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Original Article

Dimethylfumarate ameliorates high-fat diet/streptozotocin-induced type 2 diabetes in liver and aorta of rats

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ABSTRACT

In the current study, the possible curative effects of dimethylfumarate (DMF) on high-fat diet (HFD) and low-dose streptozotocin (STZ)-induced type 2 diabetes in rats were evaluated.
Methods: T2DM was induced by feeding rats with HFD/25% fructose solution for 30 days followed by STZ (35mg/kg, i.p.), DMF effects were investigated on fasting blood glucose, insulin, oral glucose tolerance test (OGTT), insulin tolerance test (ITT), liver biomarkers, lipid profile, oxidative stress biomarkers, serum advanced glycation end products (AGEs) and its receptors (RAGE) in aorta. Additionally, the mRNA expression of hepatic insulin signalling pathway genes and aortic nitric oxide synthase3 (eNOS) and NADPH oxidase4 (NOX4) were determined.
Results: Treatment with DMF for 30 days decreased fasting glucose, ameliorated insulin resistance, improved lipid profile, decreased liver enzymes activities in serum, reduced serum AGEs and aortic RAGE contents, decreased malondialdehyde content, increased superoxide dismutase activity in liver and aorta and increased the reduced glutathione content in liver. Additionally, the mRNA expression of hepatic insulin signalling pathway genes and aortic eNOS was elevated and the mRNA expression of NOX4 was decreased as a result of DMF treatment.
Conclusion: Our study demonstrated that DMF possess the potential ability to ameliorate type 2 diabetes in rats.

Keywords: Type 2 diabetes, dimethylfumarate, oxidative stress, streptozotocin, high fat diet, advanced glycation end products, insulin signalling pathway

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is the most common serious metabolic condition in the world that results from impaired β-cell function and a subnormal response of tissues to insulin (insulin resistance)[1].

The liver has an important role in the control of the whole body metabolism of energy nutrients. One of the hallmarks of T2DM is the alteration of the hepatic metabolism, where the liver losses its ability to control the glucose homeostasis leading to hyperglycemia and dysregulation of the insulin pathway. In T2DM, the insulin target tissues are damaged, which aggravates the ability of insulin to trigger downstream metabolic actions, which appear to be critical lesions contributing to insulin resistance and T2DM [1].

Insulin signal transduction is initiated when insulin binds to the insulin receptor (IR), the activation of insulin receptor substrate 1/2 (IRS1/2) initiates the stimulation of the phosphatidylinositol-3-kinase (PI3K) / serine-threonine kinase (AKT) pathway that leads to the inhibition of glycogen synthase kinase-3 (GSK-3) by phosphorylation, which subsequently phosphorylates and inactivates glycogen synthase (GS), which is needed for the metabolic effects of insulin in the liver [2].

Nonenzymatic glycation of haemoglobin and albumin proteins is one of the underlying features of hyperglycaemia and T2DM. Advanced glycation end-products (AGEs) are a heterogeneous class of compounds results from a complex cascade of reactions between glucose and its derivatives with proteins [3]. Additionally, oxidative stress plays a key role in the pathogenesis of insulin resistance and β-cell dysfunction, the two most relevant mechanisms in the pathophysiology of T2DM and its vascular complications [4]. The formed AGEs in conjunction with oxidative stress causes structural and functional impairments of plasma proteins in particular albumin [5,6] and was involved in pathophysiological mechanisms of vascular diseases in T2DM [4,7]. AGEs binding to their receptor (RAGE) activate NAPDH oxidase and thus increase intracellular reactive oxygen species (ROS) formation [8,9]. Increased ROS in turn leads to AGE formation, which triggers all described damaging mechanisms mediated by AGEs [10].

Endothelial nitric oxide synthase (eNOS) is very important in regulation the endothelium-dependent relaxations in large arteries as it produce nitric oxide (NO) which is a key vasodilator, eNOS expression is significantly lower in the aortas of diabetic rats [11] as NO-dependent vasodilation is markedly impaired in insulin resistant and diabetic rats [12,13] Dimethylfumarate (DMF) is a derivative of fumarate and considered as the most powerful nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activator, it has been used as an oral treatment for patients with relapsing forms of multiple sclerosis and it presently employed for the treatment of psoriasis [14]. Several studies reported that DMF has cardioprotective, neuroprotective, antiinflammatory and antioxidant effects as it used for treatment of autoimmune or inflammatory diseases [15-17].

Previous studies have shown that Nrf2 plays a critical role in pancreatic β-cell defense against oxidative stress and DMF pretreatment protects pancreatic β-cells against acute oxidative stress-induced cell damage [18,19]. Additionally, DMF has been reported to protect endothelial cells and prevent vascular injury via Nrf2 activation [20,21].

The objectives of the current study are:

- Establish the model of HFD/fructose/STZ-induced T2DM in rats.
- Investigate the effect of DMF on type 2 diabetic rats induced by HFD/fructose/STZ.
- Examine the possible involved pathways in the pathogenesis of T2DM and to determine the possible mechanisms through which DMF can affect HFD/fructose/STZ- induced T2DM.

MATERIALS AND METHODS

Drugs and chemicals: DMF (Santa cruze, USA), streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO), cholesterol (El-Goumhouria Co. for trading medicines, research lab fine chem. Industries, India), pyrogallol, reduced glutathione, thiobarbituric acid (TBA), Tris (hydroxymethyl) aminomethane, Ellman's reagent [5,5'-dithio-bis(2-nitrobenzoic acid)] and pentobarbital sodium, were purchased from Sigma Aldrich chemical Co. (St. Louis, MO). Fructose powder (Safety Misr, Specialized Food Industry co., Cairo, Egypt).

Experimental animals: Male Wister rats, with average age of 6-8 weeks were purchased from VACSERA medical center, Cairo, Egypt. Rats were kept in light: dark schedule (12 h:12 h), standard rat diet and drinking water and maintained at 25°C. After acclimatization for 2 weeks, rats with weight of 190 ± 10 g were selected for the experiment. This study protocol was approved by the ''Research Ethics Committee'' of Faculty of Pharmacy, Mansoura University, Egypt in accordance with ''The Principles of Laboratory Animal Care'' (NIH publication No. 85-23, revised 1985).

Development of T2DM and experimental design: Rats were randomly divided into two groups: normal control (NC, n=12), and HFD + 25 % fructose in drinking water/ fed group (HFD, n=36). Rats in the NC group were fed normal pellet diet and rats in the HFD group received HFD (67.5% normal pellet, 20% sucrose, 10% refined lard fat and 2.5% cholesterol) [22-24] and 25% fructose in drinking water [25,26].

After 30 days, HFD group was injected with STZ (35 mg/kg i.p.) [27,28] dissolved in citrate buffer (pH 4.4), while animals in the NC group were fed with normal drinking water and injected with citrate buffer, as described previously. Measurement of fasting blood glucose (FBG) levels were performed 72 h after STZ injection using a portable glucometer on blood collected from tail veins. Rats were considered as diabetic if FBG level > 180 mg/dL, then, rats were randomly divided into four groups, with six rats in each group as follows: Group (1): Control group; rats were given vehicle (CMC, 1mL/250g); Group (2): diabetic group: rats received HFD and 25 % fructose in drinking water; Group (3): DMF group: rats received DMF (25 mg/kg, orally) [29-31]. Group (4): (diabetic + DMF group): rats received DMF (25mg/kg orally). The treatment regimen was continued for another 30 days to the four groups.

Oral glucose tolerance test: Animals were fasted for 14-18 h at 48 h prior to the end day and conscious rats were administrated 40 % glucose solution (2 g/kg body weight) orally by gastric tube. Blood glucose level was measured at 0 (before glucose administration and assessed as the fasting blood glucose level), 30, 60, 120 and 180 min after glucose administration. Blood glucose was measured via glucose dehydrogenase method using tail blood and a portable glucometer (Accu-Chek Active, Roche).

Insulin tolerance test: At 24 h before the end day, conscious rats were fasted for 14-18 h, and injected subcutaneously with a dose of 0.5 IU/kg human soluble insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark). Blood glucose level was determined at 0, 30, 60, 120 and 180 min after insulin administration using tail blood and a portable glucometer.

Sample preparation: At the end of the experiment, rats were fasted for 12 h and anesthetized using pentobarbital sodium (40 mg/kg, i.p.), and blood samples were collected to prepare serum. In addition, the descending thoracic aorta was removed and divided into two parts, one part was immersed in phosphate buffered saline (PBS) and put in -80°C for further homogenization, and the later one was flash-frozen in liquid nitrogen and stored at -80^{o}C for quantitive RT-PCR.
Additionally, Liver tissues were immediately collected and snap-frozen in liquid nitrogen, stored at -80°C to be used later for real-time RT-PCR, another part was immersed in PBS and put in -80°C for preparation of liver tissue homogenate

Determination of fasting serum insulin, insulin resistance and β-cell function indices: The fasting rat insulin was measured in serum using a rat insulin ELISA kit (Mercodia Developing Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. Homeostasis model assessment of insulin resistance (HOMA-IR) index and β-cell function index were performed by estimating HOMA-IR and HOMA-β using the following equations [32]:

$$\text{HOMA-IR} = \frac{\text{fasting serum insulin}\left(\frac{\text{mU}}{\text{L}}\right) \times \text{fasting serum glucose}\left(\frac{\text{mmol}}{\text{L}}\right)}{22.5}$$

$$\text{HOMA-}\beta = \frac{20 \times \text{fasting serum insulin}\left(\frac{\text{mU}}{\text{L}}\right)}{\left[\text{fasting serum glucose}\left(\frac{\text{mmol}}{\text{L}}\right) - 3.5\right]}$$

Estimation of lipid profile and liver function biomarkers in serum: The serum levels of triglycerides (TG), total cholesterol (TC) and were estimated colorimetrically using commercial kits (Spinreact, Santa Coloma, Spain) and high density lipoprotein cholesterol (HDL-C) was estimated in serum using a commercial kit (Vitro Scient, Hannover, Germany). Serum very low density lipoprotein cholesterol (VLDL-C) was calculated according to the equation; VLDL-C = TG/5. Serum low density lipoprotein cholesterol (LDL-C) was calculated according to the equation; LDL-C = TC – [VLDL + HDL-C] [33]. The levels of ALT (alanine aminotransferase) and AST (aspartate aminotransferase) were colorimetrically measured in serum using commercial kits (Biomed Diagnostics, Badr city, Egypt).

Estimation of serum AGEs by quantitative fluorescence spectroscopy: Serum was diluted 500 fold with PBS, 20 µL were used and diluted into 10 mL then filtered through Millex-GV filters

(µm 0.22 Millipore), fluorescence spectra (corrected for background) were recorded in triplicate on a Fluoromax spectrometer (Spex instruments) at room temperature. The excitation wavelength was 370 nm and the emission maximum was 445 nm and the signal intensity was expressed in arbitrary units [34,35].

Preparation of hepatic and aortic homogenates: Liver and aortic tissues were weighed and homogenized in PBS as (1:10 w/v) using Omni-125 hand held homogenizer (Omni international, USA). The homogenates of liver and aorta were spun at 5000 g, 4ºC for 15 min and the supernatant was freshly used for assay of oxidative biomarkers MDA, GSH and SOD. For RAGE determination in aortic homogenates, centrifugation was performed at 12000 g, 4ºC for 60 min.

Estimation of RAGE concentration in aorta homogenate: RAGE concentration was measured in aorta homogenate using rat RAGE picokine ELISA kit (Boster biological technology, Pleasanton, California) according to manufacturer protocol.

Determination of hepatic and aortic levels of oxidative stress-related parameters: Malondialdehyde (MDA) content, an end product of lipid peroxidation, was determined in the form of thiobarbituric acid reactive substances (TBARS), as previously described [36]. The levels of acid-soluble thiols, mainly reduced glutathione (GSH), and superoxide dismutase (SOD) activity were assayed according to previously described methods [37,38].

Quantitative real-time polymerase chain reaction (RT-PCR): RNA was isolated from liver and aorta samples using TRIZOL reagent (Life Technologies, Carlsbad, CA, USA). First-strand cDNA was synthesized from 1 µg of total RNA in 20 µL volume using SensiFast cDNA synthesis kit (Bioline reagents Ltd, United Kingdom) according to the manufacturer's procedures. RT-PCR was performed with a thermocycler Rotor Gene Q (Qiagen, Hilden, Germany), using Hot Firepol Evagreen qPCR mix plus kit (Solis BioDyne, Tartu, Estonia).

The mRNA levels of insulin receptor substrate 1 (IRS1), phosphatidylinositol 3-kinase regulatory subunit1 (PI3K-P85 subunit) and AKT serine/threonine kinase 2 (AKT2) in liver and NADPH oxidase 4 (NOX4) and nitric oxide synthase 3 (eNOS) in aorta were normalized relative to 18S ribosomal RNA (Rn18S) in the same sample. Primers for the IRS1, PI3K-P85 subunit, AKT2, eNOS, NOX4 and Rn18S were as the following:

IRS1 (forward: GGCACCATCTCAACAATC, reverse: GTTTCCCACCCACCATAC, amplicon size = 105), PI3K-P85 subunit (forward: ACTACTGGGGAGAGGGGAGA, reverse: AACATCAGGAGGGGCAAAC, amplicon size = 198), AKT2 (forward: CACAGAGAGCCGAGTCCTACA, reverse: GGCATACTCCATCACAAAGCA, amplicon size = 103), eNOS (forward: GACCAGCACCAGACCACAG, reverse: GCCCACTTCCCAGTTCTTT, amplicon size = 83) NOX4 (forward: CTTTTTATTGGGCGTCCTC, reverse: GGTCCACAGCAGAAAACTCC, amplicon size = 92), and Rn18S (forward: AGTTGGTGGAGCGATTTGTC, reverse: GAACGCCACTTGTCCCTCTA, amplicon size = 122).
The results were expressed as n-fold change of the relative expression level of target genes from control group using $\Delta\Delta C_t$ method.

Statistical analysis: Data are expressed as mean ± standard error of mean (SEM). GraphPad software Prism V 5.02 (GraphPad Software Inc., San Diego, CA, USA) was used to carry out statistical analysis and graphing. In OGTT and ITT, the change in glucose was calculated as the difference between each point and the initial baseline for each rat. One-way analysis of variance (ANOVA) followed by Tukey–Kramer's multiple comparisons post hoc test was used to measure significant differences between groups. Statistical significance was considered at $p < 0.05$.

RESULTS

Effect of DMF on body weight gain, mean daily food intake, ALT and AST levels in rats: Both body weight gain and mean food intake were not significantly different among groups after 60 days (Table (1)). Additionally, in the diabetic group, ALT and AST levels were significantly ($p<0.05$, n=6) increased by 1.95 and 1.55 folds, respectively when compared to control group ($p<0.05$, n=6) (Table (1)).

Oral administration of DMF for 30 days significantly decreased ALT and AST activities by 1.77 and 1.55 folds, respectively when compared to diabetic rats. DMF treatment had no significant effect on body weight changes and liver function biomarkers in normal rats.

Table (1): Effect of DMF supplementation on initial body weight, final body weight, body weight gain, daily food intake and liver biomarkers levels in serum of diabetic rats. (Mean values with their standard errors of six rats from each group)

Groups / Parameters	Control	Diabetic	DMF	Diabetic + DMF
Initial body weight (g)	192.8 ± 2.19	186.3 ± 1.76	186.3 ± 1.76	193.5 ± 3.36
Final body weight (g)	276 ± 6.69	285 ± 2.22	268.8 ± 10.93	287.3 ± 3.18
Body weight gain (g)	83.2 ± 5.89	98.7 ± 2.97	71.0 ± 6.39	94.0 ± 4.36
Daily food intake (g/day/rat)	31.04 ± 0.58	31.20 ± 0.70	30.42 ± 0.60	31.42 ± 0.72
Serum ALT (U/ml)	32.00 ± 3.10	62.50* ± 3.32	36.60$^{\$}$ ± 3.12	35.25$^{\$}$ ± 3.79
Serum AST (U/ml)	26.00 ± 1.00	40.40* ± 3.18	27.60$^{\$}$ ± 1.78	25.00$^{\$}$ ± 1.03

Data are expressed as mean ± SEM, n=6.
* $P<$ 0.05 vs. Control group, $^{\$}$ $p<0.05$ vs. T2D group using one-way ANOVA followed by Tukey-Kramer multiple comparisons *post hoc test*.
DMF: dimethylfumarate; T2D: type 2 diabetic group; ANOVA: analysis of variance; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

Effect of DMF on OGTT and ITT: Oral glucose tolerance test performed at the end of the study revealed the impaired glucose tolerance, where the area under the curve (AUC) in the diabetic group was significantly (p<0.05, n=6) increased by 2.06-fold as compared to the control group. Treatment with DMF greatly reversed the impairment in glucose tolerance, showing significant decrease in the AUC of OGTT curve by 40% relative to diabetic group. These results are shown in Figure 1(A). Type 2 diabetic rats also compromised in-vivo insulin sensitivity measured by ITT, the ITT_{AUC} in the diabetic group was significantly (p<0.05, n=6) elevated by 1.63-fold, relative to the control group (Figure 1(B)). DMF improved in-vivo insulin activity, exhibiting 32% decrease of the AUC of ITT relative to diabetic group.

Effect of DMF on fasting blood glucose, serum insulin, HOMA-IR and HOMA-β indices: Type 2 diabetic rat model showed a significant state of hyperglycemia as elucidated by an increase in fasting glucose level by 2.24-fold compared to the control group (figure 2 (A)). It also showed a significant state of insulin resistance and reduction in β-cell function indicated by a significant (p<0.05, n=6) elevation in fasting insulin level, HOMA-IR index and decrease in HOMA-β index by 1.82- , 3.27- and 2.01-folds, respectively compared to the control group (figure 2 (B, C, D)).
A 30 day treatment of diabetic rats with DMF reduced the elevated fasting glucose by 45.2 % when compared to diabetic group (Figure 2(A)). DMF caused a non significant (p<0.05, n=6) decrease in the elevated fasting insulin level and significantly (p<0.05, n=6) decreased HOMA-IR by 16.3 % and 56.9 %, respectively (Figure 2(B)) and prevented the diabetic-induced reduction in HOMA-β index when compared with diabetic group. DMF treatment had no significant effect on fasting glucose, insulin, HOMA-IR or HOMA-β index in normal rats.

Effect of DMF on serum lipid profile: In diabetic group, the levels of TG, TC, LDL and VLDL significantly (p<0.05, n=6) increased by 1.82-, 1.68-, 3.76- and 1.84- folds (Figure 3(A, B, D, E)), respectively, while HDL levels in the diabetic group were significantly (p<0.05, n=6) lower than control group by 49.06 % (Figure 3(C)). DMF treatment for one month markedly reduced the blood levels of TC, TG, LDL and VLDL, and increased the HDL concentration as these parameters were almost restored to normal levels of control rats. DMF treatment had no significant effect on serum lipid profile in normal rats.

Effect of DMF on serum AGEs and aortic RAGE levels: In diabetic group, serum AGEs and RAGE content in aorta were significantly (p<0.05, n=6) elevated by 1.22- and 7.56-fold when compared to control group (Figure 4(A, B)). DMF treatment for 30 days almost restored both the serum AGEs and the elevated aortic RAGE concentration to normal level of the control group (Figure 3(A)). DMF treatment had no significant effect on serum AGEs and aortic RAGE contents in normal rats.

Effect of DMF on oxidative stress markers in liver and aorta tissues: Malondialdehyde content in liver and aorta homogenates was significantly (p<0.05, n=6) increased by 2.06- and 2.39- folds, respectively when compared to control group (Figure 5 (A)), while hepatic and aortic GSH content was significantly (p<0.05, n=6) reduced by

50.10 % and 28.02 %, respectively (Figure 5 (B)), and SOD activity was decreased by 59.61 % and 51.61 % in liver and aorta, respectively in comparison to control group (Figure 5 (C)). Hepatic MDA, GSH contents and SOD activity were almost restored to normal levels of control rats by DMF treatment, while in aorta, DMF administration significantly ($p<0.05$, n=6) reduced the elevated MDA content by 45.34%, had no significant effect on the reduced GSH content and significantly increased SOD activity to normal level when compared to the diabetic group ($p<0.05$, n=6). DMF treatment had no significant effect on oxidative stress parameters in normal rats.

Effect of DMF on mRNA expression of hepatic IRS1, PI3K - P85 subunit and AKT2 and aortic eNOS and NOX4: In diabetic group, the mRNA expression of hepatic IRS1, PI3K-P85 subunit and AKT2 (Figure 6(A, B, C)) and aortic eNOS (Figure 6(D)) were significantly ($p<0.05$, n=6) decreased by 90.87 %, 93.38 %, 93.21 % and 95.95 %, respectively when compared to control group, while, mRNA expression of aortic NOX4 (Figure 6 (E)) was significantly ($p<0.05$, n=6) increased in by 19.2-fold when compared to diabetic group. Treatment with DMF elevated the decreased mRNA expression of IRS1, PI3K-P85 subunit, AKT2 in liver by 8.33-, 8.96- and 9.39-folds, respectively. Additionally, DMF significantly ($p<0.05$, n=6) increased aortic eNOS by 24.06-fold and reduced the elevated mRNA expression of aortic NOX4 by 77.67% when compared to diabetic group. DMF treatment had no significant effect on mRNA expression of hepatic and aortic genes in normal rats.

DISCUSSION

Type 2 diabetes mellitus is the most common metabolic disorder worldwide [39]. Insulin resistance and β-cell failure are two major mechanisms which contribute to the pathogenesis of T2DM [40]. In the present study, to explore the effects of DMF on this disorder, we tested DMF in rats with STZ/induced T2DM. Previous studies had shown that a rat model of T2DM was established by the STZ/fat + fructose fed method [25,26,41,42]. Our results revealed that feeding HFD plus 25% fructose solution in combination with a 35 mg/kg b.w. STZ injection can be an easier and quicker way for the development of T2D in rats.

In the present study, DMF treatment decreased fasting glucose levels, ameliorated insulin resistance, improved lipid profile, decreased liver enzymes activities in serum, and reduced serum AGEs and RAGE contents in aorta in diabetic rats. Additionally, DMF treatment resulted in decrease in MDA content, increase SOD activity in liver and aorta, increase GSH content in liver and had no significant effect on aortic GSH concentration. Moreover, the mRNA expression of hepatic IRS1, PI3K-P85 subunit and AKT2 and aortic eNOS was elevated and the mRNA expression of NOX4 was decreased as a result of the DMF treatment.

Effects of DMF on food intake and weight gain

The average body weight of the diabetic group was not significantly different when compared to control group, the study of [43] had shown that body weights between STZ-treated HFD-fed rats and control normal pellet diet-fed rats remained largely unaltered after STZ injection (35 mg/kg, i.p.). Additionally, previous studies confirmed that a four-week adaptation period was needed for the HFD group before it begins to have a higher growth rate and there was no significant difference between the weight of the regular chow-fed group and fat-fed group after STZ injection [22,44].

Effect of DMF on OGTT, ITT, fasting serum glucose, insulin, HOMA-IR and HOMA-β indices

Insulin resistance and β-cell failure are two major mechanisms which contribute to the pathogenesis of T2DM [40]. In T2DM, a steady rise in insulin resistance leads to a corresponding increase in fasting insulin levels as the disease progresses. Increase in fasting insulin is one of the most important indications of insulin resistance [45]. Our model was authenticated by calculating HOMA-IR (a parameter that can be calculated from serum insulin levels and is regarded widely as a reliable biomarker of insulin resistance) [46]. In addition, OGTT is an important index for evaluation of islet function [47] and the decrease of HOMA-β index indicates the decline in β-cell function which used for evaluation of its basic function that correlates well with other validated physiological methods [48,49].

In the present study, results showed that diabetic group exhibited a significant ($p < 0.05$) increase AUC_{OGTT}, AUC_{ITT}, fasting blood glucose, serum insulin and HOMA-IR and decrease in HOMA-β index when compared to control group. Hence, the established (STZ / HFD + fructose fed) rat model exhibits insulin resistance and hyperglycemia which mimics metabolic characteristics of T2DM.

DMF supplementation increased insulin sensitivity and promoted glucose disposal, as indicated by significant ($p<0.05$) reductions in AUC_{OGTT}, AUC_{ITT}, It also decreased fasting blood glucose, fasting insulin levels and HOMA-IR index when compared to diabetic rats. Additionally, DMF increased the values of HOMA-β index indicating that DMF may have beneficial effect on restoring

β-cell secretory function. Our results were in agreement with the previous study which showed that DMF (25 mg/kg) significantly reduced blood glucose level and AUC of intraperitoneal glucose tolerance in rats with l-arginine induced chronic pancreatitis [30].

Effect of DMF on serum lipid profile and liver function biomarkers

Dyslipidemia is one of the major factors linked with hyperglycemia, disruptions in lipid metabolism associated with hyperglycemia is characterized by elevated levels of TC, TG, VLDL-C and LDL-C and it has been reported in the early stage of T2DM [50,51] and these elevations are greatly influenced by insulin resistance [52].

In the present study, serum TG, TC, and LDL and VLDL levels were significantly increased, HDL level was decreased in diabetic group, and these levels were restored to normal after DMF treatment which indicated the hypolipidemic functions of DMF mediated via ameliorating the disturbance of lipid metabolism in diabetic rats.

In addition, hepatic dysfunction is a major risk factor in the pathogenesis of T2D due to IR and oxidative damage [53,54]. It is characterized by the leakage of hepatic enzymes into the blood stream from the liver cytosol leading to increase in the activities of these enzymes in plasma [55,56]. A serum ALT has been indicated as a predictor of T2D in human subjects and its level is strongly correlated to HOMA-IR scores [57].

Thus, in the present study, serum levels of AST and ALT were significantly ($p<0.05$) elevated in diabetic rats which revealed the occurrence of hepatic injury. DMF treatment decreased levels of these enzymes indicating its hepatoprotective effect; a previous study showed the hepatoprotective effect of DMF as it decreased serum ALT level in a rat model of ischemia/reperfusion- induced liver injury [29].

Effect of DMF on serum AGES and aortic RAGE content

AGEs formation with concomitant generation of oxidative stress has been reported as a major contributor to the pancreatic β-cell damage in T2DM [44]. AGEs formation is accelerated due to increased concentration of circulating glucose, AGE precursors and oxidative stress in serum and tissues of people with T2DM [58]. The damaging potential of AGEs results from direct alterations on protein structures and functions via binding of AGEs to RAGE [59] that results in NAPDH oxidase activation and thus increases intracellular ROS formation [8], which triggers all described damaging mechanisms mediated by AGEs [60].

In the present study, serum AGEs and aortic RAGE contents were significantly ($p<0.05$) increased in the diabetic group, which was in agreement with a previous study which showed that the serum level of AGEs was significantly increased in patients with T2D compared with nondiabetic control subjects [58]. Additionally, the study of Kang et al (2016) confirmed the increased mRNA expression of aortic rat RAGE and the increased concentration of aortic NADPH oxidase and RAGE by immunohistochemical analysis in T2D rats [61]. DMF supplementation decreased the elevated levels of serum AGEs and aortic RAGE levels and these results were in agreement with the study of Simpson et al (1999) which showed that DMF derivative (BTS 67582) decreased AGEs formation in BSA/D-glucose and L-lysine/glucose-6-phosphate assay systems-an in vitro study [62].

Effect of DMF on MDA, GSH content and SOD activity in liver and aorta

Oxidative stress resulting from persistent hyperglycemia appears to constitute the key in the pathogenesis of T2DM and is associated with diabetic complications [63]. Several studies indicate that oxidative stress is also causal in the development of the two hallmarks of T2D: β-cell dysfunction and insulin resistance [64]. Chronic hyperglycemia leads to elevated ROS levels that are potential to increase lipid peroxidation, alter antioxidant defense and further contribute to insulin resistance [65,66]. In the study, we found that, in type 2 diabetic rats, hepatic and aortic contents of GSH and SOD activity were significantly ($p<0.05$) decreased and MDA content was significantly ($p<0.05$) increased as compared to control rats. These findings were consistent with previous studies [66-68].

DMF was more effective in decreasing MDA content in a rat model of ischemia/reperfusion-induced liver injury [29] and l-arginine induced chronic pancreatitis in rats [30]. Additionally, DMF treatment has shown protective effects via decreasing the elevated MDA levels and also increasing the reduced antioxidant enzyme activities (SOD and GSH-Px) in brain cortex of rats-induced subarachnoid hemorrhage [69].

Consistent with these studies, treatment with DMF in our study caused a significant ($p < 0.05$) increase in both GSH content in the liver and SOD activity in the liver and aorta of diabetic rats, significantly ($p < 0.05$) decreased MDA content in liver and aorta.

Effect of DMF on IRS1, PI3K-P85 subunit and AKT2 mRNA expression in liver

In the present study, we investigated the effect of DMF on IRS1, PI3K-P85 subunit and AKT2 genes at mRNA level as these are involved in insulin

receptor signalling pathway as in diabetic condition the functions of these genes get altered. The RT-PCR results showed that the mRNA expression of IRS1, PI3K-P85 subunit and AKT2 was altered in type 2 diabetic group when compared to control group and DMF treatment increased the expression level of these genes. This indicates that DMF promotes the upregulation of IRS1, PI3K-P85 subunit and AKT2 via insulin signaling pathway (PI3K/AKT pathway).

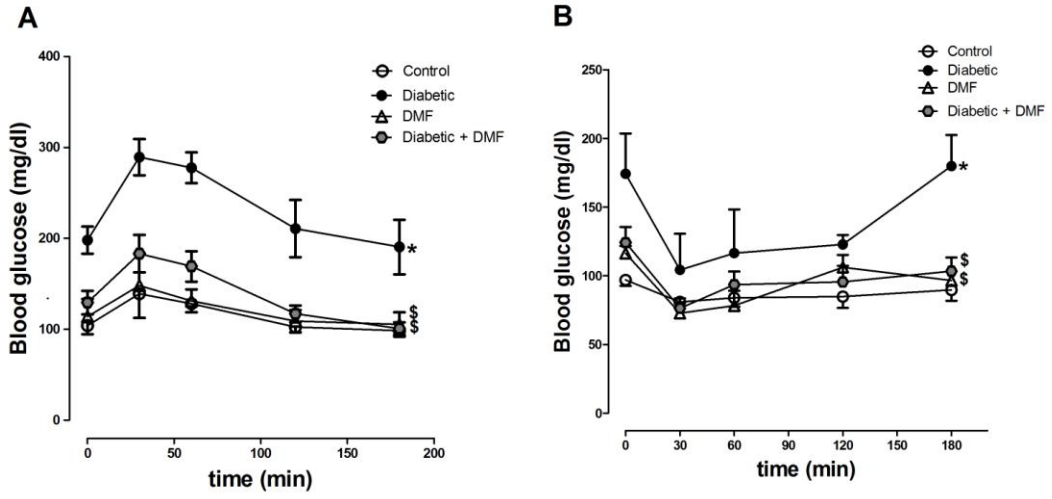
Effect of DMF on eNOS and NOX4 mRNA expression in aorta

IRS1 plays an important role in coupling signaling from the insulin receptor to PI3K and subsequent activation of eNOS [70]. PI3K was reported to play a significant role in insulin signalling pathway related to NO production. In addition, previous studies have proved direct evidence for a complete biochemical pathway involving the IRS1, PI3K and eNOS which account for physiological actions of insulin in stimulating the production of NO [71,72]. NADPH oxidase is considered as the main source of ROS in the cardiovascular tissues [73]. Emerging evidence supports the concept that NADPH oxidase 4 (NOX4) is a constitutive enzyme specialized in controlling oxidative stress response [74]. Oxidative stress and induction of mitochondrial dysfunction is mediated through upregulation of NOX4 [75] as it serves as an oxygen sensor to generate ROS from molecular oxygen [76].

The present study showed that the expression of NOX4 was significantly ($p<0.05$) increased in type 2 diabetic rats, suggesting that NADPH oxidase might play a crucial role in the development of diabetic cardiovascular problems, while the expression of eNOS was decreased significantly ($p<0.05$) in aortas of type 2 diabetic rats which was consistent with the study of Li et al (2014) which showed that the expression of NOX4 was significantly increased, while the expression of eNOS was decreased significantly in aortas of type 2 diabetic rats [77]. Additionally, eNOS expression level was decreased in aorta and second-order mesenteric arteries in Goto-Kakizaki type 2 diabetic rats [78]. Moreover, it was reported that the expression NOX4 was significantly increased in the heart of diabetic mice and rats [79]

DMF treatment decreased the aortic mRNA expression of NOX4, increased eNOS which may result in the reduced oxidative stress and the improved vascular function in diabetic rats. A previous study has shown that eNOS mRNA expression was significantly improved by DMF in the left common carotid arteries in neointimal [80].
In conclusion, DMF may attenuate T2DM through activation of the main components of the insulin signalling pathway in liver mimicking insulin activity and up-regulation of eNOS expression and down-regulation of NOX4 expression. Additionally, the anti-oxidant activity of DMF via attenuation of AGEs and RAGE elevation, DMF may present a promising therapeutic compound in T2DM.

Conflict of interest statement: None declared.



	AUC_{OGTT} (mg/dl × 180min)	AUC_{ITT} (mg/dl × 180min)
Control	20595 ± 386.2	15445 ± 1042
Diabetic	42510* ± 3688	25317* ± 2589
DMF	22656$ ± 916.5	15647$ ± 1198
Control + DMF	25095$ ± 1726	17205$ ± 1455

Figure (1): Effect of DMF on OGTT and ITT of T2D rats. (A) A curve showing the changes in blood glucose during the OGTT, (B) A curve showing the changes in blood glucose during the ITT.
Data are expressed as mean ± SEM, n=6.
* P< 0.05 vs. Control group, $ p<0.05 vs. T2D group using one-way ANOVA followed by Tukey-Kramer multiple comparisons post hoc test.
DMF: dimethylfumarate; T2D: type 2 diabetic group; ANOVA: analysis of variance; OGTT: oral glucose tolerance test; ITT: insulin tolerance test.

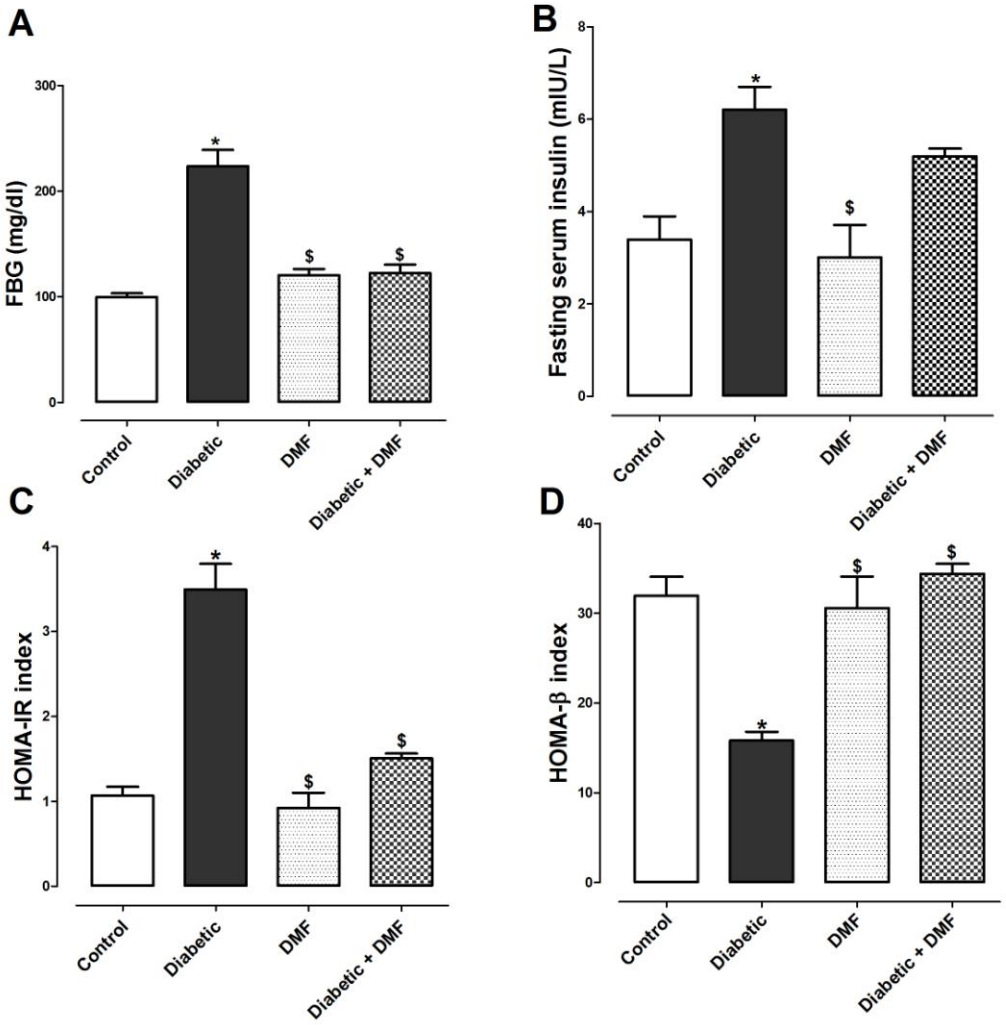


Figure (2): Effect of DMF on fasting blood glucose (A) and insulin levels (B), HOMA-IR (C) and HOMA-β (D) of T2D rats.
Data are expressed as mean ± SEM, n=6.
* *P*< 0.05 vs. Control group, $^{\$}$ *p*<0.05 vs. T2D group using one-way ANOVA followed by Tukey-Kramer multiple comparisons *post hoc test*.
DMF: dimethylfumarate; T2D: type 2 diabetic group; ANOVA: analysis of variance.
HOMA-IR index: homeostasis model assessment of insulin resistance index; HOMA-β index: homeostasis model assessment of β- cell function; FBG: fasting blood glucose.

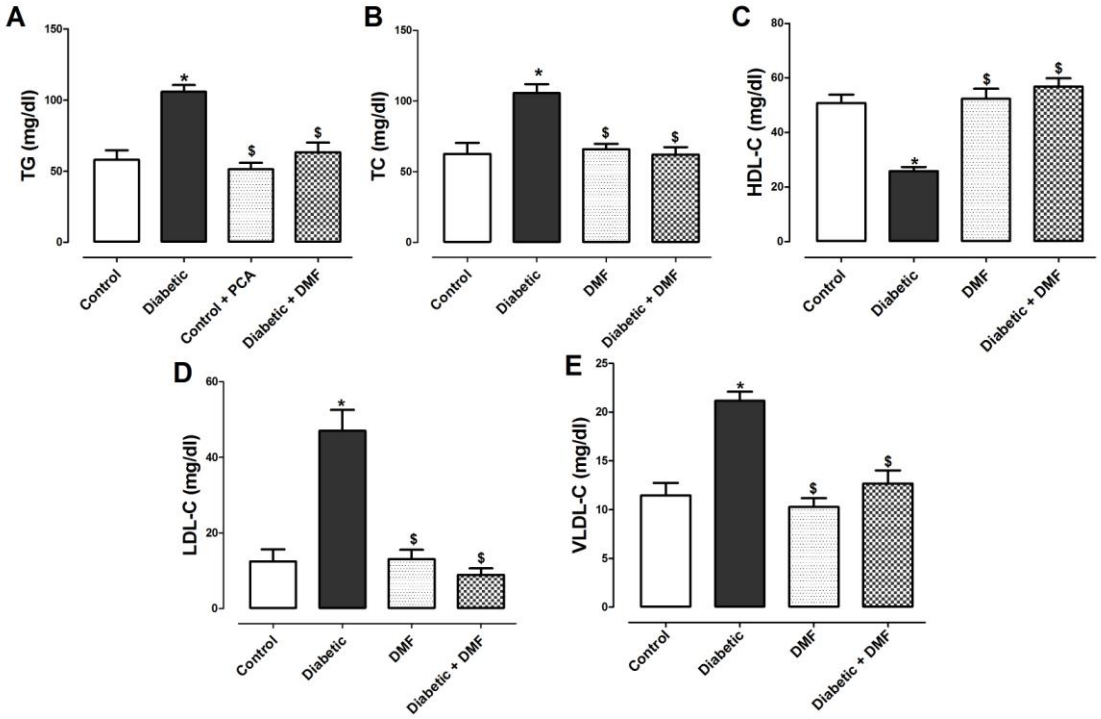


Figure (3): Effect of DMF on the lipid profile parameters of T2D rats. (A) TG, (B) Tc, (C) HDL-C, (D) LDL-C and (E) LDL-C.
Data are expressed as mean ± SEM, n=6.
* $P<$ 0.05 vs. Control group, $^{\$}$ $p<0.05$ vs. T2D group using one-way ANOVA followed by Tukey-Kramer multiple comparisons *post hoc test*.
DMF: dimethylfumarate; T2D: type 2 diabetic group; ANOVA: analysis of variance.
TC: total cholesterol; TG triglycerides; HDL-C: high density lipoprotein cholesterol; LDL-C low density lipoprotein cholesterol; VLDL-C: very low density lipoprotein cholesterol.

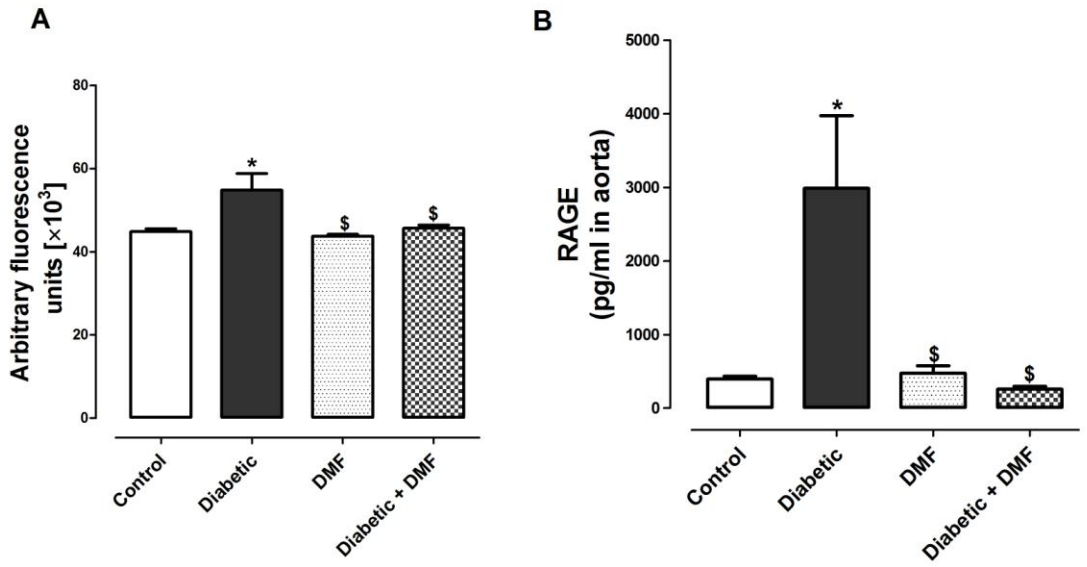


Figure (4): Effect of DMF on serum AGEs level (A) and aortic RAGE content (B) of T2D rats.
Data are expressed as mean ± SEM, n=6.
* $P<$ 0.05 vs. Control group, $^{\$}$ $p<0.05$ vs. T2D group using one-way ANOVA followed by Tukey-Kramer multiple comparisons *post hoc test*.
DMF: dimethylfumarate; T2D: type 2 diabetic group; ANOVA: analysis of variance;
AGEs: advanced glycation end products; RAGE: receptor for advanced glycation end products.

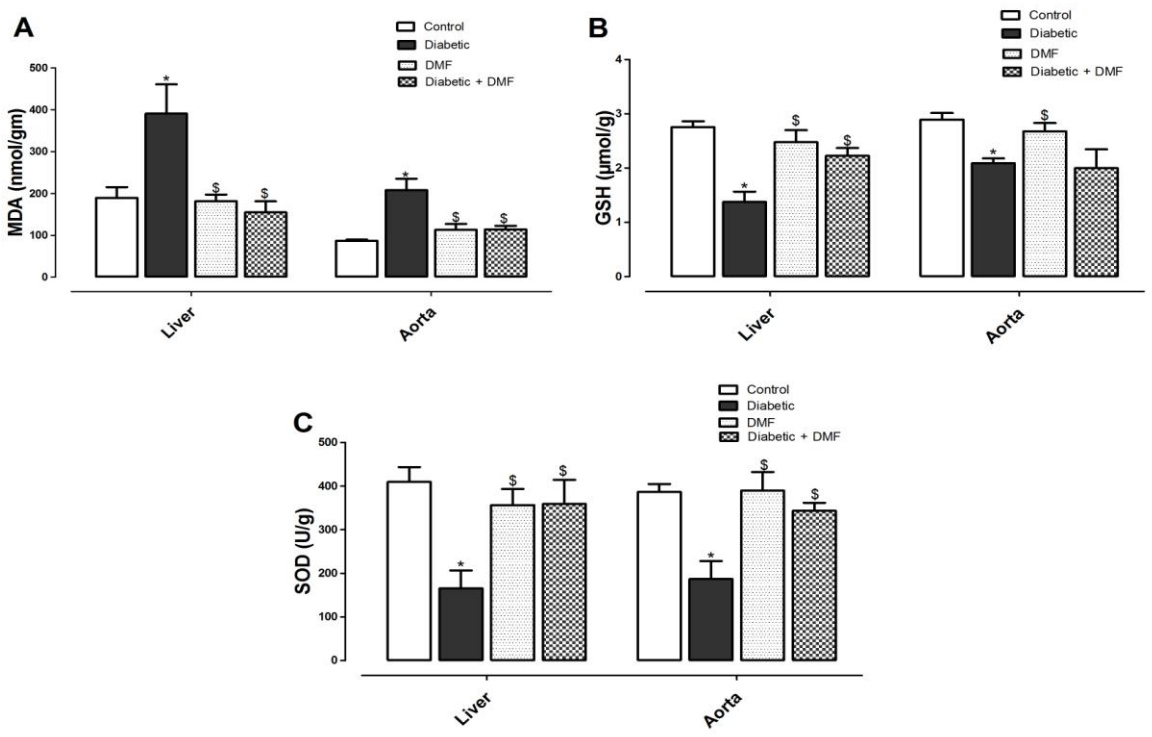


Figure (5): Effect of DMF on oxidative stress parameters in hepatic and aortic homogenates of T2D rats. (A) MDA, (B) GSH and (C) SOD.
Data are expressed as mean ± SEM, n=6.
* $P < 0.05$ vs. Control group, $^{\$}$ $p<0.05$ vs. T2D group using one-way ANOVA followed by Tukey-Kramer multiple comparisons *post hoc test*.
DMF: dimethylfumarate; T2D: type 2 diabetic group; ANOVA: analysis of variance;
MDA: malondialdehyde; GSH: reduced glutathione; SOD: superoxide dismutase**.**

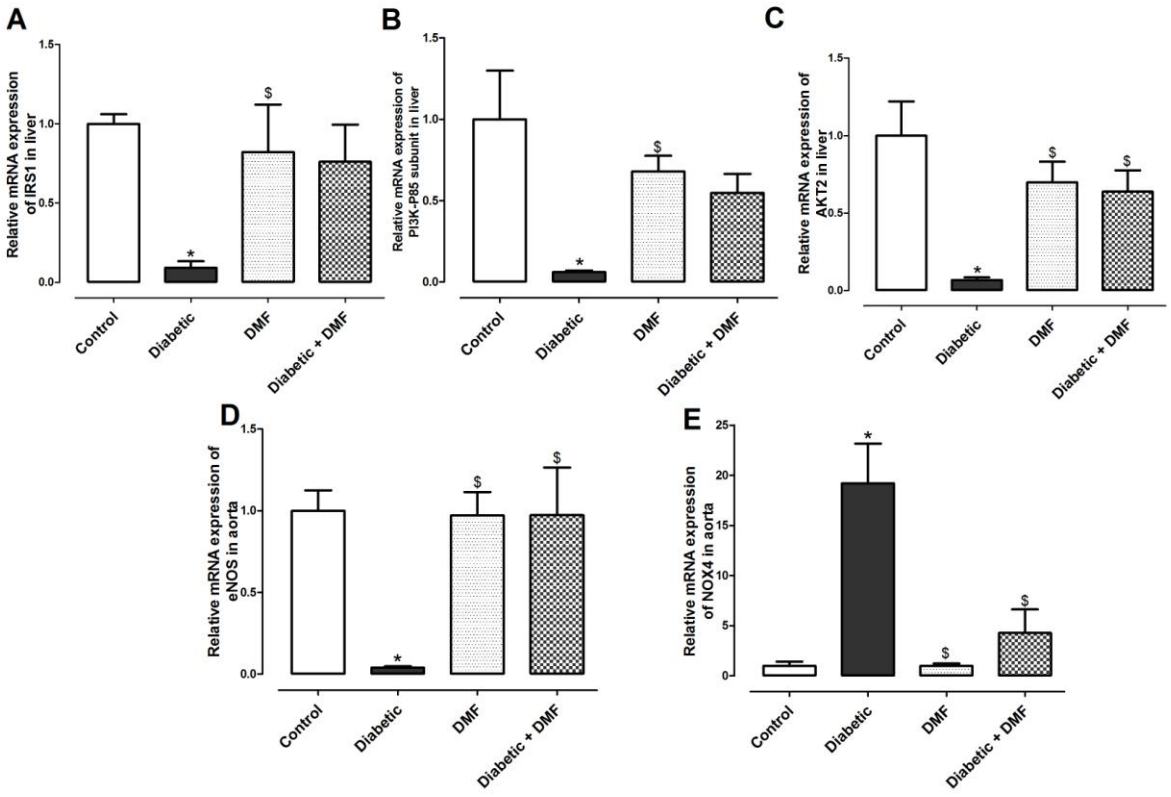


Figure (6): Effect of DMF on mRNA expression of hepatic IRS1 (A), PI3K- p85 subunit (B), AKT2 (C), aortic eNOS (D) and NOX4 (E) of T2D rats.
Data are expressed as mean ± SEM, n=6.; * $P< 0.05$ vs. Control group, $^{\$}$ $p<0.05$ vs. T2D group using one-way ANOVA followed by Tukey-Kramer multiple comparisons *post hoc test*.
DMF: dimethylfumarate; T2D: type 2 diabetic group; ANOVA: analysis of variance.
IRS1: insulin receptor substrate1; PI3K- p85 subunit: phosphatidylinositol 3-kinase regulatory subunit1; AKT2: AKT serine/threonine kinase 2; eNOS: nitric oxide synthase 3; NOX4: NADPH oxidase 4.

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