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Zataria multiflora increases insulin sensitivity and PPARγ gene expression in high fructose fed insulin resistant rats

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ABSTRACT

Objective(s): In insulin resistance, the insulin action in liver, muscles and adipocytes decreases and result in hyperglycemia, dyslipidemia and hyperinsulinemia. In this study we evaluate the effect of *Zataria multiflora* extract on insulin sensitivity in high fructose fed insulin resistant rats, since this extract was shown antihyperglycemic effect in streptozotocin induced diabetes in rats.

Materials and Methods: Experimental rats were fed with high fructose diet for 6 weeks and then were treated with *Z. multiflora* extract or a pioglitazone solution for 2 weeks. Blood and tissue samples were collected for analysis at the end of two weeks. Blood glucose, serum level of triglyceride and cholesterol were measured by auto analyzer. Insulin and adiponectin levels were assayed by enzyme-linked immunosorbent assay (ELISA) method. Plasma free fatty acids profile was studied by gas chromatography. Peroxisome proliferator activated receptor (PPAR.γ) and Glucose transporter type 4 (GLUT.4) gene expressions were assessed by real time polymerase chain reaction (PCR) and western blotting.

Results: Animals were treated by *Z. multiflora* extract showed insulin (43±11pmol/l), adiponectin (5.3±0.5 µg/ml), glucose (144±9.8 mg/dl), and triglyceride (120±10 mg/dl) levels significantly improved as compare with the control group [insulin (137±34 pmol/l), adiponectin (3.9±0.15 µg/ml), glucose (187±15mg/dl), and triglycerides (217±18 mg/dl)]. PPARγ protein level, also significantly increased in *Zataria multiflora* treated group.

Conclusion: This study demonstrates the beneficial effects of *Zataria multiflora* extract on insulin resistance in rats fed with a high-fructose diet through at least three mechanisms including direct insulin like effect, increasing in adiponectin and of PPARγ protein expression.

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Introduction

Metabolic syndrome is characterized by insulin resistance, central obesity, hypertension and dyslipidemia and increases the risk of cardiovascular disease and type 2 diabetes mellitus (1). Insulin resistance is the main defect associated with the metabolic syndrome (2) and obesity is the critical factor that induces insulin resistance. In obesity, inflammation that initiates from adipose tissue, increases secretion of free fatty acids. Moreover, pattern of adipokines secretion is modified in obesity which can affect the insulin functions in the liver and muscles. Defects in glucose transporter 4 (GLUT.4) expression and translocation have been determined in the striated muscle cells and adipose tissues in insulin resistance (3).

In diabetes and pre-diabetes states, the plasma free fatty acids (FFAs) concentrations are increased, which could be due to the enlarged adipose tissue in obesity. These findings demonstrate that FFAs may be involved in the complications of diabetes (4). On the other hand, , FFAs level elevation inhibit insulin's actions (5), including inhibition of lipolysis, which in turn will further elevate FFAs release into the circulation (6).

Adipose tissue not only releases FFAs, but also it is an active metabolic tissue that participates in insulin resistance occurrence in liver and muscles (7). Adipose tissue produces different inflammatory molecules, such as TNF-α and IL-6, which may affect other tissues. Overproduction of leptin, TNF-α and IL-6 and reduction of adiponectin were identified in obesity which are involved in insulin resistance (8).

Decline in adiponectin level which occurs in obesity has a positive correlation with insulin resistance and weight loss results in significant elevation of adiponectin level (9). Adiponectin after binding to its receptors can mediate phosphorylation

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of AMP-activated protein kinase (AMPK) and also peroxisome proliferator activated receptors (PPARs) and thereby increase fatty acid oxidation and glucose uptake. Thiazolidinediones (TZDs) (agonists of PPARs) also improve insulin sensitivity via increasing adiponectin concentration (9).

PPARs modulate expression of the genes involved in metabolism of lipids (9, 10). Its activation stimulates lipid oxidation and lipogenesis, induces differentiation of adipocytes and increases insulin sensitivity in mature adipocytes (11). Therefore, synthetic PPARγ ligands such as TZDs are applied clinically to control diabetes (12).

Zataria multiflora is a plant of Laminaceae family that grows only in Iran, Pakistan and Afghanistan (13, 14). This plant local name in Iran is Avishan-e-Shirazi (15). *Z. multiflora* is widely used in Iran as a flavor component in a wide variety of foods. The main constituents of this plant are phenolic compounds including carvacrol, thymol and gamma-terpinene (16). This plant has several traditional uses in Iran including as a carminative, stimulant, diuretic and antiseptic in (15). There is not any report on the antihyperglycemic effect of this plant in the literature. In our previous study, we demonstrated that water extract of *Z. multiflora* has a strong inhibitory effect on alpha glucosidase *in vitro* (17). This extract also significantly decreased the postprandial glucose level in diabetic rats after administration of 2 g/kg maltose, but no effect on postprandial glucose level in non-diabetic rats (18). *Z. multiflora* also decreases the postprandial glucose level after administration of glucose (unpublished data).

These findings that mentioned above, demonstrate that *Z. multiflora* affects the blood glucose concentration through mechanism(s) independent of its inhibitory effect on alpha glucosidase. In this study we evaluated the effect of *Z. multiflora* on insulin resistant rats to assess the effect of this extract on muscle and hepatic insulin resistance and possible mechanism(s) involved. Skeletal muscle cells are the main site for insulin function and development of insulin resistance (5, 19) and the liver is another insulin sensitive organ that plays a critical role in energy homeostasis (8).

Materials and Methods

Preparation of extract

Z. multiflora was collected from Fars province, Iran, and authenticated by Department of Botany, Bahonar University, Kerman, Iran. The aerial parts of *Z. multiflora* were separated, washed and air dried. Plant tissues (300 g) were milled and extracted by maceration method in 1000 ml of distillated water at room temperature for 48 hr (20). After filtration water was evaporated at 40°C in an oven and dried extract was stored at -20°C.

Experimental animals and protocol

Male Wistar rats weighing 250–300 g were obtained from animal house of Kerman University of Medical Sciences. Rats were housed in animal room at 22±3°C for 12 hr light. Rats were fed with standard chow and fresh tap water for 2 weeks. After 2 weeks, rats were divided randomly into 4 groups (n=8). three groups was fed a 60% fructose diet and fourth group as the healthy control group (HCG) with standard diet (21). After 6 weeks insulin resistance in fructose fed rats was confirmed by oral glucose tolerance test (OGTT) and was compared with the healthy control group (results not shown). The fructose-treated groups were treated by:

1: *Z. multiflora* extract group: a group who received intragastric injection of *Zataria multiflora* extract (1000 mg/kg) (18).

2: Pioglitazone group (Pio): this group received intragastric injection of pioglitazone (10 mg/kg) (22).

3: Control group (Con): used as insulin resistant control group and did not receive any injections.

Treatment was continued for 2 weeks. Water and food intake were measured daily in this period. Body weight was also measured weekly throughout the 8 weeks of treatment.

Blood and tissue collection

At the end of the treatment period, animals were fasted for 12 hr overnight, blood samples were collected from heart under ether anesthesia. Blood sample was divided into two vials with or without EDTA. Vials were centrifuged at 4000 rpm for 10 min at 4°C and Plasma (for FFA analysis) or serums (for other biochemical analysis) were separated immediately. Hind limb skeletal muscles and liver were excised. Tissue samples were immediately frozen in liquid nitrogen, and subsequently stored at −75°C until evaluation.

Measurement of serum parameters

Serum glucose, triglyceride, cholesterol and high density lipoprotein- cholesterol (HDL-c) concentrations were measured in an RA-1000 autoanalyser. Blood insulin and adiponectin levels were measured by ELISA using commercial assay kits according to the manufacturer's directions (Mouse/Rat Adiponectin or insulin ELISA kit, USCN. China). Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated using the equation: [(insulin (_U/ml)×glucose (mmol/l))/22.5].

Measurement of plasma free fatty acids

Plasma free fatty acids (FFAs) were extracted and analyzed by the method explained by Kangani *et al* with slight modifications (23). 500 µl of plasma was mixed with 20 µl of pentadecanoic acid (1 mg/ml) as an internal standard. Lipids were extracted from

plasma by reagent which constituted from isopropanol–heptane–hydrochloric acid (1M) (40:10:1, v/v/v). FFAs were separated by TLC on silica gel plates using a heptane–ether–acetic acid [60:40:3] solvent system. FFAs were visualized by Iodine on TLC plates. FFA bands were scrapped and free fatty acid methyl esters (FAME) were prepared by a reaction with BF3 containing methanol (Sigma). FFA methyl esters were separated using an Agilent GC-7890A system equipped with a flame ionization detector. The injection volume was 1 µl in the split 30:1 injection mode. A capillary column (DB-225 20 m×0.1 mm I.D., 0.1 µm film thickness, J&W GC columns, USA) was employed.

Real time PCR

Total RNA from the skeletal muscle (for GLUT.4 assay) and liver (for PPARγ assay) tissues was extracted with RNeasy mini kit (Qiagen) and according to manufacturer's guideline. The RNA concentration was determined by the ultraviolet (UV) light absorbency at 260 nm and 280 nm (ND-1000 nanodrop). The quality of the RNA was confirmed by ethidium bromide staining of 18S and 28S ribosomal RNA after electrophoresis on 2% agarose gel.

Then cDNA synthesis was performed by Quanti Tect Reverse Transcription Kit (Qiagen) and thoroughly according to the manufacture's procedure. Relative Quantitative real time PCR was performed on a Qiagen Thermal Cycler (Rotor-Gene Q 5plex HRM System, Qiagen) using the corresponding QuantiFast SYBR Green PCR kit (Qiagen) according to manufacturer's protocol. The primers that used in this study were shown in Table 1.

Cycle of threshold (CT) for each sample was determined. ΔCT was calculated via formula: ΔCT = CT (target gene) – CT (endogenous reference gene (GAPDH))

Results were analyzed by $2^{-\Delta\Delta CT}$ method.

In the real time PCR all suggestions that published by Qiagen in "Critical Factors for Successful in Real Time PCR" were regarded.

Western blotting

Total protein was extracted from muscles or liver by homogenization in the RIPA (Radio Immuno Precipitation assay) buffer (sigma, cat number: R0278). Homogenate was centrifugated at 14,000 rpm for 20 min and was removed supernatant that contain proteins. Total protein was estimated by Bradford method. Using SDS-PAGE, proteins were separated by loading of 120 µg/lane and transferred on PVDF membrane by overnight incubating with 5% non-fat skim milk in TBST buffer at 4°C. Non-specific binding sites were blocked. Then, the membrane was washed with TBST 3 times (each time for 20 min). The membrane was incubated with appropriate polyclonal primary antibodies for PPARγ (ab27649, Rabbit polyclonal to PPAR gamma, abcam) or GLUT.4 (ab33780, Rabbit polyclonal to GLUT4, abcam) antibody in TBST buffer for 1hr. The membrane was then washed with TBST, as mentioned above, followed by incubation with anti-rabbit secondary antibody (Goat polyclonal HRP conjugated Antibody to Rabbit IgG, , ab6112, abcam), for 1 hr at room temperature. Then PVDF was washed and incubated with substrate (western lightening plus ECL, Perkin-Elmer) for 1 min. Then, PVDF was exposed to Hyblot
film (Denvill) for 30 sec, in a dark room. Density of bands was analyzed by image j software.

Statistical analysis

All data are presented as mean±SEM. Statistical analysis was performed by analysis of variance (ANOVA) and *Post-hoc* Tukey test; *P*-values of less than 0.05 were considered to be significant.

Results

Body weight gain and water and food intake

There was no significant difference in the mean body weight of groups at the start of treatment. Body weight gain that was calculated by subtracting initial body weight and weight at the end of 8th week, had significant difference between *Z. multiflora* and control groups ($P<0.0001$). There was also significant difference between *Z. multiflora* and pioglitazone groups weigh gain ($P<0.05$) and *Z multiflora* reduced weight gain in comparison with pioglitazone.

There was no significant difference in water or food intake between *Z. multiflora* group and pioglitazone or control group. (Results are shown in Table 2).

Table 1. Primers used in this study

Gene	Primers	PCR product	Accession number
GLUT.4	F: ACTGGCGCTTTCACTGAACT R: CGAGGCCAAGGCTAGATTTTG	106	NM_012751
PPAR.γ	F: CATGCTTGTGAAGGATGCAAG R: TTCTGAAACCGACAGTACTGACAT	131	NM_001145367
GAPDH	F: TGGAGTCTACTGGCGTCTT R: TGTCATATTTCTCGTGGTTCA	138	NM_017008

Table 2. Effect of *Zataria multiflora* on body weight, water and food intake and other insulin resistance related parameters

Parameter	Groups				*P*-value¥
	HCG	Con	Pio	ZME	
initial weight (g)	275±8	272±9	272±12	281±12	0.586
weight after 8 week (g)	285±9	307±8	294±16	282±15	0.832
Weight gain (g)	10±2*	35±2#	22±5#	11±6*	<0.0001
Water intake (ml)	35±0.8*	47±2#	60±2#*	54±3#	0.221
Food intake (g)	21.6±0.7*	13.4±0.4#	13.5±0.3#	13±0.2#	0.779
Insulin (pmol/L)	50±4.8*	137±34#	40±2.7*	43±11*	0.007
Adiponectin (µg/ml)	2.9±0.16	3.9±0.15	5.6±0.4#*	5.3±0.5#*	0.029
Glucose (mg/dl)	132±4*	187±15#	129±5.8*	144±9.8*	0.029
HOMA.IR	2.7±0.37*	9.7±2.1#	2.1±0.12*	2.6±0.74*	0.001
Cholesterol (mg/dl)	71±12.1	59±3.2	63±4.2	54±2.9	0.955
Triglyceride (mg/dl)	85±13*	217±18#	200±51#	120±10#*	0.011
HDL-c (mg/dl)	24.86±1.4	29.25±1.8	29.38±2.3	26.35±1.2	0.668

HCG was fed standard chow and three other groups were fed 60% fructose diet for 8 weeks. In Pio and *Z. multiflora* groups after primary 6 weeks, animals were treated by pioglitazone (10 mg/kg/day) or *Z. multiflora* extract (1000 mg/kg/day) for 2 week. Biochemical parameters were assayed by auto analyzer and hormones by ELISA kits. HOMA-IR was calculated by the formula. All data are Mean± SEM. (n=8)

HCG: healthy control group, Con: control, Pio: pioglitazone, ZME: *Zataria multiflora* extract

¥ *P*-value demonstrates the difference significance between *Zataria multiflora* and control group.

* Significant difference with control group $P<0.05$)

Significant difference with HCG group ($P<0.05$)

Blood glucose, triglyceride and cholesterol

Blood glucose in *Z. multiflora* group (144±9.8 mg/dl) had significant difference with control group (187±15 mg/dl) ($P<0.05$). Moreover, *Z. multiflora* significantly decreased serum triglyceride concentration (120±10 mg/dl) as compare with control group (217±18 mg/dl) ($P= 0.011$). Pioglitazone did not have any effect on triglyceride in this study (200±51 mg/dl). There is nonsignificant difference in cholesterol and LDL-c between *Z multiflora* and other groups (all results are summarized in Table 2).

Serum level of insulin and adiponectin

Serum level of insulin (43±11 pmol/l) in *Z. multiflora* group was significantly lower in comparison with the control group (137±34 pmol/l) ($P<0.01$). However, adiponectin level in *Z. multiflora* group (5.3±0.5 µg/ml) significantly increased in comparison with control group (3.9±0.15 µg/ml) ($P<0.05$). There was no significant difference in insulin and adiponectin level between *Z. multiflora* and pioglitazone treated groups (all results are summarized in Table 2).

Homeostasis model assessment of insulin resistance

There was a significant difference in HOMA-IR between *Z. multiflora* (2.6±0.74) and control groups (9.7±2.1) and treatment with *Z. multiflora* decreased the HOMA-IR as compared with control group ($P=0.001$). Also HOMA.IR in *Z. multiflora* group did not have significant difference with pioglitazone treated group (Table 2).

Plasma free fatty acids

There was no significant difference in total free fatty acids or free fatty acids fractions between *Z multiflora* treated and control group, butpioglitazone significantly decreased the level of total free fatty acids, oleic acid, palmitic acid and palmitoleic acid as compare with control and *Z multiflora* group ($P<0.05$) (all results are presented in Table 3).

Table 3. Effect of *Zataria multiflora* on plasma free fatty acids profiles

Parameter	Groups				*P- value
	HCG	Con	Pio	ZME	
myristic acid (µmol/l)	1.07±0.01	1.12±0.06	1.2±0.06	1.3±0.08	0.109
palmitic acid (µmol/l)	5.9±0.57	11.1±2.6	5.1±0.18	7.8±0.18	0.438
palmitoleic acid (µmol/l)	1.06±0.04	1.4±0.12	1.1±0.01	1.6±0.03	0.2
stearic acid (µmol/l)	1.3±0.12	2.7±0.12	2.06±0.11	2.6±0.07	0.961
oleic acid (µmol/l)	2.2±0.21	2.9±0.22	1.7±0.08	3.6±0.31	0.122
total free fatty acids (µmol/l)	12.53±1.95	22±2.7	15±2.3	19.44±2.8	0.791

HCG was fed standard chow and three other groups were fed 60% fructose diet for 8 weeks. In Pio and ZME groups after primary 6 weeks, animals were treated by pioglitazone (10 mg/kg/day) or *Zataria multiflora* extract (1000 mg/kg/day) for 2 weeks. FFA was analyzed by Gas Chromatography as free fatty acid methyl ester after extraction by TLC. All data were shown Mean± SEM (n=8)

HCG: healthy control group, Con: control, Pio: pioglitazone, ZME: *Zataria multiflora* extract

* *P*-value demonstrate the difference between *Zataria multiflora* and control group

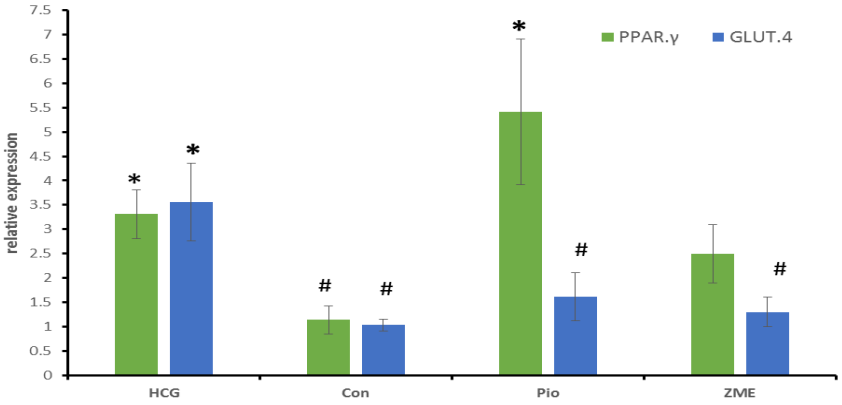

Figure 1. Effect of *Zataria multiflora* on mRNA level of liver PPARγ and muscle GLUT.4GLUT.4 and PPARγ gene expression
Our result demonstrated that *Z. multiflora* did not have any effect on PPARγ and GLUT.4 gene expression at mRNA level (*P*=0.928 and *P*=0.995, respectively). However pioglitazone significantly increased the mRNA level of PPARγ (Figure 1)
HCG was fed standard chow and three other groups were fed 60% fructose diet for 8 weeks. In Pio and *Z multiflora* groups after primary 6 weeks, animals were treated by pioglitazone (10 mg/kg/day) or *Zataria multiflora* extract (1000 mg/kg/day) for 2 weeks. PPARγ and GLUT.4 mRNA level in liver and muscle, respectively, were quantified by real time PCR. All data are Mean± SEM (n=8)
HCG: healthy control group, Con: control, Pio: pioglitazone, ZME: *Zataria multiflora* extract
* Significant difference with control group (*P*<0.05)
# Significant difference with HCG group (*P*<0.05)

GLUT.4 and PPARγ gene expression

Our result demonstrated that *Z. multiflora* did not have any effect on PPARγ and GLUT.4 gene expression at mRNA level (*P*=0.928 and *P*=0.995, respectively). However pioglitazone significantly increased the mRNA level of PPAR.γ. (Figure 1).

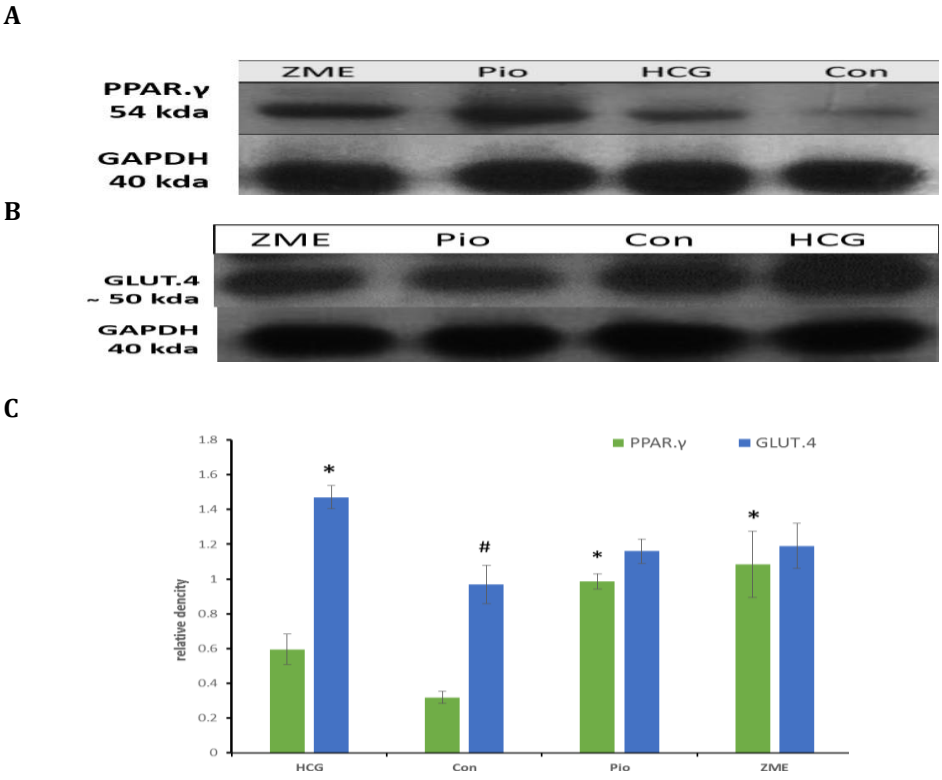

Figure 2. Effect of *Zataria multiflora* on protein level of liver, PPARγ and muscle GLUT.4
HCG was fed standard chow and three other groups were fed 60% fructose diet for 8 weeks. In Pio and *Z multiflora* groups after primary 6 weeks, animals were treated by pioglitazone (10 mg/kg/day) or *Zataria multiflora* extract (1000 mg/kg/day) for 2 weeks. PPARγ and GLUT.4 protein level in liver and muscle, respectively, were assayed by Western blotting
HCG: healthy control group, Con: control, Pio: pioglitazone, ZME: *Zataria multiflora* extract
* Significant difference with control group (*P*<0.0001)
# Significant difference with HCG group (*P*<0.05)

This study showed that, there was a significant difference in PPARγ protein level between *Z. multiflora* and control group. Moreover, pioglitazone significantly increased the level of PPARγ protein as compare with control group. There was no significant difference in GLUT.4 protein level between *Z. multiflora* and control or pioglitazone groups (Figure 2).

Discussion

In this study we found that *Z. multiflora* has beneficial effect on insulin resistance and this extract increased the insulin sensitivity in fructose fed insulin resistant rats. *Z. multiflora* significantly decreased glucose, insulin and HOMA.IR in insulin resistant animal model. The effect of *Z. multiflora* on these parameters was comparable with pioglitazone and there was no significant difference between *Z. multiflora* and pioglitazone treated groups. *Z. multiflora* also decreased the weight gain of animals in comparison with control group. To the best of our knowledge, there was not any study about *Z multiflora* effects on insulin resistance in the literature but these results clearly suggest that *Z. multiflora* improves insulin resistance. Reduced HOMA-IR and glucose level along with reduction in insulin level, suppose that antihyperglycemic effect arises from improving insulin action rather than insulin secretion (24) and decrease in insulin and glucose level propose that *Z. multiflora* has a direct insulin like effect (25).

Based on our results, TFG showed lowering effect on triglyceride level in insulin resistant rats but *Z. multiflora* did not have any effect on total cholesterol, HDL-C and water and food intake. In recent years, ATPIII guidelines suggest therapies that lower triglycerides in patients with metabolic syndrome (26). Therefore, TFG is more suitable than pioglitazone for this purpose.

Z. multiflora significantly increased the level of serum adiponectin concentration. Adiponectin level is modulated by a number of hormones and factors that participated in regulation of metabolic processes. Insulin diminishes the adiponectin expression in mice and humans (27) and thiazolidinediones, as mentioned above, increase the expression of adiponectin (28). Adiponectin inhibits gluconeogenesis in the liver and increases β-oxidation of free fatty acids in muscles and therefore increases insulin sensitivity and energy homeostasis (29). These findings are sufficient to conclude that antihyperglycemic effect of *Z. multiflora* may be due to the enhancing of adiponectin production.

In molecular level, *Z. multiflora* increases the protein of PPARγ in the liver without rising PPARγ mRNA level, and it also has no effect on GLUT.4 protein and mRNA in the striated muscles. PPAR-γ agonists exert their antidiabetic effect by PPAR-γ activation resulting to increase the sensitivity of insulin receptors (30). Thiazolidinediones significantly increase insulin sensitivity and adiponectin concentration in diabetic patients (30, 31). In Yadav *et al* study, it was shown that PPARγ mRNA level increased in liver of insulin resistant rats that treated with rosiglitazone (32). PPARγ protein decreased in fructose fed rats and increased after treatment with pioglitazone and *Z. multiflora*. Based on the role of PPARγ activity in improvement of insulin resistance, it is possible that increasing the PPARγ protein level could be another mechanism for antihyperglycemic effect of *Z. multiflora*.

PPARγ activation increases fatty acid uptake, adipogenesis and fat deposition (12). Decrease of serum triglycerides that induced by *Z. multiflora* can be correlate with triglyceride storage in insulin-sensitive tissues resulting from PPARγ over expression (33).

PPARγ increase the expression and translocation of GLUT4 in adipose tissue, and increase the catabolism of glucose in the liver along with the reduction in hepatic glucose output (34). We did not find any difference between *Z. multiflora* and control group in GLUT.4 levels. Besides, pioglitazone did not have any effect on GLUT.4 protein and mRNA levels. We evaluated in this study, total GLUT.4 in striated muscle cells. It is possible that translocation of GLUT.4 to the cell membrane is increased after treatment with *Z multiflora* and pioglitazone despite the total GLUT.4 protein is not significantly different between two groups (3). In shih *et al* study that was assayed membrane GLUT.4 instead of total GLUT.4, showed increase in GLUT.4 simultaneously with elevation of PPARγ gene expression after treatment with *Momordica charantia* (21).

Total FFA or FFA fraction did not significantly change with *Z. multiflora* in insulin resistant rat. However there was a significant difference in serum total free fatty acids and its fractions between fructose and control groups. Increase of FFA levels in the blood is an important sign of diabetes and there is an association between increased plasma FFA levels and reduction of glucose catabolism in skeletal muscles (12). PPARγ agonists as mentioned above enhance insulin sensitivity in adipose tissue and reduce serum FFA levels (11). PPARγ agonists play their insulin sensitizing activity by increasing FFA uptake and storage in adipose tissues (35). But in our study, FFA level has no significant difference between two groups whereas PPARγ protein increased. We evaluated PPARγ gene expression in the liver to evaluate hepatic insulin resistance and it is possible that PPARγ gene expression in adipocytes did not increase.

Conclusion

The findings explained in this study clearly demonstrated that *Zataria multiflora* water extract

can improve insulin resistance in insulin resistant animal model. This antihyperglycemic effect and increase in insulin sensitivity may be exerted by at least three mechanisms including direct insulin like effect, increase in adiponectin and increase of PPARγ protein expression.

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