Antioxidant Effects of *Eryngium carlinae* in Diabetic Rats

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ABSTRACT— *Metabolic diseases have increased considerably such as diabetes mellitus (DM). Since diabetes is a systemic disease, it implies high cardiovascular risks. It has been widely established that cardiac injury is related to mitochondrial dysfunction through increment of reactive oxygen species (ROS). Synthetic antioxidants can have important side effects; therefore natural sources may represent a better option. Traditional Mexican medicine has been using Eryngium carlinae (EC) for medical treatment. Also our group showed that hexanic extract possesses in vitro antioxidant capacity. Experimental diabetes in Wistar rats was generated by streptozotocin (STZ) and hexanic extract of EC was supplied for 7 weeks (30 mg/kg). Cholesterol, triacylglycerides, glucose, and thiobarbituric acid reactive substances (TBARS) levels were determined in serum. Mitochondria from left ventricle were used in the quantification of TBARS, reduced glutathione, nitric oxide (NO) levels and activity of superoxide dismutase (SOD) enzyme was performed. Biochemical parameters of glucose and triacylglycerides, as well as TBARS levels in serum show a significant reduction in diabetic group supplied with EC hexanic extract. Thus, we can conclude that the EC hexanic extract possesses antioxidant activity in vitro, and in vivo, by reducing glucose and triacylglycerides levels during hyperglycemia, which may eventually reduce the risk of developing diabetic cardiomyopathy.*

Keywords— Antioxidants, Cardiovascular disease, Diabetes, Hypoglycemia, Oxidative stress

1. INTRODUCTION

Diabetes mellitus is a metabolic disease in which glucose is not used efficiently, resulting in hyperglycemia due to insufficient insulin action. The incidence of DM has increased significantly in recent decades[1]. Risk factors associated to DM include cardiovascular diseases[1]. Diabetic vasculopathy is related to endothelial dysfunction and impaired vascular function through ROS overproduction, observed in both humans and experimental models[2]. Adults with diabetes are 2 to 4 times more susceptible to heart disease or stroke than adults without diabetes, even when the disease is controlled[3]. Approximately 65% of patients with DM die from a cardiac complication.

DCM is linked to specific anatomical and functional alterations, such as, increased muscle mass or decreased systolic function of the left ventricle[4]. Diabetic cardiomyopathy (DCM) is one of the macrovascular complications of DM that develops as a consequence of vascular and lipid metabolism abnormalities[5]. Diabetic heart damage during DM is intimately linked with mitochondrial dysfunction as well as with an increase in the generation of ROS[6]. The consistent mitochondrial damage observed in the diabetic heart indicates a failure in its quality control. Dysfunctional mitochondria may generate increased ROS production and trigger factors that favor the death of cardiomyocytes[7]. According to several reports hyperglycemia, hyperlipidemia, inflammation, apoptosis, and oxidative stress are involved in DCM pathophysiology. Reactive oxygen and nitrogen species (ROS and RNS) derived from hyperglycemia play a central role in DCM[8]. Treatments that may reduce oxidative stress, or inflammation and nitrosative stress have been shown to be effective in attenuating the development of DCM[9]. The external supply of antioxidants can counteract the effect of such species on the body and prevent the onset of many underlying diseases. Natural sources of antioxidants may be safer to use than synthetic antioxidants, due to lower toxicity and side effects[10].

Eryngium carlinae (EC) is a plant used in traditional Mexican medicine. The decoction of the aerial parts of the plant is used to treat cough, indigestion, lipid disorders and diabetes, as well as to regulate blood pressure. Its ethanolic extract has been shown to possess *in vitro* antioxidant capacity and *in vivo* hypolipidemic effects[11]. In this study our objective in the use of hexane extract of EC was to obtain different polar compounds like terpenes and sesquiterpenes, which have been related to the antioxidant power of various plant extracts[12]. Consequently, the present study was aimed

to investigate the antioxidant activity of the hexanic extract of EC and the capacity to lower glucose and triacylglycerides levels in diabetic rats.

2. METHODS

2.1. Preparation of EC hexanic extract

The plant material was collected in a locality of Morelia, Michoacán, and then dried at room temperature. Three hundred grams (dry weight) of the inflorescence of EC were weighed. This vegetable material was crushed and macerated with hexane (1:10 w / v) for 7 days at 4°C, protected from light in hermetically sealed containers. The macerate was filtered on cotton, then concentrated under reduced pressure on a rotavapor at 40°C and 90 rpm in a constant weight flask. The extract obtained was resuspended in hexane, transferred to a container and taken to dryness. It was then stored at 4°C protected from light until its use[11].

2.2. Evaluation of antioxidant capacity of EC in vitro

The dried inflorescences of E. carlinae (300 g) were triturated and macerated with n-hexane (triturated plant solvent ratio: 1:10 in n-hexane) and kept at 4°C for seven days.

2.2.1. Free radical scavenging activity on DPPH˙

Ascorbic acid (0.3 mg/mL^{-1}), or the different concentrations (0.3, 1.0, 10 and 30.0 mg/mL^{-1}) of the hexanic extract were added in 1 mL of deionized water and mixed with 1 mL of a freshly DPPH solution (0.2 mM). After a 30 min of reaction at room temperature, the absorbance was measured at 517 nm in a Perkin Elmer Lambda 18 UV/VIS Spectrophotometer[13].

2.2.2. Anti-lipid peroxidation

Egg yolk homogenate (0.5 ml, 10% in deionized water, v/v) and ascorbic acid (0.3 mg/mL^{-1}), or the different concentrations (0.3, 1.0, 10.0, and 30.0 mg/mL^{-1}) of the hexanic extract were added and made up to 1 mL with deionized water and 0.35 mM of $FeSO_4$ was added to induce lipid peroxidation and incubated 30 min. Then, 1.5 mL of 20% acetic acid and 1.5 mL of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulfate and 0.5 mL 20% TCA were added and incubated in a boiling water bath for 60 min, and centrifuged at 3500 rpm for 10 min. The absorbance was measured at 532 nm in a Perkin Elmer Lambda 18 UV/VIS Spectrophotometer[14].

2.2.3. Hydrogen peroxide scavenging activity

Ascorbic acid (0.3 mg/mL^{-1}) or the different concentrations (0.3. 1.0, 10.0 and 30.0 mg/mL^{-1}) of the hexanic extract were dissolved in 50 mM phosphate buffer (7.4 pH) and mixed with 6.45 mM of H_2O_2 and incubated 5 min at room temperature. The absorbance value of the reaction mixture was recorded at 230 nm in a Shimadzu UV-2550 UV/VIS Spectrophotometer[15].

2.3. Experimental animals

The animals were cared for according to the Mexican Official Standard (NOM-062-ZOO-1999), "Technical specifications for the production, care and use of laboratory animals", and were also approved by the Institutional Bioethics and Biosecurity Committee of the Instituto de Investigaciones Químico Biológicas, Universidad Michoacana de San Nicolás de Hidalgo No. 07/2018. In male Wistar rats (270-330 g), diabetes was induced by a single administration of streptozotocin (45 mg/kg; i.p.). Rat heart mitochondria were obtained according to Moreno and Hansford[16]. After 5 days, blood glucose concentration was measured using an *Accu-Check Performa* (Roche) glucometer. Only subjects with a glucose concentration $\geq$ 300 mg/dL were included (control animals were treated in the same way, injected intraperitoneal with 0.1 M citrate buffer, pH 4.5).

The animals were randomly divided into 4 groups. Two of the groups were controls, one of which were given 30 mg/kg of hexanic extract of EC, respectively. The remaining two groups were the diabetic groups, one of which were given 30 mg/kg of the extract, respectively. The extract was emulsified (water / Ethanol 9:1) and administered orally with an intragastric cannula (Merck) daily for 7 weeks. The final extract contained none hexane solvent. Therefore, no toxic side effects due to hexane were detected. At the end of the treatment, the rats were fasted for 12 h and subsequently sacrificed by decapitation.

2.4. Measurement of biochemical parameters

The measurement and quantification of glucose, total cholesterol, and triacylglycerides present in the serum of the specimens was performed in vitro on the VITROS DT60 automatic analyzer.

2.5. Oxidative and nitrosative stress measurements

Lipid peroxidation (assayed as thiobarbituric acid reactive substances, TBARS) levels were determined in serum and rat left ventricular heart mitochondria, according to the method of Buege and Aust[17]. Reduced glutathione and nitric

oxide (NO) were measured in left ventricle heart mitochondria, according to the methods of Jollow et al.[18] and Green et al.[19], respectively. The activity of superoxide dismutase (SOD) enzyme was measured using the kit for determination of SOD (19160, SIGMA-ALDRICH, 2014).

2.6. Statistical Analysis

Results are expressed as mean ± standard error (SEM). Statistical analysis was performed using the Student's *t*-test for data points, with the level of significance chosen at $p < 0.05$.

3. RESULTS

3.1. Effects of antioxidant capacity of EC in vitro

Antioxidant properties of the hexanic extract of the inflorescences of *E. carlinae* were evaluated by three different methods and are present in the table 1.

Table 1: Antioxidant capacity properties *in vitro*

	Ascorbic acid (control) 0.3 mg/mL^{-1}	Hexanic extract of *E. carlinae*			
		0.3 mg/mL^{-1}	1.0mg/mL^{-1}	10 mg/mL^{-1}	30 mg/mL^{-1}
DPPH scavenging	93.8 ± 0.08	45.02 ±0.31*	47.67 ±0.75*	57.95 ±0.80*	92.09 ±0.81
Anti-lipid peroxidation	25.99 ±0.53	20.48 ±1.75*	24.17 ±0.66*	28.53 ±1.193	34.2 ±1.29*
H_2O_2 scavenging	83.52 ±2.24	62.7 ± 3.76*	77.97 ±1.99	80.8 ±1.35	88.24 ±1.60

Values are means ±SEM. *p<0.05

3.2. Effects in biochemical parameters

Glucose and triacylglycerides showed a significant decrease (p<0.05) from the diabetic group treated with extract at 30 mg/kg, with respect to the diabetic group without treatment (table 2). Total cholesterol did not show a significant difference (table 2). This shows hypoglycemic and hypolipidemic effects by the extract.

Table 2: Levels of glucose, total cholesterol and triacylglycerides in serum

	Glucose (mg/dL)	Total cholesterol (mg/dL)	Triacylglycerides (mg/dL)
Control	75.8 ± 2.8	31.2 ± 10.8	68.6 ± 6.2
Control + EC	54.8 ± 10.5	16.0 ± 1.0	57.5 ± 14.2
Diabetic	355.2 ± 95.9	45.8 ± 17.7	274.8 ± 80.2
Diabetic + EC	120.0 ± 33.9*	58.4 ± 2.1	111.8 ± 14.9*

Diabetic group without treatment vs. diabetic group treated with 30 mg/kg of the extract. $p < 0.05$.

3.3. Effects in lipid peroxidation levels

To evaluate the oxidative damage in the membrane lipids present in the serum and left ventricle heart mitochondria of the specimens, the levels of lipid peroxidation were measured. In the serum, the diabetic group treated with the extract at a concentration of 30 mg/kg body weight showed a significant decrease ($p <0.05$) in lipoperoxidation levels compared to the untreated diabetic group (figure 1A). The above demonstrates an antioxidant effect of EC hexanic extract in the serum of diabetic rats. On the other hand, in the left ventricle heart mitochondria, the diabetic group treated with the extract at a concentration of 30 mg/kg body weight showed a significant increase in mitochondrial lipoperoxidation levels compared to the untreated diabetic group (figure 1B). The above shows that the hexanic extract of EC has no antioxidant effect in left ventricle heart mitochondria.
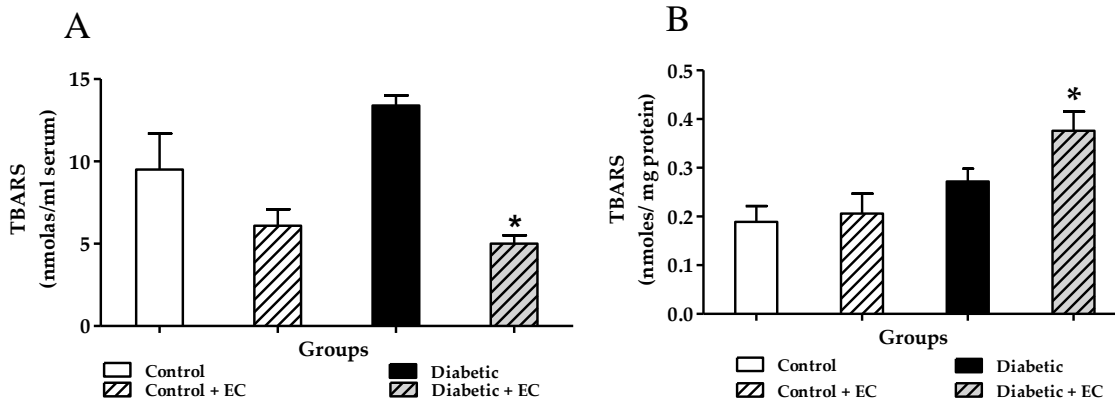
Figure 1: Lipoperoxidation levels in the serum (A) and in left ventricle heart mitochondria (B) at the end of the treatment with hexanic extract of EC.
* Diabetic group without treatment vs. diabetic group treated with 30 mg/kg of the extract.

### 3.4. Effects in reduced glutathione

For the evaluation of the mitochondrial antioxidant capacity, reduced glutathione (GSH) of the control and diabetic groups under treatment was quantified with respect to the groups without treatment. The diabetic group treated at a concentration of 30 mg/kg of the extract showed an increase in GSH levels with respect to the other diabetic groups, although this increase was not statistically significant (figure 2A). The hexanic extract of EC had no representative effects on GSH levels in the treated groups.

3.5. Effects in nitric oxide levels

To assess nitrosative stress, NO levels were determined indirectly. In the groups with diabetes, the group treated with the extract at a concentration of 30 mg/kg body weight showed a significant increase ($p < 0.05$) in NO levels compared to the untreated diabetic group (figure 2B). The increase in NO levels by the diabetic group treated at 30 mg/kg of the extract may result in increased NO bioavailability, which may contribute to the reduction of oxidative stress and this could lead to an endothelial protective effect.

3.6. Effects in SOD enzyme activity

No significant differences were observed in the antioxidant activity of SOD of the groups with diabetes and controls (figure 2C). The above shows that the hexanic extract of EC has no effect on the antioxidant activity of the SOD enzyme in diabetic rat left ventricle heart mitochondria.
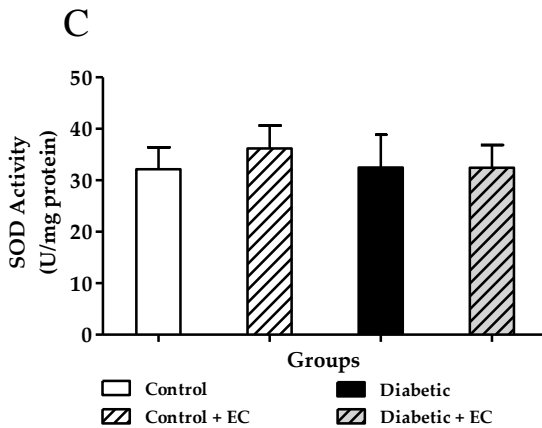
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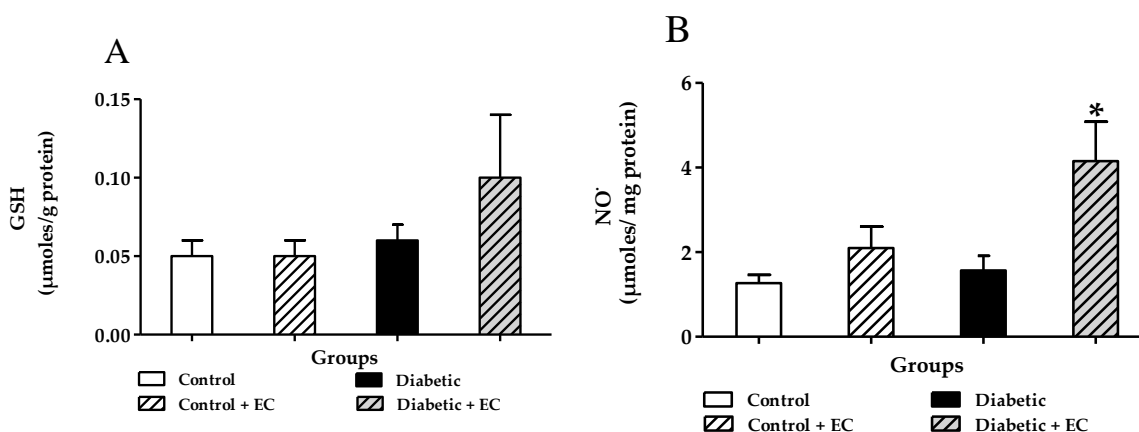
Figure 2: Reduced glutathione (A) Nitric oxide (B) and SOD activity (C) levels in left ventricle heart mitochondria at the end of the treatment with hexanic extract of EC.
* Diabetic group without treatment vs. diabetic group treated with 30 mg/kg of the extract.

4. DISCUSSION

Studies show that long-term hyperglycemia due to poor glycemic management in patients with diabetes induces permanent alterations in several tissues, particularly at the vascular level, even after the hyperglycemia has normalized[3,20]. A good control of blood glucose especially in the early stages brings with it lasting benefits; even a few years of good glycemic control of the DM patient can bring lasting health benefits[1]. On the other hand, maintaining good metabolic control can reduce the risk of CVD in patients with type 1 DM up to 57%[1,21]. Therefore, the hypoglycemic capacity of the hexanic extract of EC could be of great importance as an adjuvant in the treatment and control of diabetes.

Cholesterol levels measured in the serum were no statistically significant results compared with controls. Opposite results of reduced cholesterol and triacylglycerides in diabetic rats were obtained by our research group using the ethanol extract of EC[11], as well as by Patil et al.[22] using chloroform extract of Indian medicinal plants, such as, *Acacia arabica* bark, *Benincasa hispida* fruit, *Tinispora cordifolia* stem, *Ocimum sanctum* areal parts and *Jatropha curcus* leaves. Both extracts used are more polar solvents than hexane.

Diabetic group showed a significant decrease in triacylglycerides levels compared to the untreated diabetic group. This decrease exhibits a hypolipidemic effect of the extract. As mentioned above, good metabolic control may reduce the risk of CVD in patients with DM[1,23]. The hypolipidemic capacity of the hexanic extract of EC could contribute to the correct metabolic control of DM.

The antioxidant capacity of the extract in the serum, as measured by the determination of lipoperoxidation levels, showed a significant decrease compared to the untreated diabetic group. Previous studies have shown that ROS and oxidative stress are crucial for the development of DCM, as well as the fact that various agents that counteract ROS or antioxidants may have the ability to reduce the death of cardiomyocytes and attenuate diabetic heart damage in experimental animal models[9,23]. The antioxidant capacity at the serum level demonstrated by the extract is extremely important, since it may contribute to the prevention or slowing of the onset of MCD.

It has been demonstrated that certain markers like respiratory control ratios or decreased key gene expression for fatty acids oxidation for mitochondrial function in heart failure are located in left ventricular myocardium[24]. In our study, NO levels were measured in left ventricular heart mitochondria, and showed that the diabetic group treated with the hexanic extract reflected a significant increase in NO levels compared to the diabetic group with no treatment. Our results are analogous with Samarghandian et al.[25], where a type of terpene, safranal, obtained from the medicinal plant saffron had radical scavenger activity due to its lipid-soluble property and might act as a membrane-associated free radical scavenger. Reduction of NO bioavailability results in increased oxidative stress and is a hallmark of endothelial dysfunction and an important contribution to the pathogenesis of atherosclerosis[26,27]. The increase in NO levels by the diabetic group may result in increased NO bioavailability, which may contribute to the reduction of oxidative stress and may have an endothelial protective effect.

The values of the mitochondrial lipid peroxidation test in the diabetic group treated with the extract showed a significant increase compared to the untreated diabetic group. The above shows that the hexanic extract of EC has no antioxidant effect at the mitochondrial level. On the contrary, the results reflect an apparent prooxidant effect at this level, which seems to be enhanced by the presence of the disease[20].

Mitochondrial antioxidant capacity was measured through the quantification of GSH levels. GSH is a non-enzymatic antioxidant that plays an important role in protecting cells from oxidative damage by maintaining the cellular levels of the active forms of vitamins C and E by neutralizing free radicals[28]. The results showed an evident increase in GSH

levels in the diabetic group treated with the extract. The above-mentioned increases suggest an improvement in the mitochondrial antioxidant capacity due to the administration of the EC extract. As reported by Samarghandian et al., GSH levels increased in the presence of a terpene obtained from the medicinal plant saffron or its active components crocin and safranal[25].

Measurement of SOD activity as a reflection of mitochondrial antioxidant capacity revealed that, in the diabetic group and diabetic group treated with the hexanic extract, no significant differences were found in the antioxidant activity of SOD. The data described above indicate that the activity of the mitochondrial SOD enzyme is not affected by the administration of the hexanic extract of EC.

5. CONCLUSIONS

The results obtained in the present study show hypoglycemic, hypolipidemic, and antioxidant effects at the serum level of the hexanic extract of EC. However, at the mitochondrial level, lipoperoxidation levels were increased. An increase in NO levels was observed, which could increase their bioavailability and have an endothelial protective effect.

6. ACKNOWLEDGEMENT

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