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Hesperidin and Rosemary extract alleviates apoptosis and alterations of DNA methyltransferase and targeting microRNA in a rat model of diabetic cardiomyopathy

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ABSTRACT

Diabetic cardiomyopathy (DCM) is a complication of diabetes results in high mortality due to its oxidative stress. The present study was designed to investigate the effect of rosemary extract and hesperidin on hyperglycemia-induced oxidative stress, inflammation, and cardiac injury in experimentally induced DCM in rats. Fifty rats were divided into five groups. Group I: rats fed a normal diet. Group II**:** rats received a single dose of streptozotocin at a dose of 50 mg /kg body wt. Group III: rats were treated with insulin 2 U/rat per day. Group IV: rats were treated orally with rosemary extract at a dose of 200 mg/kg body weight/day. Group V**:** rats were treated with hesperidin orally at a dose of 100 mg/kg body weight/day. Blood samples and cardiac tissue specimen were collected at the end of the experiment. The obtained results showed a marked increase in blood glucose concentration and upregulation in Endothelin-1 (ET-1), Hypoxia inducing factor-1 α **(**HIF-1α), miR-29 and miRNA130b-3p gene expressions with hypermethylation in global DNA methyltransferase (DNMT) in cardiac tissue of DCM-induced group. Treatment with insulin, rosemary extract and hesperidin exhibited a significant decrease in blood glucose concentration and downregulation in Endothelin-1, miR-29 and miRNA130b-3p expressions with hypomethylation in DNMT, while HIF-1α showed a significant upregulation as compared to DCM non treated group. In conclusion, these results indicated that rosemary extract and hesperidin may have great therapeutic potential in the treatment of DCM.

1. INTRODUCTION

Hyperglycemia, a metabolic condition known as diabetes mellitus (DM), can permanently harm and impair many organs (Al Hroob *et al.,* 2018). Diabetes causes a degenerative change in the myocardium known as diabetic cardiomyopathy (DCM), which is unrelated to hypertension or coronary artery disease (Soares *et al.,* 2016).

The pathophysiological processes that underlie cardiac damage in diabetes are complicated and multifaceted, and include oxidative stress brought on by hyperglycemia, inflammation, and the activation of cell death pathways. Heart failure may eventually result from these procedures (Zhang *et al.,* 2017). Different inflammatory and cell death mechanisms that are involved in the development of DCM may be activated by hyperglycemia-mediated oxidative stress (Rajesh *et al*., 2010)

Excess reactive oxygen species (ROS) stimulate the apoptotic signaling pathways in the diabetic heart, including the mitochondrial apoptotic pathway, in addition to membrane lipid peroxidation and protein carbonylation (Othman *et al.* 2017). Changes in the energy metabolism and cardiac structure during the diabetic cardiomyopathy's latency period cause diastolic dysfunction, which advances to concentric ventricular hypertrophy and a decrease in its contractile reserve until reaching systolic dysfunction (Yilmaz *et al*., 2015).

Rosmarinus officinalis Linn. (Rosemary) leaves extract is one of the most well-known herbs used as an antioxidant and flavoring in food preservation and cosmetics (Cui *et al.* 2012). In many nations, rosemary is frequently utilized as a medicinal plant in both conventional and contemporary treatments for hypertension and diabetes problems (Amel, 2013; Martynyuk *et al.* 2014).

Citrus fruits including limes and lemons, tomatoes, and cherries contain large amounts of hesperidin, a saturated oxidized aglycon that is 5,7,3'-Trihydroxy-4'-methoxyflavanone (Parhiz *et al.,* 2015). Hesperidin and its glycoside have vascular, neuroprotective, anti-allergic, anti-inflammatory, anticarcinogenic, and antioxidant actions in addition to their effects on diabetes (Visnagri *et al.*, 2014).

MicroRNAs (miRNAs) are single-stranded, noncoding RNA molecules with lengths ranging from 18 to 25 nucleotides and are encoded by endogenous genes (Shukla *et al.,* 2011). The initiation and progression of diabetes are influenced by miRNAs, which are important regulators of a number of biological processes, including apoptosis, oxidative stress, and inflammatory factors (Pordzik *et al.,* 2019). Poy *et al*., (2004) provided the first explanation of the functions of miRNAs in type-2 diabetes by demonstrating the critical functions of miR-375 in insulin secretion. Later,

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accumulating studies further confirmed the regulatory roles of miRNAs.

The goal of the current study was to examine the protective effects of rosemary extract and hesperidin on DCM and its involvement in the alterations of cardiac function and associated mechanisms in a rat model of DCM, taking into account the potential therapeutic properties of two natural agents (rosemary extract and hesperidin).

2. MATERIAL AND METHODS

2.1. Experimental animals:

In this investigation, 50 white male albino rats measuring 180-200 g at 5-6 weeks of age were employed. The ambient and dietary parameters for the rats were kept consistent in separate metal cages. The rats received food and water at will. Prior to the start of the trial, all rats were acclimated for 15 days. Benha University Animal Care and Use Committee authorized the experimental protocols, which adhere to the National Institutes of Health's guide for the care and use of laboratory animals (Approval no. BUFVTM 02-8-21).

2.2. Chemicals and antioxidant agents:

2.2.1. Streptozotocin:

Streptozotocin (STZ) purchased from Sigma Chemical Co. (P.O. Box. 14508, St. Lowis, U.S.A.) was used to induce hyperglycemia by a single intraperitoneal (i.p) injected at dose of 50 mg /kg body wt. freshly dissolved in citrate buffer, PH 4.5 (Ramanathan *et al.,* 1999).

2.2.2. Insulin:

Long-acting insulin was purchased from (Lantus Solostar, Sanofi-Aventis, Germany). It is subcutaneously injected (2 U/rat per day) (Shiju *et al.,* 2012).

2.2.3. Rosemary extracts preparation:

About 250 g of the dried rosemary leaves were milled into a fine powder after being dried in the shade to prevent the chemical contents from decomposing.

The plant powder was placed in a stoppered container with ethanol (ethanol/water (70:30)) and allowed to stand at room temperature for at least 3 days. After that, the mixture was filtrated to obtained liquid extract. Then, the extract was concentrated using a rotary evaporator at 50 ℃ under reduced pressure. This process was repeated at least 3 times. Finally, the extract was weighted and stored at -20 ℃ till usage. Each rat was orally administered a daily dose 0.5 ml of rosemary extract (Abdul- Rahim and Taha, 2011).

2.2.4. Hesperidin:

Hesperidin (95%) HSP was purchased from Al-dawlya Company. Hesperidin (100 mg/kg/ day) was dissolved in saline and was administered orally (50 mg/ ml) (Pires Das Neves *et al*., 2004).

2.3. Induction of diabetic cardiomyopathy:

A single intraperitoneal (i.p) injection of 50 mg/kg body weight of STZ experimentally induced hyperglycemia in male rats, and after 6 weeks of the condition, experimental DCM occurs. Rats used as controls only received a vehicle (citrate buffer) in a quantity that was comparable. After a week, rats that had received STZ were given a 12-hour fast before blood samples were taken to measure their blood glucose levels. Rats in the diabetic group were classified as diabetic and enrolled in subsequent studies when their blood glucose levels exceeded 250 mg/dl. (Ramanathan *et al.,* 1999).

2.4. Experimental design:

All rats were divided into five groups after six weeks of DCM induction. Therapeutic treatment with insulin, Rosemary extracts and Hesperidin were given six weeks after DCM induction and continued for six weeks as following:

- Group I: Control normal group: 7 rats were given only a regular diet and no further treatments.
- Group II: DCM non treated group: A single intraperitoneal (i.p) injection of STZ (50 mg/kg body weight) was given to 13 rats.
- Group III: STZ + Insulin treated group: 10 rats were treated for 6 weeks with daily subcutaneous injections of long-acting insulin at a dose of 2 units per rat after receiving a single intraperitoneal (i.p.) injection of STZ (50 mg/kg body weight).
- Group IV: STZ + R.E. treated group: For six weeks, 10 rats were given STZ (50 mg/kg body weight) intraperitoneally (i.p.) in a single dosage and rosemary extract (200 mg/kg body weight/day) orally once daily.
- Group V: STZ + Hesperidin treated group: 10 rats were given with Hesperidin orally once daily at a dose of (100 mg/kg body weight/day) for 6 weeks after receiving a single intraperitoneal (i.p.) injection of STZ (50 mg/kg body weight).

2.5. Sampling:

2.5.1. Blood samples:

After an overnight fast, blood samples for serum separation were obtained at the end of the experiment (12 weeks). Serum was separated by centrifugation at 2500 rpm for 15 minutes. Until it was used for glucose determination, the serum was stored at -20 ℃.

2.5.2. Tissue specimens (For molecular analysis):

Rats were sacrificed at the end of the experiment (12 weeks), and the abdomen was opened. The heart tissues were then collected from all animal groups, placed in Eppendorf tubes, and immediately maintained in liquid nitrogen and frozen at –80 ℃ until RNA extraction for determination of gene expression of miR-29 and miRNA130b-3p, IL-1 and HIF-1α in addition to global DNA methylation (DNMT) level in cardiac tissue.

2.6. Analysis:

2.6.1. Biochemical analysis:

Serum glucose concentration was determined enzymatically according to Tietz, (1995).

2.6.2. Molecular analysis:

Evaluation of the degree of global DNA methylation as per the procedure outlined in (Colorimetric) Base Catalog # P-1030. As previously mentioned, DNMT was assessed using a Methyl Flash TM Global DNA Methylation (5-mC) ELISA Easy Kit from EpiGentek, Farmingdale, NY, USA (Li et *al*., 2018).

Real-time quantitative polymerase chain reaction analysis (real-time qPCR) was used to assess the mRNA expression levels of IL-1 and HIF-1 in the rat heart (Table 1). The load was managed with β-actin. Following the manufacturer's instructions, total RNA was extracted from the heart using the High Kit for extraction of pure RNA (Thermo Scientific, Fermentas, #K0731) RNA Extraction kit. RevertAid TM First Strand CDNA synthesis kit (#EP0451, Thermo Scientific, Fermentas, USA) was used to reverse transcribe each cDNA sample. Then, using the Faststart Universal SYBR Green Master, real-time quantitative PCR amplification was carried out (Roche, GER). Using the 2-Ct

technique, the target gene was normalized with β-actin (Livak and Schmittgen, 2001).

miR-29 and miRNA130b-3p Using U6 as an internal control, the expression of miRNAs in the heart was measured using Real-time PCR and SYBR Green. Thermo Scientific, USA, # K0221), a miRNA-specific forward primer (Table 2), and a universal reverse primer supplied with the Quanti-Mir RT kit were used to amplify the extracted cDNA in accordance with the manufacturer's instructions.

Table 1 Forward and reverse primers sequence for primers used in qPCR

Gene	Forward primer (′5 ------ ′3)	Reverse primer (′5 ------ ′3)
ET-1	TCTCGGAGAGCAGAGACACA	TGGACTTTGGAGTTTCTCCCT
HIF-1a	ACTATGTCGCTTTCTTGG	GTTTCTGCTGCCTTGTAT
B-actin	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC

Table 2 Forward and reverse primers sequence for real time PCR

Gene	Primer sequence (′5 ------ ′3)
miRNA29a	TAGCACCATCTGAAATCGGTTA
miRNA130b-3p	CAGTGCAATGATGAAAGGGCAT
U6	TGACACGCAAATTCGTGAAGCGTTC
Universal reverse primer	CCAGTCTCAGGGTCCGAGGTATTC

2.7. Statistical analysis:

Means and SEM were used to express all the data. One-way analysis of variance (ANOVA) was used to assess the statistical significance, and Duncan's multiple range test was used to get individual comparisons (DMRT). SPSS, version 18.0 software was used. When P< 0.05, values were deemed statistically significant (Steel *et al*., 1997).

3. RESULTS

A significant increase in blood glucose concentration was observed in STZ-induced DCM when compared with normal control group (Table 3 and Figure 1).

Treatment with insulin, rosemary extracts or hesperidin to STZ-induced DCM in rats exhibited a significant decrease in blood glucose concentration as compared with untreated group. With highest decrease in insulin group (G3).

Table 3 Effect of insulin, rosemary extract or hesperidin treatment on blood glucose concentration of STZ-induced diabetic cardiomyopathy in rats:

Parameter	Animal groups				
	Control Normal (G1)	DCM (G2)	DCM + Insulin (G3)	DCM + R.E. (G4)	DCM + HES (G5)
Glucose (mg/dl)	95.33 ± 4.03^{e}	321.33 ± 9.19^{a}	163.33 ± 5.19^{d}	247.4 ± 7.32^{b}	209.6 ± 6.68^{c}

Data are presented as (Mean ± S.E.M). S.E = Standard error. Mean values with different superscript letters in the same row are significantly different at (P≤0.05).

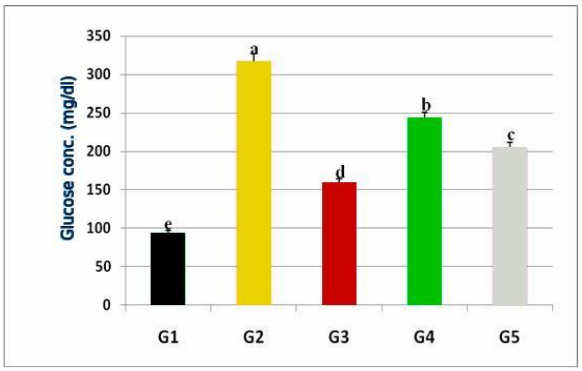

Fig. (1): Effect of insulin, rosemary extract or hesperidin treatment on blood glucose concentration in experimental model of diabetic cardiomyopathy in rats.

Table (4) and figures (2,3) showed a significant upregulation of miR-29 and miRNA130b-3p gene expressions of cardiac tissue in STZ-induced DCM when compared with normal control group.

Treatment with insulin, R.E or hesperidin to STZ-induced DCM in rats exhibited a significant downregulation of miR-29 and miRNA130b-3p gene expression as compared with untreated group. With highest downregulation of miR-29 in (G3) as compared with (G4) and (G5). However, miRNA130b-3p showed highest downregulation in (G3) and (G5) as compared with (G4).

Table 4 Effect of insulin, rosemary extract or hesperidin treatment on cardiac tissue miRNA-29 and miRNA130b-3p gene expressions of STZ-induced diabetic cardiomyopathy in rats.

Animal groups	miRNA-29 Fold change ±SEM	miRNA130b-3p Fold change ±SEM
Control Normal (G1)	1.00 ± 0.07^{d}	1.00 ± 0.06^{d}
DCM (G2)	24.93 ± 1.57^{a}	6.54 ± 0.32^{a}
DCM + Insulin (G3)	3.03 ± 0.15^{c}	2.51 ± 0.11^{c}
DCM + R.E. (G4)	14.42 ± 0.73^{b}	4.76 ± 0.18^{b}
DCM + HES (G5)	14.72 ± 0.76^{b}	2.62 ± 0.12^{c}

Data are presented as (Mean ± SEM). SEM = Standard error mean. Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

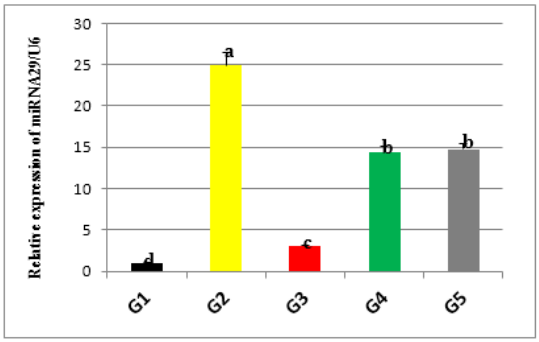

Fig. (2): Effect of insulin, rosemary extract or hesperidin treatment on miRNA-29 gene expression in experimental model of diabetic cardiomyopathy in rats.

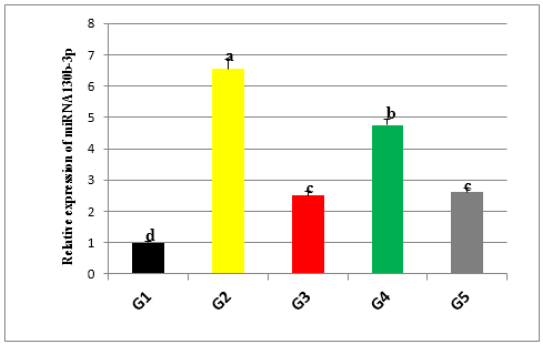

Fig. (3): Effect of insulin, rosemary extract or hesperidin treatment on miRNA130b-3p expression in experimental model of diabetic cardiomyopathy in rats.

Table (5) and figures (4,5) showed a significant upregulation of Endothelin-1 and HIF-1α gene expression in STZ-induced DCM when compared with normal control group. However, Treatment with insulin, rosemary extracts or hesperidin to STZ-induced DCM in rats exhibited a significant downregulation of Endothelin-1 gene expression as compared with untreated group. While treatment with insulin, R. E. or hesperidin significantly induces upregulation in HIF-1α as compared with non-treated group with highest increase in (G3).

Table 5 Effect of insulin, rosemary extract or hesperidin treatment on cardiac tissue ET-1 and HIF-1α gene expressions of STZ-induced diabetic cardiomyopathy in rats.

Animal groups	ET -1 Fold change ±SEM	HIF-1α Fold change ±SEM
Control Normal (G1)	1.00 ± 0.06^{e}	1.00 ± 0.07^{d}
DCM (G2)	8.06 ± 0.37^{a}	1.38 ± 0.1^{d}
DCM + Insulin (G3)	2.77 ± 0.09^{d}	6.59 ± 0.26^{a}
DCM + R.E. (G4)	5.98 ± 0.27^{b}	3.20 ± 0.14^{c}
DCM + HES (G5)	3.86 ± 0.15^{c}	5.06 ± 0.21^{b}

Data are presented as (Fold chain Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05)

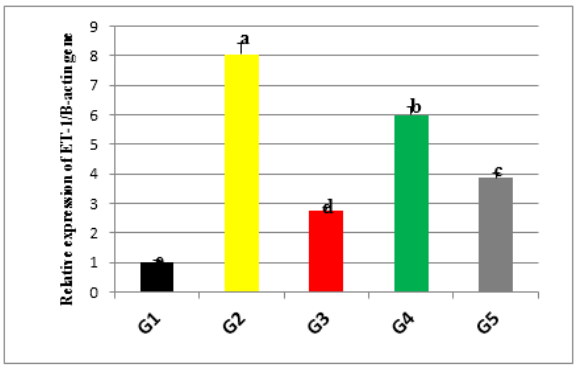

Fig. (4): Effect of insulin, rosemary extract or hesperidin treatment on ET-1 expression in experimental model of diabetic cardiomyopathy in rats.

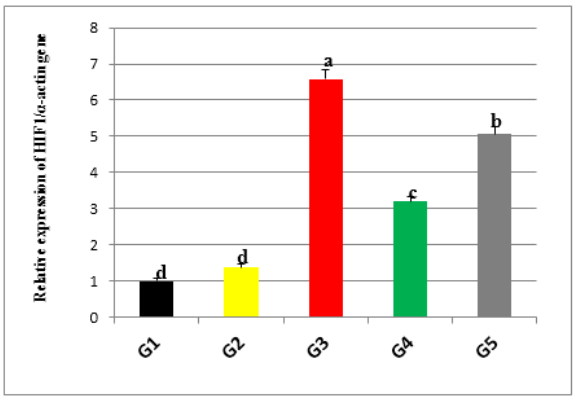

Fig. (5): Effect of insulin, rosemary extract or hesperidin treatment on HIF-1α expression in experimental model of diabetic cardiomyopathy in rats.

Table (6) and figure (6) showed a significant global hyper methylation in STZ-induced DCM when compared with normal control group. However, Treatment with insulin, rosemary extracts or hesperidin to STZ-induced DCM in rats exhibited a significant global hypomethylation as compared with untreated group with highest level in (G3).

Table 6 Effect of insulin, rosemary extract or hesperidin treatment on Global DNA methylation level (5-mC %) of STZ-induced diabetic cardiomyopathy in rats:

Animal groups	DNMT
Control Normal (G1)	1.11 ± 0.07^{e}
DCM (G2)	3.25 ± 0.12^{a}
DCM + Insulin (G3)	1.52 ± 0.09^{d}
DCM + R.E. (G4)	2.38 ± 0.09^{b}
DCM + HES (G5)	1.96 ± 0.08^{c}

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

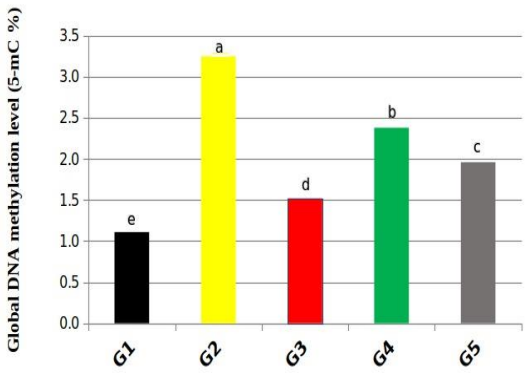

Fig. (6): Effect of insulin, rosemary extract or hesperidin treatment on global DNA methylation level (5-mC %) in experimental model of diabetic cardiomyopathy in rats.

4. DISCUSSION

Diabetes mellitus results in heart's structural, functional, and regulatory remodeling, which is where DCM first appears. Various renovation stages, including the early, advanced, and late stages, have been proposed (Chavali *et al.*, 2013). In the early stages of DCM, myocardial systolic function and structure are not significantly altered in conjunction with metabolic abnormalities including hyperglycemia and insulin resistance (Adeghate and Singh, 2014). However, magnetic resonance imaging (MRI) and echocardiography are able to identify poor myocardial relaxation. Initial signs of DCM include worsening deficiencies in relaxation and heart stiffness, as well as increased atrial filling and decreased early diastolic filling (Falcao and Leite, 2012). The loss of myocardial blood flow reserve as a result of impaired insulin signaling can be detected utilizing a variety of imaging modalities (Ernande and Derumeaux, 2012).

The collected results showed that the glucose level in control group increased significantly. These outcomes resemble those were obtained by Mestry *et al.* (2017) They found that 8 weeks after induction, STZ treatment significantly raised the serum glucose level in the diabetes group compared to the normal control group. The injection of STZ was found to increase blood glucose in numerous reputable recent investigations (He *et al.*, 2018).

When compared to the DCM non-treated group, treatment with rosemary extract considerably reduced the serum glucose concentration. These results were consistent with Bakirel *et al.*, (2008), who linked the anti-diabetic properties of numerous Labiatae species, including rosemary, to their essential oils, which contain mono sesquiterpenes, phenolic compounds, and flavonoids with hypoglycemic properties (caffeic acid and rosmarinic acid concentration). When compared to the DCM non-treated group, treatment with hesperidin dramatically lowered serum glucose concentration in STZ-induced DCM in rats. This outcome is consistent with that of Akiyama *et al.*, 2010 who used rats with type 1diabetes to show that hesperidin can lower blood sugar. The high glucose level in STZ-induced DCM in rats was dramatically reduced in the current study when treated with insulin, rosemary extract, and hesperidin, demonstrating the anti-hyperglycemic efficacy of these compounds.

In STZ-induced DCM, there was a notable increase of the miR-29 and miRNA130b-3p gene expressions. This outcome was consistent with Fernando *et al.* (2021), who stated that mir-29a was the most often reported miRNA in hypertrophic cardiomyopathy (HCM), was mostly expressed in upregulated, and was associated with both fibrosis and left ventricular (LV) hypertrophy as determined by transthoracic echocardiogram (TTE). Because miRNAs can be quantified in blood serum, a valuable biomarker candidate, the study of miRNAs in cardiovascular illnesses has expanded during the past few decades (Barwari *et al.*, 2016). The creation of extracellular matrix proteins and apoptotic processes are connected to the mir-29a (Bargaje *et al.*, 2012). Mir-29a was linked to sudden cardiac death in patients with coronary artery disease in a major experiment, likely because of its connection to fibroblast activation (Silverman *et al.*, 2020). The connection between mir-29a and fibrosis and hypertrophy in HCM may imply that several processes have a common biological base and pathogenic pathways (Olivotto *et al.*, 2015). In rats treated with insulin, R.E., or hesperidin for STZ-induced DCM, DNMT activity was significantly downregulated. This outcome is consistent with Tian *et al.*, (2021) who claimed that hesperidin increased the expression of miR-149 by lowering the degree

of DNMT1-mediated promoter methylation. Hesperidin has also been discovered to be a DNA hypomethylating agent that modifies the patterns of gene expression (Fernández *et al.*, 2017). Hesperidin, for example, could increase SFRP2 expression in adjuvant arthritic rats by lowering DNMT1 (Liu *et al.,* 2017).

The expression of ET-1 and its receptors, as well as their functional consequences, are noticeably changed as cardiovascular illnesses progress. It was shown that oxidative stress increased the production of ET-1 and autocrine ET-1 activity in vascular smooth muscle cells, which may be a mechanism contributing to endothelial dysfunction (Ruef *et al*., 2001). Additionally, ET-1 overexpression has been observed in individuals with oxidative stress-related illnesses as type II diabetes, central obesity, and hypertension (Chuang *et al*., 2012). Contrary to the control group, insulin, R. E., or hesperidin treatment to STZ-induced DCM in rats suppresses strain-induced endothelin (ET)-1 secretion. These findings were consistent with the theory that rosmarinic acid administration improves endothelial function and remodeling via reducing ET-1 production and receptor-mediated effects. Extracellular signal-regulated kinase (ERK) activation has been shown to be redox sensitive (Fujisaw *et al.,* 2002) also showing that reducing ROS prevents strain-induced ET-1 gene expression (Liu *et al.,* 2003). Therefore, the capacity of hesperidin to reduce ROS generation may be one explanation for its inhibitory effect on strain-induced ET-1 expression in the current investigation. There have been claims that hesperidin has antioxidant properties (Cypriani *et al.,* 1993). Fraga *et al*., (1987) reported that hesperidin's anti-oxidant and free radical-scavenging properties may be responsible for this action, which supported the antioxidant activity of hesperidin against cyclic strain-induced ROS generation.

In comparison to the untreated group, treatment with insulin, R. E., or hesperidin dramatically increases HIF-1 activation. This agreed with Caroline et al. (2002), who made that report; Insulin encourages the controlled component HIF-1 to build up in retinal pigment epithelia (ARPE-19) cells that have arisen on their own. The activity of the transcription factor HIF-1 is directly associated with an increase in HIF-1 expression. In fact, they demonstrate that insulin causes elevated HIF-1 protein levels, increased HIF-1 DNA binding activity, and boosted transcription of HIF-1-mediated reporter genes. During hypoxia or ischemia, the activation of HIF-1 in endothelial cells is equally important for the eventual recovery of the heart. By increasing the production of angiogenic factors, HIF-1 mediates the angiogenic response to hypoxia in addition to having metabolic oxygen sparing effects on the endothelium.

5. CONCLUSION

In conclusion, the pathophysiology of complex diseases like diabetes is increasingly being linked to epigenetic mechanisms. The results of the current investigation have demonstrated that DNA methylation and histone modifications play a significant role in the regulation of numerous genes related to diabetes. In STZ-induced diabetic rats, treatment with hesperidin and rosemary extract decreased DCM due to their anti-inflammatory, antioxidant, and anti-apoptotic properties. To determine the precise mechanisms underlying the preventive effect of hesperidin and rosemary extract, more in-depth research is needed.

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