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Eugenol ameliorates insulin resistance, oxidative stress and inflammation in high fat-diet/streptozotocin-induced diabetic rat

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ABSTRACT

Background: Eugenol, a phenolic compound present in many plant essential oils, demonstrated anti-diabetic activity but the underlying mechanisms are not fully understood. The aim of the present study was to examine the anti-diabetic, anti-oxidative and the anti-inflammatory effect of eugenol in high-fat-diet (HFD) and streptozotocin (STZ)-induced diabetic rats. Additionally, the effect of eugenol on the insulin sensitivity and on skeletal muscle protein contents of glucose transporter-4 (GLUT4) and AMP-activated protein kinase (AMPK) was investigated.

Materials and methods: HFD/STZ-induced diabetic rats were treated orally with eugenol (10 mg/kg) for 45 days. After the end of the experiment, blood and skeletal muscle samples were collected. Metformin was used as positive control.

Results: The anti-diabetic effects of eugenol were demonstrated by the significant reduction in the levels of serum glucose, triglyceride, cholesterol, Low-density lipoprotein, malondialdehyde and interleukin-6 in the treated group compared to the diabetic group. Additionally, eugenol treatment significantly restored the decreased serum levels of insulin and glutathione when compared to that of the diabetic control rats. The homeostasis model assessment of insulin resistance (HOMA-IR) was significantly lower in rats treated with eugenol than in the diabetic rats. The skeletal muscle protein contents of GLUT4 and AMPK were higher in the eugenol treated group than in the diabetic control group.

Conclusion: Eugenol possesses potent anti-oxidative and anti-inflammatory effect in HFD/STZ-induced diabetic rats. Moreover, eugenol facilitates insulin sensitivity and stimulate skeletal muscle glucose uptake *via* activation of the GLUT4-AMPK signaling pathway. Eugenol could represent a promising therapeutic agent to prevent type 2 diabetes.

1. Introduction

Diabetes mellitus is one of the most common lifestyle metabolic diseases around the world with 552 million people could have diabetes by 2030 [1]. Diabetes mellitus can be classified as type 1 due to inherited or immune-mediated β-cells destruction, leading to an inability of the β-cell of the pancreas to secrete insulin or type 2 results from insulin resistance and/or reduced insulin secretion [2].

The majority of insulin-mediated glucose uptake occurs in muscle at all glucose levels [3]. The principal glucose transporter protein that mediates this uptake is the insulin-sensitive glucose transporter-4 (GLUT4) [4]. Existing evidence indicates that activation of the AMP-activated protein kinase (AMPK) stimulates GLUT4 translocation to the cell surface, thus glucose uptake in the skeletal muscle is stimulated in an insulin-independent mechanism. Insulin resistance, a key feature of type 2 diabetes, is characterized by the inability of insulin to inhibit hepatic glucose output and to stimulate glucose uptake into peripheral tissues including skeletal muscle and adipose tissue [5]. Impaired insulin-stimulated glucose disposal process into the skeletal muscle that occurred through GLUT4 translocation to the plasma membrane is one of several mechanisms that cause insulin resistance [4]. Therefore, the activation of the AMPK-GLUT4 pathway may enhance insulin sensitivity and is effective for the treatment of type 2 diabetes mellitus.

Oxidative stress and inflammation are widely accepted to be involved in the pathogenesis of type 2diabetes and in its complications [6,7]. The baseline levels of interleukin-6 (IL-6), a major pro-inflammatory cytokine, was significantly higher among type 2 diabetes mellitus patients than among disease-free controls, suggesting a

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potential role for IL-6 in the development and progression of type 2 diabetes mellitus [7,8]. On the other hand, increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of insulin resistance and β-cell failure in the developing stage of type 2 diabetes [9].

Phytotherapy research is increasing the list of medicinal plants that have a potential treatment of diabetes by interfering with the metabolism of glucose [10]. Eugenol is a phenolic compound that can be extracted from clove tree, cinnamon and many other plants [11]. Eugenol has shown strong analgesic, anti-inflammatory, antifungal, antibacterial, neuro-protective and antihypertensive activities [11]. In diabetic animal models, eugenol lowers blood glucose by inhibiting α-glucosidase [12], enhancing glucose utilization [13], decreasing hepatic glucose production *via* gluconeogenesis [13,14] and by inhibiting of pancreatic enzyme activities [15]. Findings from these studies support the concept that eugenol exerted anti-diabetic effects in animal models. However, further preclinical researches regarding the anti-diabetic effects of eugenol are required to specify its usefulness as an effective therapy in this disease. Reports concerning the effect of eugenol on the insulin sensitivity, skeletal muscle glucose uptake through the AMPK-GLUT4 pathway and on the oxidative stress and inflammation in high fat-diet (HFD)/streptozotocin (STZ)-induced diabetic rat are not available in the literature. Therefore, the aim of this study was to investigate the effects of eugenol on HFD/STZ-induced diabetic rats.

2. Material and method

2.1. Induction of type 2 diabetes mellitus and experimental design

All animal experimental procedures were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals and approved by the committee of animal ethics at Yarmouk University (permission number: YU-4/1/2016); Irbid-Jordan. Thirty weeks-old male adult Sprague-Dawley rats were maintained in the animal house of Yarmouk University under the standard conditions of a 12-light-dark cycle and a temperature at 23 ± 1 °C. Diabetes was induced by fed the experimental rats with HFD (45% fat) for 3 weeks followed by intraperitoneal injection of freshly prepared STZ (40 mg/kg). Rats with plasma glucose concentration > 200 mg/dl were selected for the subsequent experiments. Negative control rats received normal pellet diet.

The rats were randomly divided into four groups (n = 10 each) as follows: normal control group (non-diabetic, ND), a diabetic group (D) received vehicle (corn oil), a diabetic group treated with 10 mg/kg eugenol (D + EU-10;Sigma-Aldrich, ST. Louis, USA) and a diabetic group treated with 200 mg/kg metformin (D + MET; Merck, Germany). All treatments were given orally, once per day. At the end of 45 days of treatment, rats were anesthetized with ether, blood and skeletal muscle (soleus muscle) samples were collected for further analysis. The dose of eugenol treatment was chosen based on previous work [13] and a preliminary pilot study. In the pilot study and by using the same experimental design that mentioned before, the effect of two different daily oral doses of eugenol (10 and 20 mg/kg) were evaluated. Depending on the blood glucose levels, no dose-response effect was observed when the eugenol dose was doubled from 10 to 20 mg/kg (data not shown).

2.2. Biochemical investigations

2.2.1. Measurement of serum glucose, insulin levels, and lipid profile

Serum glucose level was determined using commercial kit (linear chemicals SL, Joaquim costa, Barcelona, Spain). Serum insulin levels were determined by ELISA using a commercially available kit (Sigma-Aldrich, ST. Louis, USA). Triglyceride, Low-density lipoprotein (LDL) and cholesterol were measured using commercially available kits (linear chemicals SL, Barcelona, Spain) according to the manufacturer's instructions.

2.2.2. Intraperitoneal glucose tolerance test (IPGTT) and homeostasis model assessment of insulin resistance (HOMA-IR)

Overnight fasted rats were injected intraperitoneally with glucose (0.5 g/kg of body weight) and their blood glucose levels were determined from tail vein after 0, 30, 60, and 120 min using glucometer (Accu-Chek Performa, Roche Diagnostics). The area under the curve (AUC) for the IPGTT glucose levels was calculated by using the trapezoidal method. A homeostasis model assessment of insulin resistance (HOMA-IR), a method to quantify insulin resistance, was calculated from the fasting levels of insulin and glucose using the following formula: HOMA-IR = {Fasting glucose (mmol/L) × fasting insulin (mU/L)} / 22.5 [16].

2.2.3. Measurement of serum glutathione (GSH), malondialdehyde (MAD) and IL-6 levels

Reduced glutathione (GSH) was measured in the serum using the commercially available kit (Cayman, Ann Arbor, Michigan, USA). Lipid peroxidation levels were measured by the thiobarbituric acid reacting with the serum MDA [17]. The serum level of inflammatory cytokine IL-6 was measured by commercially available ELISA kit (Invitrogen-Thermo Fisher Scientific, Vienna, Austria) according to the manufacturer's instructions.

2.2.4. Western blotting

Skeletal muscle tissues were homogenized in phosphate buffer saline containing protease inhibitor cocktail (Sigma-Aldrich, ST. Louis, USA). The homogenate was centrifuged at 13,000 rpm for 20 min at 4 °C and the supernatant was collected. Total protein content was determined using bicinchoninic acid assay (Sigma-Aldrich) and aliquots of the supernatant containing equal amounts of protein (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and then transferred to a nitrocellulose membrane. The membranes were then incubated for 1 h with blocking solution and then incubated overnight with the AMPK and GLUT4 primary antibodies (Sigma-Aldrich, ST. Louis, USA). The blots were washed three times with washing buffer (Tween-20/Tris-buffered saline), followed by incubation with goat anti-rabbit secondary antibodies (invitrogen-Thermo Fisher, Rockford, USA) for 1 h at room temperature. Then, blots were washed three times, and the immune reactive protein bands were visualized with diaminobenzidine (DAB) substrate. The protein band intensity was measured relative to the control group using the ImageJ software (NIH, Bethesda, MD). Equal gel loading was demonstrated in Ponceau S stain (Supplementary Fig. 1).

2.3. Statistical analysis

All data are expressed as the mean ± SEM. Differences between groups were calculated by one-way analysis of variance (ANOVA) using SPSS software (SPSS Inc., Chicago, IL). The significant value of difference was considered when the P value < 0.05.

3. Result

3.1. Serum glucose, insulin levels, and HOMA-IR

As shown in Fig. 1A, blood glucose was significantly higher in the D than in the ND group and treatment with eugenol or metformin for 45 days significantly reduced blood glucose levels in diabetic rats as compared to the diabetic control (P < 0.05). Insulin levels were significantly lower in the D group as compared to the ND group (Fig. 1B; P < 0.05). Oral administration of 10 mg/kg eugenol or 200 mg/kg metformin for 45 days in the diabetic rats resulted in a significant increase in the serum insulin level.

To determine the effect of eugenol on insulin resistance and

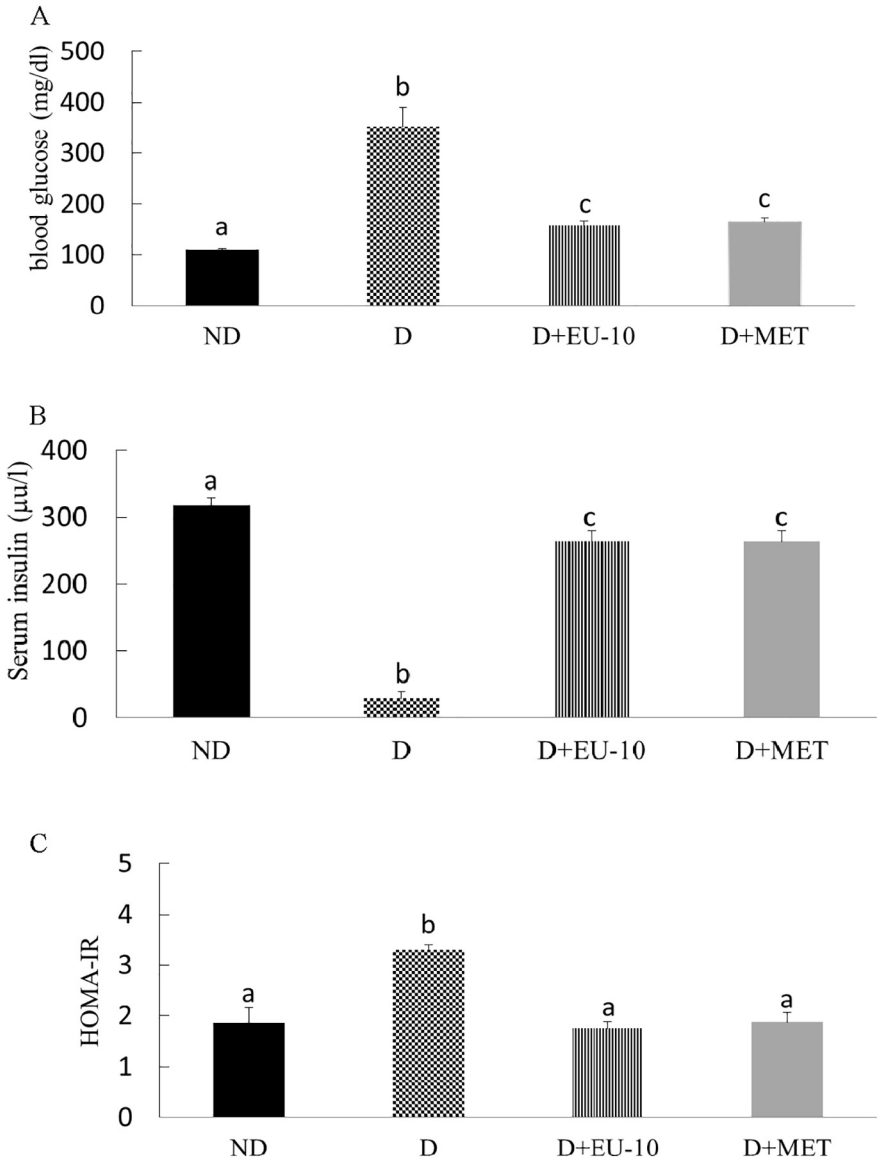

Fig. 1. Effects of eugenol treatment on blood glucose (A) insulin (B) and HOMA-IR (C) in HFD/STZ-induced diabetic rats. Data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: ND: non-diabetic; D: diabetic; D + EU-10: diabetic +10 mg/kg eugenol; D + MET: diabetic +200 mg/kg metformin; HOMA-IR: homeostasis model assessment of insulin resistance.

peripheral tissue glucose metabolism, the HOMA-IR and IPGTT were performed, respectively. As presented in Fig. 1C, the HOMA-IR was significantly decreased in the D group compared to the ND group ($P < 0.05$). However; eugenol and metformin treatment fully improved the HOMA-IR. Blood glucose level and AUC during IPGTT were significantly lower in D + EU-10 and D + MET than in D group (Fig. 2A and B; $P < 0.05$).

3.2. Lipid profile, serum oxidative stress, and inflammatory markers

Dyslipidemia was clear in the HFD/STZ-induced diabetic rats as indicated by the increased serum triglyceride, LDL and cholesterol (Table 1; $P < 0.05$). These parameters were significantly decreased following eugenol treatment. The reduction in serum cholesterol level was greater in the D + EU-10 group than D + MET group. Eugenol treatment significantly increased serum GSH level and significantly decreased serum MDA levels, as compared with the D group (Table 2; $P < 0.05$). Additionally, the treatment of diabetic rats with eugenol significantly decreased the elevated serum level of IL-6 compared to the D group (Table 2; $P < 0.05$). No differences in the serum GSH and MDA levels were observed between the D + MET group and the D + EU-10 group. However, a significantly decreased level of serum IL-6 was evident in the D + EU-10 group compared to the D + MET group (Table 2; $P < 0.05$).

3.3. Skeletal muscle GLUT 4 and AMPK protein levels

The protein levels of GLUT4 and AMPK in skeletal muscle tissues were significantly decreased in diabetic rats compared with the normal group, and these changes were significantly attenuated by eugenol treatment (Fig. 3A and B; $P < 0.05$). In the D + MET group, the protein levels of GLUT4 and AMPK in skeletal muscle tissues were significantly increased compared to D + EU-10 group.

4. Discussion

Treatment that can prevent diabetes, the most common metabolic disorder around the world, is an urgent need. Natural anti-diabetic therapeutics may provide an alternative for new drugs with low toxicity and side effects. Recent studies reported that eugenol, a phenolic

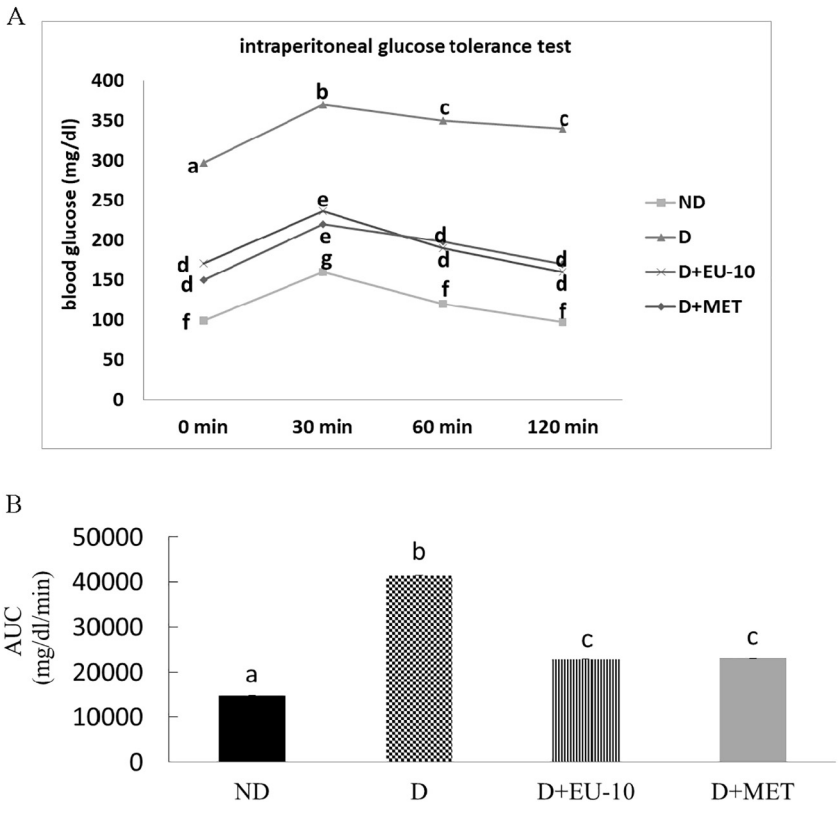

Fig. 2. Effects of eugenol treatment on intraperitoneal glucose tolerance test blood glucose levels (A) and on intraperitoneal glucose tolerance test AUC (B) in HFD/STZ-induced diabetic rats. Data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: ND: non-diabetic; D: diabetic; D + EU-10: diabetic +10 mg/kg eugenol; D + MET: diabetic +200 mg/kg metformin; AUC: area under the curve.

compound present in many plant essential oils, possesses an anti-diabetic activity in diabetic animal's model. Our current study provided further evidence that eugenol has potent anti-diabetic potential in HFD/STZ-induced diabetic rats by modulation of inflammation and oxidative stress, and by improving insulin sensitivity and stimulating skeletal muscle glucose uptake *via* activation of the AMPK-GLUT4 signaling pathway.

In the current study, eugenol significantly reduced the hyperglycemia in HFD/STZ-induced diabetic rats. This hypoglycemic effect of eugenol may be partially explained by the restoration of the activities of key enzymes involved in the metabolism and storage of glucose [13], inhibition of hepatic gluconeogenesis [13,14] and by inhibition of carbohydrate digestive enzymes activities [15]. Stimulation of glucose uptake, utilization and storage by insulin play a pivotal role in the regulation of blood glucose homeostasis. The results of the present study show that eugenol treatment restored the serum insulin levels in diabetic rats (Fig. 1B). Consistent with the ameliorated serum glucose levels, serum insulin levels, and glucose tolerance; we also found that treated diabetic rats with eugenol were characterized by low HOMA-IR value in comparison to the diabetic rats, indicating that insulin sensitivity had improved [16]. Therefore, the hypoglycemic effects of eugenol in HFD/STZ-induced diabetic rats may be related, at least in part, to the recovery of the residual β-cells in the pancreas [13,15], hence increase insulin secretion and increase insulin-dependent glucose uptake by the cells.

Table 2
Effects of eugenol treatment on blood oxidative stress and inflammatory markers in HFD/STZ-induced diabetic rats.

	ND	D	D + EU-10	D + MET
Glutathione, μmol/l	2.81 ± 0.4^{a}	0.42 ± 0.2^{b}	2.19 ± 0.2^{a}	2.18 ± 0.5^{a}
MDA, nmol/ml	0.79 ± 0.1^{a}	9.1 ± 0.4^{b}	0.87 ± 0.1^{a}	0.85 ± 0.1^{a}
IL-6, pg/ml	199 ± 15.0^{a}	2244 ± 41^{b}	282 ± 19^{c}	430 ± 30^{d}

Data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: ND: non-diabetic; D: diabetic; D + EU-10: diabetic +10 mg/kg eugenol; D + MET: diabetic +200 mg/kg metformin; MDA: malondialdehyde; IL-6: interleukin-6.

Skeletal muscle constitutes around 45% of lean body mass and is responsible for around 80% of insulin-mediated glucose uptake [3]. GLUT4 plays a key role in the process of transporting extracellular glucose into insulin-sensitive cells to keep blood glucose homeostasis [4]. In the insulin-independent pathway, GLUT4 translocation to the plasma membrane and glucose uptake in the cells is stimulated through activation of the AMPK-GLUT4 pathway [18]. Insulin resistance in obesity and type 2 diabetes is caused by impaired insulin-stimulated glucose disposal into skeletal muscle [5]. Therefore, the activation of AMPK-GLUT4 pathway enhances insulin sensitivity and is effective for the treatment of type 2 diabetes mellitus. The improvement in glucose homeostasis in the eugenol treated group in the present study was

Table 1
Effects of eugenol treatment on lipid profile in HFD/STZ-induced diabetic rats.

	ND	D	D + EU-10	D + MET
Triglycerides, mg/dl	121 ± 12.2^{a}	233 ± 12.2^{b}	148 ± 15.1^{c}	160 ± 16.3^{c}
Cholesterol, mg/dl	62.5 ± 7.6^{a}	251 ± 14.4^{b}	63.1 ± 3.8^{a}	117 ± 10.3^{d}
LDL, mg/dl	10.5 ± 1.2^{a}	174 ± 18.7^{b}	55.1 ± 4.3^{c}	52.5.1 ± 3.2^{c}

Data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: ND: non-diabetic; D: diabetic; D + EU-10: diabetic +10 mg/kg eugenol; D + MET: diabetic +200 mg/kg metformin; LDL: low-density lipoprotein.

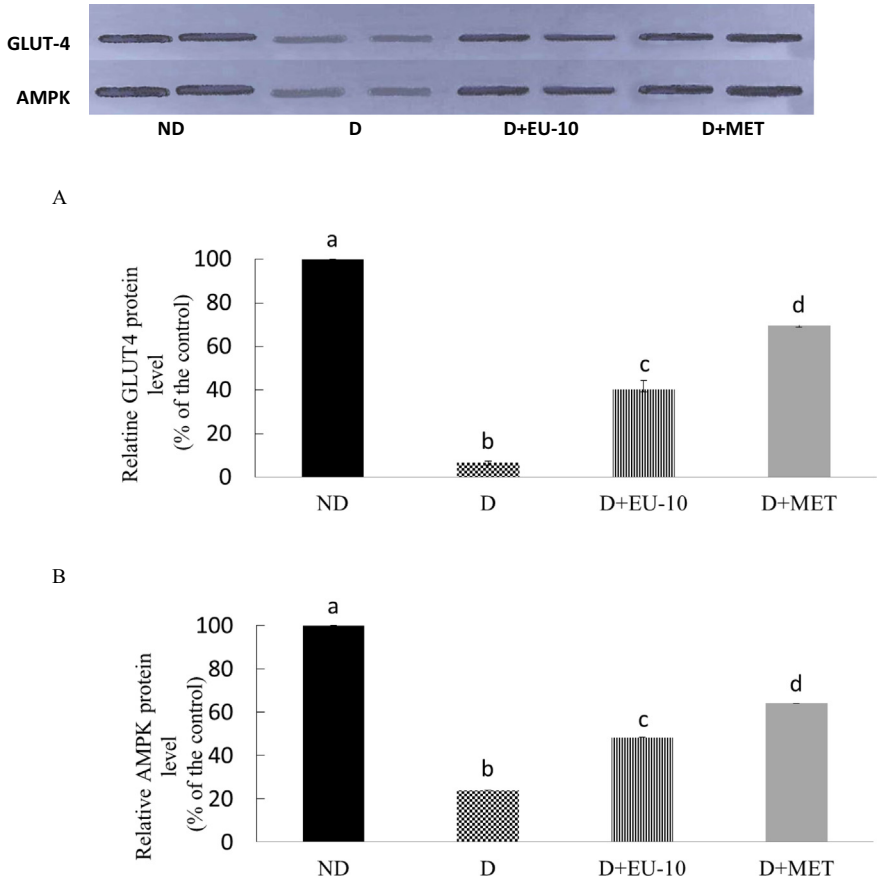

Fig. 3. Effects of eugenol treatment on skeletal muscle GLUT4 (A) and AMPK (B) protein levels in HFD/STZ-induced diabetic rats. Data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: ND: non-diabetic; D: diabetic; D + EU-10: diabetic +10 mg/kg eugenol; D + MET: diabetic +200 mg/kg metformin; AMPK: AMP-activated protein kinase; GLUT-4: glucose transporter, type 4.

associated with a significant increase in the skeletal muscle contents of GLUT4 and AMPK. Therefore, increased glucose uptake in the skeletal muscle through activation of the AMPK-GLUT4 pathway may contribute to the hypoglycemic effect of eugenol in HFD/STZ-induced diabetic rats.

Oxidative stress contributed, at least in part, in the pathogenesis of diabetes mellitus and can promote the development of its complications [9]. Oxidative stress is termed as the disturbance in the balance between reactive oxygen species (ROS) production and the antioxidant defense mechanisms. When excess glucose is available to the cell, ROS formation increased, resulting in oxidative stress [19]. ROS has been shown to reduce insulin secretion of β-cells and promote systemic insulin resistance and inflammation [9,19]. Eugenol exhibits effective antioxidant activity both *in vitro* and *in vivo* [20]. The present study found that treatment of diabetic rats with eugenol significantly decreased blood levels of MDA, one of the end products of lipid peroxidation, and increased the levels of GSH, non-enzymatic antioxidants. These findings suggest that eugenol is beneficial as a protective agent against oxidative stress induced by HFD/STZ-induced diabetes, at least in part, *via* attenuating lipid peroxidation and enhancing free radical scavenger activity.

Subclinical chronic inflammation seems to be an independent risk factor for the development of type 2 diabetes [7]. Elevated plasma IL-6 level, a multifunctional cytokine, is associated with type 2 diabetes [8]. Despite the controversial results regarding the role of IL-6 in the development of insulin resistance, elevated IL-6 cause's impaired insulin signaling in hepatocytes and inhibits glucose-stimulated insulin secretion from the β-cells in the pancreas [7]. Several studies have reported that eugenol exhibits *in vitro* and *in vivo* anti-inflammatory action [11]. Eugenol was shown to block the release of pro-inflammatory mediators (tumor necrosis factor-alpha, prostaglandin, IL-1β, and IL-6) from macrophages incubated with lipopolysaccharide [21,22]. *In vivo*, eugenol exhibits anti-inflammatory action in lipopolysaccharide-induced lung injury through a mechanism involving inhibition of tumor necrosis factor-α (TNF-α) release and nuclear factor-κB (NF-κB) activation [23]. In the present study, serum IL-6 levels were found to remarkably decrease with eugenol treatment in diabetic rats. These findings suggest that the anti-inflammatory activity may play an important role in the anti-diabetic effect of eugenol.

Finally, diabetic dyslipidemia, characterized by high plasma triglycerides, cholesterol, and LDL, contributes to the increased production of ROS, activate inflammatory pathways, induce peripheral tissue insulin resistance and accelerated macrovascular and microvascular diseases in diabetic patients [24,25]. Thus, lipid-lowering therapy is necessary for patients with type 2 diabetes. In the current study, the previously reported anti-hyperlipidemic effect of eugenol in the diabetic animal model was confirmed [15]. This indicates that eugenol can prevent lipotoxicity and subsequent, insulin resistance, oxidative stress, and inflammation in type 2 diabetes.

In conclusion, these results suggest that eugenol possess potent antioxidative and anti-inflammatory effect in HFD/STZ-induced diabetic rats. Additionally, eugenol facilitates insulin sensitivity and stimulate skeletal muscle glucose uptake *via* activation of the GLUT4-AMPK signaling pathway. Eugenol could represent a promising therapeutic agent to prevent type 2 diabetes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2018.11.034.

Conflict of interest

There are no conflicts of interest to declare.

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