



ANTI-HYPERGLYCEMIC, ANTIOXIDANT AND ANTI-INFLAMMATORY EFFECTS OF *ANISOTES TRISULCUS* IN TYPE 2 DIABETIC RATS

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Abstract

Anisotes trisulcus is a shrub rich in bioactive compounds and possesses promising pharmacological activities. This investigation explored the anti-hyperglycemic, insulin-sensitizing, antioxidant and anti-inflammatory activities of *A. trisulcus* extract (ATE) in diabetic rats. Type 2 diabetes was induced by high fat diet and streptozotocin and diabetic rats were treated with 200 and 400 mg/kg ATE for 21 days. The results showed elevated fasting and postprandial blood glucose levels in HFD/STZ diabetic rats accompanied with reduced serum insulin and increased HOMA-IR. Serum triglycerides, cholesterol, LDL, vLDL and pro-inflammatory cytokines (TNF- α and IL-6) were increased, whereas HDL-cholesterol was decreased in diabetic rats. Treatment with ATE ameliorated hyperglycemia, dyslipidemia, insulin resistance and inflammation in diabetic rats. In addition, ATE increased hepatic hexokinase, glycogen, glutathione and superoxide dismutase and decreased lipid peroxidation and the gene expression levels of phosphoenolpyruvate carboxykinase in diabetic rats. In conclusion, this study showed for the first time the anti-hyperglycemic, antioxidant and anti-inflammatory activities of ATE in type 2 diabetic rats. ATE ameliorated blood glucose, lipids and pro-inflammatory cytokines, improved insulin sensitivity and hepatic carbohydrate metabolism, and suppressed oxidative stress.

Keywords: Diabetes; Oxidative stress; Almodh; Insulin resistance; Inflammation.

Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by elevated blood glucose (hyperglycemia) and deteriorated insulin levels as well as action. Type 2 diabetes is the most common form of the disease and more than 380 million have been reported to have this disease (Cho *et al.*, 2018). Chronic elevation of blood glucose levels can cause damage to different organs and hence management of hyperglycemia is very important to prevent its serious side effects (Jellinger, 2007, Levinthal & Tavill, 1999). Hyperglycemia is well-known to induce the excessive release of reactive oxygen species (ROS) and the pro-inflammatory cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-6 (Mahmoud *et al.*, 2012). Therefore, hyperglycemia is usually associated with the development of oxidative stress and inflammation. These hyperglycemia-associated pathogenic processes can lead to lipid peroxidation (LPO), cell death, insulin resistance and many other complications (Abd El-Twab *et al.*, 2016, Al-Hroob *et al.*, 2018; Newsholme *et al.*, 2016; Tiwari *et al.*, 2013). In addition to elevated blood glucose levels, insulin resistance can increase the demand to insulin and lead to gradual destruction of pancreatic β -cells and worsen hyperglycemia (Halban *et al.*, 2014). Therefore, suppression of hyperglycemia, oxidative stress and inflammation can help increasing insulin sensitivity and preventing the complications of diabetes.

Several medicinal plants have shown very promising anti-inflammatory, antioxidant and antidiabetic effects. In this context, *Anisotes trisulcus* (family Acanthaceae) has been reported to reduce blood glucose levels in diabetic mice (Okla *et al.*, 2014). However, nothing has yet been reported on either its ability to ameliorate hyperglycemia in type 2 diabetes or the underlying mechanism of action. *A. trisulcus* is a stiff erect shrub growing in Saudi Arabia and possesses multiple pharmacological activities, including anti-bacterial, anti-hypertension and hepatoprotective (Ali *et al.*, 2001). *A. trisulcus* is rich in phenolic compounds and its methanolic

extract showed a suppressive effects on oxidative stress and inflammation (El-Shanawany *et al.*, 2014). This study evaluated the anti-hyperglycemic potential of *A. trisulcus* extract (ATE) in high fat diet (HFD)/streptozotocin (STZ)-induced diabetic rats, pointing to its modulatory role on hyperlipidemia, carbohydrate metabolism, oxidative stress and inflammation.

Materials and Methods

Collection of *A. trisulcus* and extract preparation

The plant samples were collected from the city of Sakaka (Aljouf, Saudi Arabia) and were identified and authenticated by an expert taxonomist. The leaves were separated, washed and dried in shade. The dry leaves were pulverized and macerated with 80% methanol and kept for 72 h at 4°C. After filtration, the filtrate was concentrated using rotary evaporator and kept at -20°C until used.

Experimental animals and induction of type 2 diabetes mellitus

Male Wistar rats weighing 180-200 g were housed in standard cages and maintained on a 12-h light/dark cycle at 22°C–24°C. The animals were fed a HFD (58% fat, 17% carbohydrate and 25% protein) for 4 weeks followed by a single injection of 35 mg/kg STZ (Sigma, USA) as recently reported by Elsayed *et al.* (Elsayed *et al.*, 2020). STZ was dissolved in freshly prepared citrate buffer (pH 4.5) and administered intraperitoneally (i.p.). After one week, blood glucose levels were determined and animals with blood glucose higher than 250 mg/dl were considered diabetic and selected for further investigation. Control rats were received normal diet for 4 weeks and received i.p. injection of citrate buffer.

The animals were then randomly divided into the following groups:

Group 1: Control.

Group 2 (ATE): rats received 400 mg/kg ATE orally for 21 days.

Group 3: Diabetic

Group 4 (Diabetic + 200 mg/kg ATE): diabetic rats received 200 mg/kg *A. trisulcus* extract (ATE) orally for 21 days.

Group 5 (Diabetic + 400 mg/kg ATE): diabetic rats received 400 mg/kg *A. trisulcus* extract (ATE) orally for 21 days.

ATE has been previously shown to exert anti-inflammatory activity in rats when administered at doses of 400 mg/kg (El-Shanawany *et al.*, 2014). Therefore, 200 and 400 mg/kg ATE doses have been selected in this study.

Collection of samples

At the day before the end of the experiment, blood samples were collected from overnight fasted rats and 2 h after oral administration of 3 g/kg glucose solution. At the last day of the experiment, rats were sacrificed under anesthesia and blood samples were collected to separate serum. The animals were then dissected, and liver was excised, washed and stored at -80°C.

Determination of serum glucose and lipids

Serum glucose levels were assayed following the method of Trinder (Trinder, 1969) using commercially available kit (Randox, UK). The levels of serum triglycerides (Fossati & Prencipe, 1982), total cholesterol (Allain *et al.*, 1974) and HDL-cholesterol (Burstein *et al.*, 1970) were assayed using Randox (UK) kits. vLDL- and LDL-cholesterol levels were calculated as follows:

$$\text{vLDL-cholesterol} = \text{Triglycerides}/5$$

$$\text{LDL-cholesterol} = \text{Total cholesterol} - (\text{HDL-cholesterol} + \text{vLDL-cholesterol})$$

Determination of insulin and HOMA-IR

Serum insulin was determined using RayBiotech (USA) assay kit and homeostasis model of insulin resistance (HOMA-IR) (Haffner, 2000) was calculated as following:

$$\text{HOMA-IR} = \frac{\text{Fasting insulin} \left(\frac{\mu\text{U}}{\text{ml}} \right) \times \text{Fasting glucose} \left(\frac{\text{mmol}}{\text{L}} \right)}{22.5}$$

Determination of liver glycogen and hexokinase

Liver glycogen content was determined according to the method of Seifter *et al.* (Seifter *et al.*, 1950) and hexokinase activity was assayed as described by Brandstrup *et al.* (Brandstrup *et al.*, 1957).

Determination of PEPCK gene expression by quantitative real time-PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, USA) and its quantity was determined using a nanodrop. Samples with A260/A280 higher than 1.7 were immediately reverse transcribed into cDNA. For gene expression analysis, qRT-PCR was employed using QuantiFast SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) and the following primers: phosphoenolpyruvate carboxykinase (PEPCK): F: 5'-CGTTGGGAGCTAGGAGCAAA-3' & R: 5'-CCCATCAGTGTGATGCGA-3' and GAPDH: F: 5'-AACTTTGGCATCGTGAAGG-3' & R: 5'-TACATTGGGGGTAGGAACAC-3'. qRT-PCR reactions were performed using ViiA™ 7 System (Thermo Fisher

Scientific, CA, USA) in duplicates. The transcript number was determined using the 2^{ΔΔCt} method (Livak & Schmittgen, 2001).

Determination of lipid peroxidation (LPO) and antioxidants

Liver samples were homogenized in cold 0.1 M phosphate buffer (pH 7.4), centrifuged at 6000 rpm and the supernatant was collected for analysis. Thiobarbituric acid reactive species (TBARS) as a marker of LPO was assayed according to the method of Ohkawa *et al.* (1979). Reduced glutathione (GSH) was estimated according to the method described by Ellman (1959) and superoxide dismutase (SOD) activity was assayed based on the method of Nishikimi *et al.* (1972).

Determination of pro-inflammation cytokines

Serum levels of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 were determined using commercial kits (Cusbio, China) according to the manufacturer's protocols.

Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM) and the differences between mean values of multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's Post hoc test on Graphpad Prism 7. Statistical significance was considered at P less than 0.05.

Results

ATE prevents hyperglycemia and insulin resistance in diabetic rats

Treatment of the normal rats with 400 mg/kg ATE didn't alter both fasting and postprandial glucose levels. HFD/STZ diabetic rats exhibited significant (P<0.001) increase in fasting and postprandial blood glucose levels as represented in (Fig. 1A). Treatment of the diabetic animals with ATE decreased fasting and postprandial blood glucose significantly (P<0.001).

Serum insulin levels were significantly reduced in HFD/STZ diabetic rats when compared with the control (P<0.01) (Fig. 1B). Oral administration of 200 and 400 mg/kg ATE to diabetic rats increased serum insulin significantly (P<0.05). HOMA-IR was increased in diabetic rats significantly (P<0.01) when compared with the normal rats. Treatment with ATE decreased HOMA-IR values in diabetic rats (P<0.01). ATE didn't affect serum levels and HOMA-IR values in normal rats.

ATE improves carbohydrate metabolizing enzymes and increases glycogen in diabetic rats

Hexokinase activity was significantly (P<0.001) decreased in HFD/STZ-induced rats when compared with the control rats (Fig. 2A). On the other hand, PEPCK gene expression was significantly (P<0.001) increased in the liver of HFD/STZ diabetic rats (Fig. 2B). Glycogen was significantly (P<0.001) declined in the liver of HFD/STZ diabetic rats as represented in Figure 2C. Treatment of the diabetic rats with ATE (200 and 400 mg/kg) increased hexokinase activity and glycogen content, whereas decreased PEPCK gene expression. In contrast, normal rats received 400 mg/kg ATE showed normal hexokinase, PEPCK and glycogen (Fig. 2).

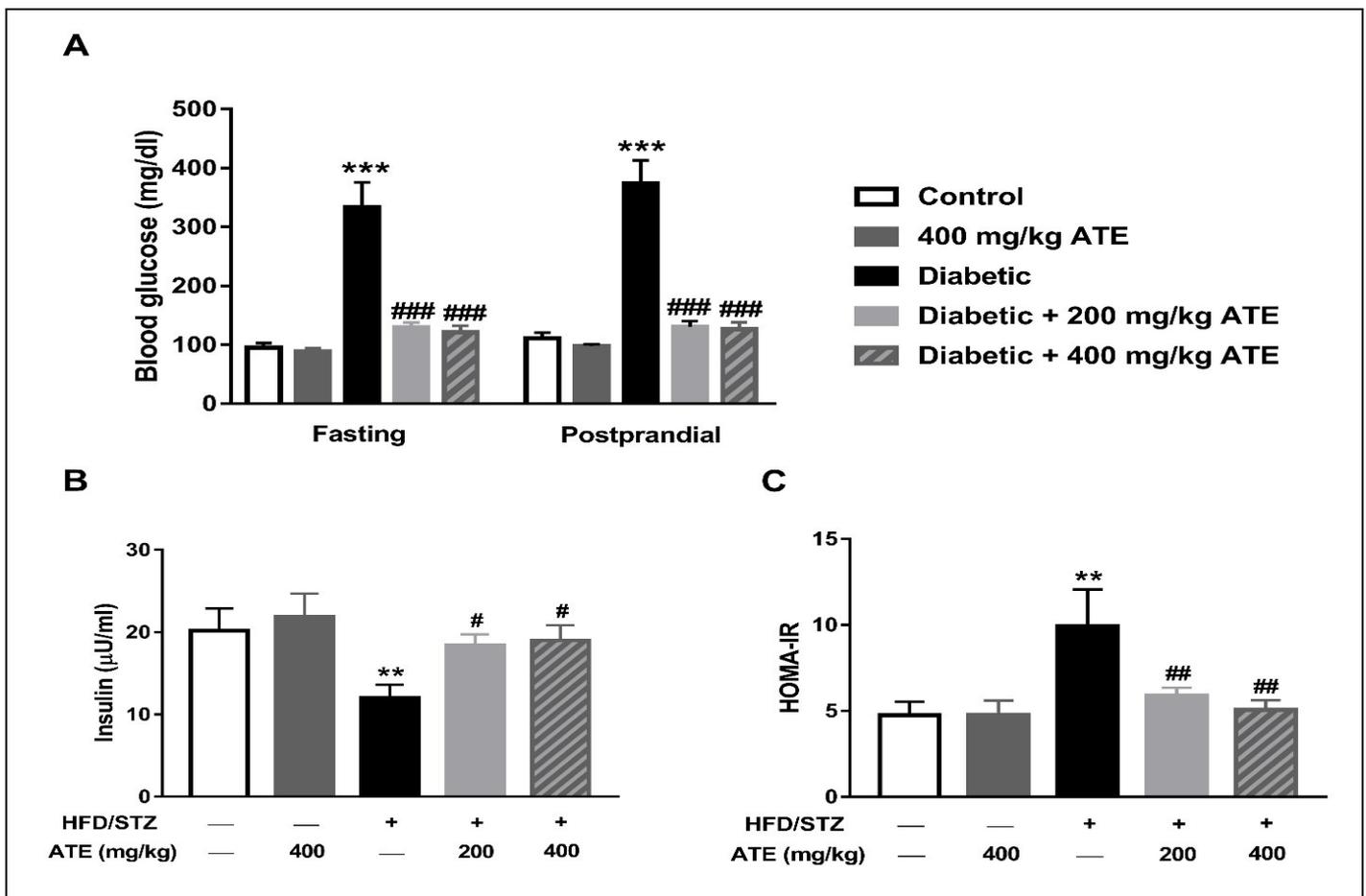


Fig. 1 : ATE decreased fasting and postprandial glucose levels (A) and HOMA-IR (C) and increased serum insulin levels (B) in HFD/STZ diabetic rats. Data are mean \pm SEM (n = 6). **P<0.01 and ***P<0.001 compared to Control. #P<0.05, ##P<0.01 and ###P<0.001 compared to Diabetic.

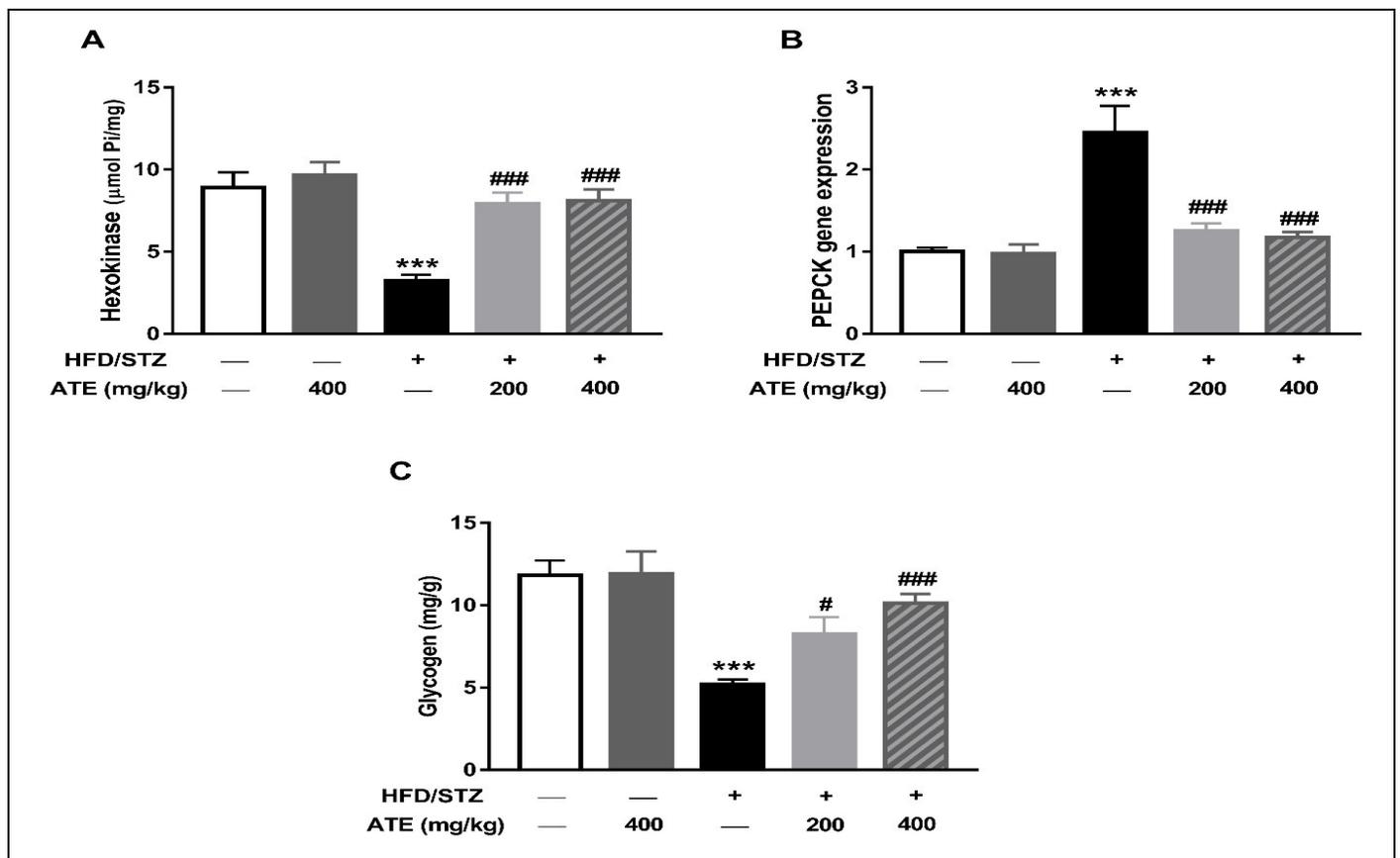


Fig. 2 : ATE increased hexokinase activity (A) and glycogen (C) and decreased PEPCK gene expression (B) in HFD/STZ diabetic rats. Data are mean \pm SEM (n = 6). ***P<0.001 compared to Control. #P<0.05 and ###P<0.001 compared to Diabetic.

ATE prevents dyslipidemia in diabetic rats

HFD/STZ diabetic rats showed significantly ($P<0.001$) increased serum triglycerides, total cholesterol, LDL-cholesterol and vLDL-cholesterol as shown in Figures 3A-D. HDL-cholesterol was decreased significantly ($P<0.01$) in serum HFD/STZ diabetic rats when compared with the control group (Fig. 3E). Treatment with ATE (200 and 400

mg/kg) decreased serum triglycerides, total cholesterol, LDL-cholesterol and vLDL-cholesterol ($P<0.001$). Although the lower dose of ATE didn't increase HDL-cholesterol in diabetic rats, the high dose increased its levels significantly ($P<0.05$). All assayed lipids showed non-significant changes in normal rats treated with 400 mg/kg ATE

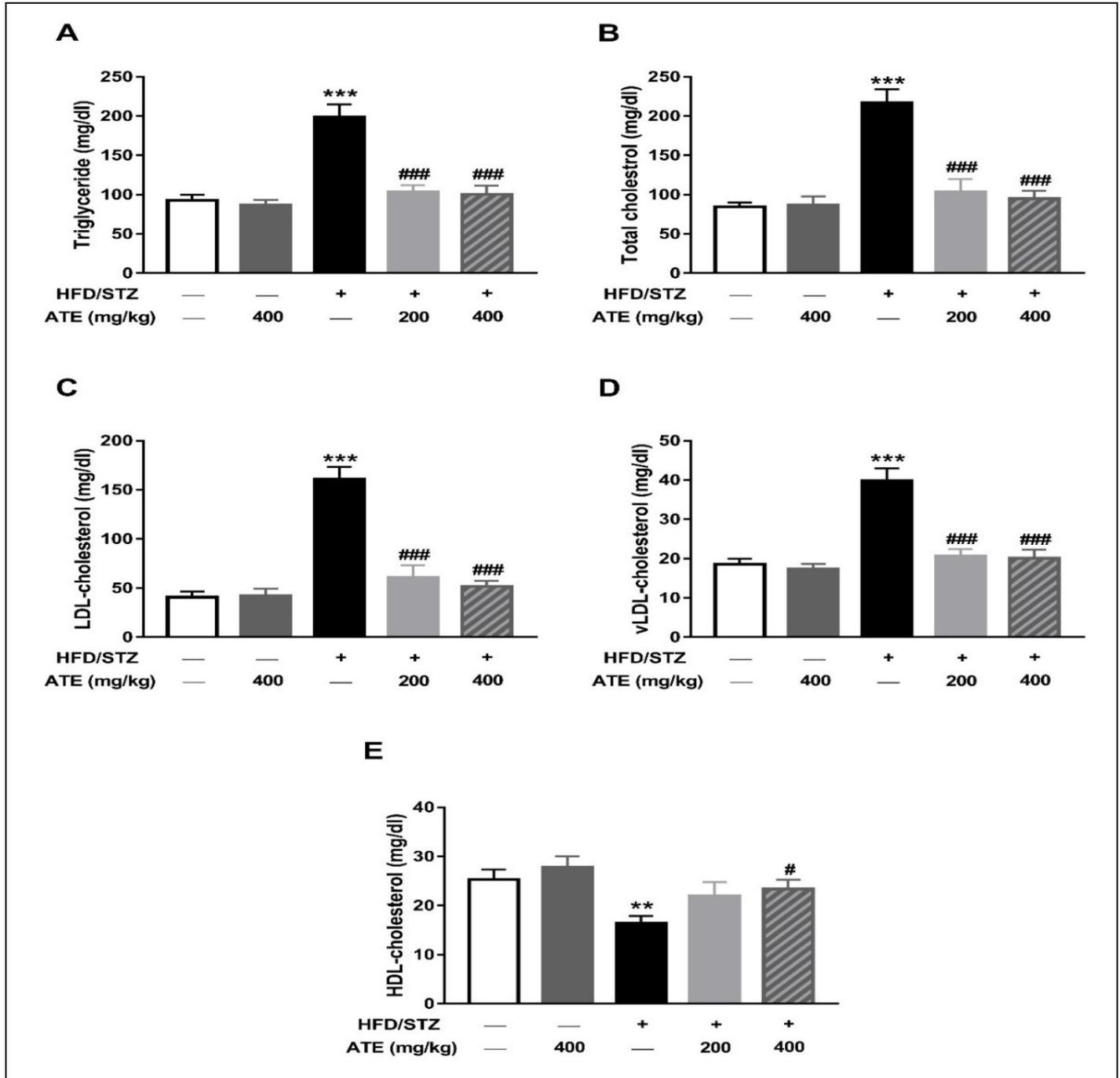


Fig. 3 : ATE prevented dyslipidemia and increased serum HDL-cholesterol in HFD/STZ diabetic rats. Data are mean \pm SEM ($n = 6$). ** $P<0.01$ and *** $P<0.001$ compared to Control. # $P<0.05$ and ### $P<0.001$ compared to Diabetic.

ATE attenuates oxidative stress in diabetic rats

Hepatic TBARS levels were elevated significantly ($P<0.001$) in HFD/STZ diabetic rats as represented in Figure 4A. On the other hand, hepatic GSH (Fig. 4B) and SOD (Fig. 4C) were decreased in HFD/STZ diabetic rats. Oral treatment with decreased TBARS and increased GSH and SOD in HFD/STZ diabetic rats. The high dose of ATE didn't alter TBARS, GSH and SOD in normal rats.

ATE prevents inflammation in diabetic rats

Serum TNF- α (Fig. 5A) and IL-6 (Fig. 5B) were elevated in HFD/STZ diabetic rats as compared to the control group ($P<0.001$). In contrast, treatment with ATE ameliorated the levels of these inflammatory mediators in diabetic rats; however, exerted no effect in normal rats.

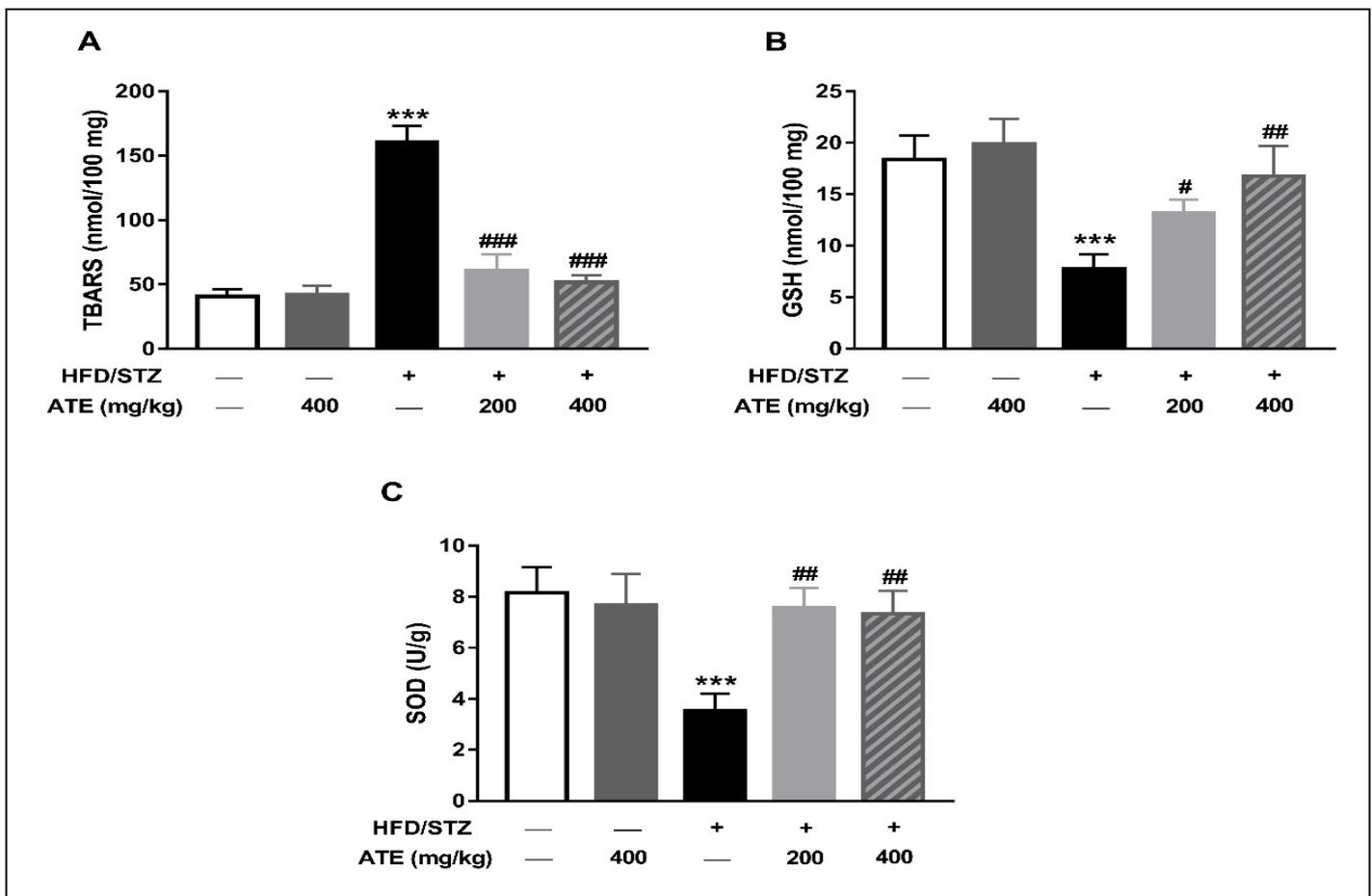


Fig. 4 : ATE decreased TBARS (A) and increased GSH (B) and SOD (C) in HFD/STZ diabetic rats. Data are mean \pm SEM (n = 6). ***P<0.001 compared to Control. #P<0.05, ##P<0.01 and ###P<0.001 compared to Diabetic.

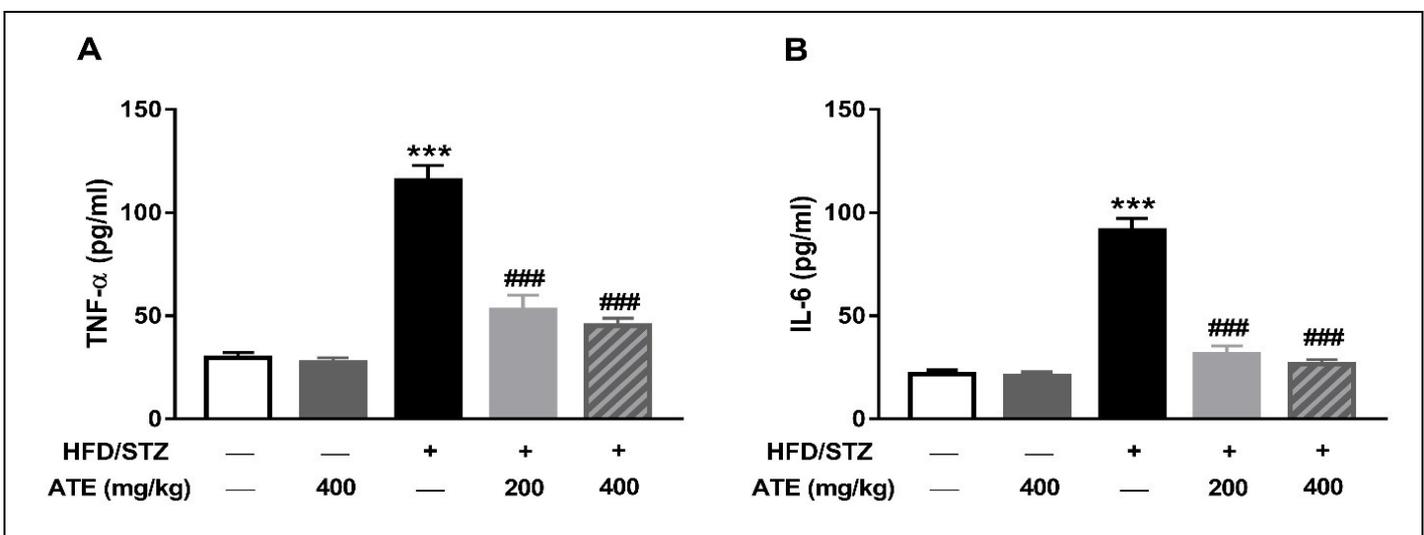


Fig. 5 : ATE decreased serum TNF- α (A) and IL-6 (B) in HFD/STZ diabetic rats. Data are mean \pm SEM (n = 6). ***P<0.001 compared to Control. ###P<0.001 compared to Diabetic.

Discussion

This study explored the ameliorative effects of ATE on hyperglycemia, dyslipidemia, oxidative stress and inflammation in HFD/STZ type 2 diabetic rats. Hyperglycemia in elevated blood glucose levels resulting from impaired insulin secretion and/or action. Hyperglycemia is the main characteristic feature of diabetes that leads to serious complications in many organs if not tightly managed (Jellinger, 2007). In this study, HFD/STZ-induced rats showed hyperglycemia manifested by the increased fasting and postprandial blood glucose levels. In addition, hyperglycemia was accompanied with decreased

serum insulin as well as insulin resistance evidenced by the significantly increased HOMA-IR. In agreement with these findings, hyperglycemia and insulin resistance have been previously demonstrated in HFD/STZ-induced rats (Elsayed *et al.*, 2020, Germoush *et al.*, 2019; Guex *et al.*, 2019; Mahmoud *et al.*, 2012). Therefore, HFD/STZ diabetes is a well-acknowledged model mimicking human T2DM (Lee *et al.*, 2011) and is an accepted model to evaluate the new therapeutic agents. Treatment of diabetic rats with ATE in the present study resulted in ameliorated hyperglycemia and insulin sensitivity. The ability of *A. trisulcus* to improve blood glucose levels has been previously reported by Okla

(Okla *et al.*, 2014) who reported decreased blood glucose levels in diabetic mice treated with *A. trisulcus* aqueous extract. However, the current study is the first to show the anti-diabetic and insulin sensitizing effects of *A. trisulcus* in type 2 diabetes.

Given the essential role of the liver in maintaining glucose homeostasis, the effect of ALE on hepatic hexokinase, PEPCK and glycogen was determined. Uncontrolled hepatic glucose production mediated via increased glycogenolysis and gluconeogenesis and suppressed peripheral glucose utilization occur consequent to impaired insulin secretion and/or sensitivity cause hyperglycemia (Nordlie *et al.*, 1999). In the present study, the activity of hexokinase was significantly decreased, whereas PEPCK expression was increased in diabetic rats. Previous investigations have reported declined hepatic hexokinase and increased PEPCK in HFD/STZ-induced diabetic (Elsayed *et al.*, 2020, Gothandam *et al.*, 2019, Mishra *et al.*, 2019). Impaired insulin secretion and insulin resistance are known causes of declined hexokinase and consequently decreased glucose oxidation *via* glycolysis (Ahmed *et al.*, 2010, Gupta *et al.*, 1999). On the other hand, PEPCK which catalyzes the initial step in hepatic gluconeogenesis (Quinn & Yeagley, 2005) has been increased. In agreement with these results, hepatic expression PEPCK was increased in HFD/STZ-induced diabetes (Elsayed *et al.*, 2020, Song *et al.*, 2019). This study pointed to increased gluconeogenesis and glycogenolysis in HFD/STZ diabetic rats as evidenced by the decreased glycogen content. These processes supported indicate insulin deficiency and insulin resistance in HFD/STZ diabetic rats because insulin stimulates glycogen synthesis (Golden *et al.*, 1979). Treatment with ATE increased insulin levels resulting in improved hexokinase activity and glycogen content as well as PEPCK expression in diabetic rats. Given that PEPCK is insulin-independent (Scott *et al.*, 1998), its decrease following treatment confirmed the added he anti-hyperglycemic effect of ATE irrespective of its insulin sensitizing effect.

HFD/STZ diabetic rats in the present study showed hyperlipidemia manifested by increased triglycerides, cholesterol, LDL and vLDL and decreased HDL-cholesterol. Hyperlipidemia is a casual risk factor for atherosclerotic cardiovascular disease and endothelial dysfunction (Mahmoud *et al.*, 2017c; Mahmoud *et al.*, 2017d; Nordestgaard, 2016). Hypercholesterolemia is a type of hyperlipidemias that elicits atherosclerosis, chronic inflammation and accumulation of hepatic lipids and has been reported in HFD/STZ-induced rats (Elsayed *et al.*, 2020; Mahmoud *et al.*, 2012). ATE significantly ameliorated dyslipidemia in diabetic rats, demonstrating its potent antidiabetic and cardioprotective effects.

Hyperglycemia can provoke oxidative stress and inflammation, leading to damage to different body organs (Mahmoud *et al.*, 2012). In addition, hyperlipidemia and accumulation of lipids in hepatocytes and endothelial cells has been associated with diminished antioxidant defenses and excess production of ROS which damage cellular components and induce cell death (Anila & Vijayalakshmi, 2003; Forstermann, 2008; Tiwari *et al.*, 2013). Also, ROS induce the release of pro-inflammatory cytokines as evidenced by increased TNF- α and IL-6. Oxidative stress in HFD/STZ diabetic rats in the current study was manifested

by increased TBARS and decreased GSH and SOD. Reduced antioxidants in the liver of diabetic rodents along with increased pro-inflammatory cytokines have been previously reported (Mahmoud *et al.*, 2012; Sahin *et al.*, 2019). In addition, serum levels of TNF- α and IL-6 are known to elevated in type 2 diabetic patients (Pickup *et al.*, 2000). These cytokines prevent insulin-stimulated peripheral glucose uptake, promote hepatic glucose production (Lang *et al.*, 1992), suppress insulin action in muscles (Del Aguila *et al.*, 1999) and increase lipolysis (Green *et al.*, 1994). Furthermore, these cytokines reduce insulin receptor substrate-1 tyrosine phosphorylation, resulting in impaired insulin signaling, insulin resistance and hyperglycemia (Müller *et al.*, 2002; Senn *et al.*, 2002). Thus, suppression of oxidative stress and inflammation can improve insulin sensitivity and glucose tolerance in diabetes. In the present study, ATE reduced TBARS, TNF- α and IL-6, and increased GSH and SOD, demonstrating its potent antioxidant and anti-inflammatory activities. These antioxidant and anti-inflammatory effects of ATE are attributed to its rich content of phenolics and other bioactive compounds. Phenolic compounds possess multiple effects, including antioxidant, anti-inflammatory, anti-hyperlipidemic and anti-diabetic (Al-Dossari *et al.*, 2019; Aladaileh *et al.*, 2019; Alhusaini *et al.*, 2019; Althunibat *et al.*, 2019; Kamel *et al.*, 2016; Mahmoud, 2012, 2013, Mahmoud *et al.*, 2017a; Mahmoud *et al.*, 2017b). ATE has been previously reported to contain veratric acid, α -amyrin, vanillic acid, and many other constituents with known antioxidant and anti-inflammatory activities (El-Shanawany *et al.*, 2014). Vanillic acid has shown protective effects against hyperlipidemia and inflammation induced by HFD in rats (Chang *et al.*, 2015). In addition, vanillic acid activated thermogenesis in brown and white adipose tissue of HFD-fed mice (Han *et al.*, 2018) and ameliorated obesity via activation of the AMPK pathway (Jung *et al.*, 2018).

In conclusion, the results of this study show for the first time the ameliorative effect of ATE on hyperglycemia, hyperlipidemia, oxidative stress and inflammation in type 2 diabetic rats. ATE ameliorated both fasting and postprandial blood glucose levels, and increased insulin sensitivity in HFD/STZ diabetic rats. ATE attenuated dyslipidemia, increased HDL-cholesterol, improved hexokinase activity and glycogen content and decreased PEPCK expression. Furthermore, ATE suppressed lipid peroxidation and inflammation and increased cellular antioxidants in diabetic rats. Therefore, TE represent a promising lead for the development of antidiabetic agent; however, further investigations are needed to explore the underlying mechanisms.

Conflict of interest

No conflict of interest

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