Scutellarin alleviates type 2 diabetes (HFD/low dose STZ)- induced cardiac injury through modulation of oxidative stress, inflammation, apoptosis and fibrosis in mice

Yan Huo
Xuzhou Medical College: Xuzhou Medical University
Abudureheman Mijiti
The Second People's Hospital in Kashgar
Ruonan Cai
Xuzhou Medical College: Xuzhou Medical University
Zhaohua Gao
Xuzhou Medical College: Xuzhou Medical University
Maierpu Aini
The Second People's Hospital in Kashgar
Abudukadier Mijiti
The First People's Hospital in Kashgar
Zhaoling Wang
Xuzhou Medical College: Xuzhou Medical University
Rui Qie (tzea51685@sina.com)
Heilongjiang University of Traditional Chinese Medicine: Heilongjiang University of Chinese Medicine

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Abstract

Background: Diabetes is a serious global health concern which severely affected public health as well as socio-economic growth worldwide. Scutellarin (SCU), a bioactive flavonol is known for its efficacious action against a range of ailments including cardiovascular problems. The present study conducted to find out possible protective effect and its associated mechanisms of SCU on experimental type 2 diabetes-induced cardiac injury.

Methods: Type 2 diabetes was induced by treating animals with high fat diet for 4 weeks and a single intraperitoneal dose (35 mg/kg body weight) of streptozotocin and diabetic animals received SCU (10 or 20 mg/kg/day) for 6 weeks.

Results: SCU attenuated type 2 diabetes-induced hyperglycemia, body weight loss, hyperlipidemia, cardiac functional damage with histo-pathological alterations and fibrosis. SCU treatment to type 2 diabetic mice exacerbated oxidative stress, inflammatory status and apoptosis in heart. Furthermore, the underlying mechanisms for such mitigation of oxidative stress, inflammation and apoptosis in heart involved modulation of Nrf2/Keap1 pathway, TLR4/MyD88/NF-κB mediated inflammatory pathway and intrinsic (mitochondrial) apoptosis pathway, respectively.

Conclusions: The current findings suggest that SCU is effective in protecting type 2 diabetes-induced cardiac injury by attenuating oxidative stress and inflammatory responses and apoptosis and it is also worth considering the efficacious potential of SCU to treat diabetic cardiomyopathy patients.

1. Introduction

Diabetes is a major global health concern which severely affected public health as well as socio-economic growth worldwide. As per estimates of International Diabetes Federation, the estimates cases of diabetes were 451 million in 2017 with unimaginable predicted rise to 693 million by 2045 (Cho et al., 2018). Diabetes affects multiple organs and tissues and cause complications including cardiovascular diseases (CVDs) (Melendez-Ramirez et al., 2010). Cardiovascular disease (CVDs) is a chronic disease with the highest morbidity and mortality (costing 17.9 million deaths every year) across the globe. Even though the etiology for this occurrence is not fully understood evidence suggests that diabetes has been considered as prime factor for CVDs (Dunlay et al., 2019).

Diabetes-induced pathological changes in the myocardium are intricate involving biochemical and molecular alterations in oxidative stress, inflammation and apoptotic mechanisms of heart and eventually lead to serious cardiac events (Rajesh et al., 2010; Soares Felício et al., 2016; Boudina and Abel, 2010; Palazzuoli and Nuti, 2010; Althunibat et al., 2019). Heart being an active organ is under severe stress during hyperglycemic condition, which after exposure experiences reduction in antioxidant defenses, elevated free radical's/lipid peroxidation levels (Althunibat et al., 2019). Previously studies reported the positive correlation between reactive oxygen species and pro-inflammatory cytokine levels which affects left ventricular function (Al-Rasheed et al., 2017; Tschope et al., 2005). Earlier reports also

highlighted the involvement of ROS in provoking internal (mitochondrial) apoptotic pathway in diabetic heart (Othman et al., 2017). Additionally, hyperglycemia-mediated oxidative stress instigates inflammatory and apoptotic pathways (Roslan et al., 2017; Gong et al., 2019).

On the other hand, Scutellarin (5,6,7,4'-tetrahydroxyflavone-7-O-β-glucuronide), the main component in the extract of herb *Erigeron breviscapus*, which has been in traditional Chinese medicine use for decades mainly for effective actions against brain and heart related ailments (Wang and Ma, 2018). The medical claim that was attributed to this extract was ant-oxidant activity based on evaluation of many assays (Wang et al., 2008). In recent years, the focus has been shifted towards finding the efficacy and mechanism of action of Scutellarin (SCU). SCU is well known for its protective potential against neurotoxicity (Guo et al., 2013), diabetic nephropathy (Xu et al., 2013; Liu et al., 2019), diabetic retinopathy (Long et al., 2019; Mei et al., 2019), diabetes-induced testicular damage (Long et al., 2015) and diabetes-induced liver injury (Wang et al., 2020) through its antioxidant (Wu and Jia, 2019) and anti-inflammatory (Wang et al., 2016) actions. Previously, studies reported that SCU protects doxorubicin-induced acute cardiotoxicity (Sun et al., 2017), isopranaline-induced myocardial infarction (Huang et al., 2018) and ischemia–reperfusion (I/R)-induced myocardial injury (Wang et al., 2016). Also, SCU was proved to be effective by alleviating interstitial fibrosis and cardiac dysfunction of infarct rats (Pan et al., 2011). Furthermore, preclinical safety evaluation of SCU by acute and sub-acute studies revealed minimal toxicity or non-toxicity in rodents (Li et al., 2011).

By considering diabetes-induced cardiac complications and SCU safety and efficacy potential, the present study is planned and executed to explore possible protective effects and mechanisms of SCU against cardiac injury in well-established experimental type 2 diabetes (high fat diet/STZ) mice model.

2. Methodology

2.1. Drugs and Chemicals

Scutellarin (SCU) and Streptozotocin (STZ) were selected as the test chemicals for the present study and purchased from Sigma Chemical Company (St. Louis, MA, USA) and other chemicals (highest grade) were bought from local commercial suppliers.

2.2. Animals

Adult healthy male Swiss mice (22 ± 03 g) were procured and allowed to acclimatize for 07 days before being used for the present study. Animals were submitted to ophthalmological and detailed clinical examination during acclimatization. Also, growth of animals was monitored regularly and the animals displaying poor growth were not included in the study. The mice were housed in polypropylene cages by using sterilized corn cob for bedding purpose and allowed *ad libitum* access to reverse osmosis autoclaved water and standard rodent feed. The rats were maintained in an automatic controlled well-controlled laboratory conditions (temperature 23 ± 2°C; 12-hour light and 12-hour dark cycle, humidity 50

± 10%). The animal experiment procedure was reviewed and approved by a panel of Institutional Animal Ethical Committee members.

2.3. Induction of Experimental Type 2 diabetes

To induce experimental type 2 diabetes in mice, animals were supplied with *ad libitum* access to high fat diet (58% energy from fat) for four weeks and given a single intraperitoneal injection of STZ (35 mg/kg body weight) in a citrate buffer of pH 4 (Cai et al., 2019). Later one week of STZ injection, animals exhibiting hyperglycemia i.e., fasting blood glucose level > 12.5 mmol/L were considered as type 2 diabetic animals and further used for the research.

2.4. Experimental Design

One-week after adaptation period, a total of 40 animals were randomly categorised into four groups of 10 in each of group 1: Normal control group - received vehicle; group 2: type 2 diabetes group; group 3: diabetic mice received 10 mg Scutellarin/ kg body weight; and group 4: diabetic mice received 20 mg Scutellarin/ kg body weight mg/kg bw of Scutellarin) for 6 weeks.

Scutellarin was dissolved in 5% DMSO and distilled water (1:9). The dosage of scutellarin was based on earlier study (Xu et al., 2020).

2.5. Determination of body weight and blood glucose levels

Weekly body weights measured using calibrated weighing balance and fasting blood glucose levels were monitored once in a week using a glucometer (Accu-Check, Roche, Germany).

2.6. Biochemical analysis

At the end of study, blood samples from the control and experimental mice were withdrawn via intraocular puncture using mild ether anaesthesia. Blood samples were allowed to clot at room temperature for atleast 40 minutes and sera were separated by centrifuging samples at 3500 rpm for 15 minutes. The serum concentrations of total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), and low density lipoprotein (LDL) were analyzed using automatic Beckman Coulter analyzer.

Analyses of serum concentrations of CK-MB, Troponin and BNP were based on ELISA kit method. Hearts were quickly separated after euthanizing animals, blotted free of blood, weighed wet to the nearest milligram by using an electronic balance and further used for histopathological, biochemical and molecular analyses. Left cardiac portion was used for biochemical and molecular analyses, while right cardiac portion was fixed in 10% neutral formalin saline for histopathological studies.

2.7. Histopathology

For histological study, hearts fixed in neutral buffer saline were taken out and trimmed off excessive tissue, processed in tissue processor with increasing series of alcohol washes, embedded in paraffin blocks, cut into 5 µm thick sections using microtome, and sections were placed on adhesive slides and stained with Periodic acid-schiff (PAS) and Masson's trichrome stains. Then, the sections were examined

under Olympus phase contrast microscope (Tokyo, Japan) and digital images were captured by microscope camera (Nikon Coolpix 5400, Nikon, Japan).

2.8. Immuno-histochemistry

To perform immunohistochemistry, heart sections from all the animals were deparaffinized, rehydration, antigen retrieved, blocked the endogenous peroxidase activity with 0.3% H_2O_2 and incubated for 14 hours at 4°C with primary antibodies (Abcam, Cambridge, MA, USA) such as NRF2, Keap1, IKB β, NFK β, TNF-α, BCL-2, and Caspase-3 at a dilution of 1:200 in 5% bovine serum albumin. After three PBS washes, sections were incubated with appropriate secondary antibody for 1 hour and were stained and counterstained with 3'-Diaminobenzidine and haematoxylin, respectively. Also, negative control was also kept to ensure antibody specificity by using normal rabbit serum instead of primary antibody to confirm the specificity.

2.9. Oxidative and anti-oxidative status in the heart

Oxidative stress parameters such as lipid peroxidation products (level of thiobarbuturic acid reactive substances as a measure of malondialdehyde levels) and protein carbonyls and anti-oxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) in 10% heart tissue homogenates samples were measured with commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as per supplier kit protocol. Protein content in the enzyme source was estimated by Bradford protein assay kit (Sigma Company, St. Louis, MO, USA).

2.10. Quantitative reverse transcription polymerase chain reaction (PCR) analysis

Immediately after removing out hearts from control and experimental animals, total RNA was extracted using Fermentas RNA isolation kit. The purity and the concentration of isolated RNA were quantified with nanodrop (NanoVue Plus Spectrophotometer, GE Health care Life Sciences, USA) and reverse transcribed with the help of cDNA synthesis kit. Real Time quantitative PCR was conducted by using SYBR Green PCR master mix kit (Invitrogen, Karlsruhe, Germany). The relative mRNA expressions of genes were quantified by normalizing with β-actin. The following primer sets were used for the reactions.

Nrf2 Forward 5’- GAAAAAGAAGTGGGCAACTGTGG-3’;

Reverse 5’- GGTGGGATTTGAGTCTAAGGAGGT-3’;

Keap 1 Forward 5’- TGCCCCTGTGGTCAAAGTG-3’;

Reverse 5’- GGTTCGGTTACCGTCCTGC-3’;

Nqo1 Forward 5’- GGTATTACGATCCTCCCTCAACATC-3’;

Reverse 5’-GAGTACCTCCCATCCTCTCTTCTTC-3’;

Ho-1 Forward 5’-GAGTGGGGCATAGACTGGGTT-3’;

Reverse 5’-GCTGGTGATGGCTTCCTTGTA-3’;

Tlr4 Forward 5’-TTCATGTCGTGTTCTCATG-3’;

Reverse 5’- TGCGCTCGCATCATGTTC-3’;

MYD88 Forward 5'- GCAAAGAACATGGTCCGATA-3';

Reverse 5'-CGTCAGTCTGTAGGTATG-3’;

Nf-kb Forward 5’-TCAGGAAGAGGTTTGGATGC-3’;

Reverse 5’-AGCCCCTAATACACGCCTCT-3’;

IκB-α Forward 5’-GGTGTTTGAATGTATTGCTGG-3’;

Reverse 5’- AGGCTGTTTGGCTGAGGT-3’;

IL-6 Forward 5’-GGACCAAGACCATCCAATTC-3’;

Reverse 5’-ACCACAGTGAGGAATGTCCA-3’;

TNF α Forward 5’-GCCTCTTCTCATTCCTGCTTG-3’,

Reverse 5’-CTGATGAGAGGGAGGCCATT-3’;

Bax Forward 5’-CCCAGAGGCGGGGTTTCA-3’;

Reverse 5’-GGAAAAAGACCTCTCGGGGG-3’;

Bcl-2 Forward 5’-TGAACTGGGGGAGGATTGTG-3’;

Reverse 5’-AAATCAAACAGAGGCCGCAT-3’;

Caspase-3 Forward 5’-TGGGTGCTATTGTGAGGCGG-3’;

Reverse 5’-GCACACCCACCGAAAACCAG-3’;

Caspase- 9 Forward 5’- GACTCTTCCTGGTCTTACCATATT-3’;

Reverse 5’- CTGCTATTGCAAGGACCCAATT-3’;

Cyt-c Forward 5’-CGTTGTGCCAGCGACTAAAAA

Reverse 5’-GATTTGGCCCAGTCTTGTGC-3’;

Parp-1 Forward 5’-GAAGCCACAGCTAGGCATGA-3’;

Reverse 5’-CGCCACTTCATCCACTCCAT-3’;

β-actin Forward 5’ –CCACACCCGCCACCAGTTCG-3’ ;

Reverse 5’-TACAGCCCGGGGAGCATCGT-3’;

2.11. Statistical analysis

Results were expressed as mean ± S.D. of ten determinants. Values of $p < 0.05$ were considered statistically significant using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test in SPSS version 21 (SPSS, Chicago, IL).

3. Results

3.1. Effect of Scutellarin on bodyweight, blood glucose levels and serum lipid profile in high fat diet and low dose of STZ induced diabetic mice

Figure 1A shows the weekly body weights in control and experimental animals. Significant higher ($p < 0.05$) in body weights of HFD-fed mice was observed when compared to normal diet fed mice. After induction of diabetes, significant body weight loss was seen in diabetic mice in comparison to control mice, while, significant decrease in body weight losses was observed in 10 ($p < 0.05$) or 20 ($p < 0.05$) mg SCU/ kg body weight treated mice when compared to diabetic mice.

The results of this study revealed diabetic mice experienced hyperglycemia through significant ($p < 0.05$) rise in FBG levels compared to normal controls. However, FBG levels in diabetic animals treated with SCU (10 or 20 mg/kg bw) for 6 weeks showed significant ($p < 0.05$) improvement in the form of decreased levels of glucose levels when compared to diabetic controls (Fig. 1B).

Lipid metabolic alterations in diabetic mice were evidenced from the study through significant increase in serum total cholesterol (TC) ($p < 0.05$) (Fig. 1C), triglycerides (TG) ($p < 0.05$) (Fig. 1D), LDL ($p < 0.05$) (Fig. 1F), and significant reduction in levels of serum HDL ($p < 0.05$) (Fig. 1E) with respect to control mice. Whereas, treatment with SCU 10 or 20 mg/kg bw to diabetic mice for 6 weeks significantly ($p < 0.05$) curbed the dyslipidemia in diabetic mice.

3.2. Effect of Scutellarin on serum cardiac markers in high fat diet and low dose of STZ induced diabetic mice

To know the protective effect of SCU against diabetes-mediated cardiac injury, we analyzed the levels of serum cardiac markers such as Troponin (Fig. 1G), CK-MB (Fig. 1H) and BNP (Fig. 1I) levels. The findings revealed that diabetic mice exhibited cardiomyocyte-injury by significantly ($p < 0.05$) elevating CK-MB, Troponin and BNP levels in diabetic mice compared to normal controls. Conversely, SCU (10 or 20 mg/kgbw) administration to diabetic mice for 42 days showed significant ($p < 0.05$) improvement in

cardiac function by decreasing CK-MB, Troponin, and BNP levels when compared to only diabetic control group without SCU treatment.

3.3. Effect of Scutellarin on histopathology of heart in high fat diet and low dose of STZ induced diabetic mice

Histological analyses of PAS (Fig. 2A) and Masson-trichrome (Fig. 2B) stained sections confirmed the cardioprotective effect of SCU in type 2 diabetes-induced myocardial injury. Control mice showed usual morphological features of heart with normal myocardial fibers. While, the diabetic heart sections showed extensive damage in myocardium through presence of necrotic myocytes, vacuolation and infiltration of inflammatory cells with interstitial fibrosis. However, SCU (10 or 20 mg/kg bw) treatment for 6 weeks ameliorated diabetes-induced histopathological alterations and myocardial fibrosis in heart.

3.4. Effect of Scutellarin on oxidative status in high fat diet and low dose of STZ induced diabetic mice

Herein, diabetic mice exhibited oxidative stress in hearts through significant ($p < 0.05$) elevation in MDA (Fig. 3F) and protein carbonyl (Fig. 3E) levels along with significant ($p < 0.05$) reduction in antioxidant defence enzyme activities such as SOD ($p < 0.05$) (Fig. 3A), CAT (Fig. 3B), GPx (Fig. 3C) and GST (Fig. 3D) compared to respective levels and enzyme activities in hearts of control mice. Nevertheless, these alterations were attenuated after treating diabetic animals with SCU at doses 10 or 20 mg/kg ($p < 0.05$) for 42 days.

To know the molecular mechanisms behind antioxidant property of SCU, a battery of genes and protein distribution levels were estimated by RT-PCR and immune-histochemsitry, respectively. The results revealed downregulated mRNA expression of *Nrf2* (Fig. 4A), *Nqo-1* (Fig. 4C), and *Ho-1* (Fig. 4D) with upregulated *Keap1* (Fig. 4B) mRNA expression in diabetic hearts of mice compared to same expression in normal healthy mice. While, diabetes-induced and SCU-treatment for 42 days significant ameliorated the alterations observed in antioxidant genes of only diabetic mice hearts.

Furthermore, Immuno-histochemistry studies supported the results of RT-PCR through reduced Nrf2 protein distribution (Fig. 4E), and elevated Keap-1 protein distribution (Fig. 4F) in heart of diabetic mice. Whereas, 10 or 20 mg/kg bw SCU treatment for 42 days has resulted in an elevated protein distribution of Nrf2 protein with lessened Keap-1 protein distribution.

3.5. Effect of Scutellarin on inflammatory markers in cardiac tissue of high fat diet and low dose of STZ induced diabetic mice

The relative mRNA expression levels of inflammatory markers such as *Tlr4* (Fig. 5A), *Myd88* (Fig. 5B), *Nf-κb 1* (Fig. 5C), *Il 6* (Fig. 5E) and *TNf-α* (Fig. 5F) were upregulated and mRNA expression level of *IkBβ* (Fig. 5D) was decreased in hearts of HFD/STZ-induced mice when compared with normal control animals. Conversely, diabetes-induced and SCU (10 or 20mg/kg bw) treated mice showed significant ($p < 0.05$) alleviation in expression levels of the selected inflammatory markers in hearts.

Additionally, protein distribution levels of NF-Kβ (Fig. 6A) and TNF α (Fig. 6C) were increased and protein distribution of IkBβ (Fig. 6B) was decreased in immuno-histochemistry sections of diabetic heart when compared to normal control mice. Conversely, SCU (10 or 20 mg/kg bw) treatment resulted in marked improvement in inflammatory protein distribution profile.

3.6. Effect of Scutellarin on apoptosis markers in cardiac tissue of high fat diet and low dose of STZ induced diabetic mice

Apoptotic profile was revealed by RT-PCR and immune-histochemistry experiments. There was an upregulation in gene expression of *Bax* (Fig. 7A), *Cyt-c* (Fig. 7C), *Caspase-9* (Fig. 7D), *Caspase-3* (Fig. 7E) and *Parp 1* (Fig. 7F) and downregulation in gene expression of *bcl-2* (Fig. 7B) in cardiac tissue of hyperglycaemic mice in comparison with normoglycemic i.e., non-diabetic mice. While, either 10 or 20 mg/kg SCU treatment to diabetic mice has shown significant improvement in apoptotic profile.

In addition, protein distribution level of pro-apoptotic protein i.e., caspase-3 (Fig. 8A) was increased with reduction in protein distribution level of Bcl2 (Fig. 8B) in cardiac tissue of diabetic mice. Whereas, treatment with either 10 or 20 mg/kg SCU resulted in marked improvement in apoptotic status.

4. Discussion

Diabetes-mediated cardiac injury has been considered as a main cause of heart failure and death in diabetic patients. Epidemiological studies have shown that diabetic patients are exposed to 2- to 5-fold increased risk of developing cardiac abnormalities compared with non-diabetics. On the other hand, Phenolic compounds possess cardio-protective potential through its modulatory effects on oxidative stress and inflammatory responses involved in cardiovascular diseases. SCU, a well-known polyphenolic flavonoid, is the major effective ingredient of breviscapine, which is extensively being used in the treatment of various ailments including cardiovascular diseases (Tang et al., 2014; Zhang et al., 2005). However, the effect of SCU against type 2 diabetes-induced cardiac complications is still not yet unveiled. Taking this into consideration, the present study is designed to elucidate the possible role and mechanisms of SCU in experimentally (HFD/STZ)-induced type 2 diabetes rat model. The results of this study revealed SCU attenuates type 2 diabetes-induced cardiac complications through its modifying effects on oxidative stress (Nrf2/Keap1/ARE pathway), inflammation (TLR4/MyD88/NF-κB pathway) and apoptosis (mitochondrial-dependent pathway). These findings indicate that SCU could be used an alternative therapy to treat and/or prevent type 2 diabetes-induced diabetic cardiac complications.

The results of present study revealed that weekly body weights of type 2 diabetic mice were significantly reduced in comparison with counterpart non-diabetic mice suggesting burning up of proteins and fatty acids as an energy source because of unavailability of carbohydrates for energy needs. The decreased body weights in diabetic animals were already reported in earlier studies (Al-Rasheed et al., 2017; Al-Rasheed et al., 2016). While, treatment of diabetic animals with SCU (10 and 20 mg/kg body weight) for 6 weeks has resulted in significant increase in body weights indicating consumption of carbohydrates for energy needs. The maintenance of high levels of blood glucose is a characteristic feature of type 2

diabetic animals (Liu et al., 2019; Hudish et al., 2019; Gomez-Banoy et al., 2019). Similarly, the present findings have revealed presence of high levels of blood glucose levels throughout the study in diabetic animals when compared to that of control animals. However, significant glycemic control was observed in 10 and 20 mg SCU/ kg bodyweight treated mice in comparison with untreated diabetic mice demonstrating the hypoglycaemic efficacy of SCU (Liu et al., 2019).

In type 2 diabetics, the prevalence of hyper-lipidemia is reported (Matsuzaka and Shimano, 2020), which later leads cardiovascular diseases (Burkhardt, 2019). Under normal biological condition, insulin stimulates lipoprotein lipase to make fatty acids and glycerol from triglycerides, the formed triglycerides are stored as fuel or can serve as energy source (Matsuzaka and Shimano, 2020; Burkhardt, 2019). Earlier it was reported that insulin resistance or its deficiency, which generally happens during diabetic condition can inactivate lipoprotein lipase and thereby shoots up triglyceride levels (Quispe et al., 2016). Low density lipoproteins transport cholesterol from liver to other tissues of body (Ference et al., 2019), and high density lipoproteins transport endogenous cholesterol from other tissues to liver for metabolization and excretion (Trajkovska and Topuzovska, 2017). In the present investigation, alterations in lipid metabolism of diabetic mice are evidenced by significant increase in serum total cholesterol, LDL, and triglycerides, and significant decrease in the levels of HDL when compared with non-diabetic mice. Considering the fact that hyperlipidemia increases the risk of diabetes-mediated cardiovascular compications, control of dyslipidemia is a key to protect cardiovascular health (Warraich and Rana, 2018). Earlier it was scientifically proved that polyphenols are effective in lowering serum lipids thereby prevent the development of cardiac diseases and its complications (Cheng et al., 2017; Othman et al., 2017). However, in this study administration of 10 or 20 mg SCU/ kg bw for 6 weeks curbed the lipid metabolic alterations in diabetic mice, which is an indication of hypolipidemic action of SCU. The results are well supported by observations of Liu et al. (2019) who reported alleviation of dyslipidemia in db/db mice after treating animals with SCU.

Evaluation of cardiac functional biomarkers has been considered as a powerfool tool to find out cardiac injury and institute therapy to myocardial damage. Analysis of serum cardiac injury biomarkers such as CK-MB, troponin and BNP is of utmost important as myocardial damage leaks these biomarkers into bloodstream (Aydin et al., 2019; Jacon and Khan, 2018). In the present study, myocardial damage in diabetic mice is evidenced from increased levels of CK-MB, troponin and BNP in serum. Similar increase in serum levels of CK-MB, troponin and BNP in serum was earlier reported in diabetic animals (Zhang et al., 2019). Alleviation of myocardial damage by means of significant decrease in serum levels of CK-MB, troponin, and BNP was observed in diabetic mice treated with SCU (10 and 20 mg/kg bw) compared to only diabetic mice demonstrating cardio-protective potential of SCU. Considering the role of lipid metabolic alterations in aggravating diabetes heart injury (Al Hroob et al., 2019), the cardio-protective potential of SCU could be attributed to its anti-hyperlipidemic effect. Earlier it was also reported that brevicapine treatment results in reversal of cardiac dysfunction in diabetic cardiamyopathy rats (Wang et al., 2010).

A large number of scientific evidances pointed out that oxidative stress phenomenon plays a significant role in the development of type 2 diabetes-induced complications including cardiac complications (Zych et al., 2019; Bigagli and Lodovici, 2019). Oxidative stress occurs when there is an overproduction of pro-oxidants relative to antioxidant defences (Sharifi-Rad et al., 2020). In hyperglycaemic condition, an increased mitochondrial glucose oxidation results in release of large amount of reactive oxygen species into cytoplasm thereby ROS outweighs the antioxidant defences, and the resulting oxidative stress can negatively impact the cardiomyocytes (Pitocco et al., 2013). In this study, increased levels of levels of MDA and protein carbonyls and decreased activities of antioxidant enzymes such as SOD, CAT, GPx and GST were observed indicating elevated oxidative stress in cardiac tissues of diabetic mice. The findings are in consonance with the earlier study of Althunibat et al. (2019), who reported elevated levels of MDA and protein carbonyls with reduced activities of antioxidant defenses in diabetic heart. Conversely, diabetic mice treated with SCU (10 or 20 mg/kg body weight) presented with attenuated oxidative stress and boosted antioxidant defence system when compared to only diabetic animals without SCU treatment. In agreement with this, SCU amerliorated ISO-induced myocardial infarction through marked improvement in antioxidant defence system and reduction in oxidative status (Huang et al., 2018).

Additionally, we suspected activation of other cytoprotective proteins might be helping in antioxidant potential of SCU. In this context, Nuclear factor erythroid 2-related factor 2 (Nrf2), a ubiquitous transcriptional factor presents in present in various organs and tissues including heart (Chen et al., 2018) which is involved in the regulation of various antioxidant defenses to protect against oxidative damage (AL Haithloul et al., 2019; Jimenez et al., 2018). During normal biological conditions, Nrf2 is tightly coupled by Keap1 (Kelch-like ECH-associated protein 1) in the cytoplasm, which facilitates its proteosomal degradation (Furukawa and Xiong, 2005). Under stimulated conditions, Nrf2 uncouples from Keap-1 and translocates to nucleus to activate various genes to protect cells against oxidative stress (Taguchi et al., 2011). In the current study, gene and protein distribution analyses of Nrf-2/Keap-1 signalling pathway by RT-PCR and immune-histochemistry, respectively, revealed that significant down regulation of Nrf2 (mRNA and protein), Nqo-1 (mRNA), and Ho-1(mRNA) genes and upregulation of Keap1 (mRNA and protein) were observed in cardiac tissue of diabetic mice. The present findings are in line with earlier reports of Wen et al. (2019). Interestingly, the current study results also revealed an upregulated gene (Nrf2, Nqo-1 and Ho-1) and protein (Nrf2) expression with downregulated Keap-1 mRNA and protein expression in diabetic mice treated with 10 or 20 mg SCU/kg body weight in comparison with only diabetic mice. In accordance, SCU showed reno-protective effects in db/db mice through modulation of Nrf2/HO-1 signaling pathway (Liu et al., 2019). These findings suggest that SCU can attenuate diabetes-induced cardiac injury through attenuation of oxidative stress and boosting of antioxidant defense system.

Several studies have claimed a strong correlation between oxidative stress and inflammation, which have been considered as critical mediators in the pathogenesis of cardiac injury in diabetes (Althunibat et al., 2019; Roslan et al., 2017). Among the various inflammatory pathways, TLR-mediated NF-kB pathway plays a pivotal role in the regulation of inflammatory responses (Hu et al., 2020; Shen et al., 2020). Upon activation, TLR signalling is carried through myeloid differentiation primary-response protein 88 (MyD88)

and inhibitor-kB (IkB) kinase, which degrades inhibitor of NF-kB i.e., Inhibitor-κB (IκB), thereby translocate NF-kB into nuclueus to activate various inflammatory cytokines including IL-6 and TNF- α (Suryavanshi and Kulkarni, 2017). In this study, the relative mRNA expression and protein distribution of inflammatory mediators (mRNA expression: Tlr4, Myd88 and NF-kB; protein distribution: NF-kB) and inflammatory cytokines (mRNA expression: IL-6 and TNF- α; protein distribution: TNF- α) were elevated with reduced mRNA expression and protein distribution of inflammatory inhibitor i.e., IkB in cardiac tissue of type 2 diabetic mice. Previously, studies also demonstrated the involvement of TLR4/MYD88/NF-kB signaling pathway in provoking inflammation and cardiac injury to promote inflammation and cardiac injury in diabetic animals (Youssef et al., 2021; Lu et al., 2021). In contrast, treatment of diabetic mice SCU (10 or 20mg/kgbw) for 42 days resulted in significant attenuation in mRNA expression and protein distribution levels of selected inflammatory markers. Supporting to this data, earlier Huang et al. (2018) reported the anti-inflammatory effect of SCU through its inhibition of NF-kB translocation from cytoplasm to nucleus and by reducing inflammatory cytokine levels. These results indicate the anti-inflammatory action of SCU through its suppression of TLR4/MyD88/NF-κB pathway, thereby limiting the inflammatory responses.

Plethora of scientific evidences revealed that several mechanisms including oxidative stress and inflammation instigate apoptosis (Althunibat et al., 2019; Gong et al., 2019). The activation of apoptosis involves sequence of events such as promotion of pro-apoptotic factors (Bax), deactivation anti-apoptotic proteins (Bcl2), destabilization of mitochondrial membrane, leakage of cytochrome c (cyt c) and further activation of caspase 9, caspase 3 and Parp1, which eventually end up in cell by activating caspase-3-activated DNase-mediated DNA fragmentation (Alarifi et al., 2017; Yang et al., 2017). Type 2 diabetic mice in this study showed upregulated mRNA expression of apoptotic factors such as Bax, cyt c, caspase 9, caspase 3 and Parp1 with downregulated expression of Bcl2 in cardiac tissue. Gene expression studies were also reflected in immune-histochemistry studies through an increased protein distribution of caspase-3 and decreased protein distribution of Bcl2 in cardiac tissue of diabetic mice. The observations in the present study are in agreement with earlier findings (Lu et al., 2021; Badalzadeh et al., 2015). SCU (10 or 20 mg/kg body weight) downregulated Bax, cyt-c, caspase-9, caspase-3 and parp-1 and increased Bcl2 in diabetic heart. This anti-apoptotic effect of fisetin against hyperglycemia-mediated activation of the mitochondrial pathways of apoptosis was supported by previous in vivo studies. Previously studies reported the anti-apoptotic protective effect of SCU against I/R (In vitro) and ISO (*In vivo*)-induced myocardial injury (Wang et al., 2016; Huang et al., 2018).

Additionally, supporting biochemical and molecular alterations observed in this study, the diabetic heart sections showed extensive damage in the myocardium by means of necrosis, vacuolation and infiltration of inflammatory cells. Additionally, interstitial fibrosis was observed in cardiac muscle fibres of HFD/STZ-induced mice. The observed hisopathological changes are supported earlier studies reporting histological and fibrotic changes in diabetic heart (Al-Rasheed et al., 2017; Othman ett al., 2017). While, SCU treatment minimised pathological changes in myocardium and attenuated myocardial fibrosis in diabetic animals. Earlier reports also observed amelioration of histopathological alterations and fibrosis after treating diabetic animals with SUC (Huang et al., 2017; Zhou et al., 2014; Pan et al., 2011). The observed effects might be due to its anti-oxidant, anti-inflamamtory and anti-apoptotic potential of SCU.

In conclusion, type 2 diabetes (HFD/STZ)- induced cardiac injury through significant alterations in metabolic parameters, lipid profile, cardiac functional parameters, oxidative and anti-oxidative status, inflammatory mediators and effectors and apoptotic profile. Moreover, treatment of type 2 diabetic mice with SCU at dose levels of 10 and 20 mg/kg body weight significantly attenuated metabolic, lipidemic and cardiac functionality markers. Furthermore, the perturbations occurred in oxidative stress, inflammation and apoptosis in type 2 diabetic heart were effectively attenuated by SCU due to activation of Nrf2/Keap1/ARE pathway and suppression of TLR/MYD88/NF-kB pathway and mitochondrial apoptotic pathway, respectively. However, clinically relevant translational studies are further warranted to further validate the efficacy of SCU for the treatment and/or prevention of diabetes-induced cardiac injury.

Declarations

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Authors' contributions

YH, AM and RQ designed that study and critical revision of the manuscript for important intellectual content. YH, AM, RC, Abudukadier.Mijiti and ZG carried out the laboratory analysis. ZG, MA and ZW analyzed the data and interpretation. YH and AM wrote the first draft of the manuscript. RQ editing final manuscript and funding. All authors contributed to the final version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of First Affiliated Hospital of Heilongjiang University of Traditional Chinese Medicine (No. DXBY2020).

Consent for publication

No personal data is noted herein.

Competing interests

The authors declare that they have no conflict of interest.

Author details

[1]Department of Endocrinology, Xuzhou Municipal Hospital Affiliated to Xuzhou Medical University, Xuzhou Jiangsu, 221000, China

[2]Department of Cardiac electrophysiology group the Second People's Hospital in Kashgar, Kashgar, Xinjiang, 844000, China

[3]Department of Emergency Medicine, The First People's Hospital in Kashgar, Kashgar,Xinjiang, 844000, China

[4]Department of Emergency, First Affiliated Hospital of Heilongjiang University of Traditional Chinese Medicine, Haerbin, Heilongjiang, 150040, China

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Figures

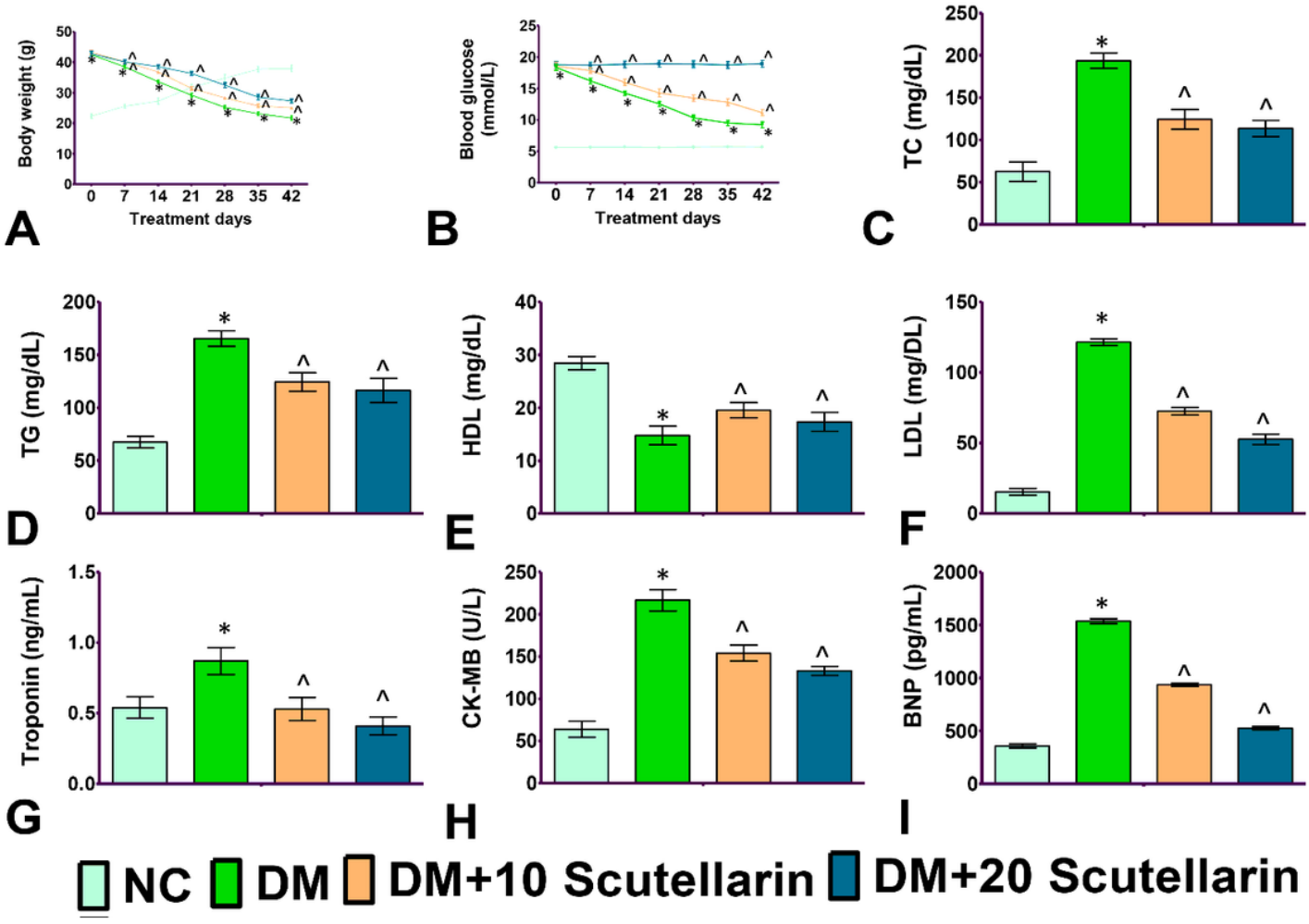

Figure 1

Effect of Scutellarin on body weight, heart weight, serum lipid profile and serum cardiac markers in high fat diet and low dose of STZ induced diabetic mice. (A): Bodyweight; (B): Blood glucose levels; (C) Total Cholesterol (TC) (D): Triglyceride (TG). (E): High density lipoprotein (HDL); (F): Low density lipoprotein (LDL); (G): Troponin; (H): Creatinekinase-MB (CK-MB); (I): Brain natriuretic peptide (BNP). NC: Normal control mice; DM: Diabetes mellitus; DM+10 Scutellarin: Diabetic mice treated with 10 mg/kg/bw of

Scutellarin; DM+20 Scutellarin: Diabetic mice treated with 20 mg/kg/bw of Scutellarin. Data are Mean ± SEM (n=6). * p < 0.05 versus NC, and # p < 0.05 versus DM.

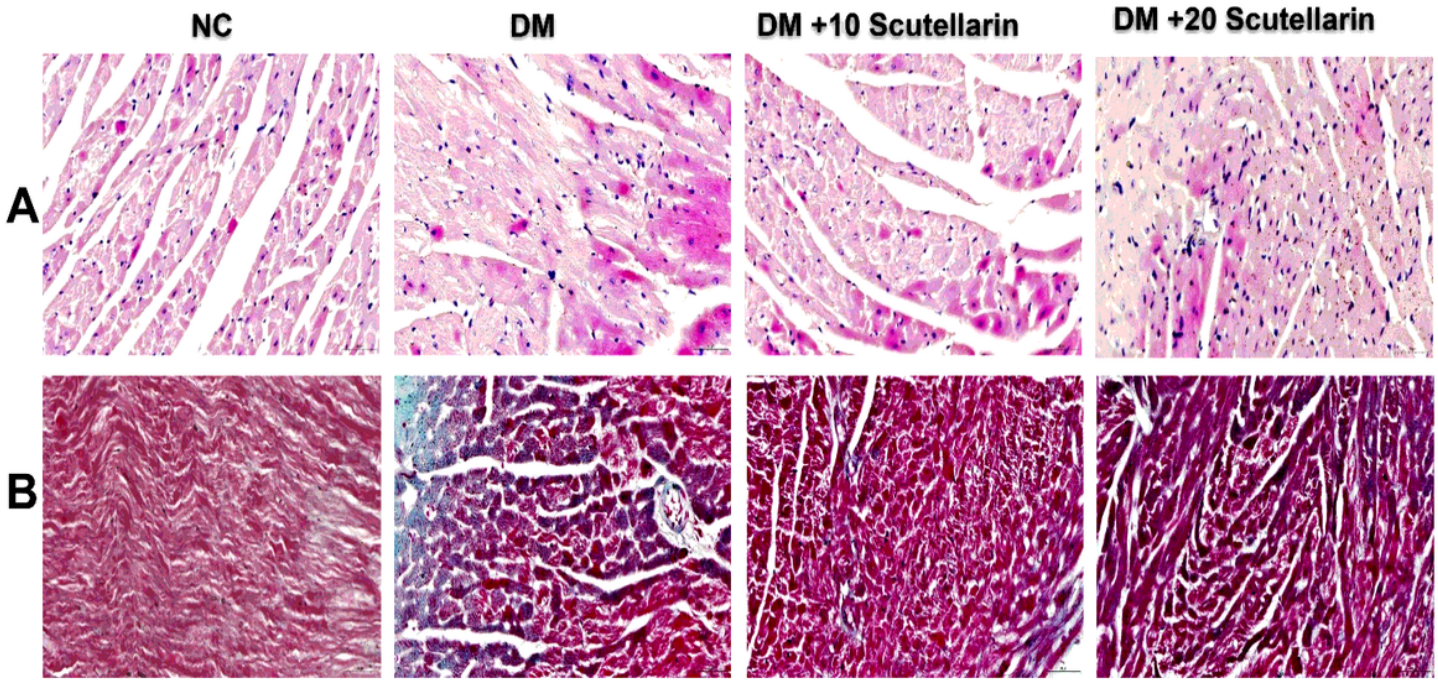

Figure 2

Effect of Scutellarin on histopathological changes in high fat diet and low dose of STZ induced diabetic mice. (A): Periodic acid-schiff (PAS); (B): Masson trichrome. NC: Normal control mice; DM: Diabetes mellitus; DM+10 Scutellarin : Diabetic mice treated with 10mg/kg/bw of Scutellarin; DM+20 Scutellarin: Diabetic mice treated with 20mg/kg/bw of Scutellarin. Scale bar: 100μm. Magnification: 40X.

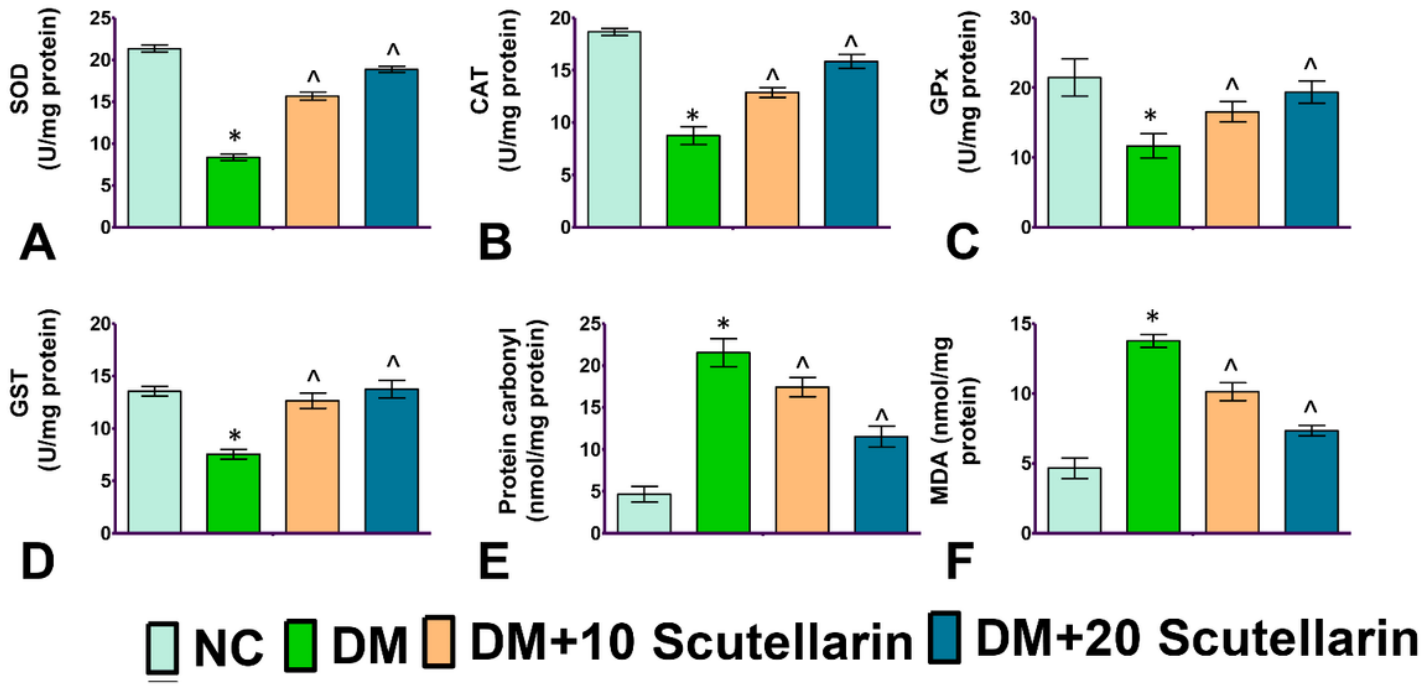

Figure 3

Effect of Scutellarin on oxidative stress and antioxidant enzyme levels in cardiac tissue of high fat diet and low dose of STZ induced diabetic mice. (A): SOD; (B): CAT; (C): GPx; (D): GST; (E): Protein carbonyl; (F): Lipid peroxidation products malondialdehyde (MDA). NC: Normal control mice; DM: Diabetes mellitus; DM+10 Scutellarin : Diabetic mice treated with 10mg/kg/bw of Scutellarin; DM+20 Scutellarin: Diabetic mice treated with 20 mg/kg/bw of Scutellarin. Data are Mean ± SEM (n = 6). *p < 0.05 versus NC, and ^p < 0.05 versus DM.

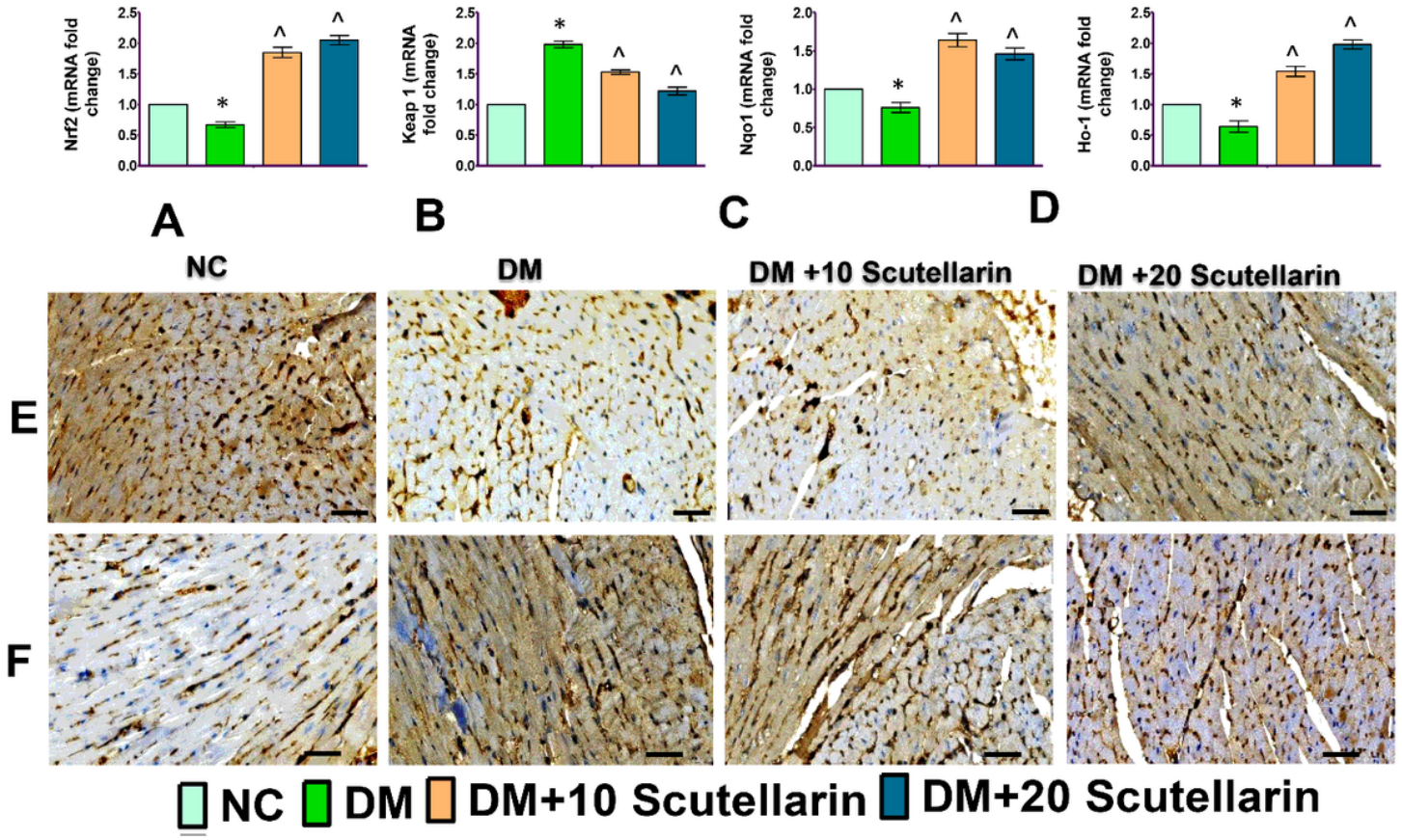

Figure 4

Effect of Scutellarin on relative mRNA expression of antioxidant enzymes in high fat diet and low dose of STZ induced diabetic mice. Relative mRNA expression of (A): Nrf2; (B): Keap 1; (C): Nqo1; (D): Ho-1; (E): Immunostaining of NRF2; (F): Keap1. NC: Normal control mice; DM: Diabetes mellitus; DM+10 Scutellarin : Diabetic mice treated with 10mg/kg/bw of Scutellarin; DM+20 Scutellarin: Diabetic mice treated with 20mg/kg/bw of Scutellarin. Data are Mean ± SEM (n = 6). *p < 0.05 versus NC, and ^p < 0.05 versus DM. Scale bar: 100μm. Magnification: 40X.

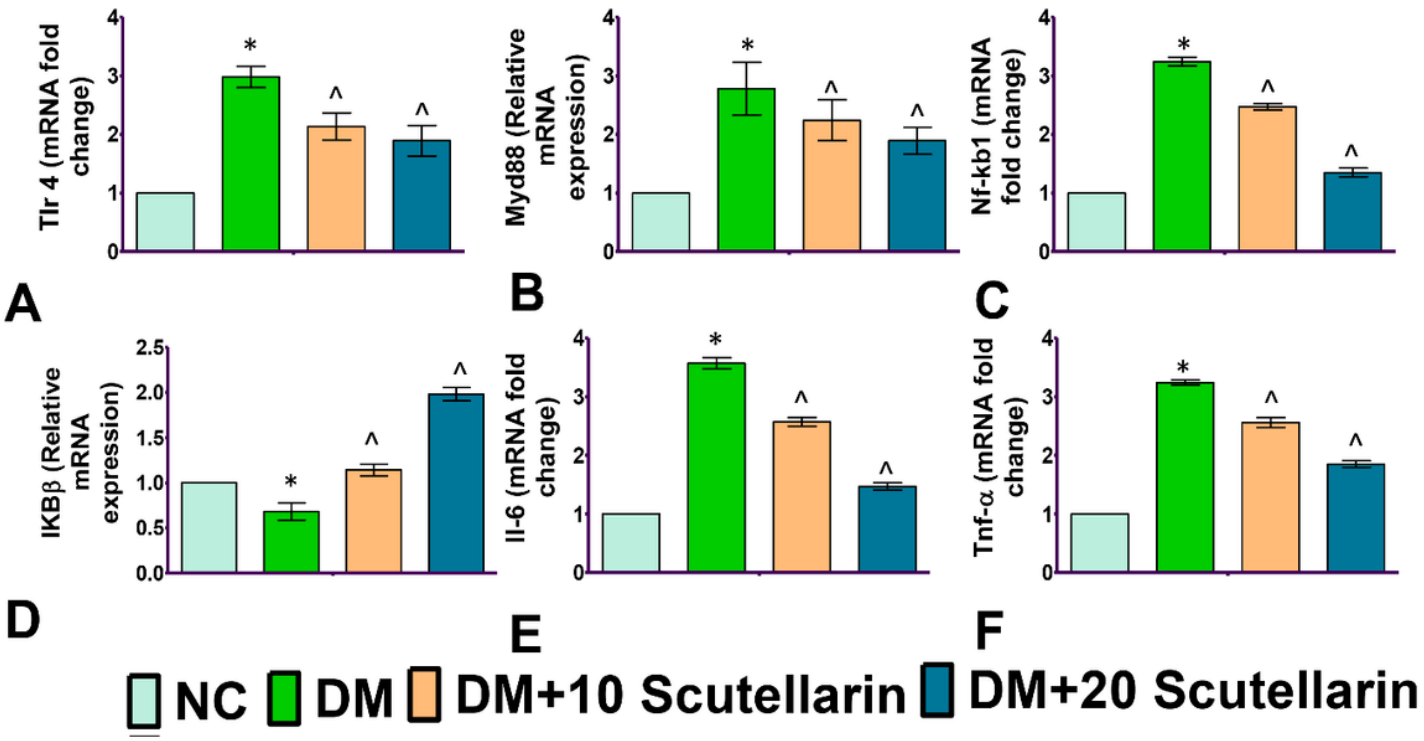

Figure 5

Effect of Scutellarin on inflammatory markers in cardiac tissue of high fat diet and low dose of STZ induced diabetic mice. mRNA expression of (A): Tlr4, (B): Myd88; (C): Nf-kb1; (D): Ikbβ; (E): Il-6; (F): TNF-α. NC: Normal control mice; DM: Diabetes mellitus; DM+10 Scutellarin : Diabetic mice treated with 10mg/kg/bw of Scutellarin; DM+20 Scutellarin: Diabetic mice treated with 20mg/kg/bw of Scutellarin. Data are Mean ± SEM (n = 6). *$p < 0.05$ versus NC, and ^$p < 0.05$ versus DM.

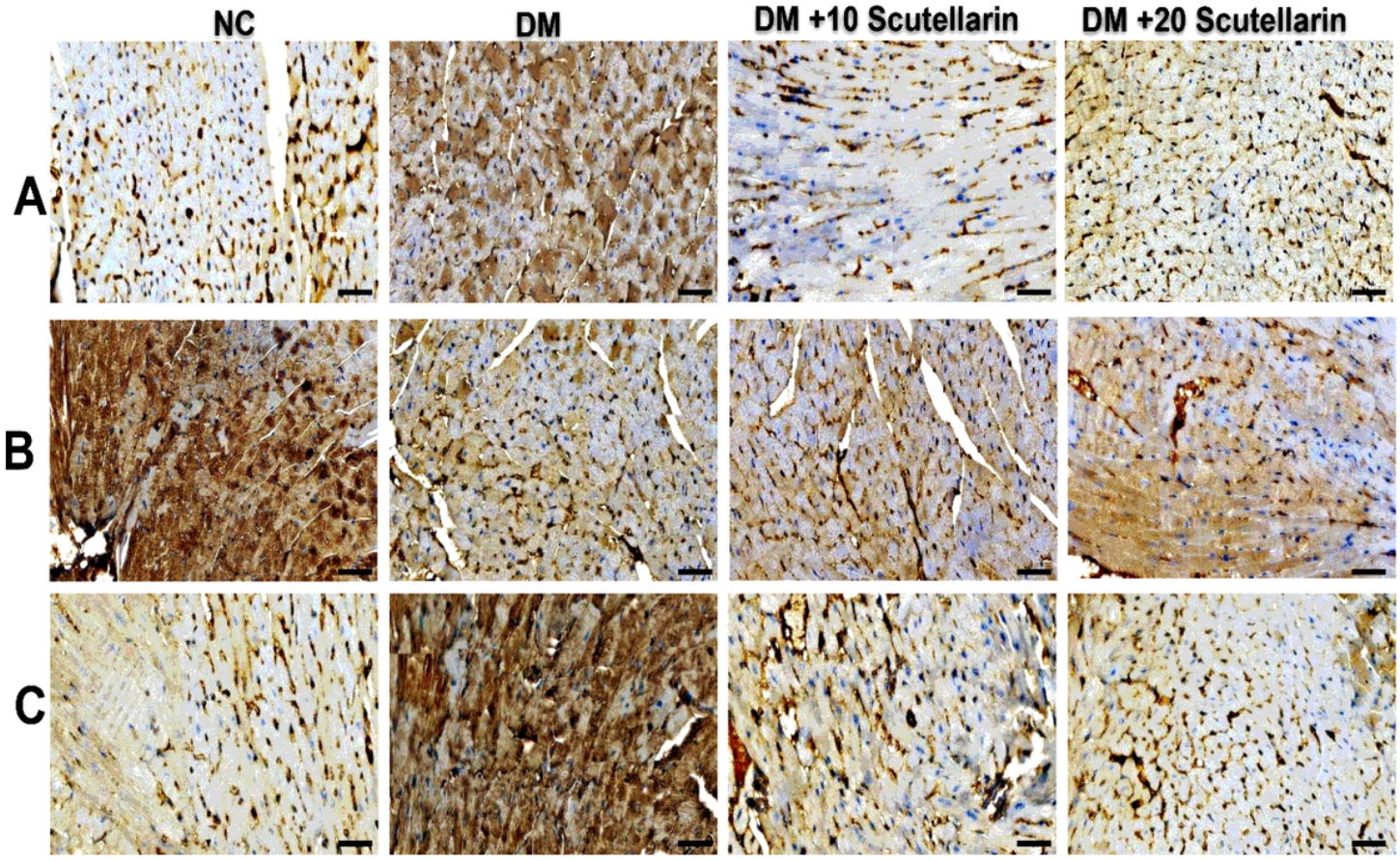

Figure 6

Effect of Scutellarin on inflammatory markers in cardiac tissue of high fat diet and low dose of STZ induced diabetic mice. Immunostaining of (A) NFKβ; (B) IkBβ (C)TNF-α. NC: Normal control mice; DM: Diabetes mellitus; DM+10 Scutellarin: Diabetic mice treated with 10mg/kg/bw of Scutellarin; DM+20 Scutellarin: Diabetic mice treated with 20mg/kg/bw of Scutellarin.. Scale bar: 100μm. Magnification: 40X.

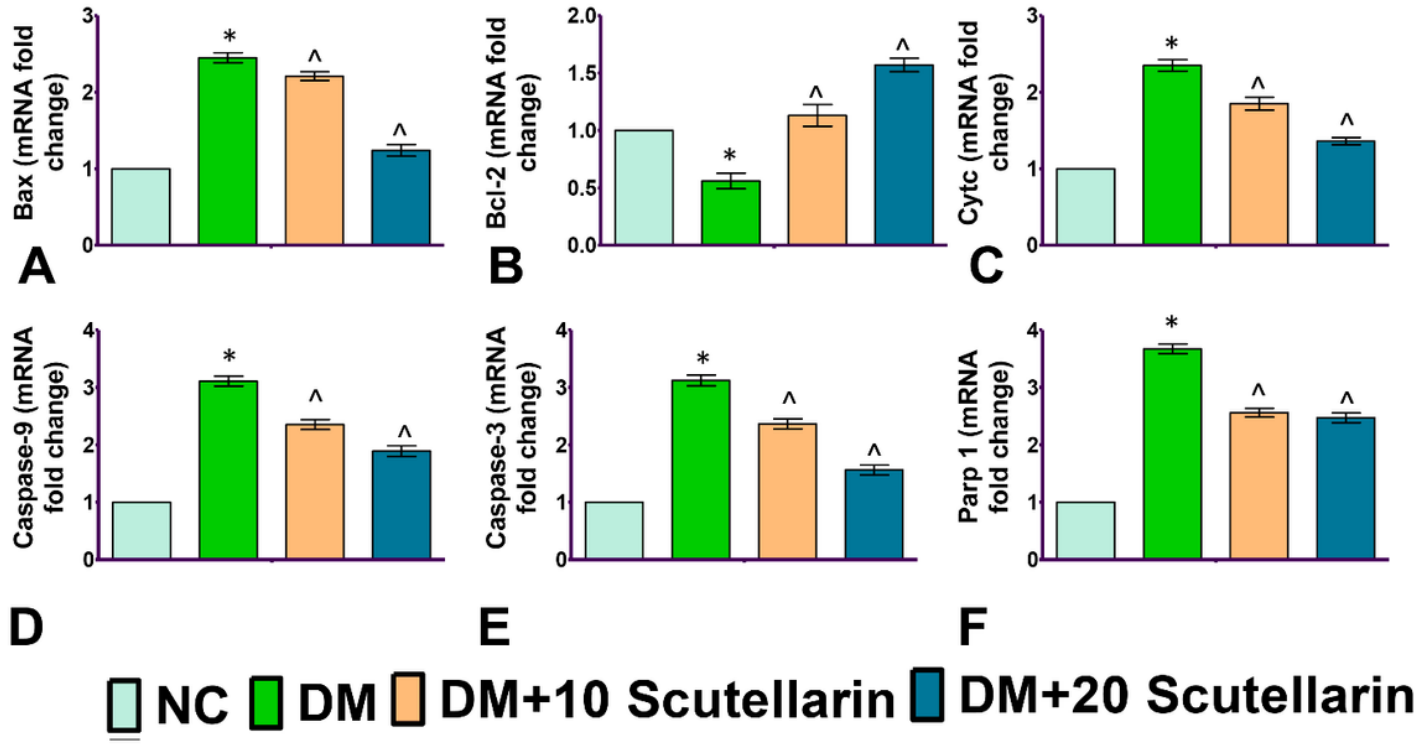

Figure 7

Effect of Scutellarin on apoptosis markers in cardiac tissue in cardiac tissue of high fat diet and low dose of STZ induced diabetic mice. Relative mRNA expression of (A): Bax; (B): Bcl-2; (C): Cytc; (D): Caspase-9; (E): Caspase-3; (F): Parp 1; NC: Normal control mice; DM: Diabetes mellitus; DM+10 Scutellarin : Diabetic mice treated with 10mg/kg/bw of Scutellarin; DM+20 Scutellarin: Diabetic mice treated with 20mg/kg/bw of Scutellarin. Data are Mean ± SEM (n = 6). *p < 0.05 versus NC, and ^p < 0.05 versus DM.

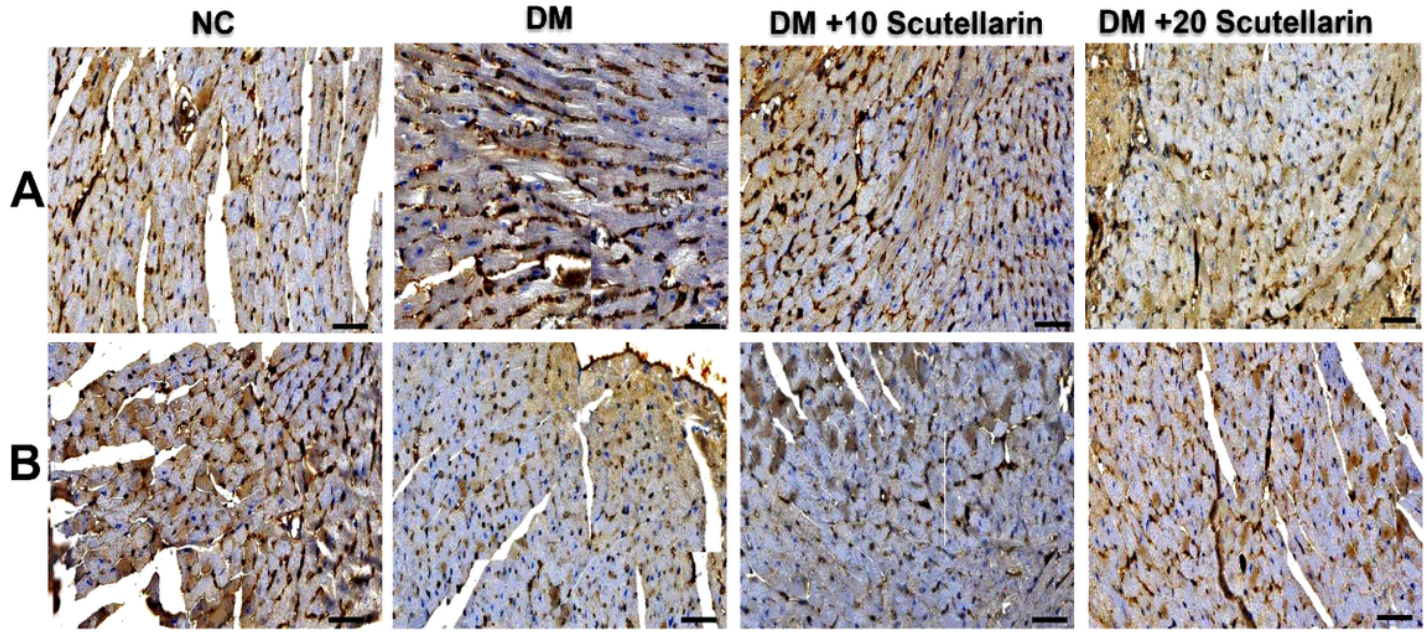

Figure 8

Effect of Scutellarin on apoptosis markers in cardiac tissue in cardiac tissue of high fat diet and low dose of STZ induced diabetic mice. Immunostaining of (A) Caspase-3 (B) BCL-2. NC: Normal control mice; DM: Diabetes mellitus; DM+10 Scutellarin : Diabetic mice treated with 10mg/kg/bw of Scutellarin; DM+20 Scutellarin: Diabetic mice treated with 20mg/kg/bw of Scutellarin. Scale bar: 100μm. Magnification: 40X.