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Original Article

Synergistic combination of taxifolin and baicalein protects against alloxan-induced diabetic cardiomyopathy by mitigating oxidative stress through the Nrf2/HO-1 signaling pathway

Qingsong Liang[1], Tingting Wu[2], Liangping Zhao[1]

[1]*Department of Cardiology, The Fourth Affiliated Hospital of Soochow University (Suzhou Dushu Lake Hospital), Suzhou 215000, Jiangsu, China;* [2]*Department of Cardiology, First Affiliated Hospital of Soochow University, Suzhou 215000, Jiangsu, China*

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Abstract: Objective: To investigate the cardioprotective effects of taxifolin (TXN) and baicalein (BCN) in an alloxan-induced diabetic rat model. Methods: Diabetic rats were treated with TXN, BCN, or their combination for 30 days. Blood glucose, lipid profile, cardiac enzymes, oxidative stress markers, and gene expression were assessed. Histopathologic and immunohistochemical analyses were performed to evaluate cardiac structure and protein expression. *In vitro* cytotoxicity assays were conducted on H9c2 cardiomyocytes. Results: TXN and BCN significantly reduced blood glucose levels, improved lipid profiles, and decreased oxidative stress in diabetic rats. They also enhanced nuclear factor erythroid 2-related factor 2 (Nrf2) signaling and upregulated antioxidant gene expression. Histologic analysis revealed reduced myocardial damage and fibrosis in treated rats. *In vitro* studies demonstrated no cytotoxicity for TXN and BCN. Conclusion: TXN and BCN exhibit cardioprotective effects in diabetic rats by improving glucose metabolism, modulating lipid profiles, and enhancing antioxidant defense through the Nrf2 signaling pathway. These findings suggest that TXN and BCN hold promise as potential therapeutic agents for the prevention and treatment of diabetic cardiomyopathy.

Keywords: Baicalein, cardioprotection, diabetic cardiomyopathy, Nrf2, oxidative stress, taxifolin

Introduction

In 2024, the estimated global prevalence of diabetes among adults aged 25-70 years exceeded 520 million [1]. As predicted by Caraturano et al. (2024), the rate of occurrence will rise to 11% (0.578 billion) by 2030 and also 11% (0.700 billion) by 2045 [2]. Cardiovascular problems account for more than 80% of diabetes-related fatalities, highlighting the significant health ramifications of this disorder [3]. The conventional criterion necessitating the absence of coronary artery disease, hypertension, or other cardiac ailments for the diagnosis of diabetic cardiomyopathy (DCM) is increasingly regarded as impractical, since diabetes often coexists with these disorders [4]. DCM's pathophysiology is complex, involving glucose toxicity, lipotoxicity, apoptotic and necrotic cell death, calcium homeostasis disruption, Advanced Glycatin End-product (AGE) cross-linking, oxidative stress, inflammation, and mitochondrial dysfunction [5, 6].

Mitochondria are pivotal in cellular metabolism, and current research suggests that mitochondrial failure directly leads to oxidative stress and cell death, hence promoting the advancement of DCM [7]. The complexes of the mitochondrial electron transport chain (ETC) are a principal source of mitochondrial reactive oxygen species (mt-ROS) [8]. Recent findings indicate that both overexpression and downregulation of the same ETC subunit can result in higher ROS production, implying that mitochondrial imbalance, rather than specific gene function changes, may be responsible for heightened ROS levels in neural cells [9, 10].

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Notwithstanding these discoveries, the specific pathways causing mitochondrial imbalance in DCM remain predominantly unexamined [11]. Additional study is required to clarify these mechanisms and create specific therapeutics that may alleviate the effects of DCM on diabetic persons [12].

Nuclear factor erythroid 2-related factor 2 (Nrf2) functions as an essential transcription factor in the cellular antioxidant defense mechanism, significantly contributing to the mitigation of oxidative stress [13]. The expression and activity of Nrf2 are influenced by multiple stresses [14]. In acute stress situations, Nrf2 separates from its inhibitor, Kelch-like ECH associated protein 1 (Keap1), and moves into the nucleus [15]. Upon entering the nucleus, Nrf2 associates with the Antioxidant Response Element (ARE) to initiate the expression of many protective genes, such as heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO-1), and peroxiredoxin 1 (Prdx1) [16]. Ge et al. have demonstrated a correlation between reduced Nrf2 expression and activity and the beginning and progression of DCM. Therefore, targeting Nrf2 activity may provide a unique therapeutic approach for the treatment of DCM [17]. Nevertheless, existing therapeutic modalities for DCM are controversial because of the possible harmful consequences linked to these medications [18]. Consequently, it is essential to investigate natural bioactive chemicals as alternative therapies that could aid in preventing the advancement of DCM [19]. Although Nrf2 plays a promising function in facilitating antioxidant responses, the specific processes leading to mitochondrial imbalance in DCM remain inadequately known [20]. Additional research on Nrf2 activators and natural substances may yield significant insight into effective treatments for DCM while reducing the adverse effects linked to traditional medicines [21].

Numerous diseases have been effectively averted and ameliorated through the use of biocompounds derived from plants [22]. Flavonoids are notable for their substantial health benefits, which include anticancer, antioxidant, neuroprotective, cardioprotective and hepatoprotective properties [23]. According to Khadrawy et al. (2024), the flavonoid taxifolin (TXN), which is also referred to as dihydroquercetin, is an essential component found in olive oil, onions, camphor pine and larch [24]. In accordance with Das et al.'s research from 2021, TXN features a complicated molecular architecture that is composed of 2 phenyl rings that are joined to one another by a heterocyclic ring [25]. The vast medicinal history of this substance, as well as its substantial antioxidant and antibacterial capabilities, may be attributed, in part, to its distinctive structure [26]. Unlike the polyphenol quercetin, TXN is non-mutagenic and is generally considered safe for consumption. According to research conducted by Moore (2009), TXN has hepatoprotective properties in mouse models of alcohol-induced inflammation and apoptosis, as well as decreasing oxidative stress and liver damage [27]. Research has confirmed its antihyperglycemic effects in mice, leading to improvements in fasting glucose levels, insulin sensitivity, and overall insulin regulation [28]. Additionally, TXN has demonstrated efficacy in alleviating diabetes-related complications, such as neuropathy [29].

Baicalein (BCN) is a flavonoid derived from the roots of *Scutellariabaicalensis*, recognized for its broad pharmacologic properties [30]. This chemical possesses a 1,2-catechol functional group and is acknowledged in Traditional Chinese Medicine (TCM) for its hepatoprotective characteristics and capacity to alleviate inflammatory illnesses. Earlier studies have discovered a wide range of bioactivities linked to baicalein, such as strong anti-inflammatory and antioxidant properties, significant inhibition of 12-lipoxygenase, and involvement in autophagy activation in cancer cells [31].

Considering the significant roles of ROS and autophagy in cardiac hypertrophy, it is posited that BCN's antioxidant qualities and autophagy-inducing effects may mitigate the onset of cardiac hypertrophy by removing ROS and impaired mitochondria [32, 33]. Earlier studies indicates that BCN can mitigate cardiac hypertrophy by blocking critical signaling pathways, including the MAPK/ERK Kinase and Extracellular signal-regulated Kinase 1 and 2 (MEK-ERK1/2), AKT/mTOR, nuclear factor kappa B (NF-κB), and calcineurin pathways [34]. Moreover, BCN has demonstrated an

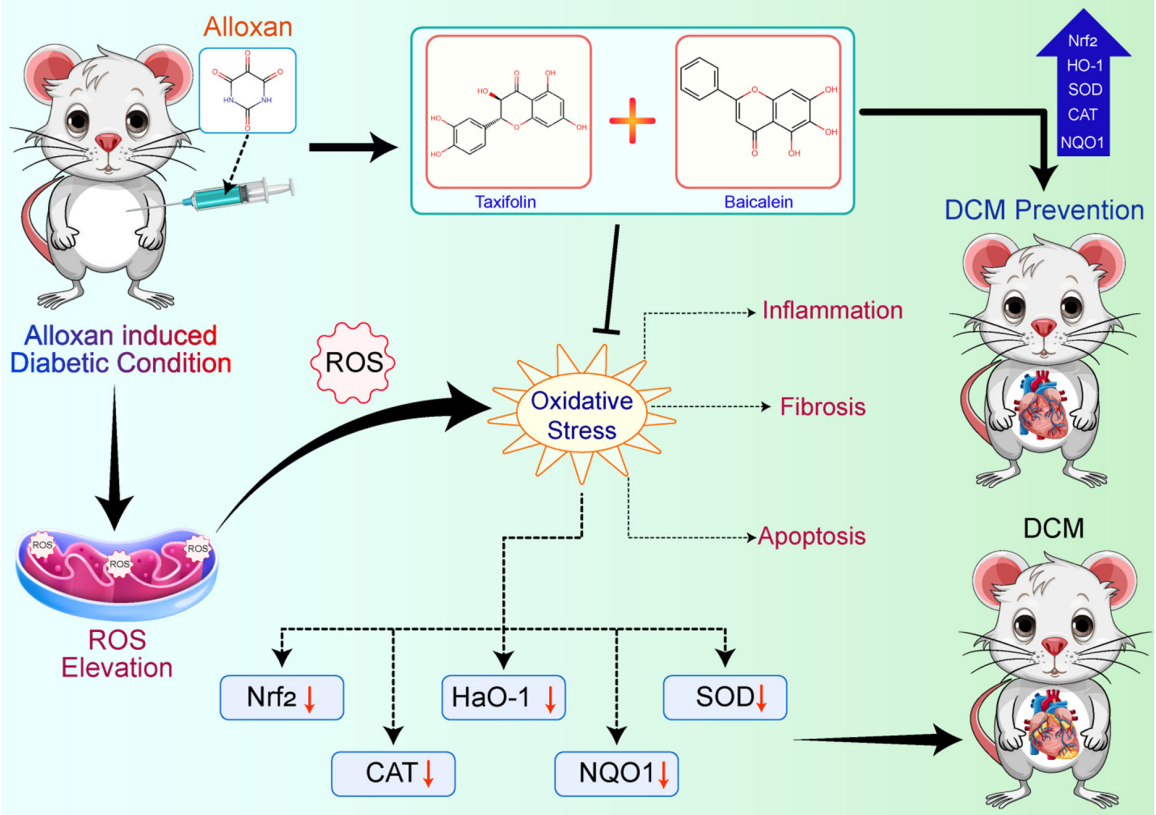

Figure 1. Illustration of the regulatory effects of taxifolin (TXN) and baicalein (BCN) on the activation of Nrf2/HO-1 proteins in alloxan (AXN)-induced diabetic cardiomyopathy (DCM). Red down head arrow:downregulation.

ability to inhibit the release of High Mobility Group Box 1 (HMGB1) and diminish the expression of Matrix Metalloproteinase-9 (MMP-9) and MMP-2 in cardiac tissues exposed to lipopolysaccharide (LPS)-induced hypertrophy [35, 36].

In this study, we demonstrate that TXN and BCN effectively attenuate alloxan (AXN)-induced DCM in a rat model. The synergistic combination of these two flavonoids induced DCM (**Figure 1**). Notably, our findings reveal alterations in the Nrf2/HO-1 signaling pathway during treatment with TXN and BCN, both of which are critical for antioxidant defense and autophagy regulation.

Materials and methods

Animals

The animal handling procedures were conducted according to the ethical norms approved by the Ethical Committee of the Fourth Affiliated Hospital of Soochow University (Suzhou Dushu Lake Hospital) (Ethical Approval no: 241145). For the purpose of this investigation, male Wistar rats weighing between 180 and 240 grams were used. Within the confines of a laboratory that adhered to established protocols, the animals were kept in individual cages. These circumstances consisted of a light and dark cycle that lasted for half a day, a temperature of 23 ± 2°C, and humidity levels ranging from 65 to 70%.

Experimental protocol

Animals were divided into eight groups, each containing six rats. The first group served as a control and was given regular saline orally for a month. Diabetes was induced in Groups II, III, and IV by a single injection of Alloxan monohydrate, 98% purity, catalog # A7413-25G at 150 mg/kg body weight [37]. Diabetes was diagnosed by measuring blood glucose 72 hours post-injection and observing symptoms like increased thirst, urination, hunger, and weight loss. Group II functioned as the diabetic control

and was administered normal saline, whereas Groups III, IV and V received TXN (25 mg/kg), BCN (200 mg/kg), and a combination of TXN and BCN, respectively, over a duration of 30 days. Groups VI, VII, and VIII served as non-diabetic control groups and were administered TXN, BCN, and a combination of TXN and BCN, respectively, for a duration of 30 days. After the 30-day treatment, blood samples were collected by heart puncture and spun at 6000 rpm/600 sec to isolate serum. The myocardial tissue was collected for histological, immunohistochemical, and gene expression analyses. All of the animals were anesthetized with pentobarbital sodium following 30 days of continuous therapy. Euthanasia was conducted by delivering a deadly dosage of pentobarbital sodium (200 mg/kg body weight, intraperitoneally), for a swift and painless death. Death was established by the observation of extended cessation of breathing and heart activity, followed by a secondary physical approach to certify irreversible death before tissue collection. All measures were taken to mitigate animal suffering during the investigation. The heart and myocardial tissues were harvested under sterile settings promptly after death was confirmed.

FTIR analysis

FTIR was employed to identify the functional groups present in TXN and BCN. A PerkinElmer FTIR spectrometer was used to obtain spectra in the wavelength range of 4000 to 400 cm^{-1}.

Blood glucose test

Rats were subjected to overnight fasting for the collection of blood samples. The Tail vein puncture method was used to collect the blood samples, and fasting blood glucose (FBG) was measured with One Touch Select Plus Simple™ blood glucose monitoring technology on days 0, 5, 10, 15, and 30 across all experimental groups. After completing the 30-day study period, the rats underwent overnight fasting before undergoing an oral glucose tolerance test (OGTT).

Biochemical marker analysis

Kits purchased from Sigma Aldrich were used to measure the serum lipid profiles, which included total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low density lipoprotein (VLDL), as well as the cardiac marker enzymes creatine kinase-N-acetyl-L-cysteine (CK-NAC) and creatine kinase-muscle/brain (CK-MB).

Lipid peroxidation (LP) determination in heart tissue

The assessment of LP involved measuring the levels of malondialdehyde (MDA) to assess LP following a specific method [38]. In this process, MDA is a byproduct of LP, interacts with thiobarbituric acid (TBA) to form a pink-colored compound called thiobarbituric acid (TBA) reactive substance. Then, 50 µL of heart tissue homogenate was mixed with 100 µL of cold 10% trichloroacetic acid (TCA) and left on cold for 20 minutes for protein precipitation. The solution was then spun at 15,000 rpm for 20 minutes at a temperature of 4°C. The supernatant (100 µL) was combined with an equal volume of 0.7% TBA and then heated in a boiling water bath for 15 minutes. 1,1,3,3'-tetramethoxypropane was used as the standard for comparison. The thiobarbituric acid reactive substance (TBARS) level was measured with a spectrophotometer at 532 nm.

mRNA expression analysis

Extraction of RNA was performed from cardiac tissue samples with the Hi-cDNA synthesis Kit. cDNA was generated by moloney murine leukemia virus (MMLV) reverse transcriptase. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed for the specific genes SOD, HO-1, Nrf2, catalase and NAD(P)H quinone oxidoreductase 1 (NQO1) using SYBR Green PCR Master Mix by the standard comparative Ct method on a QuantStudio 5, ThermoScientific. The relative gene expression levels were calculated by the $2^{(-\Delta\Delta Ct)}$ method using the internal reference gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Histologic analysis

10% buffered formalin was used to preserve the heart tissues from the experimental groups. After fixing, alcohol was used to dehydrate the tissues. Xylene was used to clean the tissues, and the tissues were embedded in paraf-

fin wax. Microtome-based thin slices of tissues (5 μm thick) were stained with hematoxylin and eosin (H&E) and Masson's trichrome staining to identify the fibrotic areas in the tissues.

Immunofluorescence staining

H9c2 cardiomyocytes were cultured on sterile coverslips and subjected to treatment with TXN (25 μg/mL), BCN (200 μg/mL), or their combination in the presence of alloxan (AXN) to create an *in vitro* diabetes model (DIA), or in the absence of AXN as non-diabetic controls (ND). Post-treatment, cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, neutralized with 45 mM NH_4Cl, permeabilized with 0.25% Triton X-100, and blocked with 0.25% gelatin. Cells were treated with primary antibodies targeting HO-1 and Nrf2 for 2 hours at room temperature, subsequently followed by a 2-hour incubation with fluorophore-conjugated secondary antibodies. Following washing, the samples were affixed to slides utilizing Aqua-Poly/Mount (Polysciences) for examination with a Zeiss LSM510 META/FCS confocal laser scanning microscope. Nuclear DNA was stained with Draq5 (BioStatus) to enhance the visibility of cell nuclei.

Intracellular ROS measurement in H9c2 cardiomyocytes

Intracellular ROS levels were quantified using the cell-permeable fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). H9c2 cardiomyocytes were seeded in 6-well plates or on sterile coverslips and allowed to adhere overnight under standard culture conditions. Cells were then divided into the following experimental groups: control, alloxan (AXN), baicalein (BCN, 200 μg/mL), taxifolin (TXN, 25 μg/mL), BCN/TXN combination, and AXN in combination with BCN/TXN. To induce oxidative stress, cells were exposed to alloxan (AXN) for the indicated duration, followed by treatment with BCN, TXN, or their combination. After treatment, cells were washed twice with phosphate-buffered saline (PBS) and incubated with DCFH-DA (10 μM) diluted in serum-free DMEM for 30 min at 37°C in the dark. Following incubation, excess dye was removed by washing the cells three times with PBS. Fluorescence intensity corresponding to intracellular ROS generation was immediately visualized using a fluorescence microscope or confocal laser scanning microscope with excitation at 488 nm and emission at 525 nm. Quantitative analysis of ROS levels was performed by measuring mean fluorescence intensity using ImageJ software, and values were expressed relative to the control group.

Measurement of MDA and antioxidants

Commercially available assay kits (Sigma-Aldrich) were employed to quantify glutathione (GSH), total superoxide dismutase (SOD) activity, and malondialdehyde (MDA) levels in the samples. With the GSH and catalase (CAT) Assay Kit, SOD activity was measured using the Total Superoxide Dismutase Assay Kit, and lipid peroxidation was evaluated by means of MDA levels measured with the Lipid Peroxidation MDA Assay Kit. GSH and CAT Assay Kit specifically determined these activities. Every test was carried out in line with manufacturer guidelines.

In vitro cytotoxicity analysis

Different concentrations of TXN at 25 μg/ml, BCN at 200 μg/ml, and a combination of TXN (25 μg/ml) and BCN (200 μg/ml) were administered to H9c2 cardiomyocyte cells that were inoculated at 1.5×10^4 cells density per well microtiter plates. Cell Counting Kit-8 (CCK-8) reagent was added to each well after 6 and 12 h of incubation, and then the wells were incubated for 2 h at 37°C. Using a Thermo Scientific MultiskanSkyHigh microplate reader, absorbance was measured at 450 nm.

Inverted phase contrast microscopy was used to identify the changes in morphology of H9c2 cardiomyocyte cells after they were treated with TXN, BCN, and their combination for 6 and 12 h. Acridine Orange/Ethidium Bromide (AO/EB) and DAPI staining were used to analyze the morphological changes and cell death of AXN, TXN, and BCN exposed cells. Viable cells have bright green nuclei, whereas dead cells have brilliant orange/red nuclei, which is how AO/EB labeling distinguishes between live and dead cells based on fluorescent dye uptake. 100 μg/mL AO/EB and 10 mM DAPI stained cells were then allowed to sit at room temperature for 20 minutes in the dark. After that, stained cells were viewed under an Olympus fluorescent microscope.

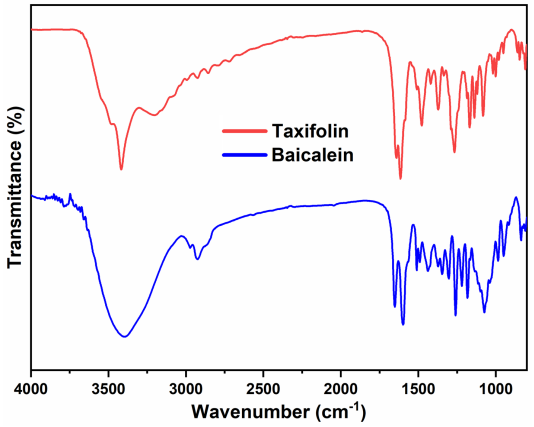

Figure 2. FTIR spectra of taxifolin (TXN) and baicalein (BCN).

Statistical analysis

Statistical analysis was performed using one-way ANOVA, followed by Tukey's post-hoc test for multiple group comparisons. Analysis of the data was conducted with GraphPad Prism version 9. The statistical significance of the comparisons between the diabetes group and the DIA+TXN, DIA+BCN, and DIA+TXN+BCN groups was determined as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 vs control. Control and diabetes groups were compared using the following *p*-values: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 vs control. Furthermore, denoted by * are comparisons between control groups and ND+TXN, ND+BCN, and ND+TXN+BCN groups. All data are presented as mean ± SD.

Results

Functional group characterization for TXN and BCN

In order to identify the functional group of TXN and BCN, FTIR analysis was performed. The FT-IR spectral results suggest that pronounced peak at 3420 cm^{-1} corresponds to the free O-H stretching vibration, indicative of hydroxyl groups in phenolic compounds; a peak at 1620 cm^{-1} is associated with C=O stretching in flavanones, suggesting the presence of a carbonyl group; a peak at 1360 cm^{-1} is situated between the O-H bending and C-O stretching vibrations, reflecting interactions involving hydroxyl groups; a peak at 1265 cm^{-1} pertains to C-O-C stretching in ether linkages, particularly in structures containing =C-O-C groups; and a peak at 1165 cm^{-1}, commonly observed in 5,7-dihydroxysubstituted flavonoids. This confirms the characteristic substitution pattern of these compounds (**Figure 2**).

Effect of TXN and BCN on food intake, water, and body weight loss

TXN and BCN treatment in diabetic rats significantly increased the body weight in comparison to the untreated diabetic group. This indicates that TXN and BCN may have positive effects on weight management in relation to diabetes. Diabetic rats demonstrated significantly higher levels of water and food intake than control rats (**Figure 3A-C**), suggesting a possible compensatory mechanism in reaction to the metabolic disturbances linked to diabetes. Diabetic rats with TXN and BCN treatment provided a significant decrease in food and water intake relative to the untreated diabetic group (**Figure 3A-C**).

This reduction may indicate enhanced metabolic regulation and a reduction in hyperphagia commonly observed in diabetic conditions. Nondiabetic rats administered TXN and BCN showed slight differences in body weight, water intake, and food consumption relative to control rats (**Figure 3**). This finding indicates that the treatments do not negatively effect normal physiological functions in healthy animals, thereby reinforcing the safety profile of these compounds. The results indicate that TAX and BCN could play a significant role in body weight management and appetite regulation in diabetic conditions while also preserving normal physiologic functions in nondiabetic individuals.

Effects of TXN and BCN on FBG and tolerance in diabetic rats

In control rats, the FBG level was within the usual range of 80 to 120 mg/dl. Diabetic rats exhibited significantly increased FBG levels during the entire experiment when compared to the nondiabetic group (**Figure 4A**). Comparing diabetic rats treated with TNX and BCN to diabetic rats treated with alloxan, it was remarkable that both groups had significantly lower FBG levels. Interestingly, FBG levels in non-diabetic rats with TNX and BCN treatment were not significantly different from control rats. OGTT was performed to assess the impact of TXN and BCN on glucose tolerance in both

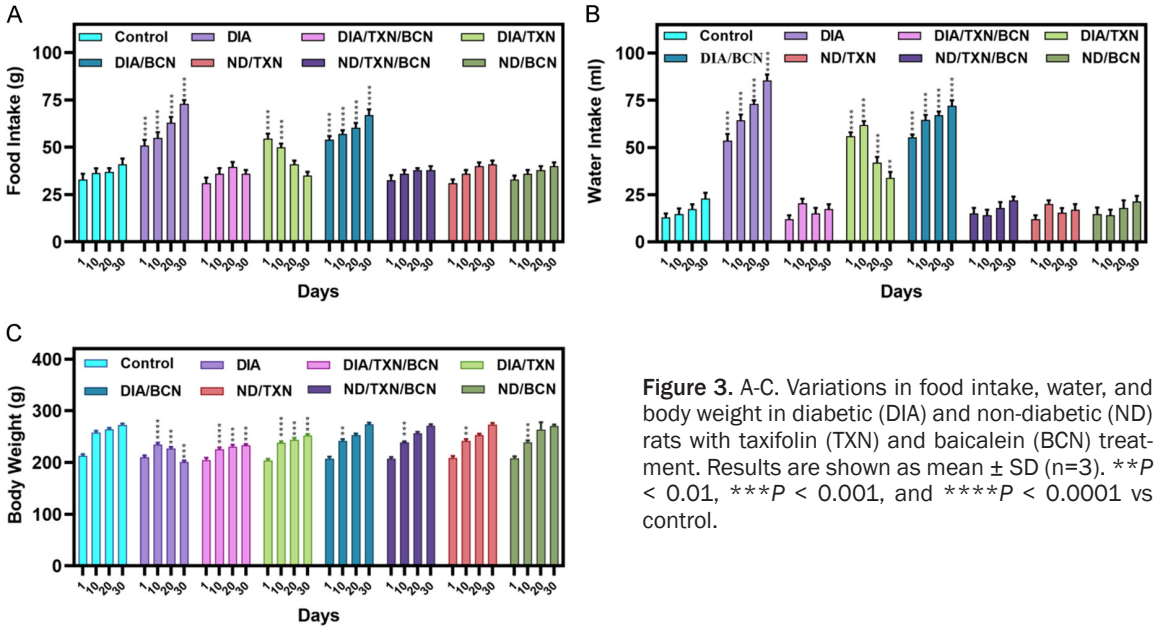

Figure 3. A-C. Variations in food intake, water, and body weight in diabetic (DIA) and non-diabetic (ND) rats with taxifolin (TXN) and baicalein (BCN) treatment. Results are shown as mean ± SD (n=3). $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs control.

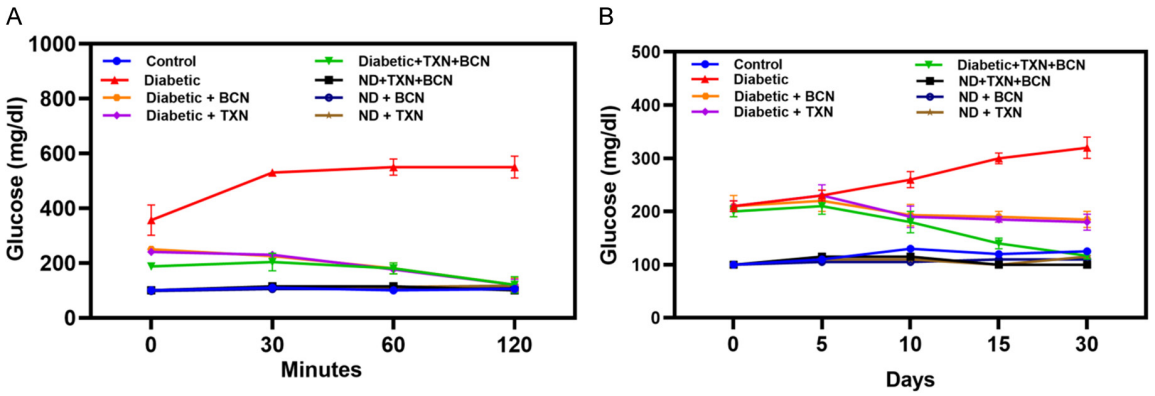

Figure 4. Blood glucose levels and glucose tolerance in nondiabetic (ND), diabetic, diabetic and non-diabetic treated with taxifolin (TXN) and baicalein (BCN) (alone and combination). A. Levels of glucose. B. Glucose tolerance.

diabetic and nondiabetic groups (**Figure 4B**). The findings indicated that diabetic rats exhibited significantly elevated FBG levels at 0-, 30-, 60-, and 120 min following glucose administration, suggesting impaired glucose metabolism. TNX and BCN significantly reduced the FBG levels after 2 h in diabetic rats. These results demonstrates that TNX and BCN reduce FBG levels in diabetic rats and enhance overall glucose tolerance. This suggests their potential as therapeutic agents for the management of hyperglycemia in diabetes. Investigating the mechanisms underlying these effects may yield important insight into their role in diabetes management and aid in the development of new treatment strategies.

Effect of TXN and BCN on lipid profile and cardiac enzyme levels

Assessment of lipid profile demonstrated substantial changes in diabetic rats, marked by increased levels of VLDL, LDL, TC, and TG, coupled with decreased levels of HDL relative to control rats (**Figure 5**). Conversely, diabetic rats with both TNX and BCN demonstrated significantly lowered levels of VLDL, LDL, TC, and TG, alongside elevated HDL levels, in comparison to the untreated diabetes cohort. In addition, non-diabetic rats administered TXN and BCN exhibited no significant alterations in lipid profile measures relative to control rats. Moreover, blood cardiac marker enzymes, including

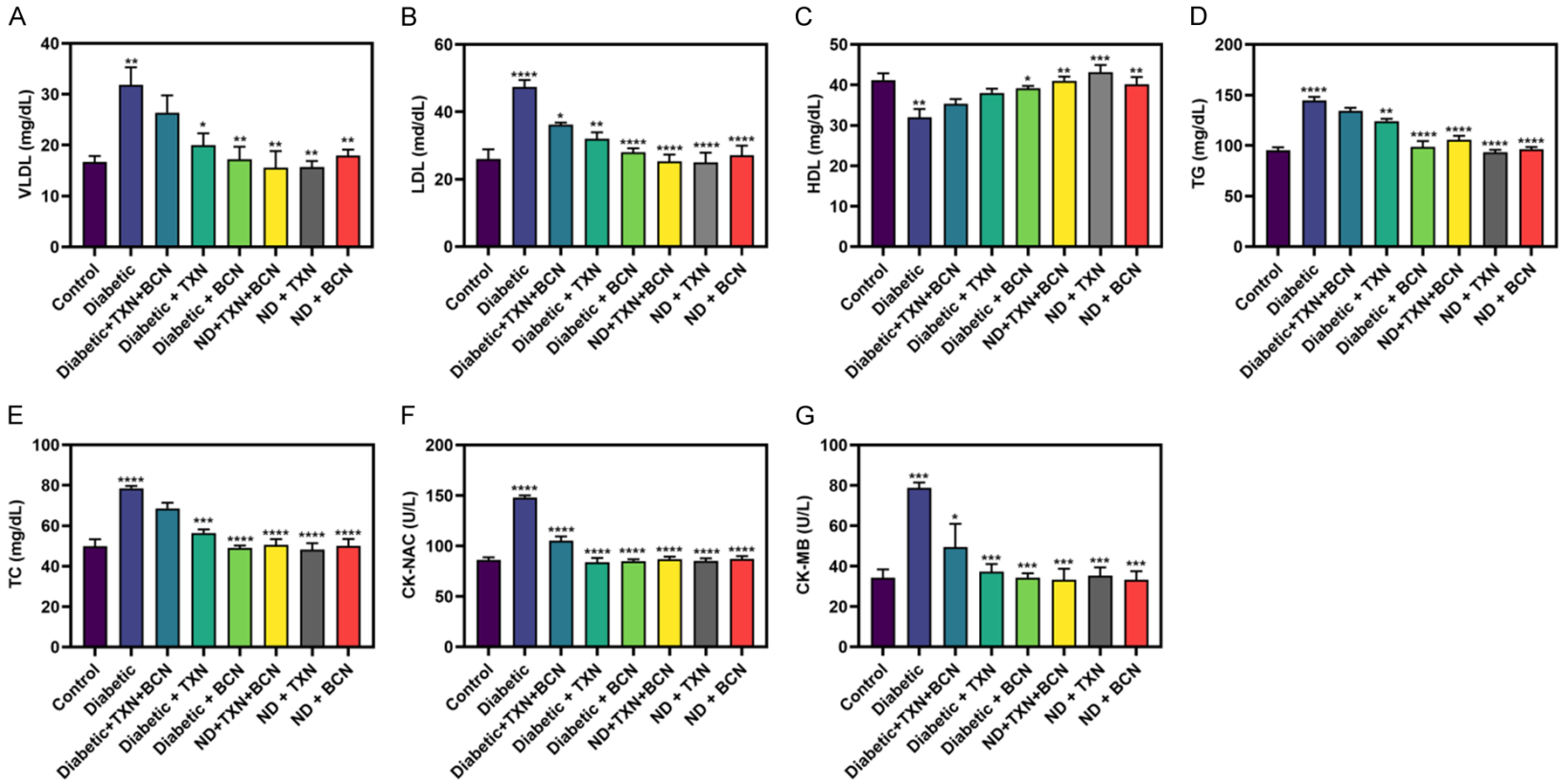

Figure 5. Impact of taxifolin (TXN) and baicalein (BCN) on blood lipid profiles and cardiac enzyme markers in diabetic rats. (A) VLDL, (B) LDL, (C) HDL, (D) TG, (E) TC, (F) CK-NAC and (G) CKMB. Results are ahown as mean ± SD (n=3). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ vs control.

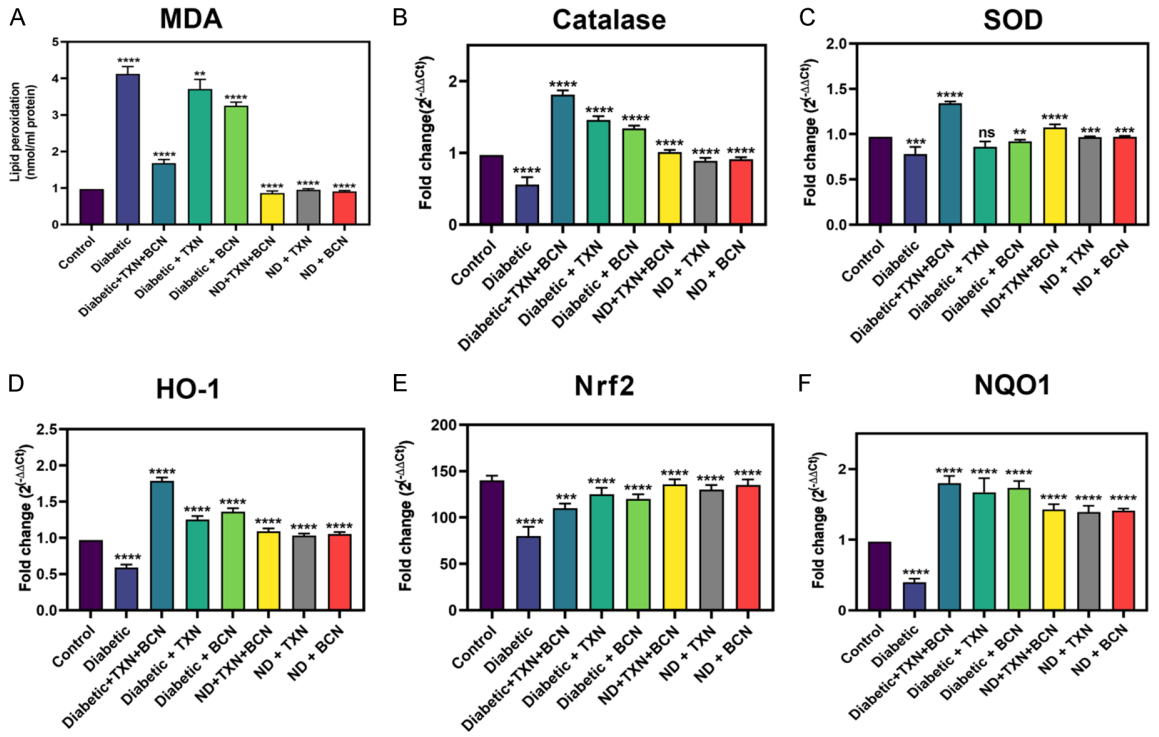

Figure 6. Oxidative stress response in the myocardium of control, diabetic, diabetic and non-diabetic rats treated with taxifolin (TXN) and baicalein (BCN) (alone and combination). (A) Levels of MDA, (B-F) mRNA expression of Catalase, SOD, HO-1, Nrf2 and NQO1. Results are shown as mean ± SD (n=3). $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs control, not significant (ns).

CK-MB and CK-NAC, were significantly higher in AXN-induced diabetic rats when compared to the control rats.

Diabetic rats administered dosages of TXN and BCN exhibited a substantial reduction in cardiac enzymes levels relative to the untreated diabetes cohort. Significantly, non-diabetic rats with TXN and BCN treatment showed no notable alterations in CK-NAC and CK-MB levels compared to control rats. The findings indicate that TXN and BCN treatment enhances lipid metabolism in diabetic conditions and provides a protective impact on heart functioning by reducing the activity of cardiac enzymes linked to myocardial damage.

Effects of TXN and BCN on MDA levels and oxidative stress

The study found that rats with diabetes had higher levels of MDA in comparison with the control rats. Conversely, diabetic rats with TNX and BCN demonstrated significantly reduced MDA levels compared to the untreated diabetic cohort (**Figure 6A**). Additionally, non-diabetic animals administered TNX and BCN exhibited no significant alterations in MDA levels relative to control rats. MDA is a recognized biomarker of lipid peroxidation, and its increased levels signify oxidative stress, frequently linked to diabetes. The decrease in MDA levels in diabetic rats administered TNX and BCN indicates that the treatment could increase antioxidant defences or mitigate LP processes induced by hyperglycemia. This finding aligns with previous research highlighting the critical role of oxidative stress in the development of diabetes, which may lead to serious complications such as cardiovascular disease [12]. The absence of notable alterations in MDA levels in non-diabetic rats administered TNX and BCN supports the assertion that these chemicals do not worsen normal physiologic states.

Enhancement of Nrf2 target gene expression by TXN and BCN

We assessed the cardioprotective effects of TXN and BCN by quantifying the expression of

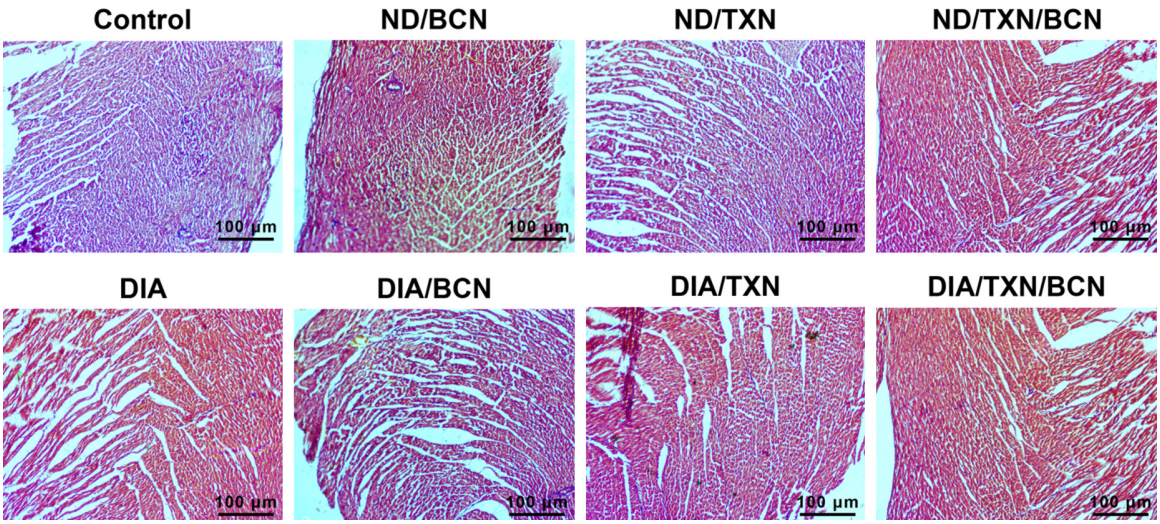

Figure 7. Impact of taxifolin (TXN) and baicalein (BCN) on histopathological changes in the myocardium of alloxan-induced DCM. Masson's trichrome staining of myocardial tissue from control, diabetic, and diabetic/non-diabetic rats treated with TXN and BCN, both alone and in combination. DIA - Diabetics and ND - Non-diabetics.

pivotal Nrf2 target genes, including catalase, SOD, HO-1 and NQO1, utilizing qRT-PCR. The findings demonstrated that the expression levels of the antioxidant genes were significantly downregulated in diabetic rats relative to non-diabetic control rats (**Figure 6B-F**). Diabetic rats administered TXN and BCN demonstrated a significant elevation of SOD, HO-1, Nrf2, NQO1 and catalase expression levels in comparison to untreated diabetic rats. Significantly, non-diabetic rats administered TXN and BCN exhibited no substantial alterations in the expression of SOD, HO-1, Nrf2, NQO1 or catalase relative to control rats.

The overexpression of these genes suggests that TXN and BCN may stimulate the Nrf2 signaling pathway, enhancing cellular defense mechanisms against oxidative damage. The absence of notable alterations in non-diabetic rats further substantiates the idea that these chemicals primarily address diseased situations rather than modifying normal physiologic processes.

Histopathologic evaluation of cardiac structure in TXN and BCN treated diabetic rats

Histopathologic study indicated that control rats displayed a normal cardiac architecture, distinguished by systematically organized myocardial fibers. The myocardium of alloxan-induced diabetic rats exhibited notable abnormalities, such as disordered myocardial cells, ruptured myocardial fibers, uneven cytoplasmic distribution, and abnormally shaped nuclei. The TXN and BCN treated diabetic rats showed a significant reduction in myocardial injury and fibrosis relative to untreated diabetic rats (**Figure 7**). This indicates that TXN and BCN may provide protective benefits to heart tissue in the context of diabetes. Furthermore, non-diabetic rats treated with TXN and BCN retained a normal cardiac structure, akin to that of control rats. The data indicate that the medication does not negatively impact heart structure in healthy individuals while offering substantial advantages in diabetes circumstances. The MT staining showed that there is increased collagen in the myocardium tissues treated with TXN and BCN when compared to the control and diabetic conditions (**Figure 8**).

Nrf2 and HO-1 protein activation by TXN and BCN

The gene expression study revealed that TXN and BCN confer cardioprotective effects through the activation of the Nrf2/HO-1 signaling pathway. To validate these findings, we conducted protein expression analyses on H9c2 cardiomyocytes cell lines with specific antibodies for Nrf2 and HO-1.

The findings demonstrated a notable reduction in the levels of HO-1 and Nrf2 proteins in the

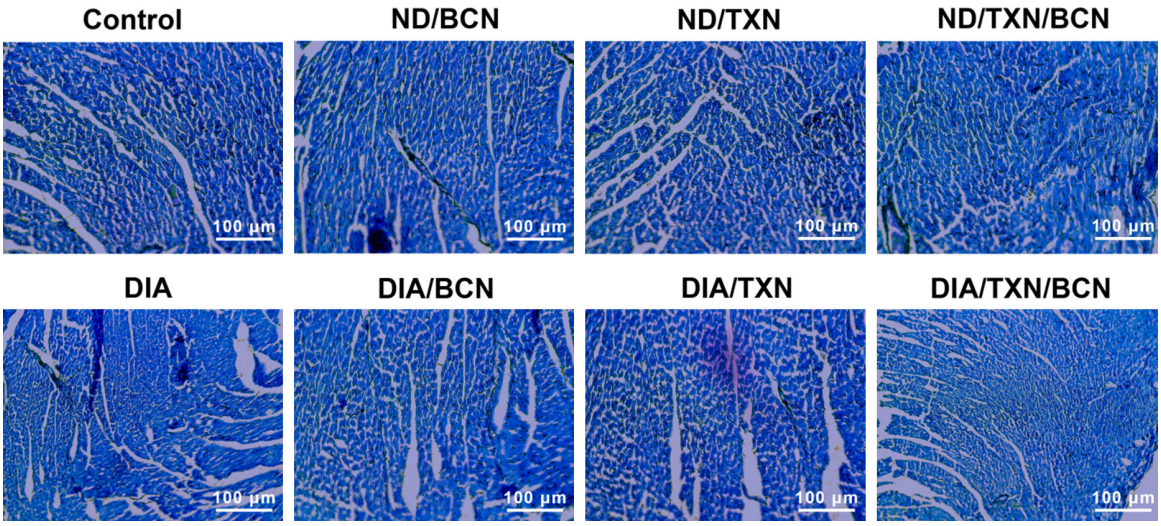

Figure 8. Masson's trichrome staining of myocardial tissue from diabetic/non-diabetic controls and diabetic/non-diabetic rats treated with taxifolin (TXN) and baicalein (BCN), both individually and in combination. DIA - Diabetics and ND - Non-diabetics.

AXN-induced diabetes groups relative to the experimental groups. Conversely, H9c2 cardiomyocytes subjected to TXN and BCN exhibited a statistically significant elevation in Nrf2 and HO-1 expression relative to the untreated condition (**Figures 9**-**11**). The increase in Nrf2 and HO-1 levels after treatment with TXN and BCN indicates that these compounds may improve the capacity to alleviate oxidative stress, thereby offering cardioprotection in diabetes conditions. No alterations in Nrf2 and HO-1 expression were seen in control cells treated with TXN and BCN, suggesting that these drugs do not affect cellular shape. The findings underscore the promise of TXN and BCN as therapeutic agents for addressing oxidative stress-related issues linked to diabetes, by activating protective pathways that may mitigate cardiovascular risk.

Nrf2-mediated transcriptional activation is probably affected by changes in the subcellular location of Nrf2 and Keap1 brought about by disturbance of Keap1's nuclear export signal (NES). Immunostaining was done to look at the nuclear translocation of Nrf2 in cells created in an alloxan-induced state. Alloxan treatment clearly lowered Nrf2 expression, as seen in **Figure 11**. Especially, Nrf2 expression was much raised in cells treated synergistically with TXN and BCN as compared to both the control group and non-diabetic cells treated with TXN and BCN.

Cytotoxicity analysis of TXN and BCN treatment in H9c2 cardiomyocytes

In vitro cytotoxicity analyses were employed to evaluate cell survival, proliferation, and migration to assess the effects of a combination treatment involving TXN and BCN. The results demonstrated that the TXN and BCN combination did not display cytotoxic reactions on H9c2 cardiomyocytes after 6 and 12 hours of treatment, indicating that these medicines are suitable for cellular applications (**Figure 12**). AO/EB staining was conducted to further validate the viability of cells (**Figure 13**). This approach effectively differentiates between viable and non-viable cells based on membrane integrity; the findings demonstrated no adverse effects on cell viability, suggesting that TXN and BCN are not negatively affecting cellular integrity.

Furthermore, intracellular concentrations of ROS were evaluated through microscopic analysis. In control cells, only a limited number displayed green fluorescence, signifying reduced ROS levels. Treatment with Alloxan, however, significantly increased ROS levels (**Figure 14**). The TXN and BCN combination significantly reduced free radical levels in a dose-dependent manner, reinforcing its promise as a therapeutic agent for improving cellular health and facilitating wound healing processes.

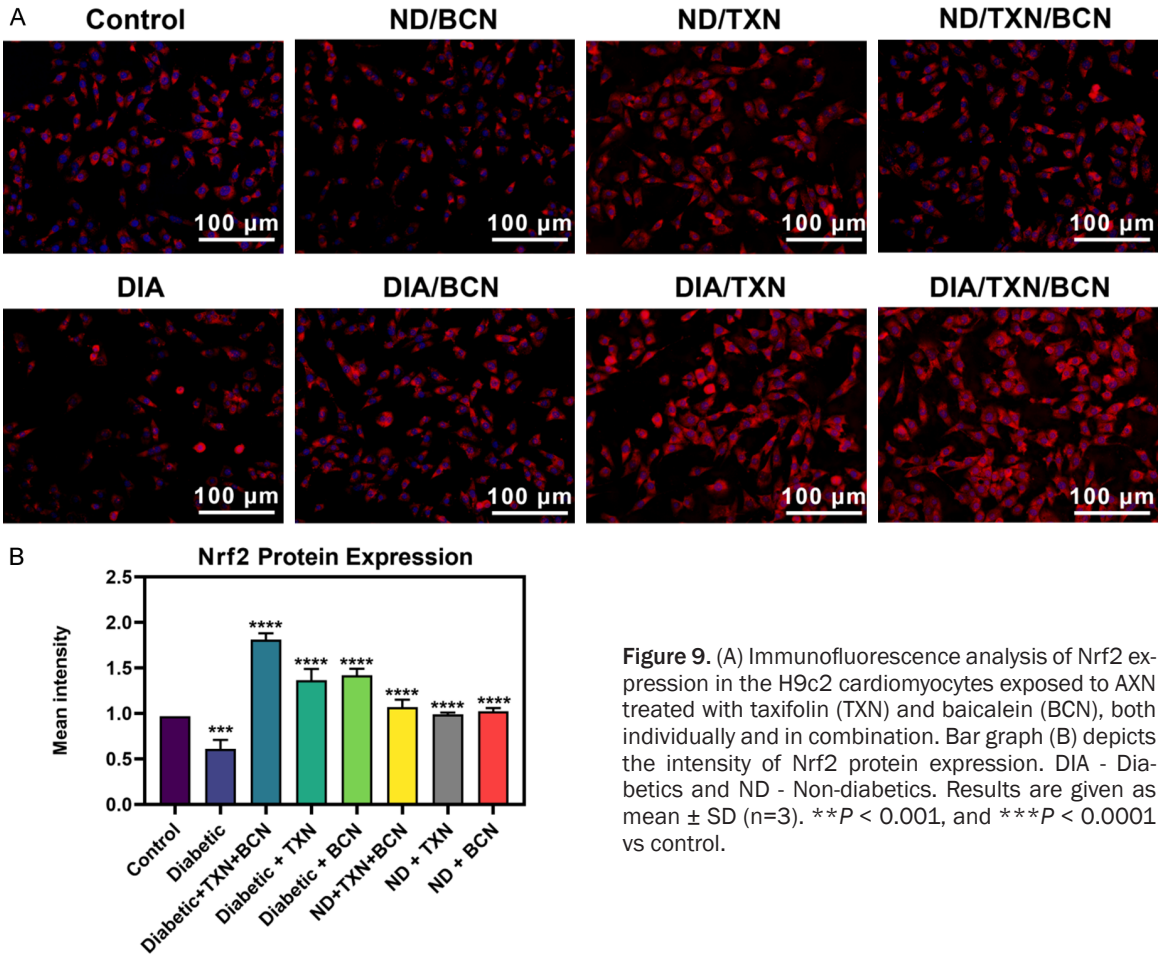

Figure 9. (A) Immunofluorescence analysis of Nrf2 expression in the H9c2 cardiomyocytes exposed to AXN treated with taxifolin (TXN) and baicalein (BCN), both individually and in combination. Bar graph (B) depicts the intensity of Nrf2 protein expression. DIA - Diabetics and ND - Non-diabetics. Results are given as mean ± SD (n=3). $^{**}P < 0.001$, and $^{***}P < 0.0001$ vs control.

To assess the impact of TXN and BCN co-treatment on reactive oxygen species (ROS) levels, we measured the concentrations of malondialdehyde (MDA), catalase (CAT), glutathione (GSH), and superoxide dismutase (SOD). As demonstrated in **Figure 15**, alloxan administration led to a significant increase in MDA levels, indicating elevated oxidative stress; however, this increase was markedly reduced following co-treatment with TXN and BCN. In non-diabetic conditions, MDA levels remained comparable to those observed in the control group. Furthermore, antioxidant markers - including CAT, GSH, and SOD - were significantly decreased in the alloxan-treated group, but their levels were significantly restored upon treatment with the combination of TXN and BCN.

Discussion

Diabetic cardiomyopathy (DCM) is a characteristic complication of diabetes mellitus, highlighted by cardiac dysfunction and anatomic alterations that may occur independently of coronary artery disease or hypertension [39]. This syndrome is mostly attributed to metabolic irregularities, including increased dyslipidemia, hyperglycemia, insulin resistance, and free fatty acid release [39, 40]. Moreover, persons with type 2 diabetes frequently encounter comorbidities that elevate the risk of left ventricular hypertrophy and ischemic damage, exacerbating the burden of heart failure [40]. Contemporary diabetes care approaches, encompassing biguanides, PPAR agonists, sulfonylureas, and glucosidase inhibitors, proficiently regulate blood glucose levels but often result in undesirable consequences, including hypoglycemia, weight gain, diminished therapeutic efficiency, and gastrointestinal distress [41, 42]. As a result, there is an urgent need for the development of new, effective, and well-tolerated antidiabetic drugs.

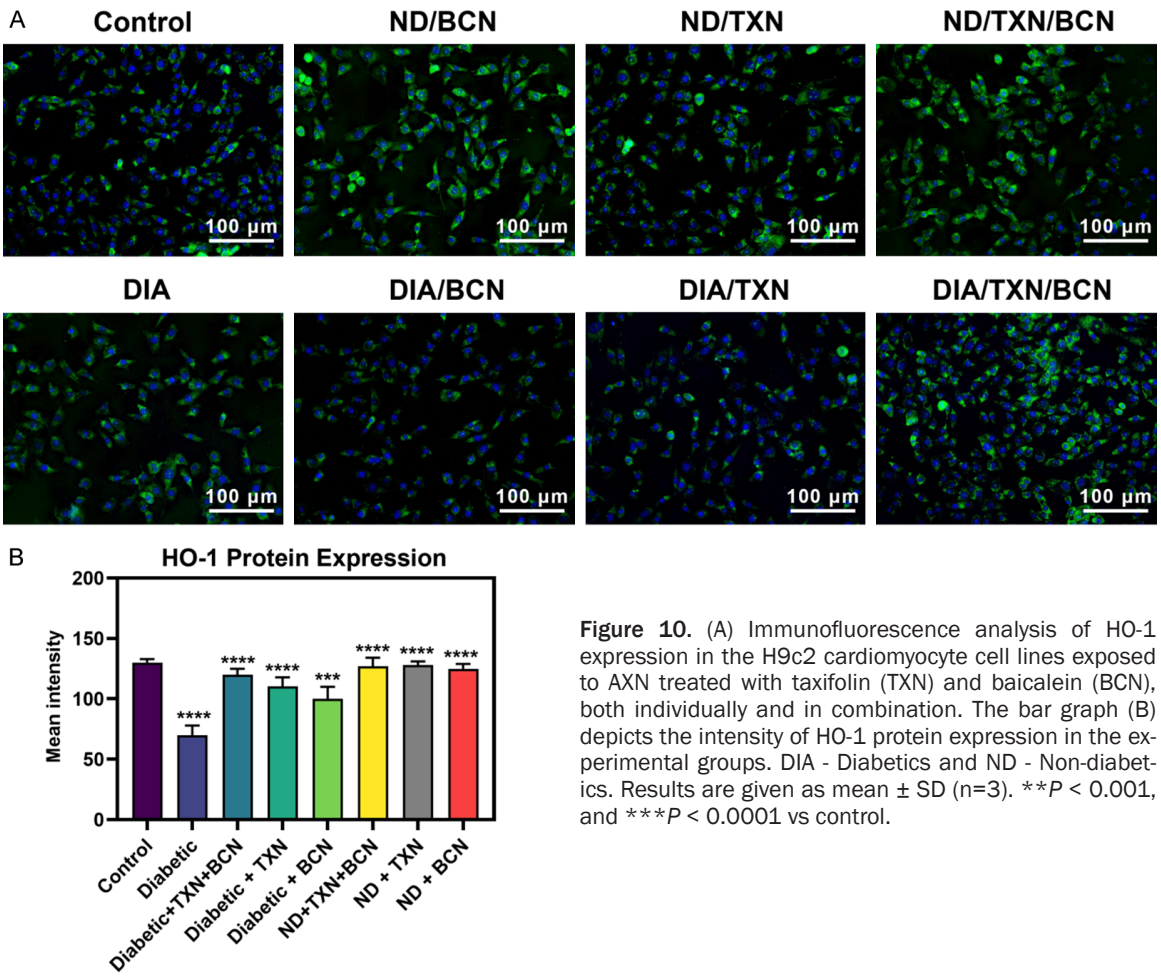

Figure 10. (A) Immunofluorescence analysis of HO-1 expression in the H9c2 cardiomyocyte cell lines exposed to AXN treated with taxifolin (TXN) and baicalein (BCN), both individually and in combination. The bar graph (B) depicts the intensity of HO-1 protein expression in the experimental groups. DIA - Diabetics and ND - Non-diabetics. Results are given as mean ± SD (n=3). $^{**}P < 0.001$, and $^{***}P < 0.0001$ vs control.

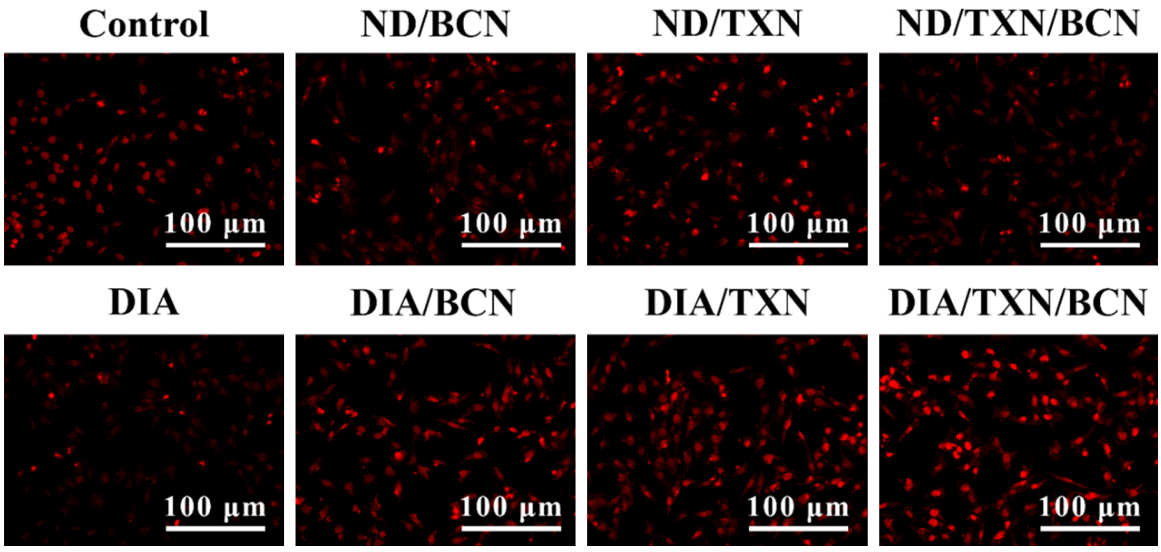

Figure 11. Nuclear translocated NRF-2 expression in H9c2 cardiomyocytes treated with alloxan and, synergistic combination of taxifolin (TXN) and baicalein (BCN). ND - non-diabetic; DIA - Diabetes.

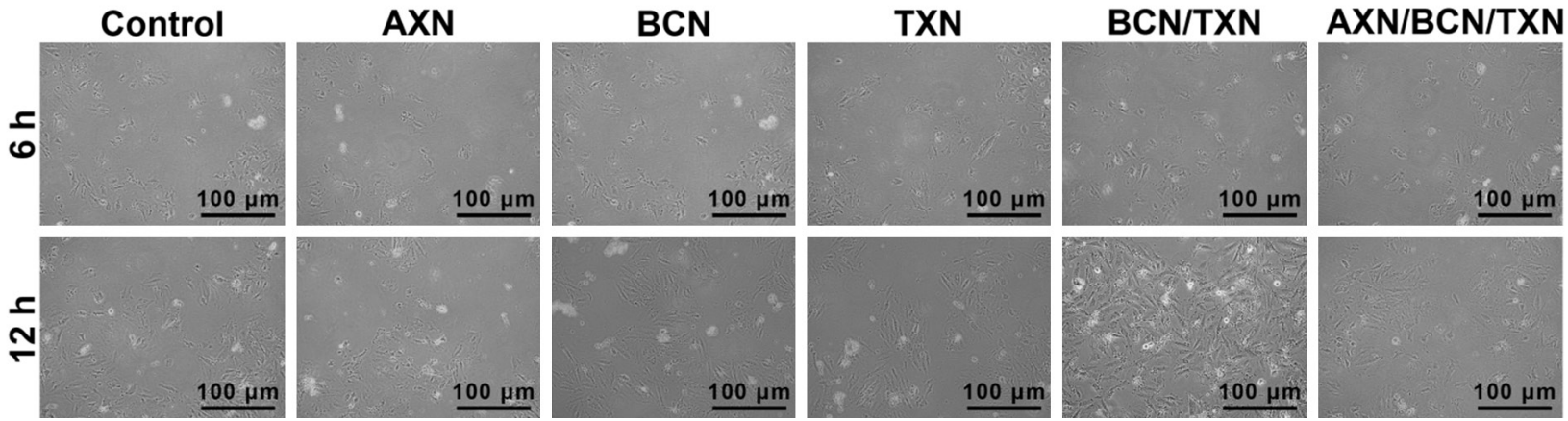

Figure 12. Cytotoxicity analysis of H9c2 cardiomyocytes treated with Alloxan (AXN), Baicalein (BCN), Taxifolin (TXN), combination of BCN/TXN, and AXN combination with BCN/TXN.

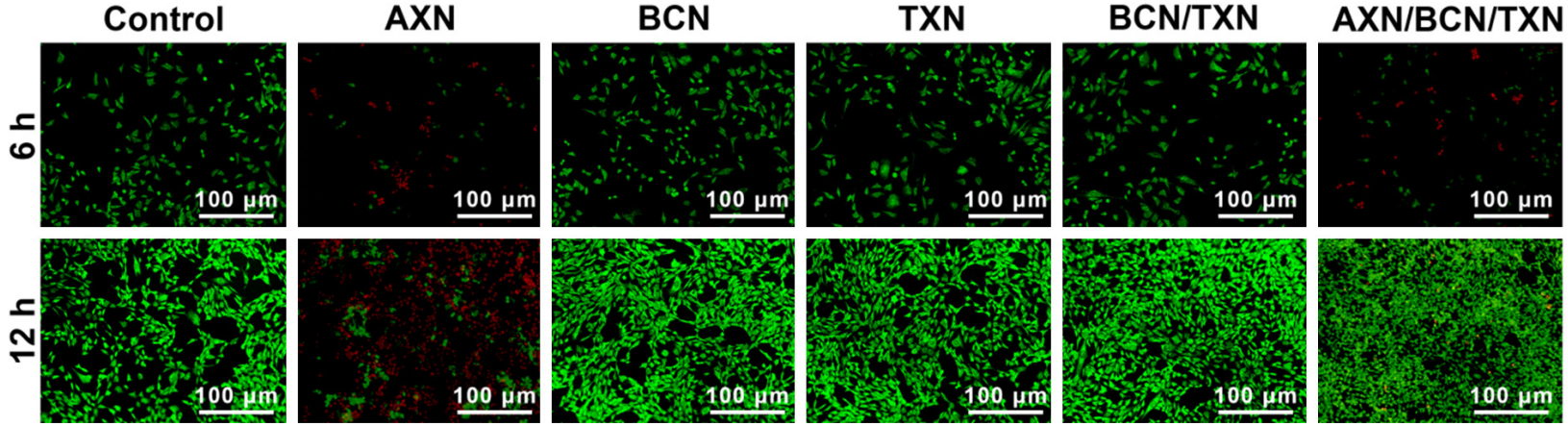

Figure 13. Cell viability analysis of H9c2 cardiomyocytes treated with Alloxan (AXN), Baicalein (BCN), Taxifolin (TXN), combination of BCN/TXN, and AXN combination with BCN/TXN.

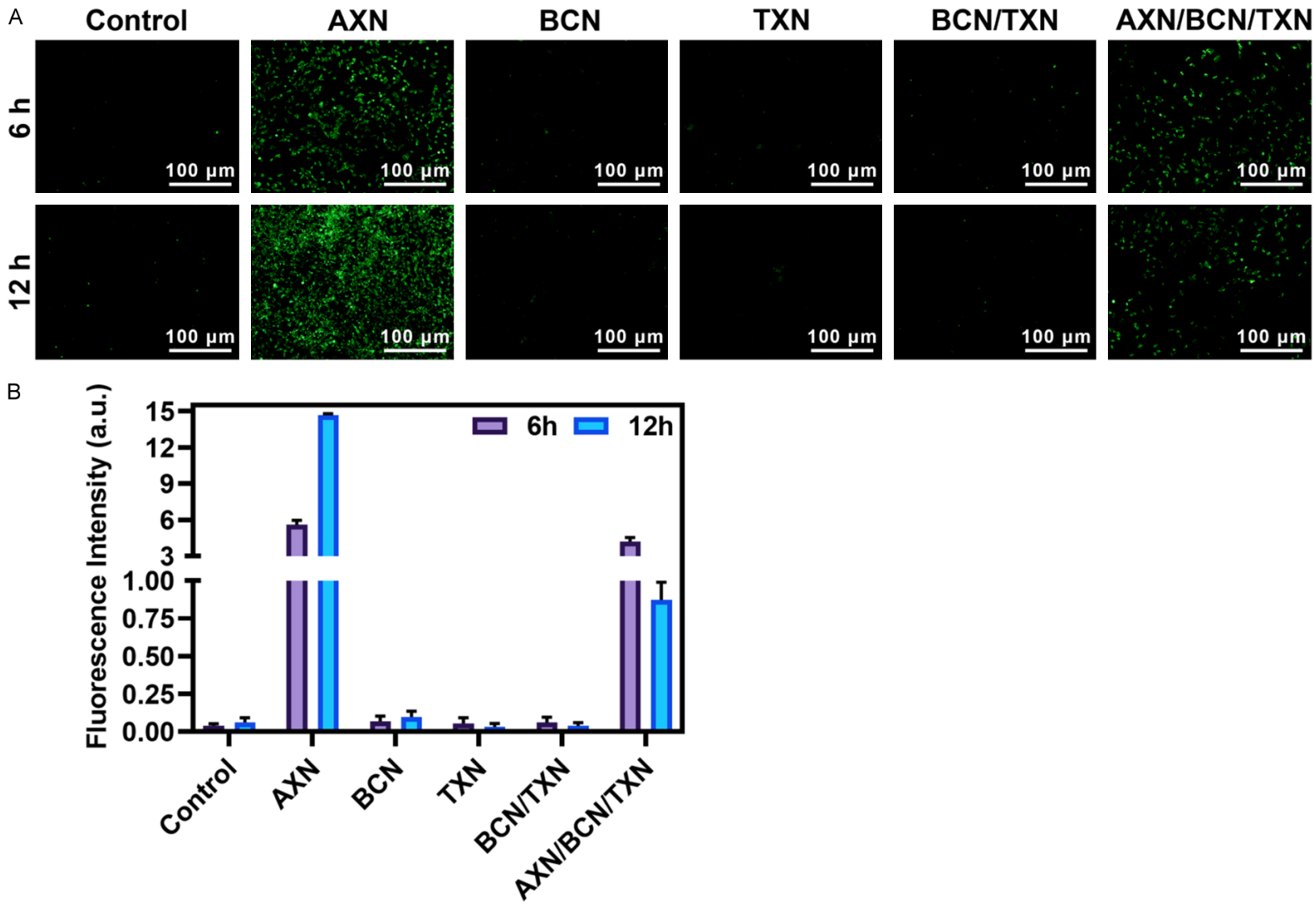

Figure 14. ROS analysis of H9c2 cardiomyocytes with Alloxan (AXN), Baicalein (BCN), Taxifolin (TXN), combination of BCN/TXN, and AXN combination with BCN/TXN.

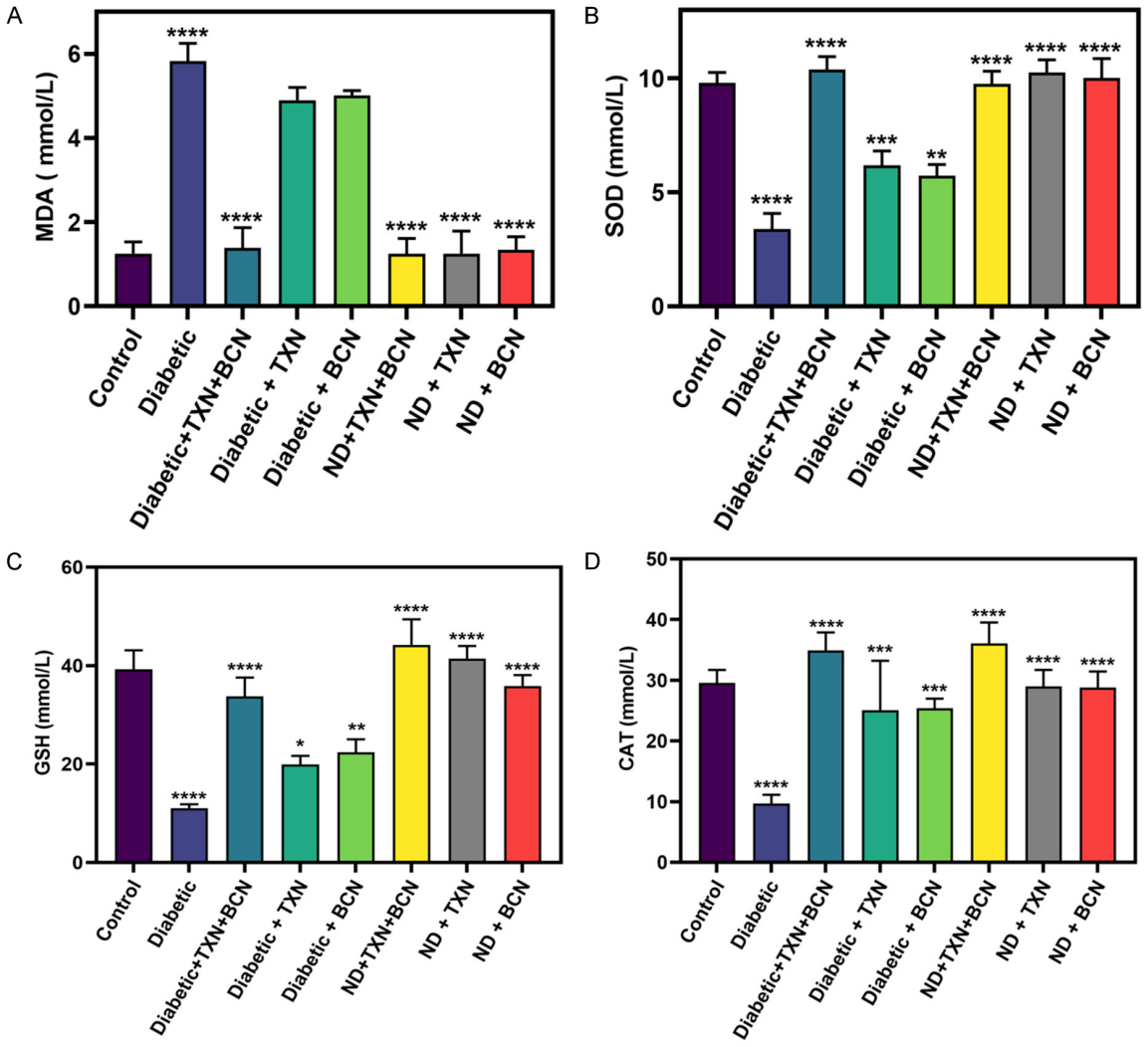

Figure 15. MDA and antioxidant expression in rats treated with synergistic combination of Baicalein (BCN) and Taxifolin (TXN). Level of (A) MDA, (B) CAT, (C) GSH and (D) SOD. Results are given as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ vs control.

Recently, plant flavonoids have been widely used for their various health advantages, including antioxidant and anticancer effects [43]. This study intends to assess the cardioprotective properties of the TXN and BCN combination in relation to alloxan-induced diabetic cardiomyopathy in rat models. The results we obtained suggest that alloxan-induced diabetic rats had characteristic symptoms of chronic hyperglycemia, including polyphagia, polyuria, substantial weight loss, and polydipsia, associated with elevated food and water consumption. AXN induced-diabetic rats with TXN and BCN combination treatment provided a significant reduction in food and water intake, indicating its antidiabetic effectiveness. Moreover, FBG levels significantly decreased in diabetic rats administered the TXN and BCN combination in comparison to untreated DCM rats. These findings align with contemporary research highlighting the importance of blood glucose regulation in diabetes management and its related cardiovascular implications.

OGTT demonstrated a slow increase followed by a rapid reduction in glucose levels in the blood of diabetic rats administered TXN and BCN, thereby substantiating its glucose-lowering properties. Diabetic rats exhibited significant dyslipidemia, marked by increased TC, TG, LDL, and VLDL alongside diminished

high-density lipoprotein levels [44]. Conversely, diabetic rats with TXN and BCN treatment at dosages of 25 and 200 mg/kg demonstrated substantial improvements in their lipid profiles, characterized by reductions in LDL, TC, VLDL, TG, and levels, coupled with elevated HDL levels. Increased levels of cardiac enzymes, specifically CK-NAC and CK-MB, indicated cardiac damage in diabetic rats. Treatment with TXN and BCN led to significantly reduced levels of these markers in comparison to untreated diabetic rats. This indicates that TXN and BCN have cardioprotective attributes. Alloxan is recognized for inducing inflammation and oxidative stress in pancreatic cells, resulting in cytotoxicity [45, 46]. In the current study, alloxan-treated diabetic rats demonstrated increased MDA levels, an indicator of oxidative stress, in comparison to control rats [47, 48]. Treatment with TXN and BCN dramatically decreased MDA levels, underscoring its antioxidant properties.

We examined the Nrf2 signaling pathway to understand the molecular processes that contribute to the cardioprotective effects of TXN and BCN. Nrf2 is an essential transcription factor that plays a pivotal role in sustaining cellular redox equilibrium [49]. In oxidative stress situations, Nrf2 separates from *keap1* and migrates to the nucleus to activate ARE, thereby triggering the transcription of several antioxidant genes, including HO-1 and NQO1 [50, 51]. Our findings demonstrated that diabetic rats displayed reduced Nrf2 expression and its target genes relative to controls; yet, TXN and BCN therapy significantly restored these levels.

Histopathologic assessment indicated that control rats had normal cardiac architecture, but diabetic rats demonstrated disordered myocardial fibers and cardiac fibrosis. Treatment with TXN and BCN significantly reduced myocardial damage and fibrosis in diabetic rats. This study reveals that TXN and BCN provided considerable cardioprotective effects against alloxan-induced diabetic cardiomyopathy through the crucial mechanisms of improving glucose metabolism and lipid profiles while increasing antioxidant defences by Nrf2 signaling pathway activation. The results indicate that TXN and BCN could function as potential therapeutic agent for alleviating the detrimental effects of diabetes on heart health while maintaining normal physiologic capabilities in non-diabetic persons.

Conclusion

The study reveals that TXN and BCN have significant therapeutic benefits in addressing diabetes-related complications. The combination treatment reduced hyperglycemia, improved body weight management, and improved appetite regulation in diabetic rats, while preserving normal physiologic functions in healthy subjects. TXN and BCN also showed protective effects on lipid profiles and cardiac health by lowering oxidative stress markers and enhancing antioxidant gene expression through the Nrf2 signaling pathway. The absence of cytotoxic effects on cell viability further supports their safety for clinical use. The study suggests that TXN and BCN not only mitigate diabetes-related effects on cardiac health but also enhance overall metabolic function, making them valuable candidates for future DCM treatment strategies.

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Disclosure of conflict of interest

None.

Address correspondence to: Liangping Zhao, Department of Cardiology, The Fourth Affiliated Hospital of Soochow University (Suzhou Dushu Lake Hospital), Suzhou 215000, Jiangsu, China. Tel: +86-13913187252; Fax: +86-13913187252; ORCID: 0009-0008-4494-830X; E-mail: zhaoliangping01@outlook.com

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