**Original Paper**

**Rosemary and hesperidin alleviate cardiac dysfunction and alter cellular signaling pathways in experimental model of diabetic cardiomyopathy**

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**ARTICLE INFO**

**ABSTRACT**

Alterations in the cardiac structure and function are the hallmarks of diabetic cardiomyopathy (DCM), separate coronary heart disease in diabetic patients. In a streptozotocin-induced diabetic rat model, the current research aimed to investigate the preventive effects of rosemary extract and hesperidin on DCM. Fifty male Rats were divided into five main groups. Group I: rats received no drugs. Group II: STZ (50 mg/kg b.wt.) was given intraperitoneally (i.p.) to rats just once. Group III: Rats treated with insulin at a dose of (2 U/rat/day). Group IV: rats treated with Rosemary extract at a dose of (200 mg/kg b.wt. /day). Group V: rats treated with Hesperidin at a dose of (100 mg/kg b.wt./day). The results showed a marked increase in serum total cholesterol and triacylglycerol concentrations and downregulation in Peroxisome proliferator-activated receptor-γ (PPAR-γ) and Sarcoplasmic reticulum Ca2+-ATPase (SERCA) genes expressions with upregulation in Collagen type IV (COL4) in cardiac tissue of DCM-induced group. Treatment with insulin, rosemary extract, or hesperidin to STZ-induced DCM in rats exhibited a substantial reduction in the levels of triacylglycerol and total cholesterol with an enormous downregulation of COL4 gene expression level in cardiac tissue. However, PPAR-γ and SERCA genes exhibited an enormous upregulation. In conclusion, the results indicated that rosemary extract and hesperidin have a beneficial effect in preventing lipid metabolic disorders and diabetes complications and modify several genes such as PPAR-γ, SERCA, and COL4 participating in cellular pathways which altered in DCM.

**1. INTRODUCTION**

Epidemiological studies showed that persons with diabetes are more likely to get heart failure, and researchers concluded that diabetes and heart failure are closely related (Drozd and Kearny, 2017). Diabetes-related cardiac abnormalities are the cause of the clinical condition known as diabetic cardiomyopathy (DCM) (Boudina and Abel, 2007). About 12% of diabetic people are predicted to get DCM, which can cause heart failure and death (Lorenzo-Almoros et al., 2017). The main characteristics of DCM are myocardial interstitial fibrosis, oxidative stress, inflammation, apoptosis, and cardiac hypertrophy (Saisho, 2014). Reactive oxygen species (ROS) and inflammation are overproduced when there is persistent hyperglycemia in diabetes, which is a major factor in DCM (Bhattacharjee et al., 2016). Numerous studies revealed that the myocardium of people with DCM had a marked rise in pro-inflammatory cytokines (Atta et al., 2018). Reduced left ventricular (LV) function and ROS were strongly linked with pro-inflammatory cytokine levels (Khanra et al., 2015).

In the etiology of complex illnesses like diabetes and its related vascular problems, it is hypothesized that there is a crucial interaction between genes and environment. Epigenetic modifications of the genome are hypothesized to facilitate gene-environment interactions, with environmental changes frequently leading to epigenetic modifications of the genome (Bird, 2007). Numerous antioxidants have received attention as a potential strategy to reduce DCM and other diabetes problems because strong evidence implicates oxidative stress in the emergence of DCM.

The evergreen aromatic plant *Rosmarinus officinalis Linn.* (Rosemary), a member of the Lamiaceae family, is found across the Mediterranean region. It grows to a height of 1 m, has straight stems, whitish-blue blooms, and dark green leaves. One of the most widely used herbal products for flavoring and antioxidants in food preservation and cosmetics was rosemary leaf extract (Cui et al., 2012). Citrus fruits including lemon, orange, lime, and grapefruit contain a lot of hesperidin, a flavanone glycoside that varies greatly depending on the species (Liu et al., 2013) and it is used in conventional medicines commonly, The beneficial function in cardiovascular, anti-inflammatory and antioxidant properties (Mao-Qiang et al., 2019). Hesperidin has multiple bioactivities which include anti-inflammatory (Wei et al., 2012), anti-oxidative (Selvaraj and Pugalendi, 2010), radioprotective (Petrova et al., 2011) and anticancer consequences (Yumnam et al., 2015). In this research, we look into the harmful complications of Streptozotocin-induced diabetes mellitus and intracellular pathways activated in the heart. Moreover, the molecular and hypolipidemic consequences of two natural agents

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(rosemary extract and Hesperidin) in experimentally induced diabetic cardiomyopathy in rats with molecular and biological characteristics are assessed in blood and cardiac tissue. In this study, we explore the harmful complications of STZ-induced DCM and intracellular signaling pathways activated and involved in pathophysiology of diabetic cardiomyopathy (DCM). Also, the ameliorating role of two natural agents (rosemary extract and Hesperidin) towards molecular alterations of cardiac tissue and lipid metabolic abnormalities has been checked.

2. MATERIAL AND METHODS

2.1. Experimental animals:
In this investigation, 50 white male albino rats’ weight 180-200 g aged 5-6 weeks were used. Throughout the course of the experiment, rats were kept in separate metallic cages under constant environmental and dietary circumstances. The rats were given a consistent ration of food, and unlimited access to fresh, clean drinking water was provided. Prior to the start of the trial, all rats underwent a minimum 15-day acclimation period. The 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. BUVTM 02-8-21).

2.2. Chemicals and antioxidant agents:
2.2.1. Streptozotocin:
We obtained streptozotocin (STZ) from (Sigma Chemical Co. P.O. Box. 14508, St. Louis, U.S.A.) uses a single intraperitoneal (i.p.) injection of 50 mg/kg body weight of freshly dissolved, citrate buffered medication for induction of hyperglycemia (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 extract 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 this period, the mixture was filtrated to obtained liquid extract. Then, the extract was concentrated using a rotary evaporator at 50 °C under reduced pressure. This process was repeated at least 3 times. Finally, the extract was weighted and stored at -20 °C till usage. Each rat was orally administered with 0.5 ml of Rosemary extract daily (Abdul- Rahim and Taha, 2011).

2.2.4. Hesperidin:
Hesperidin (95%) was purchased from Al-dawlya Company, dissolved in saline, and given orally (50 mg/ml) at a dose of 100 mg/kg per day, using intra-gastric tubes after 6 weeks of STZ induction (Pires Das Neves et al., 2004).

2.3. Induction of diabetic cardiomyopathy:
Rats were given free access to water after an 18-hour fast. To lower the animals’ blood glucose levels to a point where circulating glucose does not out-compete streptozotocin for GluT-2 binding and transport -cells, fasting is necessary prior to streptozotocin injection. Streptozotocin (STZ), freshly dissolved in citrate buffer, PH 4.5, was administered as a single intraperitoneal (i.p.) injection dosage to male rats to experimentally develop diabetes. After six weeks of starting diabetes, experimental diabetic cardiomyopathy develops. Similar doses of merely the vehicle (citrate buffer) were given to control rats. Following a week of treatment with STZ, rats were fasted for 12 hours, and blood samples were taken from the orbital venous sinus to measure glucose levels. Diabetic rats were defined as those in a group with blood glucose levels greater than 250 mg/dl and were involved in additional research (Ramanathan et al., 1999). To prevent any possible Streptozotocin-induced hypoglycemia, the animals were given access to glucose solution (5%) w/v overnight (STZ).

2.4. Experimental design:
After six weeks of diabetic cardiomyopathy induction all rats were randomly placed in individual cages and classified into five main groups as following:
- Group I: Control Normal group: Consisted of 7 rats, rats fed with ordinary diet only without any treatment during the entire experimental period of 12 weeks.
- Group II: Diabetic cardiomyopathy non treated group: Consisted of 13 rats received a single intraperitoneal (i.p) injected dose of STZ (50 mg/kg body wt.) freshly dissolved in citrate buffer, PH 4.5.
- Group III: STZ + Insulin treated group: Consisted of 10 rats received a single intraperitoneal (i.p) injected dose of STZ (50 mg /kg body wt.) and treated with daily subcutaneous injections of long-acting insulin 2 U/rat per day for 6 weeks after induction of diabetic cardiomyopathy.
- Group IV: STZ + R.E. treated group: Consisted of 10 rats received a single intraperitoneal (i.p) injected dose of STZ (50 mg /kg body wt.) and treated with Rosemary extract orally once per day at a dose of (200 mg/kg body weight/day) for 6 weeks after induction of diabetic cardiomyopathy.
- Group V: STZ + Hesperidin treated group: Consisted of 10 rats received a single intraperitoneal (i.p) injected dose of STZ (50 mg /kg body wt.) and treated with Hesperidin orally once per day at a dose of (100 mg/kg body weight/day) for 6 weeks after induction of diabetic cardiomyopathy.

2.5. Sampling:
2.5.1. Blood samples:
Blood samples were collected from all rat groups at the end of experiment after overnight fasting. Blood was centrifugation at 2500 rpm for 15 minutes for serum separation. Then, collected by an automatic pipette, in a dry, sterile Eppendorf, and stored at -20°C for estimation of total cholesterol and triacylglycerols

2.5.2. Tissue specimens (For molecular analysis):
Rats were slaughtered by decapitation and the abdomen was then opened. Heart tissues were collected, placed in Eppendorf tubes, and immediately kept in liquid nitrogen and stored at -80°C until RNA extraction for the determination of the following gene expression: Peroxisome proliferator-activated receptor-γ (PPAR-γ) and Sarcoplasmic reticulum Ca2+-ATPase (SERCA) in addition to Collagen type IV (COL4).

2.6. Analysis:
2.6.1. Biochemical analysis:
Serum total cholesterol and triacylglycerols were determined enzymatically utilizing the approach outlined by (Ellefson and Caraway, 1976) and Stein, (1987), respectively.
2.6.2. Molecular analysis:
Real-time quantitative polymerase chain reaction analysis (real-time qPCR) was used to identify the mRNA expression levels of PPAR-γ, SERCA2, and COL4 (Table 1) in rat hearts. The load was managed with -actin. Following the manufacturer's instructions, total RNA was extracted from the heart using a High Kit for extraction of pure RNA (Thermo Scientific, Fermentas, #K0731) RNA Extraction kit. The RevertAid TM First Strand cDNA synthesis kit (#EP0451, Thermo Scientific, Fermentas, USA) was used to reverse-transcribe the material for each cDNA. 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 (Livik and Schmittgen, 2001).

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR-γ</td>
<td>GCAAGGACAGCTCCAACTTCC</td>
<td>GCAAGGACAGCTCCAACTTCC</td>
</tr>
<tr>
<td>SERCA2</td>
<td>CGCCGAGGAATGGAACAGCC</td>
<td>CGCCGAGGAATGGAACAGCC</td>
</tr>
<tr>
<td>COL4a3</td>
<td>CCTAAAGGGATGAAAGCTATC</td>
<td>ACTCCGCTAAGGATGAAAG</td>
</tr>
<tr>
<td>β-actin</td>
<td>AAATTCCTACCTCACTGACC</td>
<td>AAATTCCTACCTCACTGACC</td>
</tr>
</tbody>
</table>

2.7. Statistical analysis:
The 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

3.1. Effect of treatment with insulin, rosemary extract, or hesperidin on total cholesterol (TC) and triacylglycerols (TAG) concentrations of rats with STZ-induced diabetic cardiomyopathy are depicted in table (2) and figures (1, 2): When compared to the healthy control group, a substantial rise in TC and TG concentrations was seen in STZ-induced diabetic cardiomyopathy. Injection of insulin (G3), administration of rosemary extracts (G4), or hesperidin (G5) to STZ-induced diabetic cardiomyopathy showed a noticeable decline in TC and TG concentration. With the highest decrease in (G4) followed by (G3) and finally (G5).

Table 2 Effect of insulin, rosemary extract or hesperidin treatment on total cholesterol and triacylglycerols concentrations of STZ-induced diabetic cardiomyopathy in rats:

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Total Cholesterol (mg/dl)</th>
<th>Triacylglycerols (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Normal (G1)</td>
<td>88.17 ± 3.76b</td>
<td>180.76 ± 5.50a</td>
</tr>
<tr>
<td>DCM (G2)</td>
<td>162.09 ± 6.11a</td>
<td>342.36 ± 7.45a</td>
</tr>
<tr>
<td>DCM + Insulin (G3)</td>
<td>125.63 ± 5.02a</td>
<td>305.32 ± 6.34a</td>
</tr>
<tr>
<td>DCM + R.E. (G4)</td>
<td>114.00 ± 4.39ab</td>
<td>267.67 ± 3.85ab</td>
</tr>
<tr>
<td>DCM + HES (G5)</td>
<td>138.04 ± 5.23b</td>
<td>285.55 ± 3.70b</td>
</tr>
</tbody>
</table>

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

3.2. Effect of treatment with insulin, rosemary extract, or hesperidin on Peroxisome proliferator-activated receptor-γ (PPAR-γ), Sarcoplasmic reticulum Ca2+-ATPase (SERCA), and Collagen type IV (COL4) gene expression of cardiac tissue in STZ-induced DCM in rats are presented in table (3) and figures (3, 4, 5): A significant downregulation of PPAR-γ and SERCA gene expression were noticed in STZ-induced DCM in comparison to the normal control group. However, a significant COL4 gene expression level was significant upregulation. Treatment with insulin (G3), rosemary extracts (G4), or hesperidin (G5) to STZ-induced DCM in rats exhibited a significant upregulation of PPAR-γ and SERCA gene expression as compared with the STZ-induced DCM group. With the highest upregulation in (G3) followed by (G5) and finally (G4). However, COL4 gene expression showed a significant downregulation.

Table 3 Effect of insulin, rosemary extract or hesperidin treatment on cardiac tissue PPAR-γ, SERCA and COL4 gene expression of STZ-induced diabetic cardiomyopathy in rats:

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>PPAR-γ</th>
<th>SERCA</th>
<th>COL4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Normal (G1)</td>
<td>1.00 ± 0.00a</td>
<td>1.00 ± 0.00a</td>
<td>1.00 ± 0.00a</td>
</tr>
<tr>
<td>DCM (G2)</td>
<td>0.22 ± 0.01a</td>
<td>0.15 ± 0.01a</td>
<td>11.83 ± 0.42b</td>
</tr>
<tr>
<td>DCM + Insulin (G3)</td>
<td>0.86 ± 0.03a</td>
<td>0.83 ± 0.03a</td>
<td>1.85 ± 0.08a</td>
</tr>
<tr>
<td>DCM + R.E. (G4)</td>
<td>0.46 ± 0.02a</td>
<td>0.32 ± 0.02a</td>
<td>8.40 ± 0.33a</td>
</tr>
<tr>
<td>DCM + HES (G5)</td>
<td>0.65 ± 0.02a</td>
<td>0.51 ± 0.02a</td>
<td>3.76 ± 0.16</td>
</tr>
</tbody>
</table>

Data are presented in (Fold change of mRNA). SEM = Standard error mean. Mean values with different superscript letters in the same column are significantly different at (P<0.05).

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

Fig. (2): Effect of insulin, rosemary extract or hesperidin treatment on triacylglycerols concentration in experimental model of diabetic cardiomyopathy in rats.

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

Fig. (4): Effect of insulin, rosemary extract or hesperidin treatment on SERCA gene expression in experimental model of diabetic cardiomyopathy in rats.
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4. DISCUSSION

Without any other heart conditions, cardiomyopathy is a separate consequence of diabetes mellitus (Huynh et al., 2014). The pathophysiology of DCM is likely influenced by oxidative stress, inflammation, and apoptosis that are brought on by hyperglycemia and hyperlipidemia (Falcao and Leite, 2012; Suzuki et al., 2015). In this investigation, STZ-induced diabetic cardiomyopathy was found to significantly raise TC and TG concentrations, as shown in Table (1) and images (1, 2). Similar to this, it appears that the changed lipid profile in the serum of STZ-induced diabetic rats is a key element in the emergence of atherosclerosis, a condition associated with diabetes (Chattopadhyay and Bandyopadhyay, 2005). Increased levels in serum TG and TC in diabetes are inconsistent with observations of Maiti et al. (2005) and by others (Yadav et al., 2008). A substantial reduction in TC and TG concentrations was seen in rats with STZ-induced diabetic cardiomyopathy that were treated with insulin, rosemary extracts, and hesperidin. These outcomes have been supported by Iweala and Oludare (2011) who claimed that taking a rosemary leaf extract by mouth significantly reduced blood levels of TG, LDL-C, and total cholesterol while increasing HDL-C. Due to its gradual metabolic influence over the systems involved in fat removal from the body, rosemary leaf extract has the potential to be hypolipidemic. The antioxidant properties of Rosemary’s ingredients, such as rosmarinic acid, which altered the rate of fatty acid oxidation in the liver and decreased the rate of triglycerides production in rats, may be the cause of this drop. Xinhuai et al. (2011) detected Hesperidin caused a drop in the range of total cholesterol, TG, and LDL cholesterol as well as a rise in the levels of HDL cholesterol in the serum of rats fed a high-cholesterol diet. In STZ-induced diabetic cardiomyopathy, compared to the group of healthy controls, the expression of the PPAR-γ gene was significantly downregulated, according to the data. However, treatment of STZ-induced diabetic cardiomyopathy in rats with insulin, rosemary extracts, and hesperidin resulted in a considerable elevation of PPAR-γ level in comparison to the STZ-induced diabetic cardiomyopathy group. These findings were consistent with those of Rao et al. (2006) who claimed that aqueous ethanol extract of rosemary was successful in activating PPAR-γ in 80% of cases utilizing a reporter gene experiment. Since both the phenolic diterpene chemicals carnosol and CA were identified as the active ingredients, it is possible that they are a part of the mechanism behind the anti-hyperglycemic properties of rosemary, which are similar to those of the glucose-lowering medication glitazone (Rau et al. 2006). Based on dichloromethane methanol’s anti-oxidant and relaxing effects on type 2 diabetes mellitus and obesity issues, Tu et al. (2013) outlined the mechanism of the rosemary extract’s metabolic control in HepG2 cells. By activating signaling pathways like AMP-activated protein kinase (AMPK), which induces glycolysis, and PPAR-γ. The rosemary extracts also upregulated sirtuin-1, which boosts fatty acid oxidation, Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) (which activates PPAR-γ), and low-density lipoprotein cholesterol (LDL-C) receptor, which is in charge of endocytosis of LDL-C from circulation to liver hepatocytes (Tu et al., 2013). Also, Agrawal et al., (2014) discovered that Hesperidin stimulates GLUT 4 translocation and PPAR to successfully lower blood glucose levels.

In STZ-induced DCM, a substantial downregulation of SERCA expression was seen. Similar to this, repeated tests have demonstrated that type 1 diabetes caused by streptozotocin (STZ) has a down-regulated SERCA2a mRNA expression, protein level, and activity (Zhong et al., 2001). The uptake of Ca2+ into the sarcoplasmic reticulum is a major mediator of the heart’s diastolic relaxation. Numerous investigations have demonstrated an association between poor relaxation and reduced contractility in diabetic cardiomyopathy (Belke and Dillmann, 2004). The primary factor influencing how quickly the heart relaxes is a decreased capacity to sequester calcium into the SR, which has been linked to these changes. In the current work, treatment of STZ-induced diabetic cardiomyopathy in rats with insulin, rosemary extracts, and hesperidin resulted in a substantial elevation of SERCA expression. These outcomes were consistent with (Golfman et al., 1999) and (Zhong et al., 2001) who observed that "Insulin therapy can restore the decrease in SERCA2a activity in STZ-treated rats, suggesting a direct stimulatory impact of insulin on SERCA2a." Other studies showing an upregulation of SERCA1 in skeletal muscle after stimulation with insulin provide additional evidence for this notion (Dibb et al., 2007). The current in vitro experiments demonstrating a direct influence of insulin on SERCA2a transcription in isolated cardiac myocytes further support these observations, which suggest a probable relationship between insulin and expression of SR calcium ATPase. Further evidence for insulin’s critical role in heart health comes from Kim et al., (2008) who showed that both with and without the transplantation of smooth muscle cells, insulin protects heart function in streptozotocin-induced diabetic heart failure.

In STZ-induced DCM, COL4 level was found to be significantly upregulated. In contrast to the STZ-induced DCM group, however, treatment with insulin, rosemary extracts, and hesperidin showed a significant downregulation of COL4 level. Similarly, Haneda et al. (1991) have demonstrated that glucose increases the synthesis of type IV collagen in glomerular mesangial cells from cultured rats. Patients with higher blood glucose levels have seen higher serum type IV collagen concentrations (Matsumoto et al., 1991). Increased matrix protein deposition, including collagen, and subsequent increased expression of the type I and type IV collagen genes are characteristics of DCM.

5. CONCLUSION

By reducing metabolic irregularities and cellular pathways that are disrupted in DCM, the results showed that rosemary extract and hesperidin may have potential therapeutic benefits for treating DCM and other cardiovascular diseases.
5. REFERENCES


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