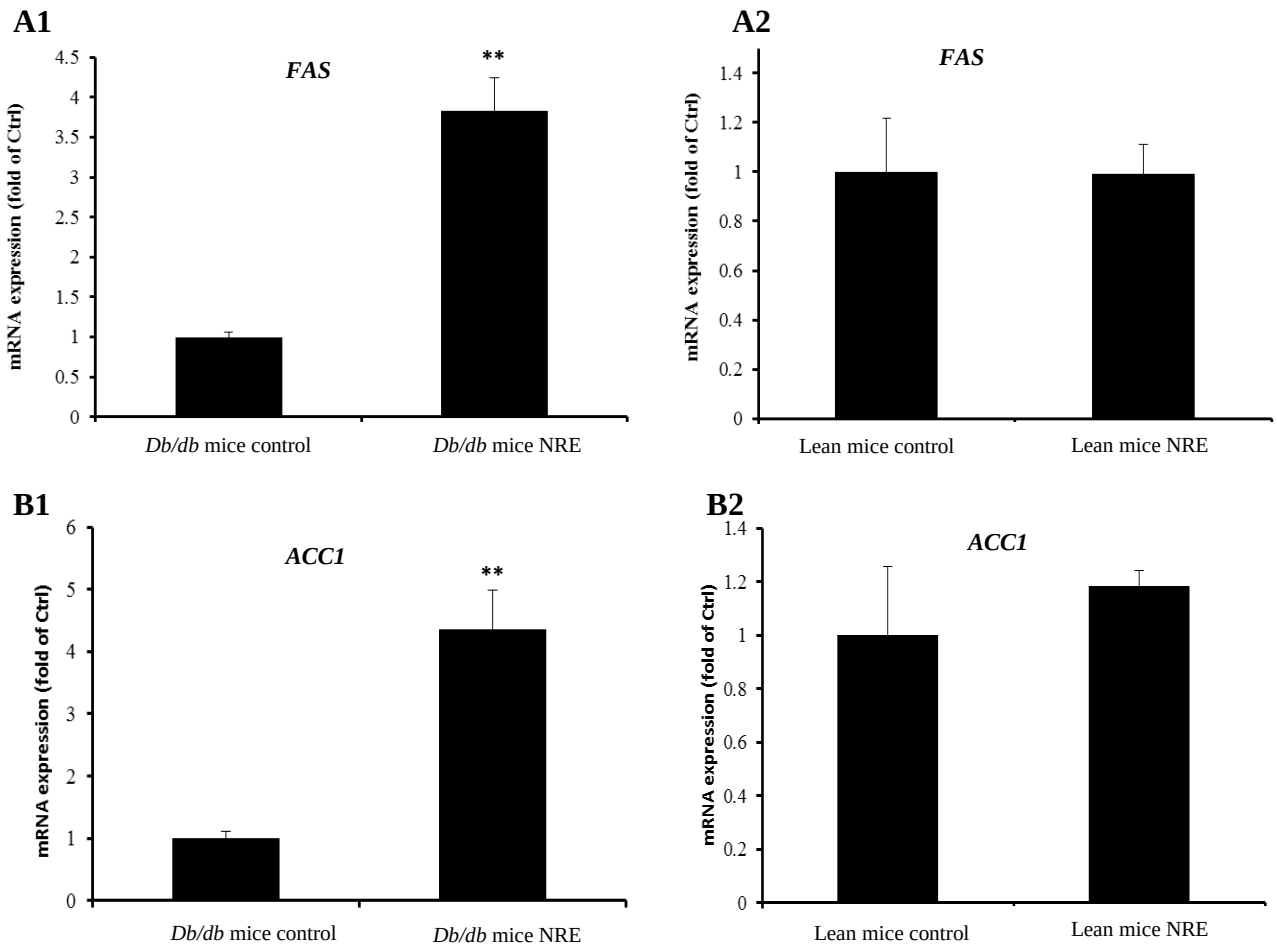

Fig. 4. 5. Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on genes regulating lipid metabolism in liver of BKS.Cg-*Dock7*^{m}+/+ *Lepr*$^{db/}$J mice. Mice were divided to 4 groups: 2 groups; *db/db* mice control and *db/db* mice NRE; were orally administrated with a daily dose of 0 and 50 mg/kg of body weight (BW) respectively for 30 days. 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. At the end of experiment, liver samples were collected and analyzed. Fatty acid synthase; *FAS*; (A; A1, A2), and Acetyl-CoA-Carboxylase; *ACC1*; (B; B1, B2). Real time PCR was conducted and result was represented as m RNA expression fold change compared to the *db/db* mice control group and lean mice control group. Bars represent mean ± SD, n=3, ** $p<0.01$ compared to *db/db* mice control group.

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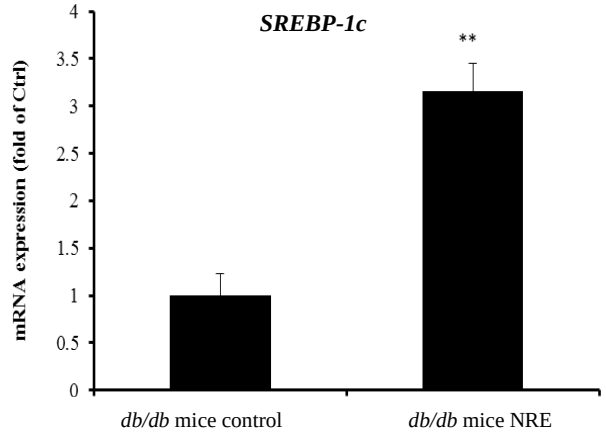

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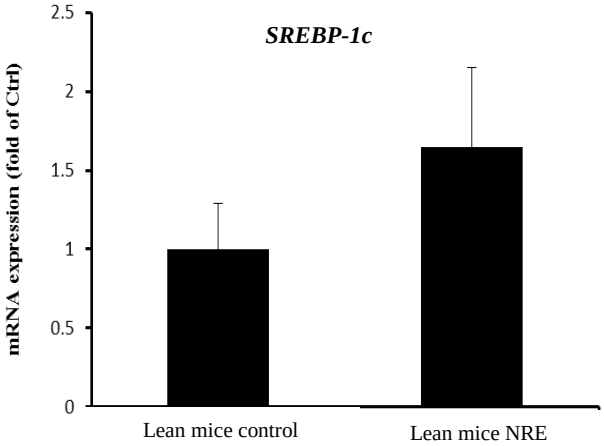

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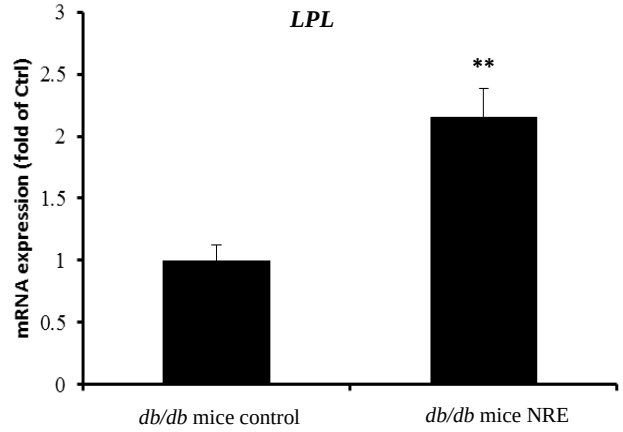

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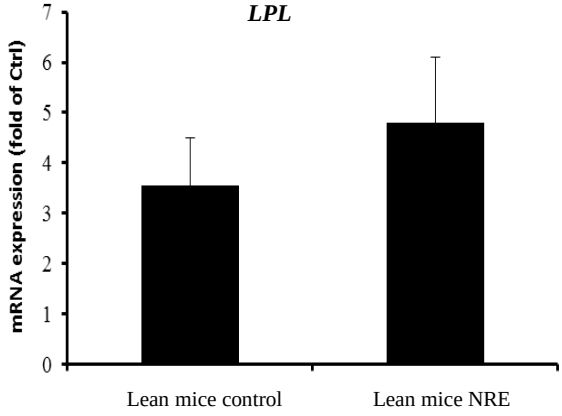

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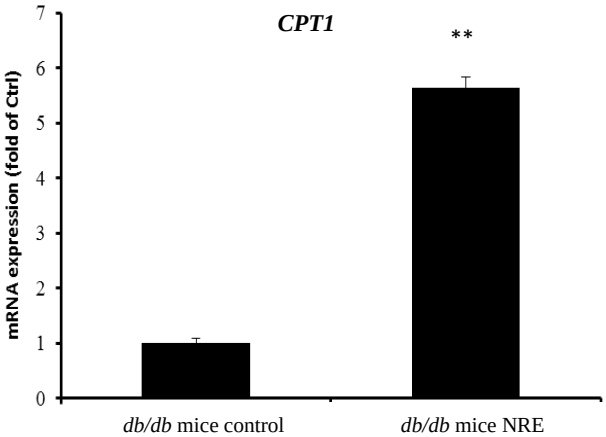

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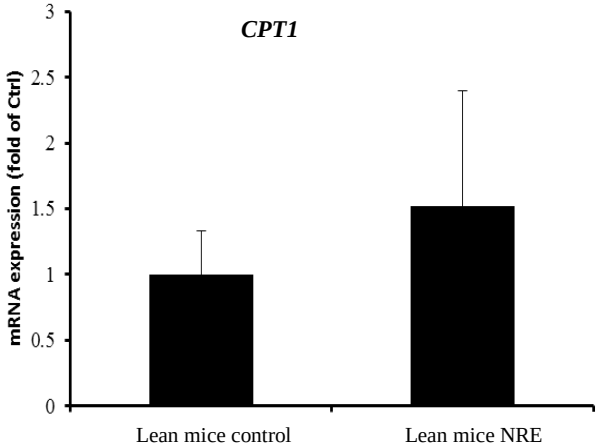


Fig. 4. 6. Effect of daily oral administration of *Nitraria retusa* ethanolic extract (50 mg/kg body weight) on genes regulating lipid metabolism in liver of BKS.Cg-*Dock7*^{m}+/+ *Lepr*$^{db/}$J mice. Mice were divided to 4 groups: 2 groups; *db/db* mice control and *db/db* mice NRE; were orally administrated with a daily dose of 0 and 50 mg/kg of body weight (BW) respectively for 30 days. 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. 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 m RNA expression fold change compared to the *db/db* mice control group and lean mice control group. Bars represent mean ± SD, n=3, ** $p<0.01$ compared to *db/db* mice control group.

CHAPTER 5

Identification of main phenolic compounds of *Nitraria retusa* extracts (stem, leaf, and their mixture) and evaluation of their anti-adipogenic activity in 3T3-L1 cell line

5.1. Introduction

The plant kingdom has become a target for the search by multinational drug companies and research institutes for new drugs and biologically active lead compounds (Li and Vederas, 2009). The world health organization (WHO) reported in 2002 that the herbal medicine serve the health need of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries (Soltan et al., 2009). As known actually, phenolic compounds are receiving increasing attention because of their health promoting effect, attributed to their various biological activities and strong capacity to improve food quality and stability (Burn and Kishore, 2000). Phenolic compounds represent the most studied phytochemicals and have been widely exploited as model systems in different areas of plant research such as pharmaceutical, food, cosmetic and chemical industries (Boudet, 2007). Improving knowledge on the use of anti-obesity medicinal preparations, and encouraging obese patients to consume them along with a healthy diet should be continued (Kazemipoor et al., 2012). In order to use safely, the medicinal plants in controlling and treating obesity, further chemical, biological, and clinical studies are needed on the efficiency of selected plants. Plant extracts may be the whole plant, or plant organs (stem, leaf, flowers, and roots). Several investigations should focus on the difference between the combination and the single effect of such compounds. In view of the diversity and the structural complexity of these bioactive molecules, their extraction is influenced by several parameters and technology including the nature, polarity, the extracting power of the solvent, and extraction method (Luthria, 2007). High performance liquid chromatography (HPLC) is the method of choice for the analysis of phenolic compounds, because of its versatility, precision and relatively low cost (Parejo et al., 2004). Most frequently, reversed-phase (RP) C18 columns, a binary solvent system containing acidified water and polar organic solvent (acetonitrile or methanol) and UV-Vis diode array

detection (DAD) are used and so far to constitute a crucial and reliable tool in the routine analysis of plant phenolic compounds (Naczk and shahidi, 2004).

The aim of this chapter is to analyze the phenolic composition of three extracts of *Nitraria retusa* plant; stem extract, leaf extract and whole aerial part extract (mixture of leaves and stems in equal quantity of dried matter). In a further step, we aim to test the anti-adipogenic effect on 3T3-L1 cells of organ extracts (stems, leaves) as well as some determined phenolic compounds of *Nitraria retusa*. This is in order to compare the efficiency and safety of combined effect (stem extract, leaf extract or their mixture extract) and single effect (single phenolic compounds).

5.2. Material s and Methods

5.2.1. 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 C_{18} analytical column of 4.6 x 100 mm and 3.5μm particle size (Zorbax Eclipse XDB C_{18}). 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 methanol. 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-vis 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-vis 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 qualibration equation of the corresponding standard.

5.2.2. Cell culture

Murine 3T3-L1 preadipocytes (Riken Tsukuba japan) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (5000 µg/mL)-streptomycin (5000 IU/mL) in 75-cm^2 tissue culture flasks. Medium was changed every 3 days and cell passage was carried out at 80% confluence at one on two ratio using 0.25% trypsin (1 mM EDTA). 3T3-L1 cells were cultured in a humidified incubator at 37°C and 5% CO_2.

5.2.3. Pre-adipocytes differentiation and oil-Red-O staining procedures

3T3-L1 pre-adipocytes were seeded into 96-well plates at 1.0 x 10^4 cells/well, and cultured for additional two days until full confluence. Two days later (Day 0), cells were incubated with a differentiation cocktail (MDI) containing 1/10 insulin solution, 1/10 dexamethasone solution and 1/10 3-isobutyl-1-methylxanthine solution in standard culture medium for 3 days followed by additional 48 h with standard culture medium containing insulin alone. The differentiation-maintenance medium was changed every 2 days. To investigate the effect *Nitraria retusa* stem and leaf extracts on adipogenesis in 3T3-L1, at different doses; (25, 50, 100, 200 and 400 µg/mL) were added to the differentiation-induction and differentiation-maintenance media. The

same procedure was conducted to investigate the effect of the determined phenolic compounds of NR; Luteolin-7-*O*-glucoside (5, 25, 50, 100 and 200 μM), Isorhamnetin (5, 25, 50, 100 and 200 μM) and Isorhamnetin-3-*O*-rutinoside (5, 25, 50, 100 and 200 μM). The staining procedure was conducted according to the adipogensis assay kit (Cayman chemical company). The absorbance was read at 490 nm with a 96-well plate reader. The lipid droplet content was reported as percentage of control cells.

5.2.4. Cell proliferation assay (MTT assay)

Cell proliferation was investigated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. 3T3-L1 cells were seeded in 96-well plates at 1×10^5 cells/mL. After incubation for 7 days (adipocytes), leaf and stem exracts samples diluted in medium were added at final concentrations of 25, 50, 100, 200, 400 μg/mL. The same procedure was conducted to investigate the effect of the determined phenolic compounds of NR; Luteolin-7-*O*-glucoside (5, 25, 50, 100 and 200 μM), Isorhamnetin (5, 25, 50, 100 and 200 μM) and Isorhamnetin-3-*O*-rutinoside (5, 25, 50, 100 and 200 μM). MTT was added after treatment for 7 days, and the resulting formazan was completely dissolved by 100 μL of 10% sodium dodecyl sulfate (SDS) for 24 h. The absorbance was determined at 570 nm in a multi-detection microplate reader (Power-scan HT, Dainippon Pharmaceutical, NJ, USA). Absorbance caused by the ability of the sample to reduce MTT or by its color, was corrected using plates as blanks, prepared in the same conditions in the absence of cells.

5.3. Results

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

Based on the HPLC analysis, the chromatographic profiles of *Nitraria retusa* extracts: stem part extract, leaf part extract and, mixed (leaf and stem) part extract acquired at 254 nm were presented in **Fig. 5. 1.**, **Fig. 5. 2.**, **Fig. 5. 3.**, respectively. Nine main components were identified in the leaf part extract (**Table 5. 1.)**. Nine main components were identified in the stem part extract (**Table 5. 2.)**. Ten main components were identified and quantified in the mixed (leaf and stem) part extract (**Table 5. 3.)**. 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 qualibration equation of the corresponding standard. The retention time of available standards used were presented in (**Table 5. 4.**). Three main flavonoids were common in the three different extracts; isorhamnetin, isorhamnetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside and having different chemical structure. According to quantification measurements, the concentrations in stem extract (µg/mL) of isorhamnetin, isorhamnetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside were: 1.463; 130.417; and 7.902; respectively (**Table 5. 1.**). Concerning, the concentrations in leaf extract (µg/mL) of isorhamnetin, isorhamnetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside were: 0.539; 278.010; 252.403; respectively (**Table 5. 2.**). In mixed part extract, the concentrations (µg/mL) of isorhamnetin, isorhamnetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside were: 1.141; 396.508; 198.340; respectively (**Table 5. 3.**). These Flavonoids were choosen for further experiments to test the single effects of *Nitraria retusa* components on cell viability, differentiation and lipid droplet formation in 3T3-L1 cells.

5.3.2. Effect of *Nitraria retusa* extracts and single bioactive components on cell differentiation and lipid droplet formation in 3T3-L1 cells

Adipogenesis assay was performed to investigate the effect of *Nitraria retusa* extracts (stem part extract, leaf part extract), as well as the effect of chosen bioactive components (isorhamnetin, isorhamnetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside) on the adipocyte differentiation and on the lipid droplets accumulation in 3T3-L1 cells. Differentiated 3T3-L1 cells were treated every two days with the different extracts described above, at various concentrations (25, 50, 100, 200 and 400 μg/mL), for 7 days. In another hand, in order to compare the effect of single compounds to the combined effect, differentiated 3T3-L1 cells were treated every two days with various concentartions of selected chemical compounds (5, 25, 50, 100 and 200 μM). Based on Oil-red-O content quantification, results showed that *Nitraria retusa* extracts (stem extract and leaf extract) treatments at 25, 50, 100, 200 and 400 μg/mL, in 3T3-L1 cells could not inhibit the lipid droplet accumulation compared to untreated cells, in dose dependent manner (**Fig. 5. 4. A., B.**). However their combined extract (stem and leaf) used in in previous chapters, and as shown in chapter 2, could exhibit stronger inhibition effect on adipogenesis (**Fig. 2. 1. A.**). Regarding the effect of chemical compounds identified in *Nitraria retusa* extracts on adipogensis, results showed that dependent on the chemical structure and molecular weights of all compounds tested, there is a variability of their effect on 3T3-L1 differentiation and triglyceride accumulation within cells (**Fig. 5. 5.**). Actually, isorhamnetin showed the strongest effect for all tested doses even for the lowest one (5 μM), compared to the other flavonoids glycosides (isorhamnetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside). After treating cells with isorhamnetin in various doses (5, 25, 50, 100, 200 μM), the triglyceride accumulation significantly decreased to 24.45 ±2.90%, 24.45 ±2.40%, 28.33 ±3.22%, 31.31 ±3.15%, and 20.57 ±0.62% respectively (**Fig. 5. 5. C.**).

However, high doses of isorhamnetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside (50, 100, 200 μM) exhibited a significant reduction of lipid accumulation (**Fig. 5. 5. A., B.**), accompanied with cell morphological changes based on microscopic observation. Therefore, it is necessary to correlate the obtained results with the MTT assay results in next section.

5.3.3. Effect of *Nitraria retusa* extracts and single bioactive components on cell viability of 3T3-L1 cells

Observation of the cell morphological changes of 3T3-L1 adipocytes after treatment with *Nitraria retusa* extract (leaf part extract), in various concentrations (25, 50, 100, 200 and 400 μg/mL) revealed no significant decrease in cell viability for all tested doses (**Fig. 5. 6. A.**). However, stem extract treatments (25, 50, 100, 200 and 400 μg/mL) showed a slight significant decrease in cell viability about 20 and 30% for 100, 200 and 400 μg/mL doses (**Fig. 5. 6. B.**). Concerning the single chemical bioactive compounds tested, cell viability was slightly significant affected by isorhamnetin-3-*O*-rutinoside treatments (25, 50, 100, 200 μM) with just 5% maximum of cell viability reduction for the higher dose (**Fig. 5. 7. A.**). Luteolin-7-*O*-glucoside treatments affected more significanly the cell viability by decreasing it by a maximum around 30% for the higher dose (200 μM) (**Fig. 5. 7. B.**). Nonetheless, isoramnetin strongly affected the 3T3-L1viability by 30% and 60% reduction for 100 and 200 μM, respectively (**Fig. 5. 7. C.**).

5.4. Discussion

Nitraria retusa extracts (stem, leaf) exhibited a lower anti-adipogenic activity compared to their single constituents (isorhamnetin, isorhamnetin-3-*O*-rutinoside, and luteolin-7-*O*-glucoside). This could be explained by the antagonistic effects of some compounds present in NR, and also

that these pure compounds tested in different doses (5, 25, 50, 100, 200 μM) were diluted in the original ethanolic extracts (stem, leaf) (Ling et al., 2010). In another hand, the biological activity could be attributed to the chemical structure of each compound, such as the presence of methoxyl group on the ring B of isorhamnetin-3-*O*-robinobioside that could participate in the antioxidant potential of this molecule (Boubaker et al., 2012). It has been also reported that several flavonoids with methoxyl substitutions exhibited higher anti-cancer activities (Boubaker et al., 2011). In our study, isorhamnetin was more effective compared to the other compounds (isorhamnetin-3-*O*-rutinoside, luteolin-7-*O*-glucoside) even in the lowest dose (5 μM). Moreover, this compound is present in low quantity in different NR extracts based on the RP-HPLC analysis. The strong activity of isorhamnetin could be due to the contribution of its chemical structure since it is the only compound of NR extracts possessing a methoxy group at a 3-position and glucose free. In this context, it was reported that flavonoids with methoxy groups showed stronger anti-diabetic effects particularly those with a methoxy group at the 3-position (Matsuda et al., 2011). As well, it was reported in comparison study between isorhamnetin free and isorhamnetin diglucoside on their anti-oxidant activities *in vivo* system that isorhamnetin diglucoside is metabolized *in vivo* by intestinal bacteria to isorhamnetin and that isorhamnetin plays an important role as anti-oxidant having a greater biological effect (Yokozawa et al., 2002). Thus, some previous studies confirmed that biological activity depends on the presence or absence of the glycoside residue (Hadj Salem et al., 2011; Williamson et al., 1996). However, in addition to the efficiency parameter, safety of tested doses of different compounds should be considered. Isorhamnetin in single effect showed a reduction on cell viability of 3T3-L1 cells compared to other glycosides compounds (**Fig. 5. 7.**). Generally, the composition of crude extract will depend on the solvent used and also on the quality of the original material, and its

composition (Jallali et al., 2012). In order to obtain a high quality extract with antioxidant activity that is suitable for use in the food, cosmetic, and pharmaceutical industries, the extract must be purified to remove all inert and undesirable components, so as to improve the antioxidant and biological activity of the bioactive molecules. A purification and fractionation processes that remove fractions with limited antioxidant activity might enable a good level of biological activity to be obtained from relatively small amounts of the original natural extract (Barbosa-Pereira et al., 2013). Thus, fractionation, purification, and/or the isolation of active principle(s) could increase bioactive component bioavailability in the extracts, and improve medicinal agent efficacy in weight loss (Sheng et al., 2008; Calixto et al., 2000). In this way, isolation of components inhibiting the anti-obesity compounds would be necessary. Further studies should be continued to purify phenolics from natural (and possibly safer) alternative sources of antioxidants. Because of their benefits to human health, natural antioxidants isolated from *Nitraria retusa* could be an alternative used in food industries in order to avoid or at least reduce the use of synthetic products.

5.5. Conclusion

As far the *Nitraria retusa* extracts (stem extract, leaf extract, mixed leaf and stem extract) tested in the current study were in crude form and probably contained many bioactive components which may well act in synergy. It is not possible to determine which compounds are responsible for the observed effects. However, our data suggest that the biological effects exhibited by this plant, under determined experimental conditions described throughout the dissertation, could be related to an overall effect of bioactive molecules extracted by 70 % ethanol.

Table 5. 1. Identification and quantification of phenolic compounds of stem extract of *Nitraria retusa*.

N°Peak	Retention Time (min)	Area	Identification	Quantification (µg/mL)
1	10.882	30.5	Resorcinol	77.542
2	15.882	43.2	Chlorogenic acid	25.958
3	20.010	13.7	*P*-coumaric acid	3.335
4	20.306	30.1	Sinapic acid	4.361
5	21.179	79.5	Luteolin 7-*O*-glucoside	7.902
6	21.875	63	Hyperoside	2.539
7	22.804	714.5	Isorhamnetin 3-*O*- rutinoside	130.417
8	24.559	23.6	Quercetin	1.805
9	25.840	32.3	Isorhamnetin	1.463

Table 5. 2. Identification and quantification of phenolic compounds of leaf extract of *Nitraria retusa*.

N°Peak	Retention Time (min)	Area	Identification	Quantification (µg/mL)
1	10.813	27.2	Resorcinol	69.152
2	15.685	150	Chlorogenic acid	20.690
3	20.220	66.6	Sinapic acid	9.651
4	21.191	2539.3	Luteolin 7-*O*-glucoside	252.403
5	21.756	23.5	Isoquercitrin	16.708
6	21.866	158.5	Hyperoside	6.388
7	22.839	1523.1	Isorhamnetin 3-*O*- rutinoside	278.010
8	24.473	23.9	Quercetin	1.828
9	25.840	11.9	Isorhamnetin	0.539

Table 5. 3. Identification and quantification of phenolic compounds of mixed part extract of *Nitraria retusa*

N °Peak	Retention time (min)	Area	Identification	Quantification (µg/mL)
1	10.878	31.4	Resorcinol	79.830
2	15.769	169.1	Chlorogenic acid	23.325
3	20.038	29.6	*P*-coumaric acid	7.207
4	20.248	113.8	Sinapic acid	16.491
5	21.219	1995.4	Luteolin-7-*O*-glucoside	198.340
6	21.772	374	isoquercitrin	265.921
7	21.888	184.7	Hyperoside	7.444
8	22.861	2172.3	Isorhamnetin 3-*O* rutinoside	396.508
9	24.595	66.2	Quercetin	5.065
10	25.887	25.2	Isorhamnetin	1.141

Table 5. 4. List of standards and their retention time (min).

Standards	Retention time (min)
Resorcinol	10.835
Chlorogenic acid	15.918
P-coumaric acid	20.091
Sinapic acid	20.278
Luteolin-7-*O*-glucoside	21.145
Isoquercetin	21.745
Hyperoside	21.803
Isorhamnetin-3-*O*-rutinoside	22.986
Quercetin	24.285
Isorhamnetin	25.899

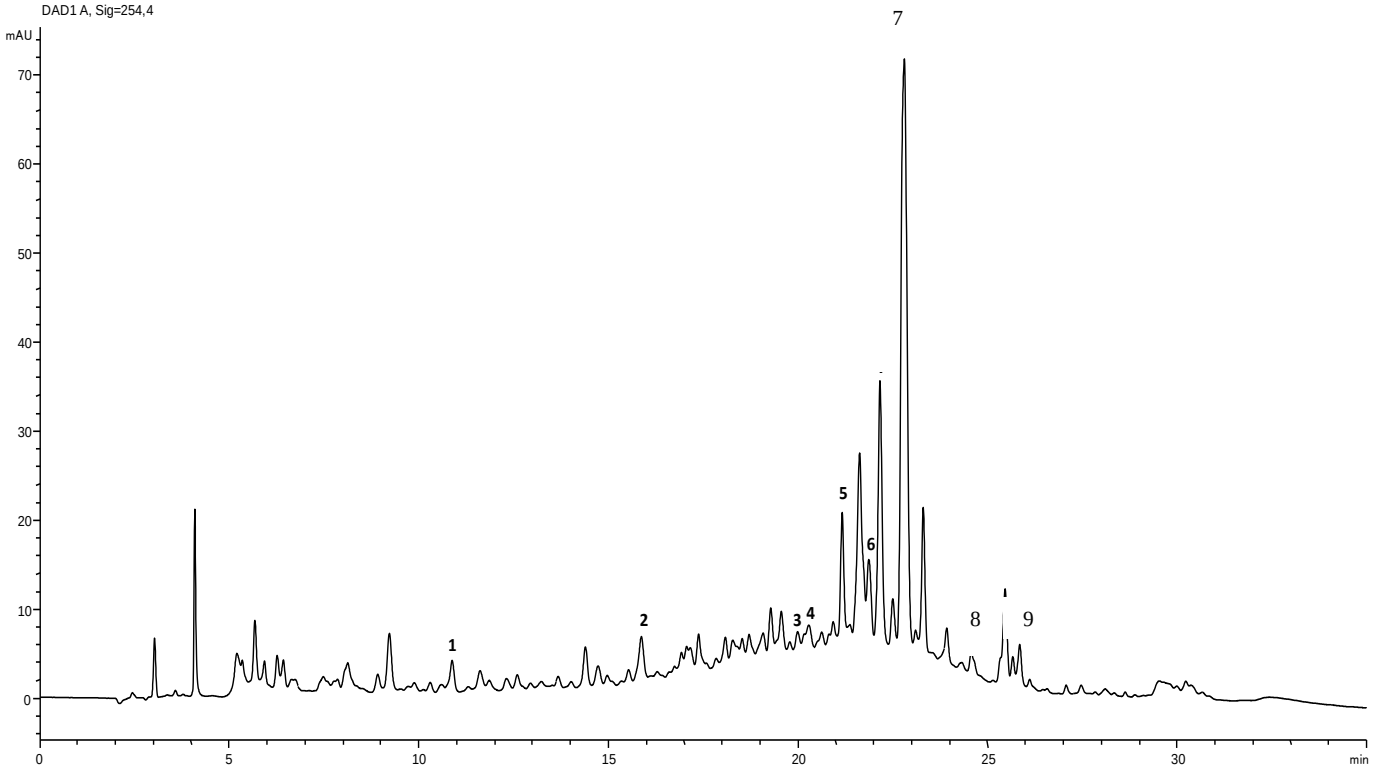


Fig. 5. 1. Chromatographic profile of stem part extract of *Nitraria retusa* acquired at 254 nm.

Peaks were identified based on the retention time of standards. 1: Resorcinol, 2: Chlorogenic acid, 3: *P*-coumaric acid, 4: Sinapic acid, 5: Luteolin 7-*O*-glucoside, 6: Hyperoside, 7: Isorhamnetin 3-*O*- rutinoside, 8: Quercetin, 9: Isorhamnetin.

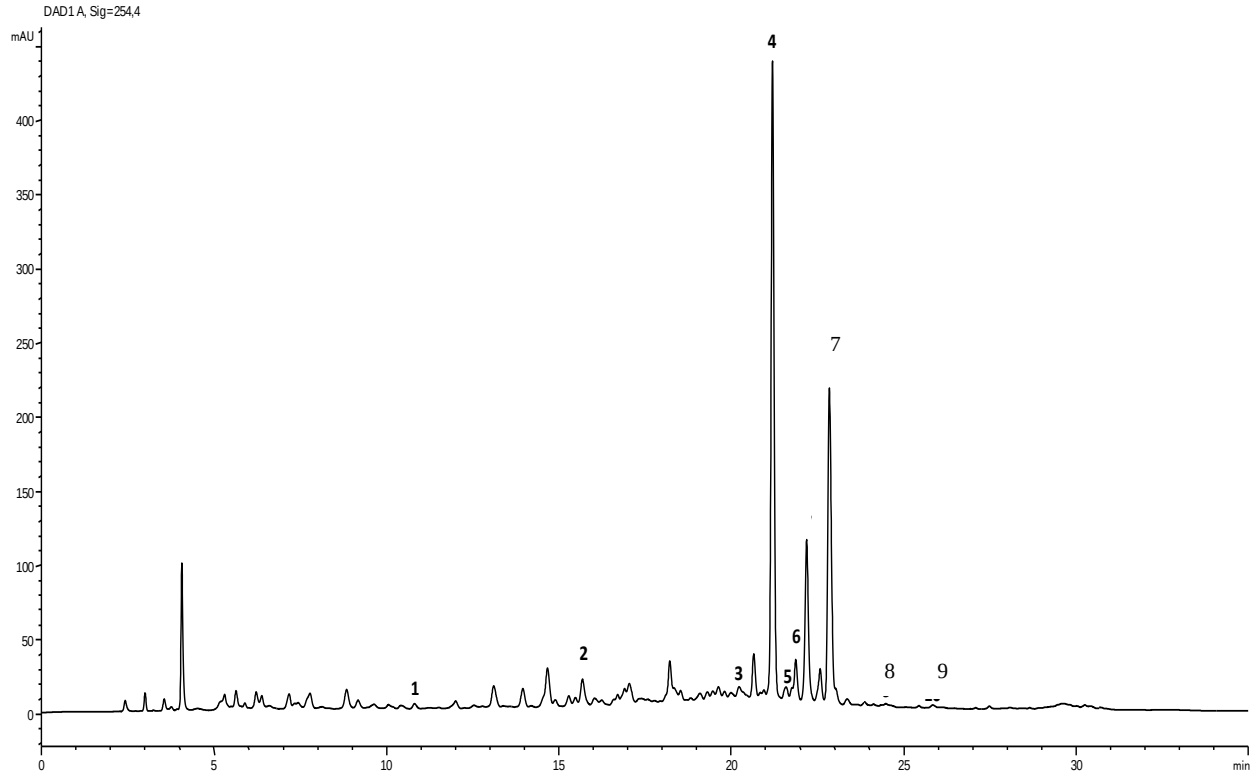


Fig. 5. 2. Chromatographic profile of leaf part extract of *Nitraria retusa* acquired at 245 nm.

Peaks were identified based on the retention time of standards. 1: Resorcinol, 2: Chlorogenic acid, 3: Sinapic acid, 4: Luteolin 7-*O*-glucoside, 5: Isoquercitrin, 6: Hyperoside, 7: Isorhamnetin 3-*O*- rutinoside, 8: Quercetin, 9: Isorhamnetin.

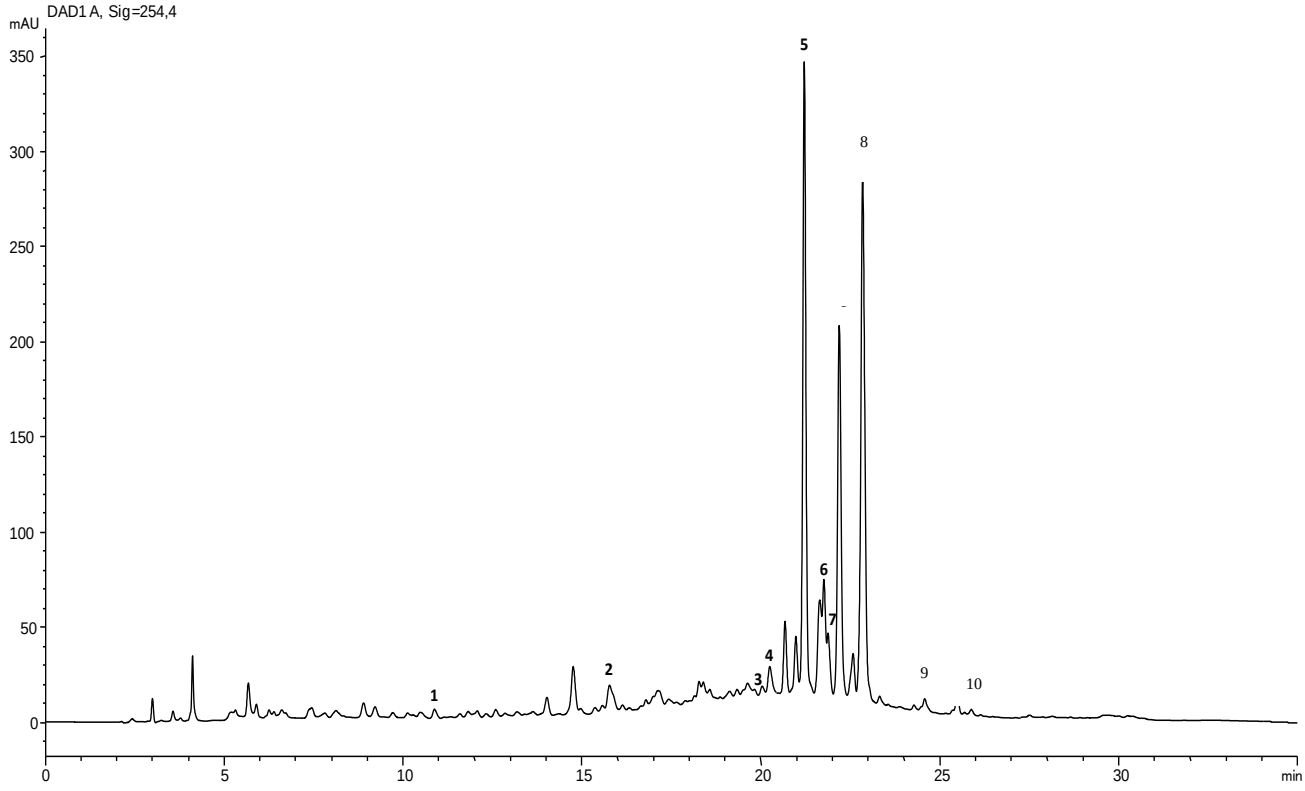
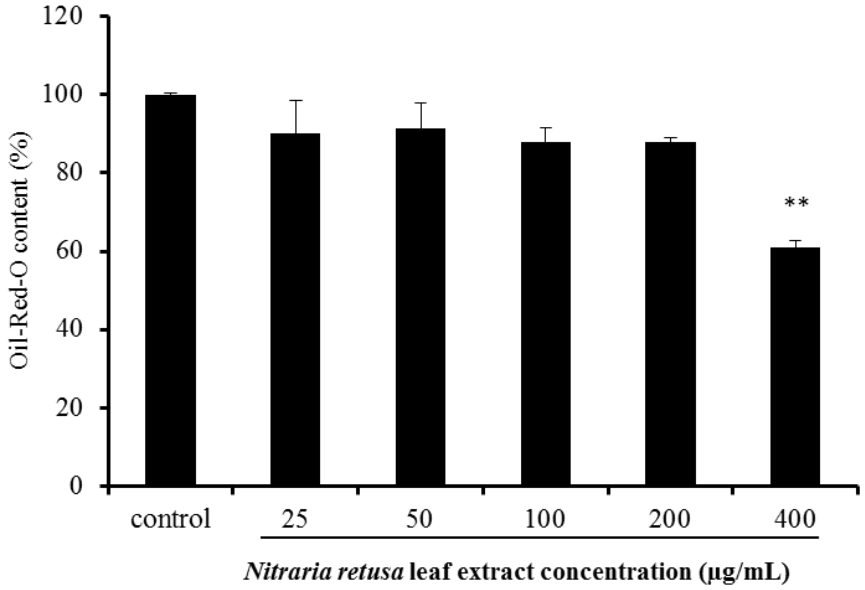


Fig. 5. 3. Chromatographic profile of mixed part extract (leaf and stem) of *Nitraria retusa* acquired at 245 nm.

Peaks were identified based on the retention time of standards. 1: Resorcinol, 2: Chlorogenic acid, 3: *P*-coumaric acid, 4: Sinapic acid, 5: Luteolin 7-*O*-glucoside, 6: Isoquercitrin, 7: Hyperoside, 8: Isorhamnetin 3-*O*- rutinoside, 9: Quercetin, 10: Isorhamnetin.

A



B

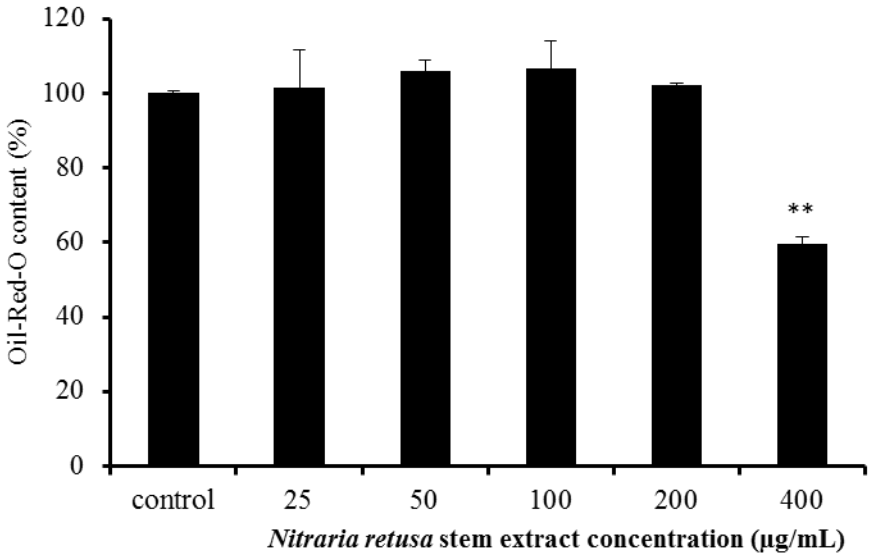


Fig. 5. 4. Effects of *Nitraria retusa* extracts; Leaf extracts (25, 50, 100,200 and 400 µg/mL): (A) and Stem extracts (25, 50, 100, 200 and 400 µg/mL): (B) on lipid droplet content in 3T3-L1 cells. Lipid droplet accumulation in treated cells was expressed as a percentage of control (untreated cells). Fat droplets in adipocytes differentiated for 7 days with or without treatments, were stained with oil Red-O dye and relative Oil-Red-O absorbance was measured at 490 nm. Bars represent mean ± SD, n=3, ** $p<0.01$ vs. control.

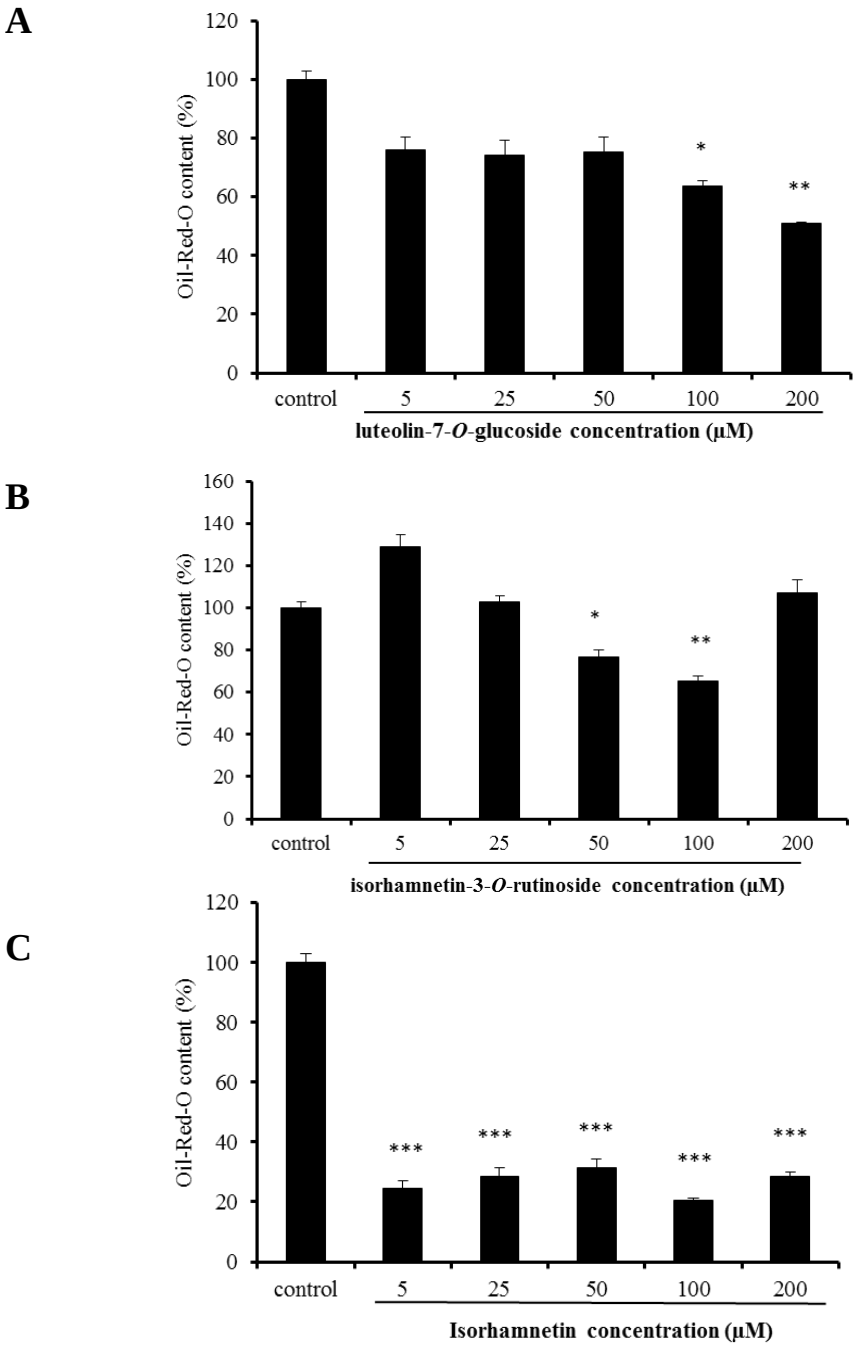
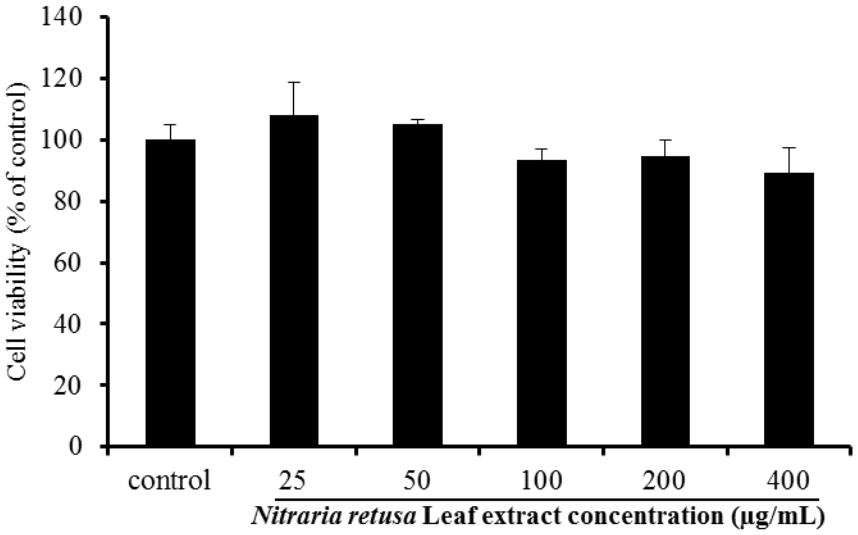


Fig. 5. 5. Effects of luteolin-7-*O*-glucoside (5, 25, 50, 100 and 200 μM): (A), isorhamnetin-3- *O*-rutinoside (5, 25, 50, 100 and 200 μM): (B) and isorhamnetin (5, 25, 50, 100 and 200 μM): (C) on lipid droplet content in 3T3-L1 cells. Lipid droplet accumulation in treated cells was expressed as a percentage of control (untreated cells). Fat droplets in adipocytes differentiated for 7 days with or without treatments, were stained with oil Red-O dye and relative Oil-Red-O absorbance was measured at 490 nm. Bars represent mean ± SD, n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control.

A



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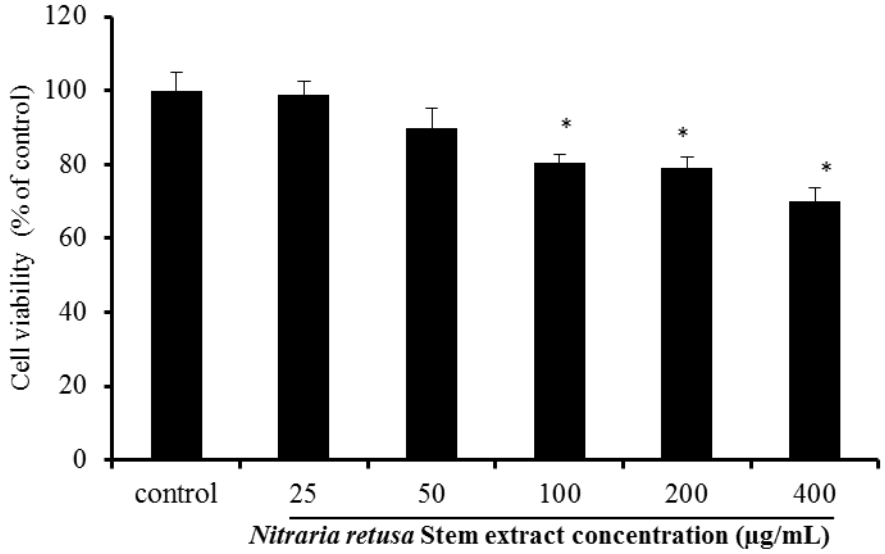

Fig. 5. 6. Effects of *Nitraria retusa* extracts; leaf extract (25, 50, 100,200 and 400 µM): (A) and stem extract (25, 50, 100, 200 and 400µg/mL): (B) on 3T3-L1 pre-adipocytes viability during differentiation (7 days treatments). Bars represent mean ± SD, n=3, * $p<0.05$.

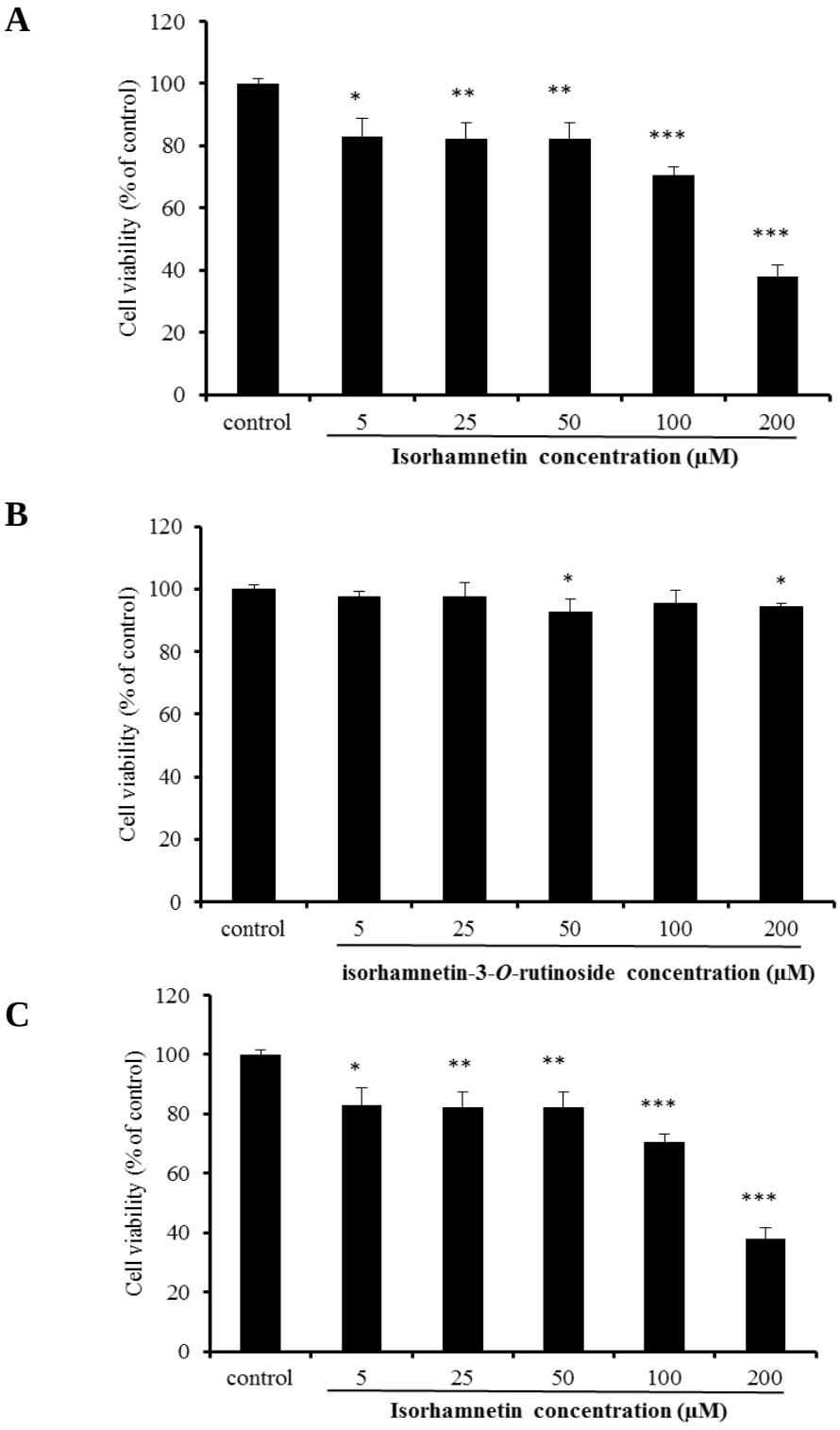

Fig. 5. 7. Effects of luteolin-7-O-glucoside (5, 25, 50, 100 and 200 µM): (A), isorhamnetin-3- O-rutinoside (5, 25, 50, 100 and 200 µM): (B) and isorhamnetin (5, 25, 50, 100 and 200 µM): (C) on 3T3-L1pre-adipocytes viability during differentiation (7 days treatments). Bars represent mean ± SD, n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

CHAPTER 6

General conclusion and perspectives

General conclusion and perspectives

This chapter aims to give a summary of the main findings in this dissertation and to address several questions as future perspectives to the application of *Nitraria retusa*, as a food supplement. 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. Halophytes, salt-tolerant species, are considered a potent source of various chemical secondary metabolites since they have the ability to overcome the severe and extreme environmental conditions due to their powerful antioxidant system. Mechanisms of anti-obesity actions of medicinal plants have been outlined, either by using *in vitro* or *in vivo* models. 3T3-L1 is a pre-adipose cell line widely used in obesity research. Suppression of pre-adipocyte differentiation or reduction of cell viability is considered as possible ways to treat obesity. In the other hand, many *in vivo* models are used for the prevention or treatment of obesity and obesity-related complications, such as monogenic mice models and high-fat diet induced-obese mice models. In current study, our main first target was first to perform a screening assay of 6 halophytes extracts (*Nitraria retusa*, *Tamarix gallica*, *Mesembryanthemum edule*, *Atriplex inflata*, *Arthrophytum scoparium* and *Rhanterirum sueavelons*) on basis of their phenolic composition, antioxidant activities and anti-adipogenic activity in 3T3-L1 cell line model. Adipogenesis assay was performed to investigate the effect of halophytes extracts on the adipocyte differentiation and on the lipid droplets accumulation in 3T3-L1 cells using Oil red O staining. Differentiated 3T3-L1 cells were treated every two days with halophyte samples extracts at various concentration and with 25 μM isorhamnetin (as a positive control), for 7 days. Based on Oil-red-O content quantification,

results showed that *Nitraria retusa* ethanolic extract (NRE) treatment at 25, 50, 100, 200 and 400 μg/mL, could inhibit the lipid droplet accumulation in 3T3-L1 cells compared to untreated cells, in dose dependent manner. Compared to other samples, *Nitraria retusa* extract was shown to possess a potential reduction of lipid droplet accumulation within cells, with modulation of hypertrophy (cell size) rather than hyperplasia (cell number) and without any cytotoxicity effect on cell viability (MTT assay, Via-count assay using flow cytometry). Thus NRE treatment might induce the cell differentiation into smaller adipocytes compared to untreated cells. Evaluating genes expression related to 3T3-L1 differentiation (*CEBP-α*, *PPAR-γ*) and their target genes; adipocyte-specific genes; such as *FAS*, *LPL* and *SREBP-1c*, could be appropriate targets for preventive intervention against obesity. *Nitraria retusa* ethanolic extract (NRE), could up-regulate these genes which is correlated with the induction effect of cell differentiation but in smaller adipocytes. Therefore, it reduced the harmful effect of oxidative stress and insulin resistance caused by fat accumulation within big adipocytes. Further step was aimed to investigate the effect of oral administration NRE *in vivo*, using high-fat diet-induced C57B6J/L obese mice model. Male C57B6J/L mice were separately fed a normal diet (ND) or a high-fat diet (HFD) and daily administrated with NRE (50, 100 mg/kg BW) or once 2 days with Naringenin (10 mg/kg BW). NRE administration significantly decreased body weight gain, fat-pad weight, serum glucose, and lipid levels in HFD-induced obese mice. To elucidate the mechanism of action of NRE, the expression of genes involved in lipid metabolism were measured in liver. Results showed that mice treated with NRE demonstrated a significant decreasing in cumulative body weight and fat pad weight, a significant lowering in glucose and triglycerides serum levels, as well as an increasing in the HDL-cholesterol serum level. Moreover mRNA expression results showed an enhancement of the expression of genes related

to hepatic lipid metabolism. Our findings suggest that NRE treatment had a protective or controlling effect against a high fat diet-induced obesity in C57B6J/L mice through the regulation of expression of genes involved in lipolysis and lipogenesis thus the enhancement of the lipid metabolism in liver. We concluded that NRE administration could control or prevent obesity caused by high fat diet intake. In next step and in order to test whether the efficiency of this extract could be maintained even in obesity-related metabolic syndrome 'Diabetes', we used transgenic diabetic obese model mice BKS.Cg-*Dock7*^{m}+/+ *Lepr*$^{db/}$J (*db/db*). We orally administrated NRE (50mg/kg BW) to BKS.Cg-*Dock7m+/+ Leprdb/*J mice model for a period of 30 days. The effect was also 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-*Dock7m+/+ Leprdb/*J mice model and may relieve obesity-related symptoms including hyperlipidemia through modulating the lipolysis-lipogenesis balance. As conclusion we observed the same tendency to reduce body weight gain, to regulate blood biochemistry as well as to enhance the lipid metabolism in liver of both of mice models (C57B6J/L and BKS.Cg-*Dock7m+/+ Leprdb/*J). Taken together all results, *Nitraria retusa* ethanolic extract could exert anti-obesity actions *in vitro* and *in vivo* models. *Nitraria retusa* mixture may relieve obesity and obesity-related symptoms including hyperlipidemia through modulating the lipid metabolism balance (lipogenesis-Lypolysis) in liver of both mice models studied in this dissertation. Although not bound by theory, the *Nitraria retusa* extract disclosed

herein likely have superior anti-obesity activity which could be due to synergy brought by the combination of compounds included in the extract. *Nitraria retusa* extract used in this research, was analyzed by reverse phase- high performance liquid chromatography (RP-HPLC) system. It is likely rich in naturally present flavonoids especially glycosides ones such as; luteolin7-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, isorhamnetin, quercetin and others, that could work all in synergy. Thus, NRE mixture could be one of possible ways to control obesity and to prevent or reduce the risk of getting various obesity-related diseases.

In conclusion, reducing body weight in *db/db* mice and controlling weight gain in C57B6J/Lmice fed a high fat diet, without appetite suppression and decreasing fat accumulation within cells, as well as enhancement of lipid metabolism of mentioned mice by NRE exposure could ameliorate obesity and obesity-related metabolic diseases such as Diabetes. Dietary polyphenols may exert their pharmacological effect via synergistic interactions with multiple targets. Therefore, further studies would be needed to search for new candidates of molecules or their mixture (single and/or mixed effects of some interesting components present in NRE) as functional foods for anti-obesity effects. Thus, to study the effects of all possible metabolites becomes an enormous and fascinating target, which will be undoubtedly matter for future research. However, when polyphenols were fractionated and isolated, the benefits of the whole extract were greater than the sum of its parts. In general, polyphenols have interactive and complementary effects, which suggest a possible application in the management of complex diseases and efforts to isolate individual components might be irrelevant for clinical medicine and/or human nutrition. The botanical sources, route of administration, presence of various bioactive components and their respective functions, experimental methods used, treatment dosage, study design, treatment duration, and safety and efficacy of the plant are also crucial

factors. Further chemical, biological, and clinical studies are needed on the effectiveness of selected medicinal plants in ameliorating and treating obesity in humans, in order to improve our knowledge on the use of anti-obesity medicinal herbal preparations. Fractionation, purification, and/or the isolation of active principle(s) could increase bioactive component bioavailability in the extracts, and improve medicinal agent efficacy in weight loss (Sheng et al., 2008; Calixto et al., 2000). In this way, isolation of components inhibiting the anti-obesity compounds would be necessary. Further studies should be continued to purify phenolics from natural (and possibly safer) alternative sources of antioxidants. Because of their benefits to human health, natural antioxidants isolated from *Nitraria retusa* could be an alternative used in food industries in order to avoid or at least reduce the use of synthetic products.

SUMMARY

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. Halophytes, salt-tolerant species, are considered a potent source of various chemical secondary metabolites since they have the ability to overcome the severe and extreme environmental conditions due to their powerful antioxidant system. Mechanisms of anti-obesity actions of medicinal plants have been outlined, either by using in vitro or in vivo models. 3T3-L1 is a pre-adipose cell line widely used in obesity research. Suppression of pre-adipocyte differentiation or reduction of cell viability is considered as possible ways to treat obesity. In the other hand, many in vivo models are used for the prevention or treatment of obesity and obesity-related complications, such as monogenic mice models and high-fat diet induced-obese mice models. In current study, our main first target was first to perform a screening assay of 6 halophytes extracts (*Nitraria retusa*, *Tamarix gallica*, *Mesembryanthemum edule*, *Atriplex inflata*, *Arthrophytum scoparium* and *Rhanterirum sueavelons*) on basis of their phenolic composition, antioxidant activities and anti-adipogenic activity in 3T3-L1 cell line model. Nitraria retusa ethanolic extract (NRE) treatment at 25, 50, 100, 200 and 400 µg/mL could inhibit the lipid droplet accumulation in 3T3-L1 cells compared to untreated cells, in dose dependent manner. Further step was aimed to investigate the effect of oral administration NRE in vivo, using high-fat diet-induced C57B6J/L obese mice model. Male C57B6J/L mice were separately fed a normal diet (ND) or a high-fat diet (HFD) and daily administrated with NRE (50, 100 mg/kg BW) or once 2 days with Naringenin (10mg/kg BW). NRE administration significantly decreased body weight gain, fat-pad weight, serum glucose, and lipid levels in HFD-induced obese mice. To elucidate the mechanism of action of NRE, the expression of genes involved in lipid metabolism were measured in liver. Results showed that mice treated with NRE demonstrated a significant decreasing in cumulative

body weight and fat pad weight, a significant lowering in glucose and triglycerides serum levels, as well as an increasing in the HDL-cholesterol serum level. Moreover mRNA expression results showed an enhancement of the expression of genes related to hepatic lipid metabolism. Our findings suggest that NRE treatment had a protective or controlling effect against a high fat diet-induced obesity in C57B6J/L mice through the regulation of expression of genes involved in lipolysis and lipogenesis thus the enhancement of the lipid metabolism in liver. We concluded that NRE administration could control or prevent obesity caused by high fat diet intake. In next step and in order to test whether the efficiency of this extract could be maintained even in obesity-related metabolic syndrome 'Diabetes', we used transgenic diabetic obese model mice BKS.Cg-*Dock7m+/+ Leprdb/*J (*db/db*). We orally administrated NRE (50 mg/kg BW) to BKS.Cg-*Dock7m+/+ Leprdb/*J mice model for a period of 4 weeks. The effect was also 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-*Dock7m+/+ Leprdb/*J mice model and may relieve obesity-related symptoms including hyperlipidemia through modulating the lipolysis-lipogenesis balance. *Nitraria retusa* extract used in this research, was analyzed by reverse phase- high performance liquid chromatography (RP-HPLC) system. It is likely rich in naturally present flavonoids especially glycosides ones such as; luteolin7-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, isorhamnetin, quercetin and others,

that could work all in synergy. Thus, NRE mixture could be one of possible ways to control obesity and to prevent or reduce the risk of getting various obesity-related diseases.

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