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The Therapeutic Effect of Quercetin in Combination with Metformin on Apoptotic Pathway in the Cardiac Tissues of Experimental Diabetic Rat Model.

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ABSTRACT:

Type 2 diabetes mellitus, often known as (T2DM), is considered a serious issue for public health on a global scale. It is known as a chronic metabolic condition that is characterized by persistent hyperglycemia in response to insulin resistance. The main causes of the increased morbidity rate among diabetic individuals are typically cardiovascular conditions. Quercetin is the most prevalent bioflavonoid, which is found in foods like citrus fruits, vegetables, and tea. This study aimed to compare the effect of traditional medication metformin to quercetin on the apoptotic pathway in the cardiac tissues of experimental diabetic rats. For this study, 40 male albino rats were selected and divided into the five following groups: the control group, untreated diabetic group, metformin-treated group, quercetin-treated group, and combined group treated with both quercetin and metformin. After four weeks from the treatment, blood samples and cardiac tissues were collected for biochemical and molecular studies. It was observed that the combined use of metformin and quercetin reduced insulin resistance and improved lipid profiles. Quercetin reduces oxidative and inflammatory damage to many tissues. The results showed a significant downregulation in nuclear factor kappa B (NF-κB) and a significant upregulation in nuclear factor (erythroid-derived 2)-like 2 (Nrf2) gene expression. It was also noticed that quercetin significantly downregulated the gene expression of caspase-3 (CASP3) and Bcl-2-associated X (Bax) in the cardiac tissues. Quercetin may be used in conjunction with metformin as an adjuvant therapy to increase the effectiveness of metformin in the treatment of T2DM.

Keywords: Type 2 diabetes mellitus, quercetin, nuclear factor kappa B, nuclear factor (erythroid-derived 2)-like 2, and Bcl-2-associated X

INTRODUCTION

Persistent hyperglycemia is a hallmark of diabetes mellitus (DM), a chronic metabolic disease. It might be brought on by a decline in insulin synthesis, peripheral organ insulin resistance, or both. In more than 90% of cases, type 2 diabetes mellitus (T2DM) is the cause of the disease **(Bird et al., 2015).** It is believed that by 2045, there will be up to 700 million diabetic patients worldwide **(International Diabetes Federation (IDF), 2019)**. The main causes of the increased morbidity rate among diabetic individuals are typically cardiovascular conditions **(Einarson et al., 2018)**. According to a study on cardiovascular-associated hospitalizations, More over 35% of all hospitalisations were for individuals with diabetes mellitus, and more than 25% were for cardiovascular issues linked to diabetes **(Institute for Public Health (IPH), 2015).**

Increased oxidative stress-induced myocardial injury is highly correlated with chronic hyperglycemia. The significant association between matrix remodeling and oxidative stress in cardiomyocytes isolated from diabetic heart tissues in numerous laboratory experiments has validated this. Reactive oxygen species (ROS) levels in cardiomyocytes are elevated, which exacerbates oxidative stress. Myocardial apoptosis results from abnormal ROS production, which enhances pro-inflammatory response **(Dludla et al., 2017).**

Apoptosis processes in the body are significantly influenced by cysteine-dependent aspartate-specific proteases, which are called caspases. A crucial apoptosis-related enzyme called caspase-3 (CASP3) is the pathway's effector in the caspase apoptotic cascade. Both the death receptor apoptotic and mitochondrial pathways activate this enzyme **(Pujara & Chaudhary, 2017).** Bcl-2-associated X (Bax) extends across the mitochondria's inner and outer membranes. This causes cytochrome C to be liberated from the mitochondria and into the cytoplasm, where it forms the apoptosome complex by interacting with apoptosis protease activating factor-1 (Apaf-1). The downstream caspases are cleaved and activated, which is mediated by apoptosomes and ultimately results in cell death **(D'Arcy, 2019)**.

In eukaryotes, the nuclear factor kappa B (NF-κB) is a crucial transcription

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factor that controls a broad range of processes inside the cell, such as inflammation, immune response, growth, development, and apoptosis. The activation of inflammatory signaling pathways and aberrant cytokine production are characteristics of systemic low chronic inflammation associated with T2DM. Increased NF-kB expression plays a crucial role in the pathophysiology of a number of inflammatory diseases, including diabetic nephropathy **(Montero et al., 2016).**

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is regarded as being the main regulator of the antioxidant response. The activation of Nrf2 in conditions including metabolic syndrome, , retinopathy, neuropathy and nephropathy delays the onset of diabetes and associated consequences, according to recent studies. Natural substances from fruits, vegetables, fungi, and micronutrients, including curcumin, resveratrol, vitamin D, and quercetin have been shown to activate Nrf2 and enhance antioxidant pathways to reduce oxidative stress and hyperglycemia-related damage. Quercetin acts as a scavenger of ROS and a suppressor of peroxidation reactions. Consequently, it offers numerous medicinal benefits, such as anti-inflammatory, anti-oxidant, anti-tumor, and immunomodulatory effects **(Jiménez-Osorio et al., 2015).**

Creatine kinase (CK) in both cardiac and non-cardiac diseases nevertheless has some diagnostic relevance. Four hours after myocardial damage, the serum level of CK-MB is greater selectivity for cardiac tissue **(Aydin et al., 2019)**. Lactate dehydrogenase (LDH) is an additional possible biomarker that could be used to identify myocardial damage. In the blood, LDH rises 6 to 12 hours after acute myocardial damage **(Patibandla et al., 2023).**

This study's aim was to evaluate the possible therapeutic effect of quercetin on the apoptotic pathway in the cardiac tissues of an experimental model of T2DM and to compare its effect with a conventionally used anti-diabetic drug (metformin) through the determination of the gene expression of NF-κB, Nrf2, CASP3, and Bax in cardiac tissues.

2. MATERIALS AND METHODS

2.1. Experimental animals

Forty male Wistar albino rats aged 2 months (100-120g) were used. The animals were collected from the Animal House, Medical Research Institute, Alexandria University, Egypt. Prior to experimentation, rats were housed in standard cages with a 12-hour light/dark cycle, unlimited access to food and water, and a well-ventilated environment at 25 ± 2 °C and 43 ± 3 relative humidity.

2.2. Ethical statement

All experiments followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and were authorized by the Alexandria University Institutional Animal Care and Use Committee (IACUC) (Approval No.: AU01219101613). The study additionally followed ARRIVE standards and the National Research Council's guidelines for laboratory animal care and use.

2.3. The induction of T2DM

For four weeks, the rats were fed an obesogenic diet. The obesogenic diet´s constituents were 30 g protein (300 kcal/g diet), 26.5 g fat (195 kcal/g lard, 70 kcal/g corn oil), 36.5 g carbohydrate (105 kcal/g dextran, 106 kcal/g corn starch, 140 kcal/g sucrose), 3 g vitamin mix (30 kcal/g), and 4 g mineral mix (40 kcal/g) **(Kamel et al., 2014).** Alloxan (200 mg/kg) dissolved in distilled water was administered intraperitoneally (IP) as a single dose to induce type 2 diabetes (T2DM) **(Ighodaro et al., 2017)**. Rats that had a fasting blood glucose level of more than 200 mg/dl after two days of injection, as determined by a glucometer (ACCU CHEK Active, Roche Co.), were classified as diabetic and selected for the study.

2.4. The treatment of T2DM

Quercetin was bought from Sigma Aldrich and administered to rats intraperitoneally dissolved in dissolved in diluted dimethyl sulfoxide at a dose of 50 mg/kg daily for a duration of 4 weeks **(Gaballah et al., 2017)**. Metformin was available in tablet form under the brand name Glucophage (a Merc Pharmaceuticals product). Each tablet contains 1000 mg. After being dissolved in distilled water, they were given to the rats orally via gastric gavage at a dose of 200 mg/kg daily for 4 weeks **(Jin et al., 2017).**

2.5. The design of experiment

Forty male rats were divided into five groups (8 rats each). **Group I (control group)**: healthy rats that were kept under a normal diet and no treatment was administered. **Group II (diabetic group)**: diabetic untreated rats. **Group III**: diabetic rats that received quercetin IP at a dose of 50 mg/kg daily for 4 weeks **(Gaballah et al., 2017)**. **Group IV:** diabetic rats that received metformin orally at a dose of 200 mg/kg daily for 4 weeks **(Jin et al., 2017)**. **Group V:** diabetic rats that received both quercetin and metformin daily for 4 weeks.

2.6. Collection of Samples

All rats were sacrificed by decapitation after anesthesia with isoflurane by inhalation at the end of the experimental period. Blood samples and cardiac tissues were collected for biochemical and molecular studies after the time intervals mentioned above. Blood samples were obtained in tubes. After 20 minutes at 40°C, the samples were centrifuged at 3000 xg for 10 minutes to separate sera for analysis of fasting blood glucose level, insulin, HOMA-IR, triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT) activity, aspartate aminotransferase (AST) activity, urea, creatinine levels, creatine kinase (CK), and lactate dehydrogenase (LDH). The obtained cardiac tissues were used to determine the gene expression of Nrf2, NF-κB, CASP3, and Bax.

2.7. Serum parameters measurements:

The non-radioactive quantitative quantification of insulin in rat serum was performed using an immunoassay kit (EMD Millipore USA) according to the manufacturer's instructions, and the homeostasis model assessment index for insulin resistance (HOMA-IR) was subsequently calculated using the following formula:**(Caumo et al., 2006)**

$$HOMA-IR = \frac{Fasting\ insulin(\mu IU/ml)\,x\,Fasting\ glucose(mg/dl)}{22.5 \times 18}$$

Serum TG, TC, and HDL-C levels were assessed by an enzymatic colorimetric method with reagents provided by BioMed Diagnostics INC (USA). The absorbance was detected at 546 nm. Serum LDL-C was calculated from TG, TC, and HDL-C levels via this equation**:(Burtis et al., 2006)**

LDL-C (mg/dL) = TC – (HDL-C) –TG/5

The activity of serum ALT and AST was determined with reagents supplied by BioMed Diagnostics INC (USA), with absorbance at 340 nm **(Huang et al., 2006)**. All procedures were carried out following to the manufacturer's instructions.

Serum urea and creatinine levels were evaluated with reagents purchased from BioMed Diagnostics INC (USA). The absorbance was at 570 nm for urea **(Hansen & Vilstrup, 1985)** and 510 nm for creatinine **(Keppler et al., 2007).** All procedures were carried out according to the manufacturer's instructions.

Serum LDH and CK levels were detected by the UV kinetic method with reagents provided by Bio-Med Diagnostic INC (USA). All procedures were carried out following the manufacturer's instructions, respectively **(Gerhardt & Waldenström, 1979; Kaplan & Pesce, 1984).**

2.8. Gene expression of Nrf2, NF-κB, CASP3 and Bax.

Total RNA was extracted from cardiac tissues via the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's guidelines. The integrity and concentration of isolated RNA were measured by the nanodrop. TOPscript™ RT DryMIX (dT18/dN6 plus) kit (Enzynomics Co Ltd, Korea, cat number RT220) was used for reverse transcription, following manufacturer instructions. The tissue expression of Nrf2, NF-κB, CASP3, and Bax were quantified in the cDNA relative to the reference gene 18s rRNA using ViPrime PLUS Taq qPCR Green Master Mix (Viviantis Technologies, Malaysia, cat. Number QlMM12) which enables rapid and reliable qRT-PCR quantification on the Bio-Rad CFX cycler. The fluorescent dye SYBR Green I in the master mix allows for the examination of a wide range of targets without the need to synthesize target-specific labelled probes. The employment of the hot-start enzyme HotStarTaq Plus DNA Polymerase, in conjunction with a specialized rapid PCR buffer, results in high PCR specificity and sensitivity. The quantitative PCR amplification settings were changed to include an initial denaturation at 95°C for 5 minutes, followed by 45 cycles of PCR for amplification as follows: denaturation at 95°C for 20 seconds, annealing at 55°C for 20 seconds, and extension at 70°C for 15 seconds. The housekeeping gene 18s rRNA served as a reference gene for normalization. The primers used to determine rat genes are shown in **Table 1**. The $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative change in mRNA expression across samples **(Livak & Schmittgen, 2001)**

Table (1): Primers used for the gene expression of Nrf2, NF-κB, CASP3, Bax and reference gene 18s rRNA.

Gene Name	Accession number	Primer	
Bax	**NM_017059.2**	**Forward**	5'- GCGAATTGGCGATGAACTG-3'
		Reverse	5'- ATGGTTCTGATCAGCTCGG-3'
Nrf2	**NM_031789.2**	**Forward**	5'-CGAGATATACGCAGGAGAGGTAAGA-3'
		Reverse	5'-GCTCGACAATGTTCTCCAGCTT-3'
NF-κB	**NM_199267.2**	**Forward**	5'-CAGGACCAGGAACAGTTCGAA-3'
		Reverse	5'-CCAGGTTCTGGAAGCTATGGA-3'
CASP-3	**NM_012922.2**	**Forward**	5'-GAAATTCAAGGGACGGGTC-3'
		Reverse	5'-TTCTTTGCATGGAAAGTGGC-3'
18S rRNA	**NR_046237.2**	**Forward**	5'-GTAACCCGTTGAACCCCATT-3'
		Reverse	5'- CAAGCTTATGACCCGCACTT-3'

2.9. Statistical analysis

The data were analyzed with SPSS software version 18.0 (SPSS Chicago, IL, USA). Results were represented as means ± SD and analyzed by ANOVA for group comparisons and Pearson for correlation studies. A p-value of less than 0.05 was regarded to indicate significance **(Hagen, 2022)**.

3. RESULTS

3.1. Glucose homeostasis parameters

It is clear that untreated diabetic rats had a significant higher FBG levels as compared to normal rats. All treated groups had a significant lower FBG levels as compared to untreated group. Metformin-treated group showed a significant higher FBG than quercetin-treated group. The combined group treated with quercetin and metformin showed a significant lower FBG than metformin-treated group **(Table 2).**

The untreated diabetic rats had a significant lower insulin levels as compared to normal rats. The quercetin-treated rats and metformin-treated rats had a significant lower insulin levels than normal rats but higher than untreated diabetic rats. The diabetic combined group, which was treated with quercetin and metformin, showed significantly higher insulin levels than untreated diabetic group but still lower than the normal group **(Table 2).**

The untreated diabetic rats had a significant higher HOMA-IR as compared to normal rats. All treated rats showed a significant lower HOMA-IR than untreated diabetic rats. The diabetic combined group treated with both quercetin and metformin showed significantly lower HOMA-IR than metformin-treated group **(Table 2).**

Table (2): Glucose homeostasis parameters in the studied groups.

	Groups	FBG (mg/dl)	Insulin (µIU/ml)	HOMA-IR
		Mean ± SD.	**Mean ± SD.**	**Mean ± SD.**
	Control	106.75 ± 2.49	10.27 ± 0.13	2.72 ± 0.07
Diabetic rats	**Untreated**	430.37^{a} ± 18.74	6.0^{a} ± 0.31	6.33^{a} ± 0.41
	Quercetin	125.38^{ab}± 7.09	8.37^{ab} ± 0.12	2.61^{b} ± 0.16
	Metformin	140.38^{abc} ± 6.37	8.20^{ab} ± 0.24	2.84^{b} ± 0.17
	Quercetin and metformin	114.0^{bd} ± 6.35	9.06^{abcd} ± 0.15	2.51^{bd} ± 0.16

Data were expressed as Means ± SD, n=8, p<0.05 considered significant.

Comparison between different studied groups is carried out using Post Hoc Test (Tukey) for ANOVA test.

a: Significantly different as compared with **control group.**

b: Significantly different as compared with **diabetic untreated group.**

c: Significantly different as compared with **quercetin-treated group**.

d: Significantly different as compared with **metformin-treated group.**

3.2. Lipid profile parameters

The diabetic (untreated) rats showed significantly increased TG levels as compared to normal rats. All treated diabetic groups had significantly lower TG levels as compared to diabetic (untreated) group **(Table 3).**

Regarding the total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), the untreated diabetic group had significant higher levels as compared to the normal group. All the treated rats had significantly lower TC and LDL-C levels compared with the untreated rats. The metformin-treated group had significantly higher LDL levels in comparison with the normal and quercetin-treated groups. The diabetic rats treated with quercetin and metformin showed a significant reduction in LDL levels as compared to metformin-treated rats **(Table 3).**

The untreated diabetic group had a significant lower HDL levels as compared to normal group. All treated rats showed higher HDL levels than untreated diabetic rats. The metformin-treated rats showed significantly lower HDL levels than quercetin-treated rats **(Table 3).**

Table (3): Lipid profile parameters in the studied groups

	Groups	Triglycerides (mg/dl)	Total Cholesterol (mg/dl)	HDL-Cholesterol (mg/dl)	LDL-Cholesterol (mg/dl)
		Mean ± SD.	**Mean ± SD.**	**Mean ± SD.**	**Mean ± SD.**
	Control	105.63 ± 5.63	65.38 ± 2.26	30.38 ± 3.25	13.87 ± 1.60
Diabetic rats	**Untreated**	148.13^{a} ± 2.23	90.0^{a} ± 6.09	25.25^{a} ± 2.43	35.39^{a} ± 5.93
	Quercetin	61.37^{ab} ± 7.27	63.13^{b} ± 1.73	36.0^{ab} ± 1.51	14.90^{b} ± 3.21
	Metformin	58.0^{ab} ± 5.32	65.75^{b} ± 4.33	31.38^{bc} ± 1.77	22.77^{abc} ± 4.17
	Quercetin and metformin	63.0^{ab} ± 2.14	62.25^{b} ± 1.91	35.02^{ab} ± 3.65	14.64^{bd} ± 2.60

Data were expressed as Means ± SD, n=8, p<0.05 considered significant.

Comparison between different studied groups is carried out using Post Hoc Test (Tukey) for ANOVA test.

a: Significantly different as compared with **control group.**

b: Significantly different as compared with **diabetic untreated group.**

c: Significantly different as compared with **quercetin-treated group**.

d: Significantly different as compared with **metformin-treated group.**

3.3. Liver function tests

The untreated diabetic group showed a significant increase in ALT levels as compared to the normal group. All treated groups had significant lower ALT levels as compared to untreated diabetic group. The diabetic group treated with both quercetin and metformin demonstrated a significant reduction in ALT levels compared with the normal group **(Table 4).** The diabetic (untreated) group noticed a significant elevation in AST levels compared with the normal group. The diabetic groups treated with metformin alone or in combination with quercetin showed a significant reduction in AST levels than untreated diabetic group. The combined group showed significantly lower AST levels than quercetin-treated group **(Table 4).**

3.4. Kidney function tests

The untreated diabetic group revealed a significant increase in urea levels compared with the normal group. All treated groups had significantly lower urea levels compared with diabetic untreated groups. The quercetin-treated rats and metformin-treated rats had significantly lower urea levels than untreated rats, but metformin-treated rats had significantly higher urea levels than rats treated with quercetin. The combined group showed the lowest urea levels among the treated groups **(Table 4)**.

The untreated group demonstrated a significant elevation in creatinine levels as compared to the normal group. All treated groups had significantly lower creatinine levels as compared to the untreated diabetic group but still higher than the normal group. The metformin-treated rats had significantly higher creatinine levels than quercetin-treated rats. The combined group showed the lowest creatinine levels among all treated groups **(Table 4).**

Table (4): Liver and kidney function tests in the studied groups

	Groups	Urea (mg/dl)	Creatinine (mg/dl)	ALT (U/L)	AST (U/L)
		Mean ± SD.	Mean ± SD.	Mean ± SD.	Mean ± SD.
	Control	18.83 ± 2.32	0.18 ± 0.01	33.50 ± 2.17	106.33 ± 5.79
Diabetic rats	Untreated	40.0^{a} ± 3.46	0.54^{a} ± 0.04	40.0^{a} ± 1.31	139.63^{a} ± 18.96
	Quercetin	24.25^{ab} ± 2.05	0.38^{ab} ± 0.03	31.50^{b} ± 3.51	129.13^{a} ± 2.23
	Metformin	29.75^{abc} ± 2.71	0.46^{abc} ± 0.04	32.0^{b} ± 4.84	116.25^{b} ± 6.63
	Quercetin and metformin	20.0^{bcd} ± 1.51	0.28^{abcd} ± 0.03	27.63^{ab} ± 4.37	104.75^{bc} ± 3.28

Data were expressed as Means ± SD, n=8, p<0.05 considered significant.

Comparison between different studied groups is carried out using Post Hoc Test (Tukey) for ANOVA test.

a: Significantly different as compared with **control group.**

b: Significantly different as compared with **diabetic untreated group.**

c: Significantly different as compared with **quercetin-treated group**.

d: Significantly different as compared with **metformin-treated group.**

3.5. Cardiac function tests

The untreated diabetic group noticed a significant elevation in LDH activity in comparison with normal rats. The quercetin-treated rats had significantly lower LDH activity as compared to the untreated group. The metformin-treated rats had significantly higher LDH activity than the control rats and quercetin-treated rats. The combined group showed a significantly decrease in LDH activity than the untreated group and metformin-treated group (**Table 5).**

The untreated diabetic group showed significantly higher CK activity as compared to the normal group. The quercetin-treated rats had significantly lower CK activity than the untreated rats. The metformin-treated rats also had lower CK activity than untreated diabetic rats but had significantly higher CK activity than normal rats and quercetin-treated rats. The combined group showed significantly lower CK activity than the untreated group and metformin-treated group (**Table 5).**

Table (5): Cardiac markers (LDH and CK) in the different studied groups

	Groups	LDH (U/L)	CK (U/L)
		Mean ± SD.	Mean ± SD.
	Control	387.17 ± 34.38	543.33 ± 163.94
Diabetic rats	Untreated	1024.38^{a} ± 111.78	1893.0^{a} ± 90.35
	Quercetin	386.50^{b} ± 4.38	635.25^{b} ± 4.17
	Metformin	982.63^{ac} ± 34.20	1560.63^{abc} ± 44.79
	Quercetin and metformin	334.37^{bd} ± 4.87	538.0^{bd} ± 6.12

Data were expressed as Means ± SD, n=8, p<0.05 considered significant.

Comparison between different studied groups is carried out using Post Hoc Test (Tukey) for ANOVA test.

a: Significantly different as compared with **control group.**

b: Significantly different as compared with **diabetic untreated group.**

c: Significantly different as compared with **quercetin-treated group**.

d: Significantly different as compared with **metformin-treated group.**

3.6. Cardiac expression of Nuclear factor kappa B (NF-κB)

The untreated diabetic rats showed a significantly upregulation in NF-κB expression than control rats. The quercetin-treated rats had significantly downregulation in NF-κB expression as compared to control rats and untreated diabetic rats. The metformin-treated rats had significantly downregulation in NF-κB expression than untreated diabetic rats. The combined group had significantly downregulation in NF-κB expression compared with normal group, untreated group, and metformin-treated group **(Figure 1).**

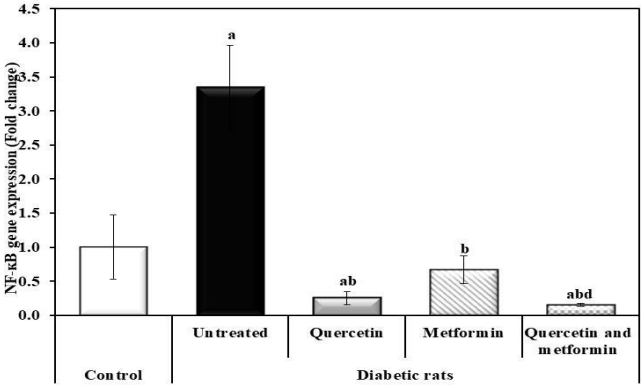

Figure (1): Cardiac NF-κB gene expression (fold change) in the studied groups.

Data were expressed as Means ± SD, n=8, p<0.05 considered significant.

Comparison between different studied groups is carried out using Post Hoc Test (Tukey) for ANOVA test
a: Significantly different as compared with **control group.**
b: Significantly different as compared with **diabetic untreated group.**
d: Significantly different as compared with **metformin-treated group**

3.7. Cardiac expression of Nuclear factor erythroid 2-related factor 2 (Nrf2)

The untreated diabetic rats have significantly downregulation in Nrf2 expression than normal rats. The quercetin-treated rats showed significantly upregulation in Nrf2 expression compared with control rats and diabetic untreated rats. The metformin-treated rats had significant upregulation in Nrf2 expression than diabetic untreated rats but lower than quercetin-treated rats. The combined group had significantly upregulation in Nrf2 expression than the control group, untreated group, and quercetin-treated group **(Figure 2).**

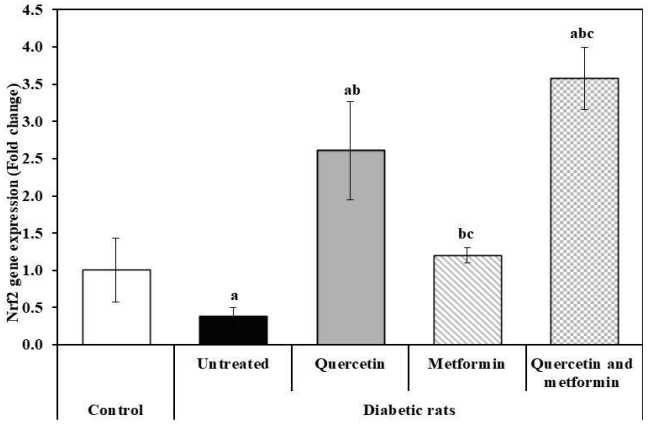

Figure (2): Cardiac Nrf2 gene expression (fold change) in the studied groups.

Data were expressed as Means ± SD, n=8, p<0.05 considered significant.

Comparison between different studied groups is carried out using Post Hoc Test (Tukey) for ANOVA test
a: Significantly different as compared with **control group.**
b: Significantly different as compared with **diabetic untreated group.**
c: Significantly different as compared with **quercetin-treated group**.

3.8. Cardiac expression of Bcl-2-associated X (Bax)

The untreated diabetic rats showed significantly upregulation of Bax expression than normal rats. All treated groups revealed significantly downregulation of Bax expression as compared to untreated group **(Figure 3)**

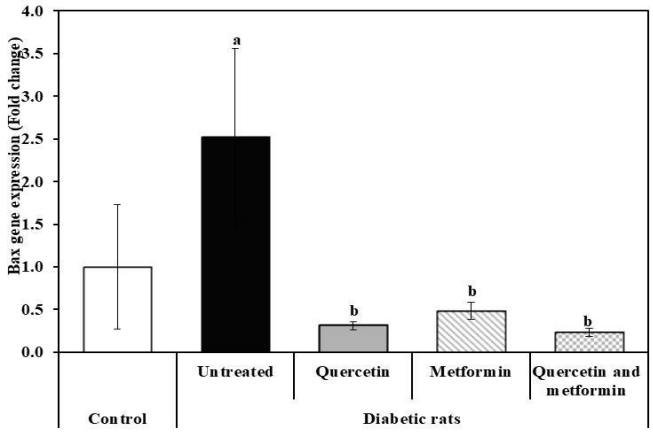

Figure (3): Cardiac BAX gene expression (fold change) in the studied groups.

Data were expressed as Means ± SD, n=8, p<0.05 considered significant.

Comparison between different studied groups is carried out using Post Hoc Test (Tukey) for ANOVA test.
a: Significantly different as compared with **control group.**
b: Significantly different as compared with **diabetic untreated group**

3.9. Cardiac expression of Caspase-3 (CASP3)

The untreated diabetic rats showed significantly upregulation of CASP3 expression than normal rats. The quercetin-treated rats showed significantly downregulation of CASP3 expression as compared to normal rats and untreated diabetic rats. The metformin-treated rats have downregulation of CASP3 expression than diabetic (untreated) rats. The combined group had significantly lower Bax expression than control group and untreated diabetic group **(Figure4)**

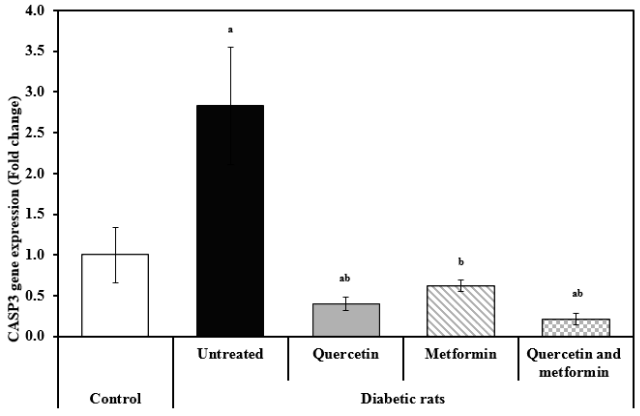

Figure (4): Cardiac CASP3 gene expression (fold change) in the studied groups.

Data were expressed as Means ± SD, n=8, p<0.05 considered significant.

Comparison between different studied groups is carried out using Post Hoc Test (Tukey) for ANOVA test.
a: Significantly different as compared with **control group.**
b: Significantly different as compared with **diabetic untreated group.**

3.10. Correlation studies

The results of the statistical study among diabetic treated groups using Pearson correlation showed the following:

Cardiac CASP3 expression was positively correlated with NF-κB expression (r=0.798, p<0.001) **(Figure 5A)**, Bax expression (r=0.789, p<0.001) **(Figure 5B)** and negatively correlated with Nrf2 expression (r=-0.824, p<0.001) **(Figure 5C)**.

Cardiac NF-κB expression was positively correlated with Bax expression (r=0.759, p<0.001) **(Figure 5D)** and negatively correlated with Nrf2 expression (r=-0.728,p<0.001) **(Figure 5E).**

Cardiac Bax expression was negatively correlated with Nrf2 expression (r=-0.756, p<0.001) **(Figure 5F)**

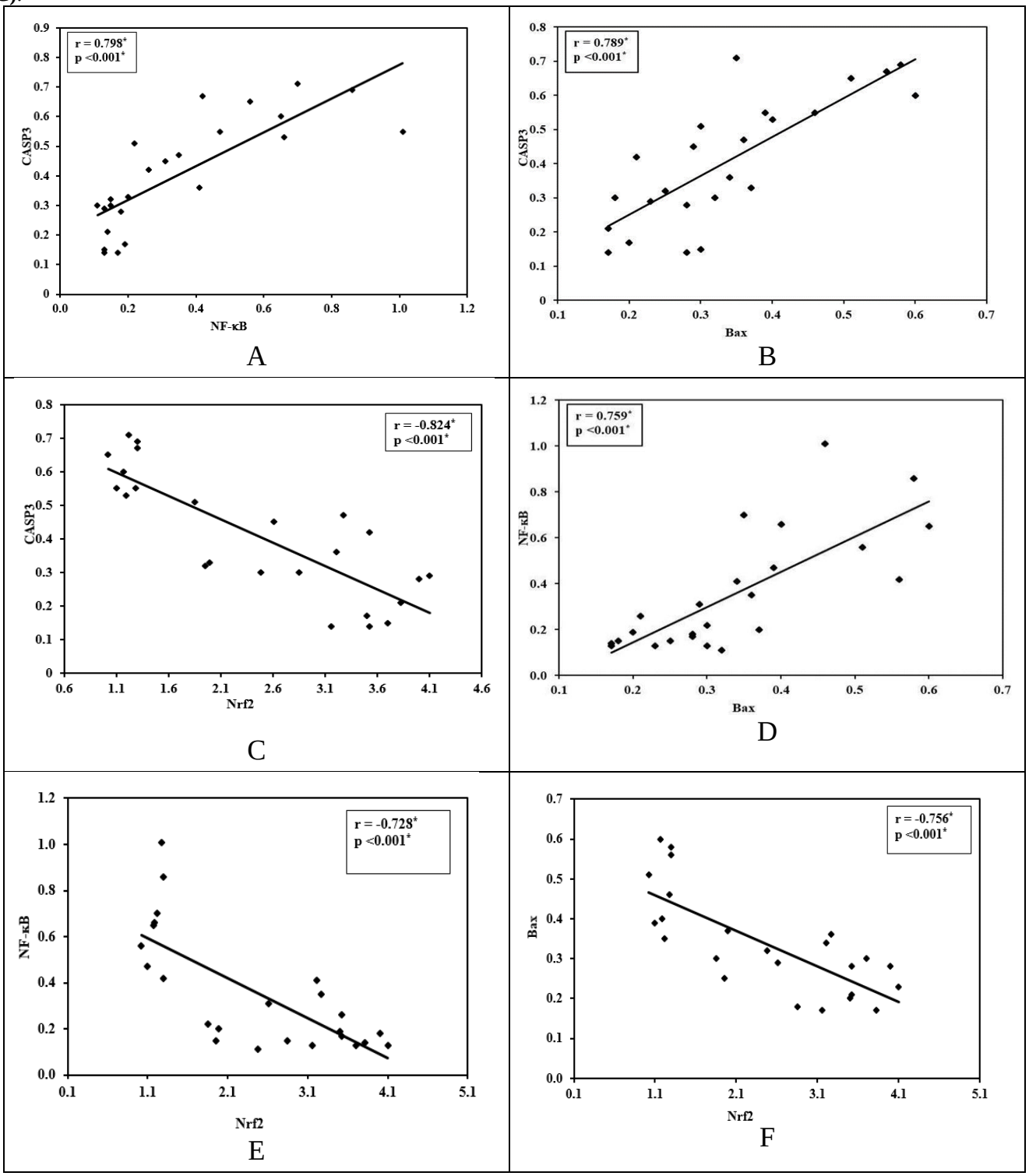

Figure (5): Cardiac correlation studies

Discussion

In the current study, the altered glucose homeostasis in obesogenic diet/alloxan experimental diabetic rat model was accompanied by changes in lipid profile, which showed significantly elevated serum triglycerides (TG), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C) as well as significantly reduced high-density lipoprotein cholesterol (HDL-C) in the untreated diabetic rats in comparison with the normal rats, a condition known as diabetic dyslipidemia. Diabetes and insulin resistance may be a cause or a result of these problems. Despite preventive treatment for those results, dyslipidemia is one of the primary causes for cardiovascular disease in T2DM, with extremely high morbidity and death **(Dake & Sora, 2016)**.

Regarding the present results, metformin administration to diabetic rats controls the glycemic parameters and corrects the dyslipidemia through a significant reduction in FBS, HOMA-IR, TGs, TC and LDL-C levels and a significant elevation in HDL-C levels which is in line with **Dallak et al. (2018)** and **Kotb et al., (2022)**. Major international treatment guidelines list metformin as the first-line glucose lowering pharmacological therapy for type 2 diabetes due to its favorable risk-benefit ratio and cost-effectiveness. Metformin inhibits lipoprotein production in diabetic rats' intestines by downregulating mRNA expression of genes involved in intestinal lipid balance (**Kender et al., 2019)**.

In our study, quercetin treatments significantly decreased fasting blood glucose in diabetic rats. Furthermore, quercetin treated rats have significantly higher levels of insulin and lower HOMA-IR as compared to untreated diabetic rats. This is in line with the results **of Arias et al., (2014)**, who reported that quercetin dramatically lowered basal glucose as well as HOMA-IR in diabetic rats versus untreated diabetic rats.

Previously, it was shown that quercetin had the capacity to increase insulin expression and insulin secretion in insulin-secreting insulinoma cells in rats **(Bhattacharya et., 2014)**. Quercetin improves insulin secretion, lowers blood glucose levels, and boosts VEGF and VEGFR2 production in diabetic rats' pancreas, which might promote β cell regeneration **(Suganya et al., 2018).** Moreover, **Kittl et al. (2016)** found that the intracellular Ca2+ signaling pathway was activated by quercetin through significantly stimulated insulin secretion. Anti-diabetic effects of quercetin may be exerted by its lipotropic effect, since they significantly modified TG, TC, HDL-C, and LDL-C levels, to the same extent or better than metformin. Given quercetin's effect on lipid profile, they can be regarded as potential hypolipidemic agents, which will be extremely beneficial for diabetics as well as those suffering from atherosclerosis or hyperlipidemia **(Yi et al., 2021).**

The current study showed that diabetic untreated rats have a significantly increased alanine transaminase (ALT) and aspartate transferase (AST) activities in comparison with control rats. However, there was a significant decrease in serum ALT and AST activities in metformin-treated group and quercetin-treated rats and the best results were noticed in the diabetic group, which treated with a combination of quercetin and metformin**.** AST and ALT activities are released into the bloodstream as a result of hepatocellular injury. These enzymes' activities can be used to detect hepatocyte or cardiac injury. This observation is in line with **Senyigit et al.**, who demonstrated that AST and ALT activities are increased in diabetic rats **(Senyigit et al., 2019).** Interestingly, metformin treatment significantly lowered AST and lactate dehydrogenase (LDH) changes and returned them to normal levels. Increased ROS generation caused by hyperglycemia damages the cell membranes of cardiomyocytes through interactions with proteins, lipids, and other components of cell and causes the release of LDH and AST from the injured heart tissues into the bloodstream **(Yoshinaga et al., 2021).** The great role of metformin in recovering LDH and AST activities demonstrated metformin's antioxidant properties, which protected cardiomyocytes from oxidative damage **(Kelly et al., 2015).**

The present findings revealed that the untreated diabetic group exhibited a significant higher LDH and CK levels than normal rats. All diabetic treated rats showed significantly reduced LDH levels as compared to diabetic untreated rats.

Currently, it is unknown what molecular mechanism underlies metformin's substantial reduction in creatine kinase (CK) activity, and more research is needed to determine whether this reduction is due to transcriptional downregulation, translational suppression, or inhibition of post-translational modification processes. Nonetheless, the antioxidant functions of these substances are thought to play key roles in cardioprotection **(Naghdi et al., 2022).**

In a previous study, it was suggested that apoptosis levels in the diabetic cardiac tissues were reduced, quercetin decreases inflammation and oxidative stress. It was also discovered that quercetin could prevent destruction of the cardiac tissues in diabetes. LDH and CK levels in untreated diabetic rats significantly reduced after treatment with quercetin **(Giribabu et al., 2016).**

The present study showed a significant increase in urea and creatinine levels in untreated diabetic rats in comparison with the normal rats. These results are matching with those of **Kandemir et al., (2018)**. Experimentally induced diabetes raises the serum levels of urea and creatinine, which are thought to be indicators of renal injury **(Ali et al., 2013).**

The effect of metformin on kidney functions in diabetic rats has been tested in this study. When compared to diabetic rats, metformin therapy significantly reduced blood creatinine and urea levels. These results are matched with **Albasher et al., (2020).**

The treatment of diabetic rats with quercetin causes a significant reduction in urea and creatinine levels. These findings are confirmed by the findings of a recently published study in which quercetin was found to have some protective effects against renal failure in rats **(Hu et al., 2022).** In another study it was postulated that the alleviation of diabetes issues, decline of oxidative stress, and stimulation of the antioxidant defense system may be related to the recovery of compromised renal function in diabetic rats after quercetin treatments **(Ali et al., 2020)**.

According to the current study, the gene expression of Bax, CASP3, and NF-κB showed upregulation in untreated diabetic rats than normal rats, but treatment of rats with quercetin and/or metformin significantly downregulated these

gene expression in all treated groups, especially the diabetic combined treated group. In contrast, the gene expression of Nrf2 showed downregulation in untreated diabetic rats than normal rats. All diabetic treated groups showed significantly upregulation in Nrf2 expression as compared to the untreated group.

It has been demonstrated that cardiac apoptosis in both in vivo and clinical tests, mostly is caused by a high glucose level which has a major impact on the cardiac function, regressing in diabetes cases **(Davargaon et al., 2019; Su et al., 2021; Su et al., 2016).** Increased ROS levels in hyperglycemic cases are assumed to inhibit AKT activation by decreasing its phosphorylation. AKT inhibits the transcription factor FOXO3, which contributes to the expression of pro-apoptotic genes such as Bax; thus, reduced phosphorylation of AKT removes the inhibitory effects on FOXO3, induces the expression of Bax, and raises the potential of Bax-Bax homodimers formation, which accelerates the apoptotic process **(Dong et al., 2018).**

Increased oxidative stress levels in cardiac tissues have been observed to induce apoptosis **(Tsai et al., 2013).** Reduced levels of lipid peroxidation products are a sign of low levels of cellular oxidative stress, which suggests that quercetin was capable of preserving the expression levels of anti-oxidative enzymes in the cardiac tissues. Quercetin has also been considered a free radical scavenger and activator of cellular antioxidant enzymes **(Sinha et al., 2014).** The capacity of quercetin to reduce cardiac oxidative stress and inflammation may be responsible for cardioprotection. Catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) are three examples of the antioxidative enzymes that quercetin has been shown to activate in the heart **(Wang et al., 2015).** It has also been shown to improve the lipid peroxidation product levels in diabetic hearts. The heart's signaling system may be impaired, which could result in the inactivation of these enzymes **(Gao et al., 2016)**.

The pathway of Nrf2/Keap1 is crucial for defense against oxidative stress. The heart's Nrf2 gene may function as a compensatory mechanism in diabetic rats to counteract oxidative damage. Keap1 protein levels were reduced by metformin, which stimulated Nrf2 and promoted its translocation into the nucleus, upregulating antioxidant enzymes such as hemeoxygenase-1 (HO-1). These findings suggested that metformin may protect against obesity-related cardiac remodeling. This is possibly due to its ability to alleviate metabolic disorders and increase endogenous antioxidant function. However, more research is needed to determine the direct targets of metformin in Nrf2/Keap1 signaling regulation **(Du et al., 2020)**.

Additionally, it has been demonstrated that quercetin protects cells from oxidative stress by triggering the Nrf2 pathway **(Saw et al., 2014)**. It was reported that quercetin application upregulated Nrf2 expression, which in turn declined the oxidative stress by elevating the levels of NQO1 and HO-1 **(Yardim et al., 2020)**. The antioxidant capabilities of quercetin and its synergistic effects on oxidative stress when combined with metformin are linked to increased expression of the Nrf2 protein and its downstream antioxidant enzymes, catalase and SOD **(Rad et al., 2022)**.

Inflammation and hyperglycemia are closely related **(Zhang et al., 2016).** Inflammation has also been connected to oxidative stress. Hyperglycemia has been stated to induce inflammation in the cardiac tissues **(Al-Harbi et al., 2016)**. It was reported that the level of NF-κB was decreased following quercetin treatment. However, the mechanism of metformin action remains unknown. Additionally, it is well known that metformin activates the energy sensor AMPK **(Luo et al., 2016),** AMPK-independent approaches, like preventing the production of reactive oxygen species by blocking the NADPH oxidase pathway have also been documented **(Batchuluun et al., 2014).** The Nrf2 antioxidant signaling pathways have been shown to be stimulated by metformin pretreatment **(Ashabi et al., 2015).**

Metformin has been linked to increased fatty acid oxidation in adipose tissue, increased muscle glucose uptake, and decreased blood glucose levels and hepatic gluconeogenesis, according to research. This was linked to improvements in dyslipidemia and reversal of fatty liver, most likely as a result of a reduction in pro-inflammatory cytokine production and consequently hepatic inflammation. Also, it was suggested that metformin has the capacity to control NF-κB, which lowers levels of TNF- and IL-1 and has an anti-inflammatory effect **(Araújo et al., 2017)**.

Quercetin's ability to keep plasma glucose and insulin levels near normal helps to protect the heart from oxidative stress and inflammation caused by hyperglycemia. Additionally, the capability of quercetin to enhance the heart's antioxidant capacity may aid in reducing inflammation brought on by oxidative stress. According to Si et al., quercetin has been revealed to prevent the lipopolysaccharide-increased NF-κB from activating the inflammatory pathway **(Si et al., 2016)**.

Additionally, It has been discovered that quercetin can reduce cardiac apoptosis, which results from a combination of oxidative stress and inflammation **(Zhang et al., 2016).** According to earlier research, quercetin can suppress apoptosis in diabetic cardiac tissues. Lower concentrations of pro-apoptotic markers (CASP3 and Bax) were observed in diabetic cardiac tissues **(Guo et al., 2016; Ouyang et al., 2014).**

Conclusion:

According to our findings, quercetin has anti-inflammatory, antihyperglycemic, and antiapoptotic properties that could be useful in the treatment of T2DM. Quercetin's antidiabetic effects in obesogenic diet/alloxan diabetic rats are evidenced by a significant reduction in fasting blood sugar, insulin resistance, total cholesterol (TC), and triglycerides (TG). Quercetin also has, antioxidant, anti-inflammatory and antiapoptotic effects, as it significantly downregulated NF-κB, significantly upregulated Nrf2, and significantly downregulated Bax and Casp3 gene expression, respectively. The best results were observed in the combined group treated with quercetin and metformin. In conclusion, the health benefits of quercetin that have been reported in this study could assist in reducing the severity of heart damage in

diabetics. Additionally, it can be combined with metformin to boost its effectiveness to treat T2DM.

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