Saudi Pharmaceutical Journal 28 (2020) 683–691

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

Radix pseudostellariae of Danzhi Jiangtang capsule relieves oxidative stress of vascular endothelium in diabetic macroangiopathy

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ARTICLE INFO

Article history:
Received 6 December 2019
Accepted 17 April 2020
Available online 6 May 2020

Keywords:
Radix pseudostellariae
Danzhi Jiangtang capsule
Diabetes
Diabetic macroangiopathy
Oxidative stress

ABSTRACT

Aim: Medicinal plants act as an alternative source of anti-diabetic agents. Recently, Danzhi Jiangtang capsule (DJC) has been clinically used for treatment of diabetes, but the effect of DJC on diabetic macroangiopathy remained unclear. The present study investigates the therapeutic role of DJC in diabetic macroangiopathy and elucidates the underlying mechanisms.

Methods: Diabetes patients were treated with DJC for 20 weeks. Blood glucose and serum parameters (insulin, FFA, SOD, GSH-Px, MDA, NO) were determined before and after treatment. Streptozotocin -induced diabetic rat model and human HUVECs cells were applied to assess the anti-oxidative capacity of DJC and its bioactive constituents. The expression levels of eNOS, JNK, GRP78, CHOP, Bcl2, and BAX were measured by qPCR and/or immunoblotting.

Results: Diabetic macroangiopathy were ameliorated by DJC administration. Radix pseudostellariae (RP) mediated the anti-oxidative stress capacity of DJC, which improved insulin resistance ($p < 0.01$) and relieved oxidative stress ($p < 0.01$) of vascular endothelium through oxidative stress signaling and apoptosis pathway. The ability of DJC to ameliorate diabetic macroangiopathy and relieve oxidative stress was mainly mediated by its bioactive constituent RP.

Conclusion: This study would provide experimental evidence for DJC in the prevention and treatment of diabetes and diabetic macroangiopathy.

1. Introduction

The diabetes mellitus and its complications have become a major global health issue. The International Diabetes Federation reported that more than 415 million adults (20–79 years old) had diabetes mellitus globally, which will rise to 642 million by 2040 (IDF, 2015; Zimmet 2017). Diabetic macroangiopathy, one of the most common complications of diabetes mellitus, is the major cause of death (nearly 80%) in people with diabetes (Zheng et al. 2017; Zimmet 2017), which is characterized by excessive or abnormal neovasculogenesis induced intra-plaque new vessel formation, increased capillary vessels permeability, and tissue edema (Andresen et al. 1996; Brownlee et al. 1988; Zheng et al. 2017). Diabetic macroangiopathy results in frequent atherosclerotic plaque hemorrhage and plaque rupture, as well as in cardiac microvascular dysfunction (Andresen et al. 1996; Madonna et al. 2018). Hence, there is crucial importance to develop the effective therapeutic treatments of diabetic macroangiopathy.

The cumulative experimental evidences demonstrate a close link between oxidative stress and diabetes through monitoring oxidative stress biomarkers in both diabetic patients and animal models (Aragno et al. 2005; Ramesh et al. 2007; Sekhar et al. 2011). Insulin resistance (IR) and elevated free fatty acids (FFA) result in lots of alterations at the cellular level that cause vascular dysfunction and boost the atherosclerotic process (Funk et al. 2012), such as the increased oxidative stress, hyperglycemia, deactivated antioxidant enzymes (SOD, GSH-Px, etc.), reduced bioavailability of nitric oxide (NO), imbalance of cellular signal

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jsps.2020.04.009
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transduction, unregulated apoptotic pathway, and overexpression of the prothrombotic factors (Funk et al. 2012; Paneni et al. 2013).

Numerous experimental, clinical, and epidemiology studies have highlighted the protective role of antioxidants in the therapy of diabetes and its complications. Kunisaki et al. reported that vitamin E, a classical antioxidant, improved retinal blood flow and protein kinase C activity in the vascular tissue of diabetic rats (Kunisaki et al. 1995). Obrosova et al. demonstrated that α-lipoic acid increased serum erythrocyte glutathione peroxidase (GSH-Px), prevented changes in superoxide dismutase and quinone reductase activities in liver, retina, and plasma of streptozotocin (STZ) induced diabetic Wistar rats (Obrosova et al. 2000). A clinical trial conducted by Reaven et al., found that 10-week of RRR-α-tocopherol administration (1600 IU) reduced approximately 60% plasma Low-density lipoprotein (LDL) oxidation in diabetes patients (Reaven et al. 1995).

Medicinal plants act as the fundamental source of potent anti-diabetic drugs, because they contain various phytoconstituents such as flavonoids, terpenoids, saponins, carotenoids, alkaloids, and glycosides (Salehi et al. 2019). Durazzo and colleagues studied and reviewed the anti-diabetes role of okra, a flowering plant in the mallow family, through inhibiting the liver X receptors (LXRs) and peroxisome proliferator-activated receptors (PPARs) pathway in both liver and adipose tissues (Durazzo et al., 201)). Danzhi Jiangtang Capsule (DJC), a traditional Chinese medicine, has been used to treat diabetes in clinic for about 10 years (Lu et al. 2018b; Zheng et al. 2016). Recently, Lu et al. reported that DJC had the protective effects on high-fat diet or palmitic acid induced vascular endothelial damages in diabetic rats (Lu et al. 2018b). However, the regulatory role of DJC in oxidative response and its effect on diabetic macroangiopathy have not been investigated. The aim of present study is to investigate the therapeutic role of DJC in diabetic macroangiopathy and elucidate the underlying mechanisms. We demonstrated the therapeutic role of DJC in diabetic macroangiopathy, and revealed that radix pseudostellariae (RP), a bioactive constituent of DJC, plays important protective role against the oxidative stress of vascular endothelium. In comparison with conventional treatment (Gliclazide and Acarbose), DJC combined administration had more robust ability on improving insulin resistance index, activating antioxidant enzymes, lowering FFA and malondialdehyde (MDA) in diabetic patients and rats. Mechanistically, DJC and RP are able to relieve oxidative stress and inhibit cellular apoptosis in vascular endothelium of thoracic aortas. These results indicate a promising therapeutic role of DJC in the treatment of oxidative stress induced diabetic macroangiopathy.

2. Methods

2.1. Subjects

A total of 60 type 2 diabetes patients (according to World Health Organization criteria) were recruited by the First Hospital Affiliated to Anhui University of Chinese Medicine. The patients were divided into two groups randomly, conventional treatment (Con, n = 30) and DJC combined with conventional treatment (Con + DJC, n = 30). Within the 20-week treatment, the Con patients were treated with Gliclazide (Diamicron 80 mg tablets, Laboratoires Servier, Suresnes, France) one tablet twice a day, and Acarbose (Precose 50 mg tablets, Bayer, Leverkusen, Germany) one pill a time thrice daily; the Con + DJC patients were additionally treated with DJC 5 pills a time thrice daily. The blood glucose and serum parameters were measured before and after treatment. Informed consent was derived from each patient. The conventional treatment, conventional plus DJC treatment, and data analysis was shown in the graphical scheme (Fig. 1). All studies were approved by the Ethics Commitment of the First Hospital Affiliated to Anhui University of Chinese Medicine.

2.2. Animal studies

The 8-week old Sprague-Dawley male rats were used in this study. The animals were bred and maintained following the standard rearing conditions of 12 h light and 12 h dark. All animals' studies were performed following the guideline established by the First Hospital Affiliated to Anhui University of Chinese Medicine Institutional Animal Care and Use Committee. The diabetic rat model was induced by streptozotocin (STZ, Sigma, St. Louis, MO, USA) which was administrated to rats through intraperitoneal (i.p.) injection at 50 mg/kg body weight for five days. The diabetes animals were treated by oral gavage as below: diabetes model (DM) group was administrated saline; the DM + DJC group; the DM + Radix pseudostellariae group was administrated radix pseudostellariae (RP); the DM + Rehmannia group was administrated rehmannia; the DM + Coxtex moutan group was administrated coxtex moutan; the DM + Rhizoma alismatis group was administrated rhizoma alismatis; the DM + Cuscuta group was administrated cuscuta; the DM + Leeches group was administrated leeches; Normal group, without STZ injected rats and administrated saline by oral gavage. The design of animal study and drug treatment was shown in the graphical scheme (Fig. 1). The blood glucose and other parameters were measured after the treatment. In order to minimalize the influence of circadian rhythm on the drug administration in this study, all feeding and blood glucose measurements were carried out between 5p.m. and 6p.m. concerning the habits of rats.

2.3. Human umbilical vein endothelial cells (HUVECs) culture and treatment

HUVECs cells were obtained from American Tissue Culture Collection (ATCC, CRL-1730™, Manassas, VA, USA). The cells were cultured in 45% Minimum Essential Medium (MEM, Thermo Fisher Scientific, Waltham, MA, USA), 45% Ham's F-10 Nutrient Mixture Media (Thermo Fisher Scientific), 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U/mL peni-cillin and 100 lg/mL streptomycin, and maintained in humidified-air atmosphere incubator containing 95% air/5% CO_2 at 37℃. The cells were treated with 0.5 mM palmitic acid (PA, Cayman Chemical, Ann Abor, MI, USA), 0.5 mM PA + DJC, 0.5 mM PA + RP; 25 μM hydrogen peroxide (H_2O_2, Sigma), 25 μM H_2O_2 + DJC, 25 μM H_2O_2 + RP; tunicamycin (TM, Sigma), TM + DJC, TM + RP. The treatment and analysis of HUVECs was shown in the graphical scheme (Fig. 1). The cells were collected and lysed for immunoblot analysis 24 h after the treatment.

2.4. Blood glucose assay

The blood glucose (BG) of type 2 diabetes patients and animals were measured using the BG meter with test strips (Bayer, Leverkusen, Germany), as described in previous study (Freckmann et al. 2012). Both fasting BG (FBG) and postprandial BG (PBG) were monitored in this study.

2.5. Serum parameters analyses

Patient and rat serum insulin concentration was measured using Human Insulin ELISA Kit (ab200011, Abcam, Cambridge, UK) and Rat/Mouse Insulin ELISA Kit (EZRMI-13 K, MilliporeSigma, Burlington, USA), respectively. Serum concentration of non-esterified fatty acids (NEFA, or free fatty acids) was measured using

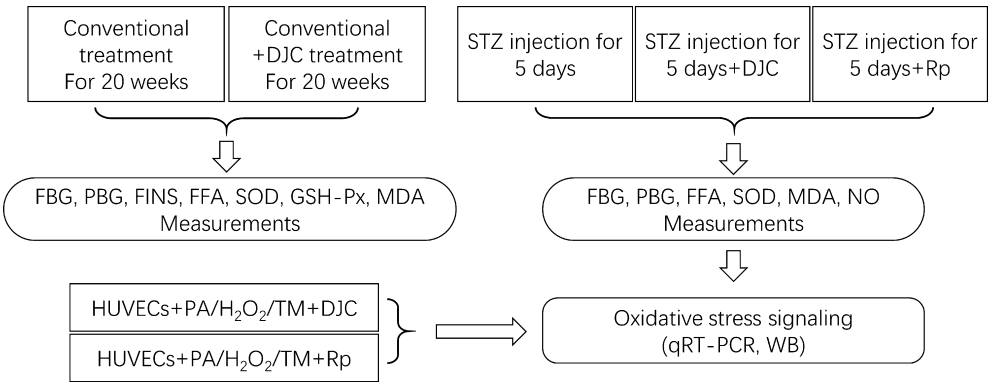

Fig. 1. A graphical scheme of study design.

Free Fatty Acid Quantitation Kit (MAK044, Sigma, St. Louis, USA). The concentrations of serum superoxide dismutases (SOD) and glutathione peroxidase (GSH-Px) were measured by using Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Abor, USA) and Glutathione Peroxidase Activity Colorimetric Assay Kit (BioVision Inc., Milpitas, USA), respectively. The serum malondialdehyde (MDA) and nitric oxide (NO) levels were tested using the malondialdehyde (MDA) Colorimetric Assay Kit (Elabscience, Wuhan, China) and Nitric Oxide (NO) Colorimetric Assay Kit (Elabscience, Wuhan, China) flowing the kit instructions.

2.6. Cytotoxicity analyses

The cytotoxicity of DJC and RP was evaluated in HUVECs cells as described previously (Lu et al. 2018a). In order to determine the mutagenic potential of DJC and RP, bacterial reverse mutation assay was performed in Salmonella TA100 and 102 strains by using the Salmonella Mutagenicity Complete Test Kit (Trinova Biochem, Giessen, Germany) flowing the manufacturer's instruction.

2.7. Immunoblotting analyses

Frozen thoracic aortas of DM rats were homogenized and were lysed using the radioimmunoprecipitation (RIPA) buffer (#89901, Thermo Fisher Scientific). The treated HUVECs cells were lysed using the RIPA buffer. Samples were subjected to immunoblotting analysis as described previously (Guo et al. 2017). The p-eNOS (pSer1176) (SAB4504393, 1:1000 dilution), and eNOS (SAB4502013, 1:2000 dilution) primary antibodies were purchased from Sigma; GRP78 (#ab21685, 1:1000 dilution), CHOP (#ab11419, 1:1000 dilution), BAX (#ab32503, 1:1000 dilution), and Bcl-2 (#ab185002, 1:2000 dilution) primary antibodies were purchased from Abcam; p-JNK (Thr183/Tyr185) (#4668, 1:1000 dilution) and JNK (#9252, 1:1000 dilution) primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The internal control β-actin antibody (#ab8226, 1:2000 dilution) were ordered from Abcam. The protein level quantification was analyzed using the ImageJ.

2.8. Real-time quantitative RT-PCR

The total RNA was isolated and extracted from frozen thoracic aortas of DM rats using the Invitrogen TRIzol (Thermo Fisher Scientific). One μg total RNA was converted into cDNA using the M−MLV (Thermo Fisher Scientific) and random hexamers. The qRT-PCR was performed in CFX Real-Time PCR Detection Systems (Bio-Rad, Hercules, CA, USA) using SYBR™ Green PCR Master Mix (Thermo Fisher Scientific). The expression of tested gene was normalized by *GAPDH*, and fold change was calculated by using the $2^{-\Delta\Delta CT}$ method. Primers for the RT-qPCR were listed below: GRP78, F: GAA CGT CTG ATT GGC GAT GC, R: GAG TCG AGC CAC CAA CAA GA; CHOP, F: TCC AAC TGC AGA GAT GGC AG, R: TCC TCC TCT TCC TCC TGA GC; XBP1, F: CTG AGT CCG CAG CAG GTG, R: TCT GCT ATC CTC CAG GCA GT; ATF6, F: CAG CAG GAA CTC AGG GAG TG, R: AAT GTG TCT CCC CTT CTG CG; eNOS, F: GAC CCA CTG GTG TCC TCT TG, R: CTC CGT TTG GGG CTG AAG AT; JNK, F: GCC CGC GTC TCT GTT ACT C, R: TCT CCC ATG ATG CAC CCA AC; GAPDH, F: GAA AGC CTG CCG GTG ACT AA, R: TTC CCG TTC TCA GCC TTG AC.

2.9. Statistical analysis

Statistical analyses were carried out by using the GraphPad Prism (https://www.graphpad.com/). The differences between groups were analyzed using one-way analysis of variance (ANOVA) with a Tukey's post hoc test or Student's *t* test. All data represented mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control or DM group, $^{\#\#\#}P < 0.001$ compared to normal group.

3. Results

3.1. DJC combined with conventional treatment reduced insulin resistance and oxidative stress in type 2 diabetes patients

In order to investigate the protective effects of DJC on type 2 diabetes, we recruited 60 type 2 diabetes patients (according to World Health Organization criteria) and divided them into conventional group (Con, n = 30; with Gliclazide and Acarbose oral administration) and DJC combined with conventional group (Con + DJC, n = 30; with additional DJC oral administration), with indicated treatments for 20 weeks (Fig. 1). The BG, insulin, and other serum parameters were monitored before and after the treatment to assess the effects of DJC administration. Although both Con and Con + DJC treatment could significantly lower the levels of fasting blood glucose (Fig. 2a), postprandial blood glucose (Fig. 2b), and fasting insulin (Fig. 2c) after 20-week administration, there was no difference between the two groups, which indicated that Con + DJC treatment can't further decrease the glucose and insulin levels of blood in term of the improved effects of Con treatment. However, the insulin resistance index was dramatically improved in Con + DJC treated patients compared to Con treatment ($p < 0.001$) (Fig. 2d). Moreover, the serum concentrations of two important antioxidant enzymes SOD and GSH-Px were significantly increased in Con + DJC treated patients ($p < 0.001$), but there was no change in conventional group (Fig. 2e-f). The serum MDA, a

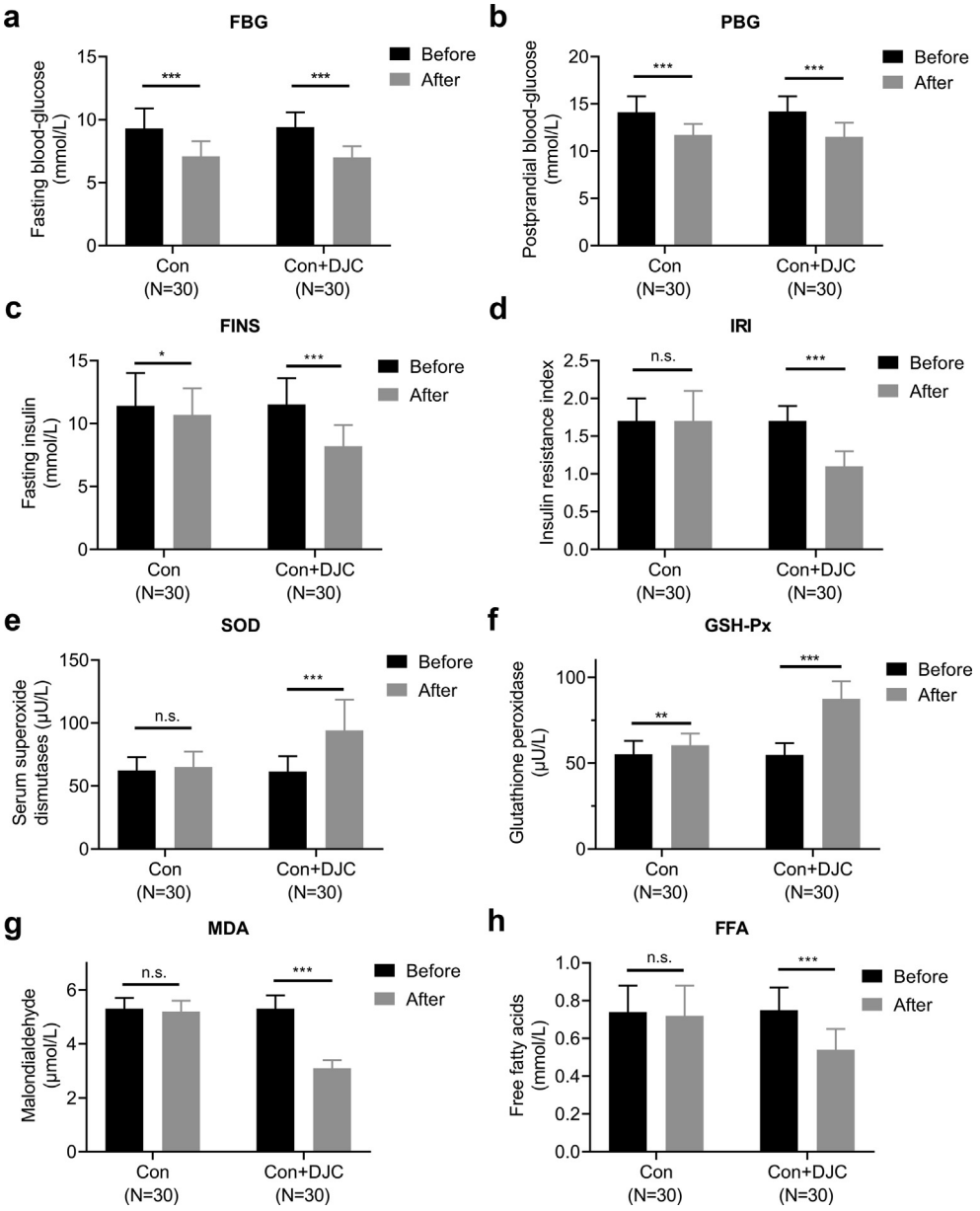

Fig. 2. DJC combined with conventional treatment reduced insulin resistance and oxidative stress in type 2 diabetes patients. (a) Fasting blood-glucose (FBG), (b) postprandial blood-glucose (PBG), (c) fasting insulin (FINS), (d) insulin resistance index (IRI), (e) superoxide dismutases (SOD), (f) glutathione peroxidase (GSH-Px), (g) malondialdehyde (MDA), (h) free fatty acids (FFA) in diabetes patients were determined before and after conventional treatment (Con, n = 30) and DJC combined treatment (Con + DJC, n = 30). The data represent the mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

lipid peroxidation marker, was correspondingly decreased in Con + DJC group compared to Con treatment ($p < 0.001$) (Fig. 2g). Furthermore, we also found the Con + DJC treatment could significantly reduce the serum level of free fatty acids (FFA), which are elevated in obese individuals and associated with decreased glucose oxidation and increased insulin resistance, in diabetes patients administrated with Con + DJC ($p < 0.001$), however, the conventional treatment had no effect on it (Fig. 2h). All these clinical data suggested that DJC combined with conventional treatment reduced insulin resistance and oxidative stress in type 2 diabetes patients.

3.2. DJC suppressed insulin resistance and oxidative stress in DM rats

To uncover the mechanism of DJC mediated improvements of insulin resistance and oxidative response, we made the STZ induced diabetic rat model (DM) and treated them with vehicle, DJC, and RP (Fig. 3). Both cytotoxicity and bacterial reverse mutation assay were carried out to evaluate the cytotoxicity and genotoxicity of DJC and RP. The 50% cell survival results showed that both are safe for the following in vitro and in vivo experiments (Fig. 3a and b). As shown in Fig. 4a and 4b, the STZ induced DM showed higher BG and lower fasting insulin level compared to normal group. Notably, DJC gavage could significantly decreased the BG level and increased fasting insulin level in DM rats ($p < 0.001$) (Fig. 4a-b). We further measured the serum parameters in the control and DJC treated DM rats, in consistent with the type 2 diabetes patients' results, DJC administration dramatically reduced serum FFA and MDA levels ($p < 0.001$) (Fig. 4c and 4e), and increased the serum SOD and nitric oxide (NO) levels ($p < 0.001$) (Fig. 4d and 4f). The above data indicated that DJC treatment suppressed insulin resistance and oxidative stress in DM rats.

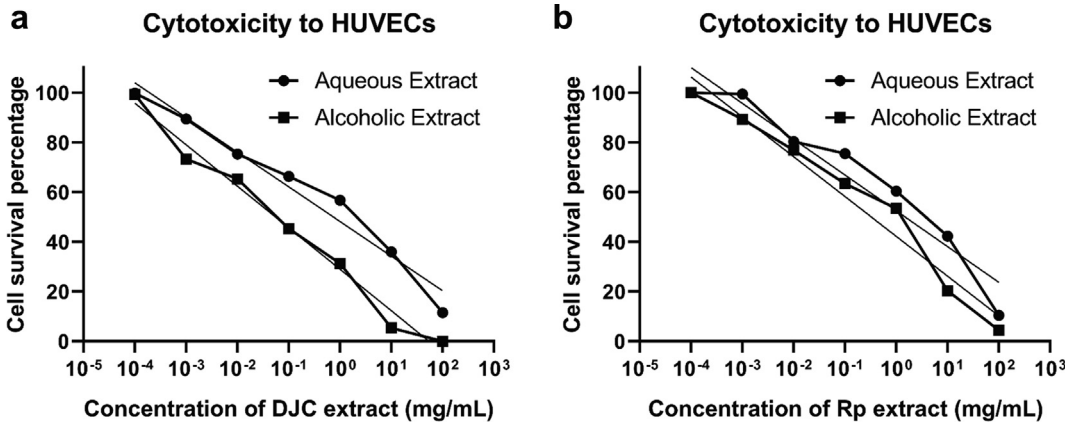

Fig. 3. Cytotoxicity of Danzhi Jiangtang capsule and Radix pseudostellariae. (a) Cytotoxicity of Danzhi Jiangtang capsule to HUVECs. Cell survival of fifty percent was exhibited by 0.70 and 0.05 mg/mL of aqueous and ethanolic extracts respectively. (b) Cytotoxicity of Radix pseudostellariae to HUVECs. Cell survival of fifty percent was exhibited by 1.40 and 0.30 mg/mL of aqueous and ethanolic extracts respectively.

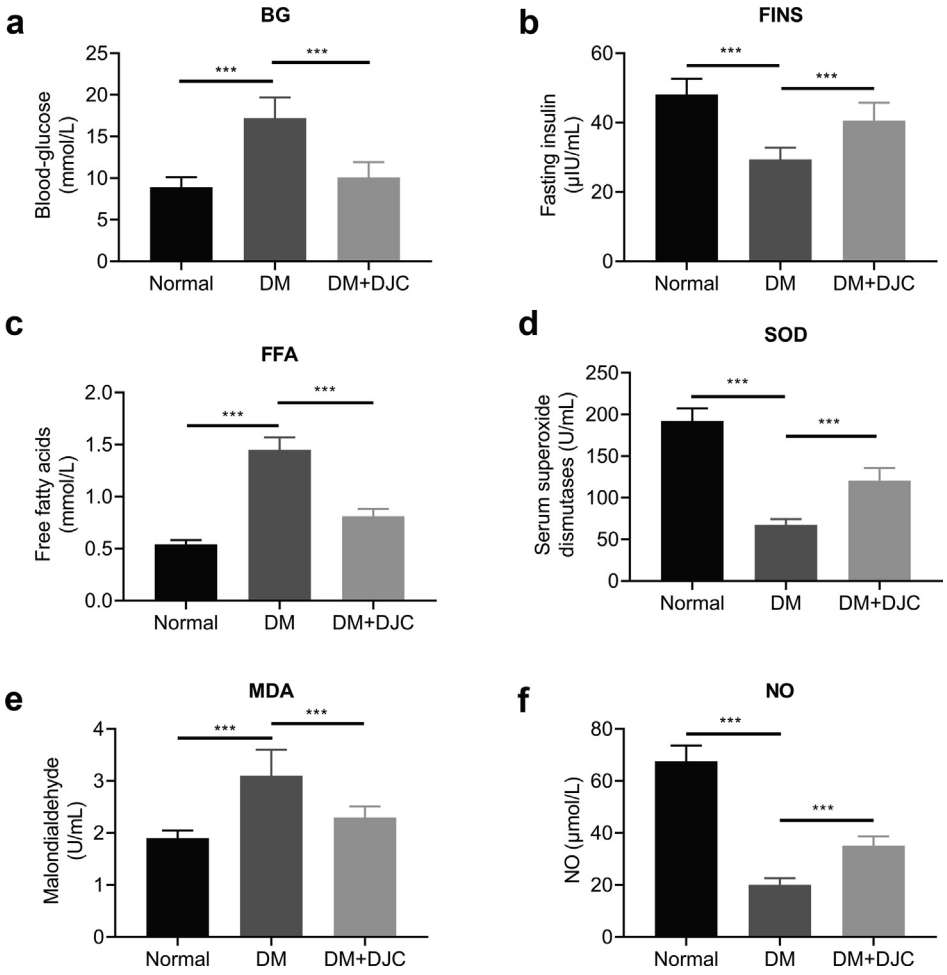

Fig. 4. DJC reduced insulin resistance and oxidative stress in type 2 diabetes rats. (a) Fasting blood-glucose (BG), (b) fasting insulin (FINS), (c) free fatty acides (FFA), (d) superoxide dismutases (SOD), (e) malondialdehyde (MDA), and (f) NO levels were measured in normal rats and diabetes rats with or without DJC treatment. The data represent the mean ± SD. ***$p < 0.001$.

3.3. RP decreased insulin resistance and oxidative stress in DM rats

DJC is composed of multiple bioactive constituents including RP, rehmannia, coxtex moutan, rhizoma alismatis, cuscuta, and leeches. Next, we tried to figure out which is the major protective factor of DJC against insulin resistance and oxidative stress in DM rats. We found that all the above bioactive constituents could lower BG in DM rats compared to saline control group (Fig. 5a). RP, cuscuta, and leeches administration significantly improved insulin resistance (Fig. 5b). The RP, rehmannia, and rhizoma alismatis treatment decreased serum FFA levels (Fig. 5c). For the oxidative stress associated parameters, in comparison with DM

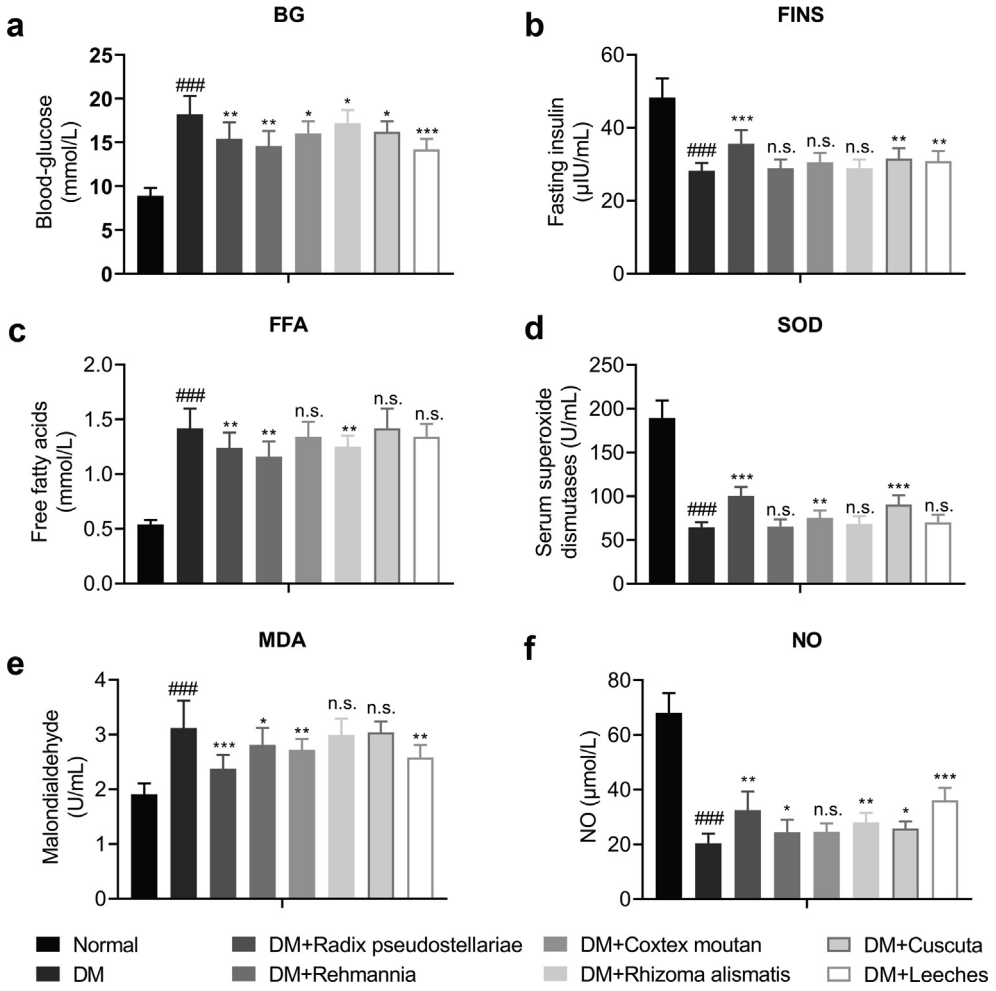

Fig. 5. RP ameliorated insulin resistance and oxidative stress in type 2 diabetes rats. (a) Fasting blood-glucose (BG), (b) fasting insulin (FINS), (c) free fatty acides (FFA), (d) superoxide dismutases (SOD), (e) malondialdehyde (MDA), and (f) NO levels were measured in normal rats and diabetes rats with or without DJC treatment. The data represent the mean ± SD. $^{###}p < 0.001$, *vs* Normal; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *vs* DM.

group, RP, coxtex moutan, and cuscuta treatment increased the concentration in serum (Fig. 5d); RP, rehmannia, coxtex moutan, and leeches reduced serum MDA levels (Fig. 5e); RP, rehmannia, rhizoma alismatis, cuscuta, and leeches increased serum NO levels (Fig. 5f). Overall, the RP was the major protective bioactive constituent of DJC against insulin resistance and oxidative stress in DM rats.

3.4. RP relieved oxidative stress of the thoracic aortas of DM rats

To investigate the molecular mechanism of RP induced antioxidative effects, we dissected the thoracic aortas of DM rats for transcriptional and translational analysis. The mRNA level of ER chaperone 78-kD glucose-regulated protein (GRP78), a major regulator of ER homeostasis (Flodby et al. 2016), and C/EBP homologous protein (CHOP), a regulator of ER stress mediated apoptosis pathway (Li et al. 2014), was elevated in the thoracic aortas of diabetic rats (DM, Fig. 6a). Notably, after DJC or RP administration, the expression of both GRP78 and CHOP was significantly decreased in the thoracic aortas compared to DM group ($p < 0.001$) (Fig. 6a). Although the mRNA level of XBP1, ATF6, eNOS, and JNK was no change, the phosphorylated eNOS (p-eNOS) was significantly increased and phosphorylated JNK (p-JNK) was decreased after DJC or RP treatment (Fig. 6b and 6c). Moreover, the protein level of Bcl-2, an anti-apoptotic protein, was dramatically elevated, and BAX, a pro-apoptotic protein, was significantly decreased after DJC or RP administration (Fig. 6d and 6e). Similarly, the protein level of GRP78 and CHOP was significantly decreased in the thoracic aortas compared to DM group (Fig. 6d and 6e). The above results suggested that JC and RP relieved oxidative stress and inhibited apoptosis in thoracic aortas of diabetic rats.

3.5. DJC and RP protected endothelial cells from oxidative injury through oxidative stress signaling and apoptosis pathway

HUVECs is an ideal endothelial model to study oxidative stress induced injury and assess the anti-oxidative effect of compound. Here we further investigated the RP induced anti-oxidative and anti-apoptosis effects using three oxidative stress induced HUVECs injury models. First, we performed PA treatment, which induces ROS accumulation and results in cardiomyocyte endothelial inflammation and apoptosis (Carta et al. 2017). As shown in Fig. 7a and 7b, co-administration PA with DJC or RP could significantly increase p-eNOS and decrease GRP78 and CHOP protein levels compared to PA treated cells. Next, we examined the co-administration DJC/RP with H_2O_2 or TM, both known inducers of oxidative and ER stress. Similarly, DJC or RP administration restored the p-eNOS protein of H_2O_2 exposure (Fig. 7c and 7d). DJC or RP administration also suppressed the TM induced GRP78 and CHOP protein levels (Fig. 7e and 7f). Taken together, these

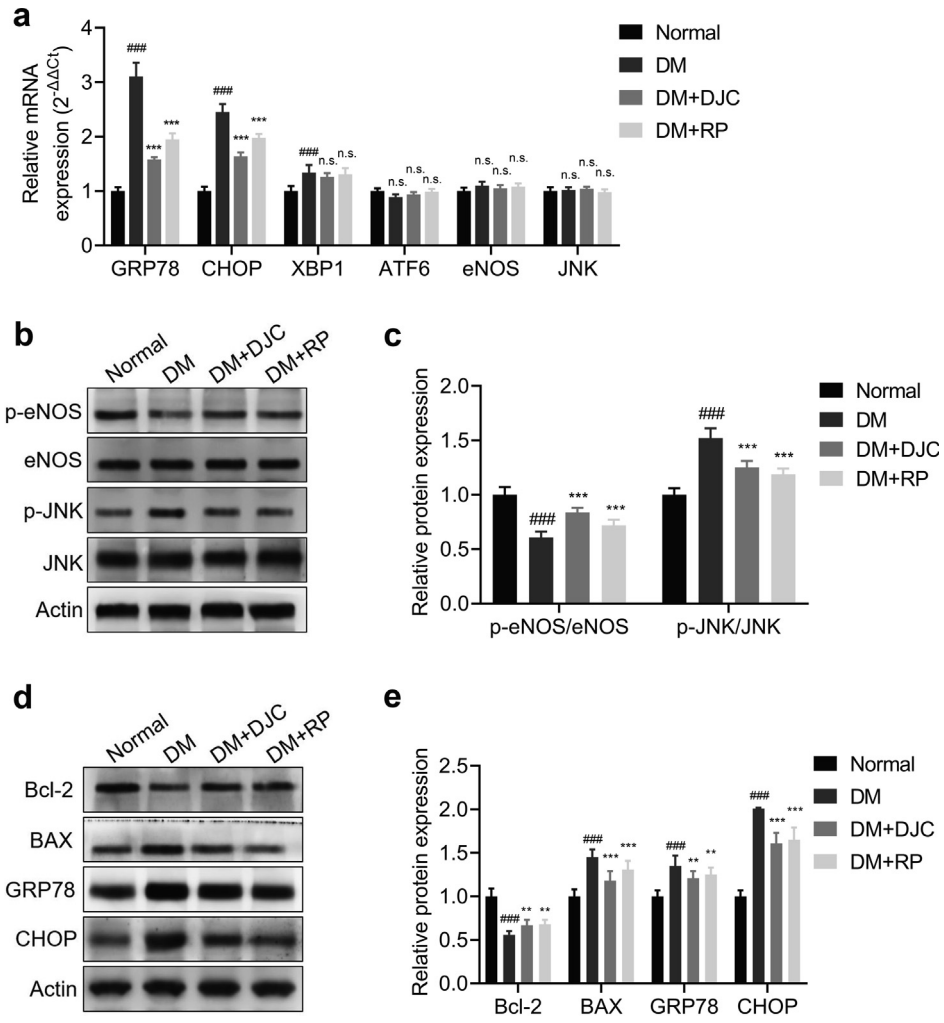

Fig. 6. Effects of RP in oxidative stress signaling in thoracic aortas of DM rats. (a) qRT-PCR analysis of indicated genes in thoracic aortas of normal, DM, DM + DJC, and DM + RP rats. (b) Western blots analysis of p-eNOS, eNOS, p-JNK, JNK expression of normal, DM, DM + DJC, and DM + RP rats. (c) Densitometric analysis of the percentage of p-eNOS to eNOS, p-JNK to JNK ratio in b. (d) Western blots analysis of Bcl-2, BAX, GRP78, and CHOP expression of normal, DM, DM + DJC, and DM + RP rats. (e) Densitometric analysis of the percentage of Bcl-2, BAX, GRP78, and CHOP in d. The data represent the mean ± SD. $^{###}p < 0.001$ *vs* Normal; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* DM.

results indicated DJC and RP relieved endothelial cell injury through inhibiting oxidative stress signaling and apoptosis pathway.

4. Discussion

As the prevalence of diabetes has risen to epidemic proportions globally, diabetic vascular complications have become one of the most challenging health issues. Diabetic macroangiopathy, a specific form of accelerated atherosclerosis, is closely associated with hyperglycemia, hyperosmolar stress, insulin resistance, and oxidative stress (Domingueti et al. 2016; Madonna et al. 2018; Zheng et al. 2017). The antioxidants have been highlighted in treating diabetes, and medicinal plants act as the fundamental source of potent anti-diabetic drugs (Durazzo et al., 2018; Kunisaki et al. 1995; Manzella et al. 2001; Matough et al. 2012; Obrosova et al. 2000; R O, L N, 2014; Reaven et al. 1995; Salehi et al. 2019; Schernthaner et al. 2004; Sekhar et al. 2011; Tavender and Bulleid 2010). As a traditional Chinese medicine, DJC showed protective effects on palmitic acids induced vascular endothelial damages (Lu et al. 2018b), and it has been used to treat diabetes accompanied with vascular complication in clinic (Zheng et al. 2016). Here, we investigated the therapeutic role of DJC in diabetic macroangiopathy, and revealed that RP, a bioactive constituent of DJC, play important role of protector against the oxidative stress of vascular endothelium. (See a graphical scheme of study design in Fig. 1) We found that DJC/RP administration had more robust ability on improving insulin resistance index, activating antioxidant enzymes, lowering FFA and MDA in diabetic patients and rats. Mechanistically, DJC and RP are able to relieve oxidative stress and inhibit cellular apoptosis in vascular endothelium of thoracic aortas. It's worth noting that there are multiple bioactive constituents in DJC (Lu et al. 2018b; Sun et al. 2019; Zheng et al. 2016), our study indicates that RP instead of other constituents paly the major anti-oxidative role in diabetic rat model.

Hyperglycemia and oxidative stress, as well as the deregulated apoptotic pathways have been recognized as key events in diabetic vascular complications (Funk et al. 2012; Paneni et al. 2013). DJC or RP administration could lower blood glucose, improve insulin resistance index, more importantly, increase the concentrations of antioxidant enzymes (such as SOD and GSH-Px) and NO, and decrease the MDA and FFA in diabetic patients and rats. Here, the DJC works like an antioxidant on releasing oxidative stress in diabetic patients and rodents. For instance, intraperitoneal administration of α-lipoic acid (an organic compound that acts as a powerful antioxidant) in STZ induced diabetic rats, decreased

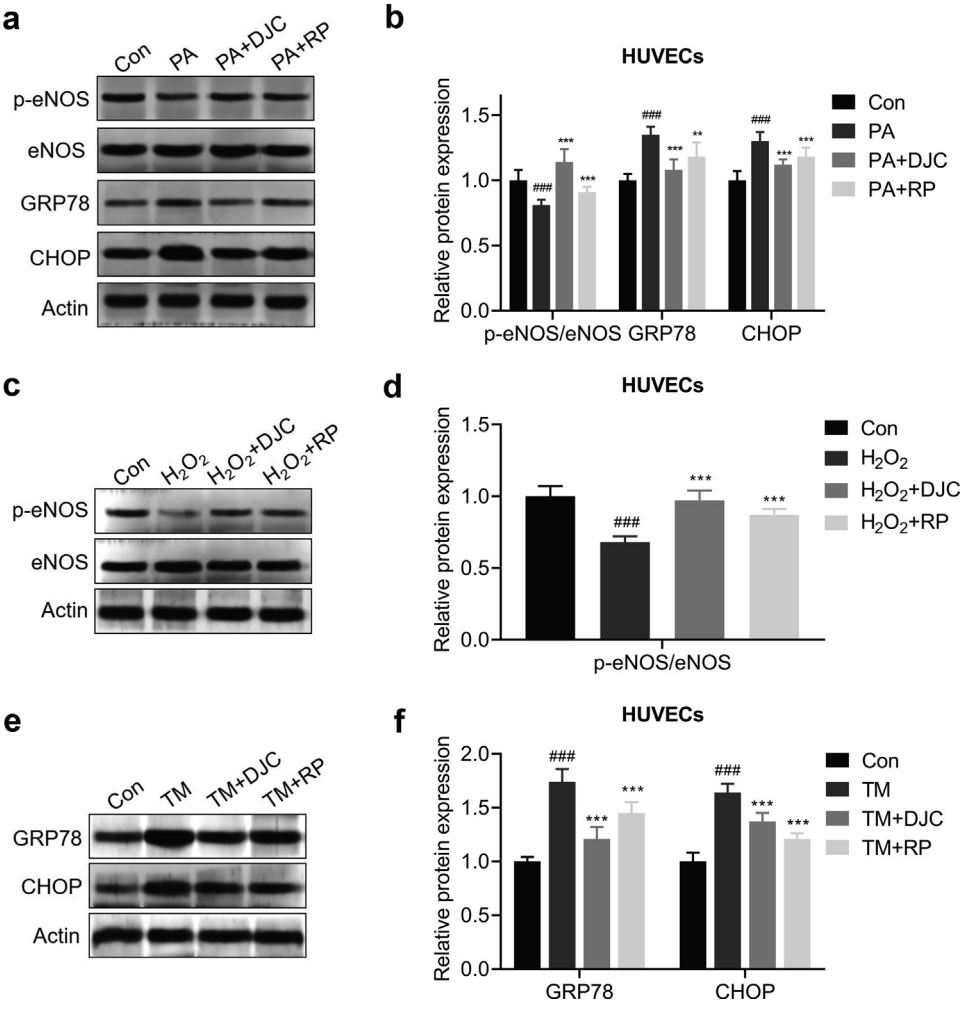

Fig. 7. DJC and RP protected human HUVECs cells from oxidative injury. (a) Western blots analysis of p-eNOS, eNOS, GRP78, and CHOP expression in HUVECs with palmitic acid (PA), PA + DJC, and PA + RP treatments. (b) Densitometric analysis of the percentage of p-eNOS to eNOS ratio, GRP78, and CHOP in a. (c) Western blots analysis of p-eNOS, and eNOS expression in HUVECs with H_2O_2, H_2O_2 + DJC, and H_2O_2 + RP treatment. (d) Densitometric analysis of the percentage of p-eNOS to eNOS ratio in c. (e) Western blots analysis of GRP78 and CHOP expression in HUVECs with tunicamycin (TM), TM + DJC, and TM + RP treatment. (f) Densitometric analysis of the percentage of GRP78 and CHOP in e. The data represent the mean ± SD. $^{###}p < 0.001$ *vs* Normal; $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* DM.

the severity of diabetic neuropathy through maintaining GSH levels (Obrosova et al. 2000). Hong et al. demonstrated that vitamin E, a classical antioxidant, reduced the accumulation of superoxide radicals, decreased the generation of oxidative damaging substances, and maintained the membrane fluidity in the diabetic rats (Hong et al. 2004).

In addition, the oxidative stress signaling pathway and ER stress associated apoptosis pathway have been implicated in the onset and progression of congestive heart failure and diabetic cardiomyopathy (Wold et al. 2005). Apoptotic cell death associated with elevated oxidative stress in many organ systems of diabetic human and rodents (Kajstura et al. 2001; Srinivasan et al. 2000; Wold et al. 2005). In this study, we found that the activation of stress-activated kinases (p-JNK), regulators (GRP78, and CHOP), pro-apoptotic protein (BAX), on the contrary, inhibition of endothelial nitric oxide synthase (p-eNOS) and anti-apoptotic protein (Bcl2) in thoracic aortas of diabetic rats. Notably, DJC treatment specifically activate p-eNOS and Bcl2, and suppress p-JNK, BAX, GRP78, and CHOP in diabetic rats. Consistent with our finding, Wu et al. reported that DJC attenuated the toxicity of high glucose load in pancreatic β cells through GLP-1/Akt signaling pathway (Wu et al. 2019). Another group also demonstrated that DJC markedly inhibited pancreatic β cell apoptosis with up-regulated Bcl-2 and down-regulated BAX in type 1 diabetic rats (Zheng et al. 2016). Lu et al. found that DJC protect vascular endothelial cells from HFD and palmitic acid induced damages through enhancing NO release, decreasing ER stress and endothelial cell apoptosis (Lu et al. 2018b). Recently, Sun et al. reported that DJC could ameliorate kidney injury through JAK-STAT signaling pathway in diabetic nephropathy rats (Sun et al. 2019). These findings suggested that DJC possesses the anti-oxidative stress properties and may act as a new strategy for diabetes complications prevention and treatment.

5. Conclusion

The findings of this study firstly demonstrated that RP is the major anti-oxidative bioactive constituent of DJC in amelioration of diabetic cardiomyopathy. DJC and RP relieved oxidative stress of vascular endothelium through regulating oxidative stress signaling and apoptotic pathway. This study would provide further evidence for clinical use of DJC in the management of diabetes. Future studies will test the optimal dosage of this combined administration and investigate the effects of other bioactive molecules in DJC.

Funding

This work was supported by National Natural Science Foundation of China (No. 81573944, 81774286).

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