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Berberis integerrima ameliorates insulin resistance in high-fructose-fed insulin-resistant rats

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ABSTRACT

Objective(s): This study was aimed to investigate the effect of *Berberis integerrima (B. integerrima)* extract on insulin sensitivity in high-fructose-fed insulin-resistant rats.

Materials and Methods: Experimental rats were randomly divided into two groups: the control group was fed a regular chow diet while other group fed with a high-fructose diet for 8 weeks. After the first six weeks, the animals were treated with *B. integerrima* extract or pioglitazone for two weeks. Insulin and adiponectin levels were measured by ELISA. Additionally, Insulin resistance was calculated using a Homeostasis Model Assessment of Insulin resistance (HOMA-IR). The plasma free fatty acid (FFA) profile was obtained by gas chromatography. PPARγ and GLUT4 gene expression were assessed by real-time polymerase chain reaction (PCR) and western-blotting.

Results: Comparing the *B. integerrima* treated group with the control group, weight gain (P=0.009) and levels of insulin (P=0.001), blood glucose (P<0.0001), and HOMA-IR (P<0.0001) were significantly reduced. Additionally, the adiponectin concentration was significantly increased (P<0.0001). Among the FFA fractions, the mean concentration of palmitoleic acid and stearic acid in the *B. integerrima* group were significantly higher than the control group (P<0.0001 and P=0.005, respectively). However, there was no significant difference at the mRNA and protein level of GLUT4 and PPAR-γ between B. *integerrima* treated group and control group.

Conclusion: The study findings revealed that *B. integerrima* might be a protective candidate against Type 2 diabetes/insulin resistance through direct insulin-like effect and an increase in adiponectin levels. However, the mechanism of *B. integerrima* was independent of GLUT4 and PPARγ.

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Introduction

Obesity is the primary disease of adipocytes and is strongly associated with serious comorbidities that impair an individual's health (1, 2). Obesity is closely related to insulin resistance and other chronic diseases including all components of metabolic syndrome, cardiovascular diseases, type 2 diabetes mellitus (T2DM), and certain types of cancers (2–4). Chronic consumption of high-fat diets leading to the development of obesity can induce insulin resistance in both human and animals (5).

Insulin resistance is defined as impaired ability of insulin to promote glucose uptake and exert its metabolic effects in its target tissues (liver, skeletal muscle and adipose tissue) (6, 7). Moreover, intensive studies have demonstrated a strong association between obesity and insulin resistance with dysregulation of adipokines (8–10). Adiponectin, one of the most abundant adipokines secreted exclusively by adipocytes, plays an influential effect in insulin-sensitizing and anti-diabetic activity (8–12). Recent findings have revealed that adiponectin exerts its effect by binding to both AdipoR1 and AdipoR2 receptors, thereby leading to the activation of peroxisome proliferator-activated receptors (PPARs) and 5′AMP-activated protein kinase (AMPK) signaling pathways (11, 13). Subsequently, PPARs and AMPK cause an increase in fatty acid oxidation and glucose uptake (14, 15).

Several mechanisms have been proposed that increased free fatty acids (FFAs) interference with insulin function (16–19). High circulating levels of FFA not only inhibit lipolysis but also prevent insulin signaling pathway through activating protein kinases such as protein kinase C (PKC), and c-JUN NH2-terminal protein kinase (JNK) (2, 20). Since insulin acts as an effective inhibitor of lipolysis, increased circulating levels of FFAs contribute to

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insulin resistance (17, 21).

It is well established that insulin resistance is associated with increase in inflammatory mediators such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), IL-10, plasminogen activator inhibitor-1 (PAI-1), macrophages and monocyte chemoattractant protein-1 (MCP-1), leptin and resistin (16, 21–23). These cytokines can interfere with the binding of IRS1/2 to the insulin receptor by activating the suppressor of cytokine signaling (SOCS) proteins and several serine/threonine kinases such as JNK, PKC, and IκB kinase β (IKK-β) (22, 24). Furthermore, an imbalance in energy metabolism of skeletal muscles plays an essential role in the development of metabolic diseases (25). The overproduction of lipids in skeletal muscles contributes to inhibition of glucose uptake through GLUT4 translocation from intracellular location to plasma membranes which can lead to insulin resistance (22, 23). In turn, regulation of GLUT4 is a major contributor of glucose metabolism in both skeletal muscle and adipose tissue (22, 23).

PPAR is a ligand-activated transcription factor which has three isotypes (α, γ, and ß/δ) (26). Activation of PPAR enhances insulin resistance and promotes lipid metabolism by increasing FFA oxidetion and repressing the expression of leptin gene (2, 27). Rosiglitazone and pioglitazone which are subclasses of thiazolidinedione (TZD) are recognized as PPAR-γ agonists (28). These drugs have been used clinically as regulators of genes involved in glucose, lipid, and protein metabolism to control hyperglycemic status in patients with T2DM (29,30). However, drugs like fibrates (the PPARα agonists), and the mentioned PPAR-γ agonist cause an increase in the risk of cardiovascular disease and bladder cancer (31, 32). Consequently, much attention has been focused on functional food ingredients and herbal extracts to reduce the mortality rate and other serious side effects (1, 31).

Berberis integerrima, a plant belonging to Berberidaceae family, has exclusively been cultivated in Iran, especially in Northern and North-eastern regions where it is referred to as Zereshk in the Persian language (33–35). In traditional medicine, *B. integerrima* was mainly used as an impressive medicine for treating diabetes in the Alamut region, in the province of Qazvin, Iran (33). Furthermore, *B. integerrima* was used to treat various type of infectious fevers, diarrhea and it has also been reported to have other effects including hypolipidemic, hypotensive, and anti-inflammatory features (33, 34). Many components of *B. integerrima* have been identified. The main constitutes of the plant include berberine, palmatine chloride, berberine chloride, oxyacanthine, berbamine, and columbamine, among them, berberine is the most important active constituent found in the bark of the root, stem, and unripe fruit (33–37). Pharmaceutical and therapeutic effects of berberine have also been reported in treating diabetes. Previous studies have indicated that berberine improves insulin sensitivity and glucose uptake by activating protein kinase B and increasing glucose uptake through AMPK and AMPK-P38 pathways (36, 38).

Nevertheless, available data regarding the effects of *B. integerrima* extract on the possible mechanisms of insulin resistance are scarce. As skeletal muscles and hepatic tissue are the chief parts involved in insulin function and energy homeostasis, we selected these two tissues. In the direction of unknown mechanisms and low evidence, the objective of the present study was to examine the effects of *B. integerrima* root extract on muscle and hepatic insulin resistance in rats to determine the possible mechanisms involved.

Materials and Methods

Preparation of extract

B. integerrima was collected from Fars province, Iran. Its genus and species were approved by the department of botany, at Bahonar University, Kerman, Iran (Voucher specimen no: KF1194). After collecting the plants, fruits were dried in the shade at 30 °C. Dried fruits were milled and converted into powder. The resulting powder was extracted by maceration method (300 g in 1000 ml distilled water) for 48 hrs at room temperature (39). After filtration water was evaporated at 40 °C in an oven and dried extract was stored at -20 °C.

Experimental animals and treatment

In this experimental study, male Wistar rats (weighing 250–300 g) were obtained from the animal house of Kerman University of Medical Sciences. The Ethics Committee of Kerman University of Medical Sciences approved the study procedure. Rats were maintained in the animal room at 22±3 °C under an automatic lighting schedule (08:00–20:00 hr). They had free access to standard chow and water for two weeks. They were then randomly divided into four groups (n=8). The first three groups were fed a 60% fructose diet, whereas the fourth group, as the healthy control group (HCG) was fed with normal chow for 8 weeks (40). The fructose-enriched diet contained 60% fructose, 21% protein, and 5 % fat, sodium 0.49%, and potassium 0.49% (40). After 6 weeks, insulin resistance was confirmed by oral glucose tolerance test (OGTT).

While rats were still on the fructose diet, they were further subdivided into three groups: Two groups received an intragastric injection of *B. integerrima* extract (1000 mg/kg) (41) and pioglitazone (Sigma, Saint Louis, MO, USA) (10 mg/kg) (42) as a *B. integerrima* extract group and pioglitazone group (Pio), respectively. The third group as a control group (Con) without any treatment. These treatment

Table 1. Primers used in this study

Gene	Primers	PCR Product	Accession Number
GLUT4	F: ACTGGCGCTTTCACTGAACT R: CGAGGCCAAGGCTAGATTTTG	106 bp	NM_012751
PPARγ	F: CATGCTTGTGAAGGATGCAAG R: TTCTGAAACCGACAGTACTGACAT	131 bp	NM_001145367
GAPDH	F: TGGAGTCTACTGGCGTCTT R: TGTCATATTTCTCGTGGTTCA	138 bp	NM_017008

periods lasted for 2 weeks. Body weight was measured weekly throughout the study.

Blood and tissue collection

At the end of the treatment period, the food was removed for 12 hr before collecting blood samples from the heart under ether anesthesia. The blood samples were divided into two tubes with or without EDTA. Tubes were centrifuged at 6000 g for 10 min at 4 °C. Plasma (for FFA analysis) or serum (for other biochemical analyses) was immediately separated and stored at -20 °C. The hind limb skeletal muscle and liver tissue were quickly removed and then frozen in liquid nitrogen and kept at −75 °C until use.

Measurement of serum parameters

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

Measurement of plasma-free fatty acids

Plasma FFAs were extracted and analyzed by the method explained by Kangani *et al.* with slight modifications (43). Five hundred microliter of plasma was mixed with 20 μl of pentadecanoic acid (1 mg/ml) as an internal standard. Lipids were extracted from plasma by a reagent constituted from isopropanol–heptane–hydrochloric acid (1M) (40:10:1, v/v/v). After separating FFAs by TLC on silica gel plates using a heptane–ether–acetic acid [60:40:3] solvent system, FFAs were visualized by iodine vapor on TLC plates. FFA bands were scrapped, and free fatty acid methyl esters (FAME) were prepared by a reaction with BF3 containing methanol (Sigma). Then, 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.

RNA extraction and real-time quantitative PCR

RNA was isolated from the skeletal muscle (for GLUT4 assay) and liver (for PPARγ assay) tissues by RNeasy mini kit (Qiagen) according to manufacturer's instruction. The concentration of RNA was quantified by the ultraviolet (UV) light spectrophotometry at 260 nm and 280 nm (ND-1000 nanodrop). The quality of the extracted RNA was confirmed by ethidium bromide staining of 18S and 28S ribosomal RNA bands after electrophoresis on a 2% agarose gel.

Reverse transcription of RNA to cDNA was performed using Quanti Tect Reverse Transcription Kit (Qiagen) according to the manufacture's procedure. Relative Quantitative real-time PCR was conducted 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 instructions. Primer sequences used for real-time PCR are shown in Table 1. The cycle of threshold (CT) value was determined for each sample. ΔCT was calculated using the equation: ΔCT= CT (target gene) –CT (endogenous reference gene (GAPDH)). Results were calculated by the equation: $2^{-\Delta\Delta CT}$. In the real-time PCR all suggestions that published by Qiagen in "Critical Factors for Success in Real Time PCR" were considered.

Western immunoblotting analysis

Total protein was extracted from muscle or liver by homogenization in the radioimmune precipitation assay (RIPA) buffer (Sigma, cat number: R0278), followed by centrifugation at 14,000 rpm for 20 min and then supernatant containing proteins was collected. Total protein was measured by a Bradford protein assay using bovine serum albumin (BSA) as standard. Proteins were separated by using SDS- polyacrylamide gel electrophoresis and transferred on polyvinylidene difluoride (PVDF) membrane. Non-specific binding sites were blocked by overnight incubating with 5 % (w/v) non-fat skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) buffer at 4 °C. Then, the membrane was washed with TBST three times (each time for 20 min) and incubated with appropriate polyclonal primary antibodies for PPARγ (ab27649, mouse polyclonal to PPAR gamma, Abcam, UK) or GLUT4 (ab33780, mouse polyclonal to GLUT4, Abcam, UK) antibody in TBS-T buffer for 1 hr. After washing, as mentioned above, membranes were incubated with the anti-rabbit secondary antibody (Goat Polyclonal HRP

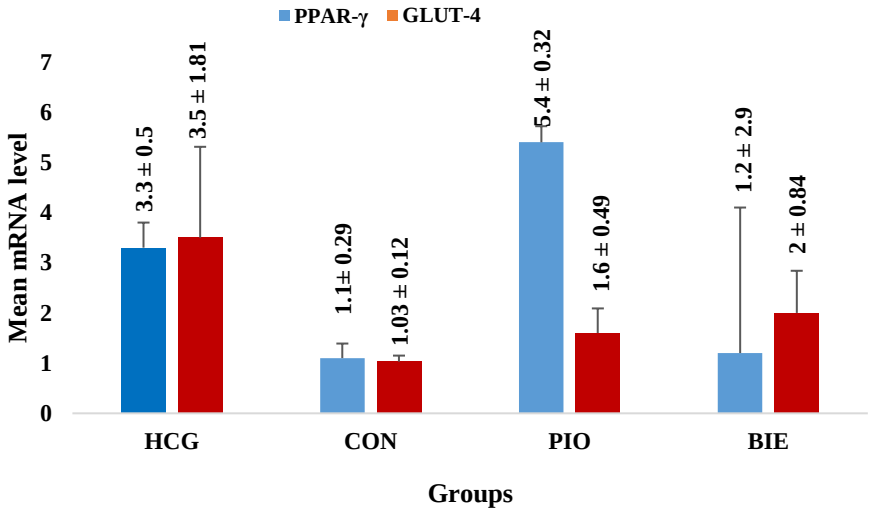

Figure 1. Effect of *Berberis integerrima* on mRNA level of liver PPARγ and muscle GLUT4 gene expression
The chart demonstrates that *B. integerrima* did not have any effect on PPARγ and GLUT4 gene expression at mRNA level (P=0.928 and P=0.995, respectively). However, pioglitazone significantly increased the mRNA level of PPARγ (Figure 1). All data are expressed as mean± SEM
HCG: healthy control group, Con: control, Pio: pioglitazone, BIE: *Berberis integerrima* extract

conjugated antibody to rabbit IgG, ab6112, Abcam, UK), for 1 hr at room temperature. Then PVDF was washed and incubated with substrate (Western lightening plus ECL, Perkin-Elmer) for 1 min. After exposing PVDF membrane to Hyblot film (Denvill) for 30 secs, in a dark room, band intensities were quantified by image j software.

Statistical analysis

All data are presented as mean±SEM (standard errors of the mean). The differences among groups were analyzed using one-way analysis of variance (ANOVA), and *Post-hoc* Tukey test was used to compare means between groups. The statistical analyses were performed using the SPSS software version 18.0 for Windows (SPSS Inc, Chicago, IL). P<0.05 were considered statistically significant.

Results

Weight gain, water and food intake

No significant difference was found among the groups in comparison of body weights at the beginning of treatment. A significant difference was observed in the body weight gain between *B. integerrima* and control groups (P=0.009). The difference between the initial body weight and body weight at the end of the 8th week were considered as body weight gain. *B. integerrima* reduced weight as compared to pioglitazone. However, no significant difference was observed in water or food intake between *B. integerrima* and controls groups (Table 2).

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

Parameters	Groups				*P*-value*
	HCG	Con	Pio	BIE	
Initial weight (g)	275±8	272±9	272±12	255±11	0.676
Weight after 8 weeks (g)	285±9	307±8	294±16	278±15	0.421
Weight gain (g)	10±2*	35±2#	22±5	14±6*	**0.009**
Water intake (ml)	35±0.8*	47±2#	60±2*#	45±1#	0.749
Food intake (g)	21.6±0.7*	13.4±0.4#	13.5±0.3#	13±0.2#	0.84
Insulin (pmol/l)	50±4.8*	137±34#	40±2.7*	25±6*	**0.001**
Adiponectin (µg/ml)	2.9±0.16	3.9±0.15#	5.6±0.4*#	6±0.3*#	**0.000**
Glucose (mg/dl)	132±4*	187±15#	129±5.8*	115±3*	**0.000**
HOMA-IR	2.7±0.37*	9.7±2.1#	2.1±0.12*	1±0.3*	**0.000**
Cholesterol (mg/dl)	71±12.1	59±3.2	63±4.2	63±2	0.967
Triglyceride (mg/dl)	85±13*	217±18#	200±51	121±9	0.179
HDL-c (mg/dl)	24.86±1.4	29.25±1.8	29.38±2.3	28±1	0.988

Biochemical parameters were measured by autoanalyzer and hormones by ELISA kits. HOMA-IR was calculated by the formula. All data were expressed as mean±SEM (n=8). HCG (was fed standard chow). Con (was fed 60% fructose), Pio (was fed 60% fructose with pioglitazone-treated (10 mg/Kg)) and BIE (was fed 60% fructose with *Berberis integerrima* extract-treated (1000mg/Kg))
HCG: healthy control group, Con: control, Pio: pioglitazone, BIE: *Berberis integerrima* extract
¥ *P*-value demonstrates the difference significance between *Berberis integerrima* and control group
* Significant difference with control group (P<0.05); # Significant difference with HCG group (P<0.05)

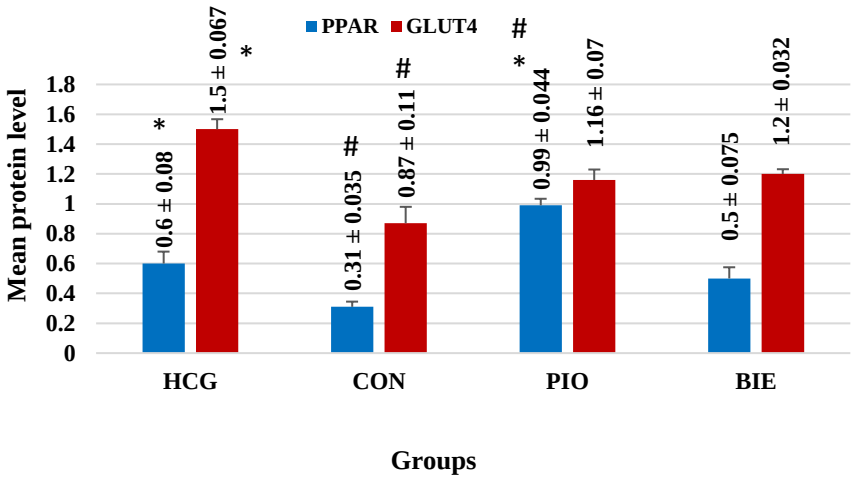

Figure 2. Effect of *Berberis integerrima* on protein level of liver PPARγ and muscle GLUT4
The chart illustrates that the level of PPAR-γ protein in pioglitazone group was significantly higher than the control group (P<0.0001). Regarding the mean protein level of GLUT4 and PPARγ, no significant difference was found between *B. integerrima* group and the control group. All data are expressed as mean ± SEM.
HCG: healthy control group, Con: control, Pio: pioglitazone, BIE: *Berberis integerrima* extract
* Significant difference with control group (P<0.0001)
# Significant difference with HCG group (P<0.05)

Blood glucose, triglycerides, and cholesterol

Table 2 shows that the mean blood glucose level was significantly decreased in *B. integerrima* group (115±3 mg/dl) compared to the control group (187±15 mg/dl) (P<0.0001). However, no significant differences were observed in mean serum levels of triglycerides, cholesterol and HDL-c between *B. integerrima* and other groups. Pioglitazone did not show any effect on triglycerides in the current study (200±51 mg/dl).

Serum levels of insulin and adiponectin

Comparison of *B. integerrima* group with controls, the mean level of insulin was significantly decreased (25±6 pmol/l and 137±34 pmol/l, respectively) (P=0.001). However, the mean level of adiponectin significantly increased in *B. integerrima* as compared to the control group (3.9±0.15 µg/ml) (P<0.0001). No significant difference was found in mean levels of insulin and adiponectin between *B. integerrima* and pioglitazone groups (Table 2).

Homeostasis model assessment of insulin resistance

As shown in Table 2, in *B. integerrima* treated animals (1±0.3), HOMA-IR was significantly lower compared to the control group (9.7±2.1) (P<0.0001). Moreover, no significant difference of HOMA-IR was observed in *B. integerrima* animals in comparison with pioglitazone treated animals.

Plasma free fatty acids

Among FFA fractions, the mean serum levels of palmitoleic acid and stearic acid were significantly higher in the *B. integerrima* group compared to the control group (P<0.0001 and P=0.005, respectively), while no significant difference was found in total FFAs between *B. integerrima* and control groups (Table 3).

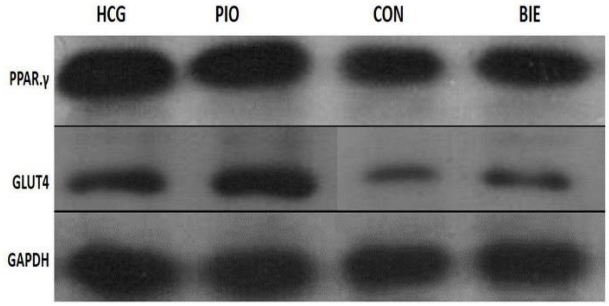

Figure 3. Western blot assay of protein level of liver PPARγ and muscle GLUT4 from HCG (was fed standard chow). Con (was fed 60% fructose), Pio (was fed 60% fructose with pioglitazone-treated (10mg/Kg)) and BIE (was fed 60% fructose with *Berberis integerrima* extract-treated (1000mg/Kg)). Immunodetection was performed using polyclonal anti- PPARγ or anti- GLUT4 antibodies
HCG: healthy control group, Con: control, Pio: pioglitazone, BIE: *Berberis integerrima* extract

GLUT4 and PPAR-γ gene expression

Findings demonstrated that *B. integerrima* did not have any effect on PPAR-γ and GLUT4 gene expression at mRNA level (P=1 and P=0.88, respectively). Similarly, no significant difference was observed in the mRNA level of PPAR-γ and GLUT4 in pioglitazone group as compared to control group (Figure 1).

As shown in Figures 2 and 3, no significant difference was found in PPAR-γ protein level between

Table 3. Effect of *Berberis integerrima* on plasma free fatty acids profiles

Parameters	Groups				*P*-value*
	HCG	Con	Pio	BIE	
Myristic acid (µmol/l)	1.07±0.01	1.12±0.06	1.2±0.06	1.4±0.04	0.013
Palmitic acid (µmol/l)	5.9±0.57	11.1±2.6	5.1±0.18	6.7±0.32	0.252
Palmitoleic acid (µmol/l)	1.06±0.04	1.4±0.12	1.1±0.01	2.1±0.11	**0.000***
Stearic acid (µmol/l)	1.3±0.12	2.06±0.11	2.00±0.08	2.1±0.1	**0.005***
Oleic acid (µmol/l)	2.2±0.21	2.9±0.22	1.7±0.08	3±0.3	0.981
Total free fatty acids (µmol/l)	12.53±1.95	22±2.7	19.44±2.8	14.1±3.13	0.132

Plasma free fatty acids profiles were measured by GC. All data were expressed as mean±SEM (n=8)
HCG (was fed standard chow), Con (was fed 60% fructose), Pio (was fed 60% fructose with pioglitazone-treated (10mg/Kg)) and BIE (was fed 60% fructose with *Berberis integerrima* extract-treated (1000 mg/Kg))
HCG: healthy control group, Con: control, Pio: pioglitazone, BIE: *Berberis integerrima* extract
* *P*-value demonstrates the difference between *Berberis integerrima* and control group

B. integerrima and control group. However, the level of PPAR-γ protein in pioglitazone group was significantly higher compared to the control group ($P<0.0001$). Regarding the mean protein level of GLUT4, no significant difference was observed between the *B. integerrima* and control groups (Figures 2 and 3).

Discussion

In the present study, findings demonstrated that *B. integerrima* clearly improved insulin resistance in high-fructose-fed insulin-resistant rats. We showed that *B. integerrima* extract alters body weight gain, insulin, adiponectin, blood glucose levels, and HOMA-IR. In agreement with several studies, the present findings indicated that hyperlipidemia, insulin resistance, and hyperinsulinemia states were induced after treating the animals with a high-fructose diet (3, 39, 40). Significant decreases in body weight, insulin, blood glucose, and HOMA-IR were observed after administration of *B. integerrima* in insulin-resistant rats compared to the control group. To the best of author's knowledge, there was no study on the effects of *B. integerrima* on insulin resistance until now; however, the current findings clearly indicate that *B. integerrima* improves insulin resistance.

In agreement with our previous study, *B. integerrima* like *Zataria multiflora* extract showed protective effects on glucose homeostasis and HOMA-IR (21). Decreases in insulin level along with the reduction in glucose and HOMA-IR suggest that *B. integerrima* has a direct insulin-like activity and acts as a hypoglycemic factor through improving insulin action rather than insulin secretion (44). Adipocyte accumulation plays a pivotal role in glucose homeostasis imbalance and adipose tissue inflammation (9, 10). Particularly, body weight is defined by the balance between food intake and energy expenditure (45). The present results have demonstrated that *B. integerrima* reduced body weight gain. Hence, decreases in visceral adiposity and adipocyte size could lead to improving insulin resistance.

Several studies have been put forward to explain the relationship between insulin resistance and dyslipidemia (46–48). Controversially, Ashraf *et al* have reported that administration of *B. integerrima* increased the serum levels of HDL-cholesterol and decreased triglycerides and total cholesterol (36). Further studies on the *B. integerrima* in different doses are needed to clarify the discrepancy.

Recently, it has been reported that lower production of adiponectin is directly linked to incidence of insulin resistance and metabolic syndrome (8–10). A reverse correlation has been recorded between adiponectin with adipocyte size and circulating adiponectin levels increased with weight loss (8, 49, 50). Consistent with other studies, Matsubara *et al.* findings indicated that adiponectin has a negative correlation with enlarged visceral fat, body mass index (BMI), and insulin resistance (51). Furthermore, adiponectin has been shown to come into play as major insulin-sensitizer adipokines by binding to their receptors in the liver and skeletal muscle (8, 9). TZDs increase the gene expression and plasma concentration of adiponectin and other genes involved in glucose uptake (52, 53). The serum level of adiponectin in the *B. integerrima* group was significantly higher as compared to the control group. Taken together, it could be deduced that *B. integerrima* exerts its antihyperglycemic effect, at least in part, by improving adiponectin production.

Most of the recent studies revealed that the improvement of insulin sensitivity has already been related to increased GLUT4 and PPAR-γ expression (40, 54). To clarify the molecular mechanisms involved in insulin resistance, we measured GLUT4 and PPAR-γ genes expression at the protein and mRNA levels. According to the recent studies on insulin sensitivity improvement, the activation of PPAR-γ can establish an important part of the molecular mechanism behind the adipogenesis. PPAR-γ is the most important member of the PPARs family that is involved in the adipogenesis adjustment. PPAR-γ, as a regulator of lipid and glucose metabolism, improves the insulin and glucose parameters and increases whole-body insulin sensitivity. PPAR-γ is predominantly expressed in the adipose tissue and it is the target of some anti-diabetic drugs such as pioglitazone. PPAR-γ agonists

contribute to enhancing the insulin sensitivity and glucose metabolism, and promoting the differentiation of pre-adipocytes into adipocytes. Comparing the *B. integerrima* treated group with the control group, no significant difference was found at the mRNA level between GLUT4 and PPAR-γ. Moreover, *B. integerrima* administrated group did not has any effect on protein level of GLUT4 and PPAR-γ. It can be assumed that the mechanism of decreased expression might be related to the composition of *B. integerrima*. However, PPAR-γ protein level was significantly higher in pioglitazone treated group in comparison with control group. Results derived from Sakamoto *et al.* demonstrated pioglitazone as a high-affinity selective PPAR-γ activator (55). PPAR-γ agonists ameliorate insulin resistance, reduce inflammation and enhance the differentiation of adipocytes and macrophages (56, 57). TZDs, particularly pioglitazone, have been extensively used in insulin resistance and T2DM.

Down-regulation of GLUT4 expression may enhance whole body insulin resistance and glucose imbalance (7). The GLUT4 content was lower in skeletal muscle fibers and fat tissue of type-2 diabetic patients. Besides, insulin begins insulin signaling cascade, which results in GLUT4 translocation and increased glucose uptake at the cell membrane in muscle cells. In this pathway, the impairment of any of these steps leads to the decrease of GLUT4 translocation and causes insulin resistance. Insulin decreases circulating glucose levels by suppressing hepatic glucose production and also activating GLUT4. GLUT4 increases insulin-mediated glucose uptake in muscle and adipose tissue (7, 58). In the present study, GLUT4 mRNA and protein levels were not significantly different among groups. Therefore, we speculate that the extracted *B. integerrima* contents do not increase the GLUT4 genes expression at protein and mRNA levels. However, it could improve GLUT4 translocation and glucose uptake. Moreover, no significant differences were found in GLUT4 protein and mRNA levels between the two groups. Expression and translocation of GLUT4 is increased in adipose tissue by PPARγ (21, 57). The reason for this issue can be administration of a single dose to these groups. In striated muscle, no significant difference was found at the total GLUT4 protein between two groups, therefore, it could be supposed that translocation of GLUT4 to the cell membrane is increased after treating animals with *B. integerrima* and pioglitazone.

Many studies in animals and humans have highlighted the association of obesity with T2DM/insulin resistance (2, 9). Although the exact mechanisms have not been clearly elucidated, it could be deduced from previous studies that in most obese individuals, FFA levels are obviously too high, also acute and chronic increases in FFA levels lead to insulin resistance (18, 19). Meanwhile, this FFA elevation causes interference in insulin signaling and function (19, 39). On the other hand, Insulin has a profound influence on the metabolic homeostasis and potently affects the metabolism of fuel molecules in adipose tissue, skeletal muscle, and the liver (58, 59). Accordingly, we analyzed the FFAs profile in high-fructose-fed insulin-resistant rats. Total plasma FFA level was not significantly different; however, palmitoleic acid and stearic acid were significantly increased in *B. integerrima* treated group in comparison with the control group. PPARγ agonists enhance the insulin sensitivity in adipose tissue and decrease serum FFA levels (57). In the present study, no significant difference was found between pioglitazone and control groups regarding the PPARγ gene expression, since we evaluated PPARγ gene expression in the liver to investigate hepatic insulin resistance. This, in turn, could be suggested that further studies on PPARγ gene expression in adipocytes are required to clarify the direct effect of PPARγ on FFA levels. In the present study, using single dose of *B. integerrima* is one of the limitations. We could not confirm the optimal dose of *B. integerrima* because of the limited relevant investigations. Moreover, the lack of enough time and budget, and inadequate literature were some limitations of the present study.

Conclusion

The *B. integerrima* water extract, significantly improved insulin resistance in insulin-resistant rats. We demonstrated that *B. integerrima* not only increased adiponectin but also significantly reduced plasma glucose level and HOMA-IR. However, the mechanism of *B. integerrima* was independent of GLUT4 and PPARγ. We suggest that *B. integerrima* might be a protective candidate against T2DM/insulin resistance. Therefore, further studies are needed to identify precisely which components of *B. integerrima* have the main role in its therapeutic effect.

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Conflict of interest

The authors declare that there is no conflict of interest.

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