

Antioxidant effects of silver nanoparticles obtained by green synthesis from the aqueous extract of *Eryngium carlinae* on the brain mitochondria of streptozotocin-induced diabetic rats

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Abstract

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia that affects practically all tissues and organs, being the brain one of most susceptible, due to overproduction of reactive oxygen species induced by diabetes. *Eryngium carlinae* is a plant used in traditional Mexican medicine to treat diabetes, which has already been experimentally shown have hypoglycemic, antioxidant and hypolipidemic properties. The green synthesis of nanoparticles is a technique that combines plant extracts with metallic nanoparticles, so that the nanoparticles reduce the absorption and distribution time of drugs or compounds, increasing their effectiveness. In this work, the antioxidant effects and mitochondrial function in the brain were evaluated, as well as the hypoglycemic and hypolipidemic effect in serum of both the aqueous extract of the aerial part of *E. carlinae*, as well as its combination with silver nanoparticles of green synthesis. Administration with both, extract and the combination significantly decreased the production of reactive oxygen species, lipid peroxidation, and restored the activity of superoxide dismutase 2, glutathione peroxidase, and electron transport chain complexes in brain, while that the extract-nanoparticle combination decreased blood glucose and triglyceride levels. The results obtained suggest that both treatments have oxidative activity and restore mitochondrial function in the brain of diabetic rats.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia and alterations in the metabolism of carbohydrates, lipids, and proteins as a result of defects in the secretion or action of insulin or a combination of both (International Diabetes Federation 2021). DM is one of the main health problems worldwide. It is estimated that the number of people with DM in 2021 will reach 537 million, and by 2045 the figure will increase to 783 million (International Diabetes Federation 2021). With development for several years, DM causes different complications that are classified as microvascular (neuropathy, retinopathy, and nephropathy) and macrovascular (peripheral arterial disease, cerebrovascular disease, and diabetic heart disease), which affect practically all tissues and organs (International Diabetes Federation 2021). One of the organs most affected by diabetes is the brain; since high glucose levels cause increased production of reactive oxygen species (ROS) that are highly reactive and can react with any macromolecule, altering its normal structure and function and exacerbating the complication and damage caused by DM (Islam 2017). Excess glucose generates a greater amount of mitochondrial substrates (Du et al. 2003; Nishikawa et al. 2000), which causes a greater electron leakage from the respiratory chain, especially in complexes I and III, and this ultimately increases ROS production (Sivitz and Yorek 2010). Increased ROS production leads to dysfunction of respiratory chain complexes activity (Chowdhury et al. 2018; Ortiz-Avila et al. 2013; Raza et al. 2015), in addition to the enzymes of antioxidant system (Peña-Montes et al. 2020; Huerta-Cervantes et al. 2020; Hemmati et al. 2018). Imbalance between ROS production/scavenging leads to oxidative stress, in this context, the brain is especially susceptible to oxidative damage due its high rate of oxygen consumption, a modest antioxidant system compared to other tissues, and its high content of

polyunsaturated lipids (Cobley et al. 2018; Hulbert et al. 2007). Long term, oxidative damage produced by DM causes apoptosis, necrosis, neuroinflammation, neurodegeneration, cognitive decline, and development of Alzheimer's disease (Islam 2017).

E. carlinae is a plant used in traditional Mexican medicine, commonly known as "herb frog" or "bighead," which has different properties, such as diuretic, hypolipidemic, antioxidant, antihypertensive, hypoglycemic, and healing properties. The biological activities of *E. carlinae* are attributed to the secondary metabolites present in its extracts, from which mainly flavonoids, terpenes, sesquiterpenes, and phenolic compounds can be isolated. Pérez-Ramírez et al. (2016) obtained an aqueous decoction of the aerial part of *E. carlinae*, where mainly flavonoids were isolated and presented anti-inflammatory activity. Also, multiple reports show that flavonoids significantly reduce oxidative stress (Teixeira et al. 2018; Li et al. 2018; Zhou et al. 2019) and prevent mitochondrial dysfunction (St-Pierre et al. 2006; Jones et al. 2012; Liu et al. 2015). Peña-Montes et al. (2019) isolated terpenes and sesquiterpenes from a hexanic extract of the inflorescences, and this extract showed antioxidant effects in the brain, liver, and kidneys of diabetic rats. Noriega-Cisneros et al. (2020) isolated sesquiterpenes from ethanolic extract of the aerial part and showed a hypolipidemic effect in diabetic rats. While, García-Cerrillo et al. (2018) reported a hypoglycemic effect in diabetic rats from a hexanic extract of the inflorescences. In recent years, the use of nanoparticles synthesized through green synthesis technique, mainly from the secondary metabolites present in plant extracts, has gained great importance in the biomedical area because is an environmentally friendly technique, as opposed to traditional synthesis that uses toxic solvents (Gupta and Xie 2018). Among the different nanoparticles synthesized through the green synthesis technique, silver nanoparticles (AgNP) have antioxidants (Prabhu et al. 2018) and hypoglycemic effects (Sengottaiyan et al. 2016; Mahmoudi et al. 2021). Also, they inhibit α -glucosidase enzymes (Johnson et al. 2018), and significantly increase serum insulin levels (Alkaladi et al. 2014; Shanker et al. 2017). In this paper, we examine the antioxidant, mitochondrial functionality, and hypoglycemic effects of the aqueous extract of the aerial part of *E. carlinae* and its combination with AgNP, in diabetic rats.

2. Materials And Methods

2.1. Vegetal material

The aerial part of *E. carlinae* was collected in Morelia, Michoacán in the fall. After harvesting, the plants were transferred to the Biochemistry Laboratory of the Instituto de Investigaciones Químico Biológicas of the UMSNH, dried and powdered. Subsequently, the powdered plant was stored protected from sunlight at room temperature until use.

2.2. Extract preparation

The aqueous extract of the plant was prepared by the method described by Villalpando and Rosas (2019) with slight modifications. Three grams of the powdered plant was added per 100 mL of deionized water (30 mg/mL). Then, the mixture was heated at a temperature of 85°C with constant stirring for 45 min.

Immediately after heating, the extract was filtered using Whatman No. 1 filter paper. The aqueous extract was stored at 5°C in the dark until use.

2.3. Phytochemical analysis of the extract

Total phenols, flavonoids, and terpenoids were quantified to determine what types of secondary metabolites were more abundant in the extract. Total phenol quantification was measured as previously described by Kim et al. (2003). 10 µL of extract at a concentration of 1 mg/mL were mixed with 750 µL of Folin-Ciocalteu reagent at 10% for 5 min with stirring. Later, 750 µL of 6% Na₂CO₃ was added and mixed for 1 min. Later, it was incubated for 1 h in the dark, and subsequently, the absorbance at 725 nm was measured in a Perkin Elmer UV/Vis Lambda 18 spectrophotometer. The concentration of total phenols was quantified from a standard curve of gallic acid at different concentrations (0–1 µmol). The results obtained were expressed as mg of gallic acid equivalents (GAE)/mL of extract.

Total flavonoid quantification was measured as previously described by Schwarz et al. (2001). 10 µL of extract (1 mg/mL) were mixed with 490 µL of methanol (MeOH) for 1 min. Later, 1 mL of MeOH, 100 µL of AlCl₃, and 100 µL of CH₃O₂K were added and mixed for 1 min. Later, the mixture was incubated for 1 h in the dark and absorbance was measured at 415 nm in a Perkin Elmer UV/Vis Lambda 18 spectrophotometer. The concentration of total flavonoids was quantified from a standard curve of quercetin at different concentrations (0–100 µmol). The results obtained were reported as mg of quercetin equivalents (QE)/mL of extract.

The presence and quantification of total terpenoids was measured as previously described by Ghorai et al. (2012). A total of 250 µL of extract (10 mg/mL) were mixed with 2.5 mL of chloroform for 3 min. Then, the tubes were placed for 10 min in an ice bath. Later, 100 µL of H₂SO₄ was added and placed for 1.5 h in the dark. Then, 100 µL of the reddish phase formed at the bottom of the tube was taken with a micropipette and placed in a cuvette with 900 µL of MeOH, and the absorbance was read at 538 nm in a Perkin Elmer UV/Vis Lambda 18 spectrophotometer. The concentration of total terpenoids was quantified from a standard curve of linalool at different concentrations (0–30 mg/mL). The results were reported as mg of linalool equivalents (LE)/mL of extract.

2.4. AgNP synthesis and characterization

The synthesis and characterization of AgNPs were performed using the method of Villalpando et al. (2020) with modifications. A 10 mM AgNO₃ solution was prepared. Later, 5 ml of this solution was reacted with 5 mL of aqueous extract of *E. carlinae* (30 mg/mL). The reaction was carried out at room temperature with constant stirring for 30 min at a pH of 4.5. AgNP synthesis was confirmed with the UV/Vis technique using a Perkin Elmer UV/Vis Lambda 18 spectrophotometer with 200 to 900 nm scan. Then, scanning electron microscopy (SEM) was performed with a Jeol JSM-7600F microscope. Later, X-ray diffraction (XRD) was used to characterize structures in the crystalline phase using a Bruker D8 Advance DAVINCI equipment (lynx eye) diffractometer.

2.5. Experimental design

Thirty-six male Wistar rats with an initial weight between 280 and 360 g were used. The rats were kept in standard cages with a 12-h light/dark cycle and with food and water provided *ad libitum*. The rats were randomly divided into six groups with n = 6. Group 1, control (vehicle, deionized water). Group 2, NEC (normoglycemic rats administered 30 mg/kg EC). Group 3, NExAgNP (normoglycemic rats administered 30 mg/kg ExAgNP). Group 4, diabetic (vehicle, deionized water). Group 5, DEC (diabetic rats administered 30 mg/kg EC). Group 6, DExAgNP (diabetic rats administered 30 mg/kg ExAgNP). Diabetes induction was performed by a single intraperitoneal injection of 45 mg/kg of body weight streptozotocin (STZ) dissolved in citrate buffer 0.1 M (pH 4.5). At the same time, the normoglycemic groups were injected with citrate buffer alone. Fourteen days after induction, diabetes was confirmed with a commercial glucometer Accu-Check, and individuals showing glucose levels ≥ 300 mg/dL were considered for treatment. The vehicle, the extract, and nanoparticles were administered orally daily for 45 days using an orogastric tube. Glucose levels and body weight were monitored weekly. Once the treatment period was over, the rats were sacrificed by decapitation. Later, the brain was removed, and blood samples were collected to measure cholesterol and triglyceride levels.

2.6. Mitochondria isolation

Brain mitochondria were isolated by the modified Sims method (1990). Once the brain was removed, it was placed in ice-cold brain mitochondria isolation medium [70 mM sucrose, 210 mM mannitol, 1 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.5% albumin, 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.4]. Later, the brain was then cut into smaller fractions. The tissue was transferred to a Potter-Elvehjem tube to homogenize with the help of a Teflon-tipped stem. The homogenate was centrifuged at 2180 rpm at 4°C for 10 min in a Beckman J2-MC centrifuge. Then, the supernatant was decanted into another tube and centrifuged at 10350 rpm at 4°C for 10 min. After centrifugation, the supernatant was discarded and the pellet formed at the bottom of the tube corresponding to the isolated mitochondria was gently resuspended in 750 μ L of isolation medium with a fine-tipped brush. Isolated mitochondria were placed in Eppendorf tubes and stored at -60°C until use.

2.7. Determination of the activity of respiratory chain complexes

To determine the activity of complexes I, II, II + III, and IV, isolated brain mitochondria were permeabilized using the freezing/defrost method previously described by Peña-Montes et al. (2020). Complex I (NADH:ferricyanide oxidoreductase) activity was determined spectrofluorimetrically. Mitochondrial protein (0.2 mg/mL) was resuspended in 50 mM phosphate buffer (PB) at pH 7.4 and incubated for 7 min with 1 μ g of antimycin A and 1 mM KCN. Then, 10 μ M $K_3[Fe(CN)_6]$ was added, and basal fluorescence was recorded for 1 min at an excitation/emission of 340/464 nm. Later, 100 μ M NADH was added, and its oxidation was recorded for 1 min. Next, 10 μ M rotenone was added, and the changes in fluorescence were recorded for 2 min. Finally, 100 μ M NADH was added, and the changes in fluorescence were recorded for 2 min in a Shimadzu RF-5301 spectrofluorophotometer (Ortiz-Avila et al. 2013). Specific activity was calculated using a standard curve of β -NADH.

For complex II (succinate:2,6-dichloroindolphenol (DCIP) oxidoreductase) activity, 0.2 mg/mL mitochondrial protein was resuspended in deionized water and incubated for 2 min. Next, 50 mM PB, 5 mM succinate, 10 μ M rotenone, 2.5 μ M antimycin A, and 1 mM KCN were added and incubated for 3 min. Then, 80 μ M DCIP was added, and the changes in absorbance were recorded for 2.5 min at 600 nm. Finally, 0.5 mM 2-thenoyltrifluoroacetone (TTFA) was added, and the absorbance was recorded for up to 5 min at 600 nm in a Perkin Elmer UV/Vis Lambda 18 spectrophotometer (modified from Cortés-Rojó et al. 2009).

For segments II + III (succinate:cytochrome *c* oxidoreductase), 0.2 mg/mL mitochondrial protein was resuspended in deionized water and incubated for 2 min. Later, 50 mM PB, 5 mM succinate, 10 μ M rotenone and 1 mM KCN were added and incubated for 3 min. Afterward, 250 μ g oxidized cytochrome *c* was added, and the changes in absorbance were recorded for 1.5 min at 550 nm. Finally, 2.5 μ M antimycin A was added, and the absorbance was recorded for up to 3 min at 550 nm in a Perkin Elmer UV/Vis Lambda 18 spectrophotometer (modified from Cortés-Rojó et al. 2009).

For complex IV (cytochrome *c* oxidase), 0.2 mg/mL mitochondrial protein was resuspended in deionized water and incubated for 2 min. Next, 50 mM PB, 10 μ M rotenone, 0.5 mM TTFA, and 2.5 μ M antimycin A were added and incubated for 3 min. Then, 125 μ g reduced cytochrome *c* was added, and the changes in absorbance were recorded for 24 s at 550 nm. Finally, 1 mM KCN was added, and the absorbance was recorded for up to 1.5 min (modified from Cortés-Rojó et al. 2009).

2.8. Determination of reactive oxygen species

ROS production was determined with the 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) fluorescence probe. Mitochondrial protein (0.5 mg/mL) was resuspended in buffer (10 mM HEPES, 100 mM KCl, 3 mM MgCl₂ and 3 mM KH₂PO₄, pH 7.4) and incubated with 12.5 μ M H₂DCFDA for 15 min in ice bath with stirring (Ortiz-Avila et al. 2013). Basal fluorescence was recorded for 1 min, then 5 mM glutamate plus malate was added, and the increase in fluorescence was monitored for 20 min. Changes in fluorescence were recorded at excitation/emission wavelengths of 485/520 nm in a Shimadzu RF-5301 spectrofluorophotometer.

2.9. Determination of SOD2 and glutathione peroxidase activity

Superoxide dismutase 2 (SOD2) activity was determined using a commercial assay kit (Sigma Aldrich, USA). Mitochondrial protein (0.2 mg/mL) was resuspended in 200 μ L assay buffer. Then, 20 μ L of enzymatic work solution were added and incubated for 20 min at 37°C with gentle agitation. The absorbance was measured at 440 nm using a Perkin Elmer UV/Vis Lambda 18 spectrophotometer. SOD2 activity results were calculated using *Escherichia coli* SOD as a standard.

Glutathione peroxidase (GPx) activity was measured spectrophotometrically. A total of 0.8 mg/mL mitochondrial protein was resuspended in 50 mM phosphate buffer + 5 mM 2,2',2'',2'', (ethane-1,2-

diethylenetriaminepentaacetic acid (EDTA). Subsequently, it was incubated for 5 min with 1 mM GSH, 1 mM NaN₃, 0.1 mg bovine serum albumin (BSA) and 100 U/mL glutathione reductase (GR). After the fourth min of incubation, 100 µM NADPH was added, and the remaining min was incubated. Next, fluorescence was measured for 30 s at excitation/emission wavelengths of 352/464 nm, 250 µM H₂O₂ was added, and the changes in fluorescence were recorded for 3 min. GPx activity results were calculated with a curve of NADPH at different concentrations (0-120 µmol).

2.10. Lipid peroxidation

Lipid peroxidation was measured according to the method previously described by Buege and Aust (1978). Then, 0.8 mg of isolated and previously washed mitochondria were resuspended in 0.1 M PBS buffer (pH 7.4). Then, 3% BHT, the reactive solution (15% trichloroacetic acid, 0.375% TBA, and 0.25 M HCl) were added and homogenized in 3 vortex/ice cycles. Later, the samples were incubated for 30 min in a boiling water bath. The resulting precipitate was centrifuged at 7500 rpm for 5 min. Finally, 1 mL of supernatant was transferred to a cuvette to determine the absorbance at 532 nm in a Perkin Elmer UV/Vis Lambda 18 spectrophotometer. The results were expressed as nmoles of thiobarbituric acid reactive species (TBARS)/mg of protein and calculated using the molar coefficient of malondialdehyde (156 mM⁻¹cm⁻¹).

2.11. Animal use statement

All procedures were carried out in accordance with the Mexican Federal Regulations for the production, care, and use of laboratory animals (NOM-062-ZOO-1999). All protocols performed were approved by Institutional Committee for the Use of Animals of the UMSNH.

2.12. Statistical analysis

The results are expressed as the mean ± standard error (SE). Differences between means were assessed using two-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. A p value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Phytochemical evaluation of the aqueous extract of *Eryngium carlinae*

The concentrations of different types of secondary metabolites present in the extract were quantified (Table 1). The results showed that different classes of secondary metabolites were present in the extract. The most abundant metabolites were flavonoids, with a concentration of 3.0332 ± 0.11 mg QE/mL, followed by terpenoids (0.0424 mg LE/mL) and phenols (0.0038 ± 0.09 mg GAE/mL).

Table 1
Concentration of phenols, flavonoids and terpenoids
in the extract.

Metabolite	Concentration
Total phenols (mg EAG/mL)	0.0038 ± 0.09
Total flavonoids (mg EQ/mL)	3.3032 ± 0.11
Total terpenoids (mg EL/mL)	0.0424 ± 0.0028

Results are expressed as the mean ± SE of three independent experiments.

3.2. AgNP synthesis and characterization

The *E. carlinae* aqueous extract and the silver ions reacted for 30 min to evaluate the AgNPs formation. SEM determined the reaction products morphology, size, and elemental chemical composition. Figure 1A shows the formation of AgNPs with semi-spherical morphology, monodisperse and with a size of 10–20 nm. The chemical mapping (Fig. 1B) and the point Energy-Dispersive Spectroscopy (EDS) (Fig. 1C) show that Ag forms NPs entirely.

A color change was observed in the reaction mixture from a light brown to the reddish-brown color, characteristic of AgNPs synthesis (data not shown). Subsequently, to verify the synthesis, the absorbance UV-Vis spectrum (Fig. 2A) of both the extract and the reaction mixture was acquired by scanning from 200–900 nm. Comparing both UV-Vis spectrums, an absorbance peak at 445 nm was observed in the reaction mixture, which is characteristic of surface plasmon resonance (SPR) of the AgNPs. In contrast, this peak was not observed in the aqueous extract spectrum, confirming the synthesis of AgNPs. The crystalline nature of the AgNPs was determined by XRD. In the diffraction pattern (Fig. 2B), Bragg reflections are observed at 2 θ -angles of 38.15°, 44.35°, 64.54°, 72.52°, and 81.60° corresponding to planes (111), (200), (220), (311) and (222) according to the crystallographic card JCPDS 99-101-3078, indicating a typical fcc structure of Ag. In addition, a relative broadening of peak in the XRD pattern is observed, which suggests a small crystal size.

3.3. Effect of treatments on body weight, glucose levels, cholesterol, and triglycerides

The diabetic group showed a significant increase in glucose levels (6.5 times higher) compared to those of the control group, as shown in Table 2. While, DEC group showed a slight decrease in glucose levels, however, it did not show significant differences with respect to diabetic group. While DExAgNP group showed a significant decrease in glucose levels with respect to the diabetic and DEC groups. Moreover, the normoglycemic groups administered the treatments did not show significant differences with respect to control. The control group showed a significant and normal gain of body weight (Table 2) with respect to diabetic, DEC and DExAgNP groups, since all diabetic groups showed a decrease in body weight. On the other hand, the normoglycemic groups administered the treatments did not show significant differences with respect to the control group. Serum cholesterol levels (Table 2) did not show significant

differences between any groups in this study. However, serum triglyceride levels (Table 2) showed a significant increase in the diabetic group compared to the control group. In contrast, the DEC group showed a slight decrease in triglyceride levels, but these were not significant compared to the diabetic group. Nevertheless, the DExAgNP group showed a significant decrease in triglyceride levels with respect to the diabetic group and did not show differences with respect to the control. Moreover, the normoglycemic groups administered the treatments did not show significant differences compared to the control group.

Table 2
Serum and body weight measurements

Group	Glucose (mg/dL)	Body weight (g)	Cholesterol (mg/dL)	Triglycerides (mg/dL)
Control	100 ± 3.33 ^c	400 ± 5.57 ^a	70.66 ± 3.07	59.16 ± 3.50 ^b
NEC	93.55 ± 1.60 ^c	397.83 ± 11.47 ^a	77.66 ± 2.20	61.66 ± 2.41 ^b
NExAgNP	102.66 ± 5.73 ^c	370.83 ± 8.23 ^a	77.5 ± 4.62	60.83 ± 3.77 ^b
Diabetic	536.16 ± 20.05 ^a	243.66 ± 10.00 ^b	72.33 ± 8.24	151.16 ± 18.47 ^a
DEC	436 ± 28.22 ^a	257.16 ± 6.36 ^b	73.83 ± 4.82	108.5 ± 11.73 ^a
DExAgNP	318.66 ± 53.52 ^b	265.66 ± 7.10 ^b	63.66 ± 2.37	95.82 ± 13.64 ^b

The results are expressed as the mean ± SE, n = 6. Different superscript letters indicate significant differences between groups (p value, $p \leq 0.05$), two-way ANOVA, Tukey's post-hoc test. NEC, normoglycemic plus extract; NExAgNP; normoglycemic plus extract-AgNP combination; DEC, diabetic plus extract; DExAgNP, diabetic plus extract-AgNP combination.

3.4. Effect of treatments on mitochondrial respiratory chain complex activities

The activities of respiratory chain complexes were evaluated to determine the impact of diabetes, as well as the impact of both treatments on the activities of complexes in isolated brain mitochondria (Fig. 3). The results showed a significant decrease in complex I (Fig. 3A), complex II (Fig. 3B), and segment II + III (Fig. 3C) activities, as well as a slight decrease in the activity of complex IV (Fig. 3D) in the diabetic group with respect to the control group. Both DEC and DExAgNP groups showed restoration of complex I, II, and II + III activities since they significantly increased their activities with respect to diabetic group and did not show significant differences with respect to the control. Moreover, NEC and NExAgNP groups did not show changes in complex I, II, and IV activities with respect to the control. Nevertheless, the NExAgNP group showed a significant increase in complex III activity compared with the control group.

3.5. Effect of treatments on ROS determination

As shown in Fig. 4, a significant increase in ROS production was observed in the diabetic group (1.6 times greater) compared to that of the control group when the mitochondria were previously incubated with glutamate plus malate. While the DEC and DExAgNP groups both showed a significant decrease in ROS production with respect to the diabetic group, they did not show significant differences compared to the control. Moreover, the normoglycemic groups that were administered the treatments showed no modifications in ROS production compared to control group.

3.6. Effect of treatments on SOD2 and GPx activity

The activity of two antioxidant enzymes, SOD2 and GPx, was measured in isolated brain mitochondria (Fig. 5A and 5B). The results showed a significant decrease in SOD2 and GPx activity in the diabetic group compared to the control. Both the DEC and DExAgNP groups restored the activity of SOD2 and GPx since both groups showed a significant increase in activity with respect to the diabetic group and did not show significant differences than the control. In addition, the NEC and NExAgNP groups did not show changes in either enzyme activity with respect to the control.

3.7. Effect of treatments on lipid peroxidation

Lipid peroxidation was measured in previously washed isolated brain mitochondria (Fig. 6). The results obtained showed a significant increase in lipid peroxidation in the diabetic group compared to the control (1.7 times higher). While the DEC and DExAgNP groups showed a significant decrease in lipid peroxidation with respect to diabetic group (1.4 and 1.5 times lower, respectively), but no significant differences were observed compared to the control. Moreover, the NEC and NExAgNP groups did not show modifications in lipid peroxidation with respect to the control.

4. Discussion

Oxidative stress and mitochondrial dysfunction play important roles in the development of diabetic complications at the brain level since they are related to cognitive decline, alterations in neurotransmission, neuroinflammation, apoptosis, and the development of Alzheimer's disease (Anderson et al. 2009; Hoehn et al. 2009; Muriach et al. 2014). The consumption of medicinal plant extracts to treat different diseases, including DM, has been carried out for several years, and positive effects have been observed on the treated health problems (Arumugam et al. 2013). Among the plants reported to have antidiabetic properties is *E. carlinae*, an endemic plant in Mexico for which works have already been previously reported in which its use as an alternative treatment against DM is promising (Peña-Montes et al. 2019; Noriega-Cisneros et al. 2020; García-Cerrillo et al. 2018). On the other hand, in recent years, new strategies have been sought to increase the effectiveness of drugs. One of these strategies is the use of nanoparticles, which act as drug carriers. Their use is very promising since, in previous reports, they have been shown to deliver drugs to specific sites, crossing biological barriers more quickly, and thus guaranteeing a greater biological effect (Zhang et al. 2020; Mulvihill et al. 2020). The phytochemical evaluation of the aqueous extract of the aerial part of *E. carlinae* carried out in this study showed that flavonoids had the greatest abundance in the extract (Table 1). More precise previous

phytochemical evaluations of the different extracts of *E. carlinae* identified different compounds, especially terpenes, sesquiterpenes (Peña-Montes et al. 2019; Noriega-Cisneros et al. 2020), phenolic acids, flavonoids and saponins (Pérez-Ramírez et al. 2016).

In this work, AgNPs were synthesized that were corroborated by SEM micrographs (Fig. 1A-B), observing AgNPs nearly monodisperse between 10–20 nm and uniformly semi-spherical. These results indicated the efficacy of the *E. carlinae* extract in reducing Ag^+ to Ag^0 and controlling growth and morphology. The results were like those of more expensive and complex conventional methods (Aziz et al. 2019; Sofia et al. 2020). Also, the results were better than those obtained by green synthesis controlled by some parameters such as pH (Manosalva et al. 2019). UV-Vis spectra were obtained to confirm the synthesis of AgNP (Fig. 2A), where both extract and AgNP were compared. An SPR band at 445 nm was observed in the spectrum of AgNPs, whose position corresponds to typical signal of spherical (isotropic) AgNPs (Kumar et al. 2018; Rajakumar et al. 2017). On the other hand, the structural characterization by XRD demonstrated the crystalline nature by identifying the fcc phase of the AgNPs and, as measured by the Debye-Scherrer equation, obtaining an average crystal size of 17 nm. Thus, the broadening of the peaks indicated the formation of silver nanocrystals (Dey and Das 2021; Gracien et al. 2019). The purity of the AgNPs was also demonstrated through chemical EDS analysis (Fig. 1C). Both the observation in the change of color, as the analysis by SEM, XRD, and UV-Vis demonstrated the formation of AgNPs by the aqueous extract of *E. carlinae*.

In this work, a significant increase in glucose levels in diabetic group (6.5 times higher) than in the control group is reported (Table 2) because of STZ administration, which is a highly cytotoxic and selective drug toward pancreatic β -cells (Furman 2015). Nevertheless, a significant decrease in glucose levels in DExAgNP group compared to diabetic group was observed (Table 2). In contrast, a slight decrease in glucose levels in the DEC group was observed, however, it was not significant with respect to the diabetic group. Therefore, the antidiabetic effect can be directly attributed to AgNPs. Previously, it has been reported that AgNPs significantly increase serum insulin levels in diabetic rats (Alkaladi et al. 2014; Hussein et al. 2018; Shanker et al. 2017). Therefore, the decrease in glucose levels observed in DExAgNP group could be related to a higher utilization of carbohydrates by insulin-dependent tissues. DM is a disorder that presents chronic hyperglycemia, but serum lipids are also strongly affected by insulin absence (Hirano 2018). The results obtained in this work showed a significant increase in triglyceride levels in diabetic group with respect to the control (Table 2). This increase may be related to the inability to use carbohydrates by insulin-dependent tissues, which causes an increase in lipolysis in adipose tissue and a subsequent increase in triglycerides in blood circulation (Noriega-Cisneros et al. 2020). In contrast, DEC group showed a slight decrease, although it was not significant compared to diabetic group. Moreover, DExAgNP group showed a significant decrease compared to diabetic group, which is related to glucose level decrease shown in this same group, since this decrease was related to a greater use of carbohydrates by insulin-dependent tissues. This results in decreased lipolysis in adipose tissue (Noriega-Cisneros et al. 2020) and therefore decreased blood triglycerides.

It has been reported that a chronic increase in glucose levels is related to an increase in reducing equivalents NADH and FADH₂ (Sivitz and Yorek 2010), which causes an increase in the flow and leakage of electrons in the respiratory chain. In this work, a decline in complex I, II, and segment II + III activities in diabetic group compared to control group was reported (Fig. 3). This is related to increase in ROS production observed in this same group (Fig. 4), since it has previously been reported that an increase in oxygen consumption in mitochondria during diabetes increases ROS production. This increase in reducing equivalents and oxygen consumption causes respiratory chain complexes dysfunction, which implies a greater ROS production due to electron leakage (Raza et al. 2015; Muller et al. 2004; Hoffman and Brookes 2009). In addition, the decreased expression of some subunits of respiratory chain complexes in the brain of diabetic rats, such as NDUFB8, which is an accessory protein of complex I, has been reported. Likewise, the decreased expression of SDHB of complex II has been reported (Chowdhury et al. 2018). These observations are related to complex III activity during hyperglycemic conditions. It has been reported that posttranslational modifications due to overproduction of ROS in complex III are related to its dysfunctional activity and increased ROS production (Ortiz-Avila et al. 2013; Chowdhury et al. 2018). Moreover, the results obtained for SOD2 (Fig. 5A), and GPx (Fig. 5B) activity showed a significant decrease in activity in diabetic group compared to control. It has been reported that decreased activity of both, SOD2 and GPx may be due to posttranslational modifications in the structure of these enzymes due to increased ROS and RNS production during diabetes (Sharma et al. 2015; Oboh et al. 2018), specifically by the reaction between O₂^{•-} and NO that produces ONOO⁻. In addition, it has been previously reported that this molecule reacts with Tyr residues, specifically Tyr34 of the active site of SOD2 (Kitada et al. 2020; Yamakura and Kawasaki 2010). Additionally, it has been reported that diabetes induces alteration in AMPK/SIRT/PGC-1 α and Nrf2 signaling pathways, inducing decreased expression of SOD2 and GPx (Chowdhury et al. 2018; St. Pierre et al. 2006). The imbalance observed between ROS overproduction and antioxidant enzyme dysfunction in brain mitochondria is related to the lipid peroxidation increase observed in diabetic group (Fig. 6). On the other hand, the DEC and DExAgNP groups showed a significant decrease in ROS production (Fig. 4), a restoration of activity of respiratory chain complexes (Fig. 3), SOD2 and GPx (Fig. 5), and this resulted in decreased lipid peroxidation (Fig. 6). These activities can be directly attributed to secondary metabolites present in both treatments, especially flavonoids. Previously, it was reported that different extracts of *E. carlinae* decreased both ROS production and lipid peroxidation (Peña-Montes et al. 2019; García-Cerrillo et al, 2018). The pathways by which secondary metabolites, especially flavonoids, can exert their antioxidant effect has been reported. Mainly by direct neutralization of ROS (Teixeira et al. 2018), decreasing the formation of metal-dependent hydroxyl radicals (Cu⁺ and Fe²⁺) through Fenton's reaction due to a chelation mechanism and increasing antioxidant enzyme activity (Kosuru et al. 2018), which is in accordance with the decrease in ROS production and lipid peroxidation as well as restoration of antioxidant enzymes and respiratory chain activities in the DEC and DExAgNP groups reported in this work. Additionally, it has been reported that flavonoids can modulate certain signaling pathways related to antioxidant system activity, such as the Nrf2 signaling pathway (Mansuri et al. 2014; Li et al. 2018; Zhou et al. 2019; Khan et al. 2020). Flavonoids can also exert activating effects on the AMPK/SIRT/PGC-1 α signaling pathway, which is the master pathway for regulation of metabolic and energetic homeostasis and mitochondrial biogenesis

(Hardie 2007; Hardie 2008; Greco et al. 2011), which could also explain the restoration of antioxidant enzymes and respiratory chain complex activity and decrease ROS production and lipid peroxidation; however, more studies are needed to confirm this hypothesis.

5. Conclusion

In conclusion, our results show that experimental diabetes increases ROS production, leading to redox imbalance, oxidative damage, and respiratory chain, SOD2, and GPx dysfunction in brain mitochondria. However, administration of both, extract and extract-AgNP combination prevented oxidative damage and respiratory chain, SOD2, and GPx dysfunction in diabetic rat brains. In addition, the extract-AgNP combination presented hypoglycemic and hypolipidemic effects in serum. The AgNPs obtained with *E. carlinae* extract were nearly monodisperse between 10–20 nm and uniformly spherical.

Declarations

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Informed consent statement Not applicable

Conflict of interest The authors declare no conflict of interest.

Institutional review board statement The Institutional Committee approved all animal procedures for the Use and Care of animals of the Instituto de Investigaciones Químico Biológicas of the Universidad Michoacana de San Nicolás de Hidalgo (UMSNH) (Number 2019-08; December 2019).

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Figures

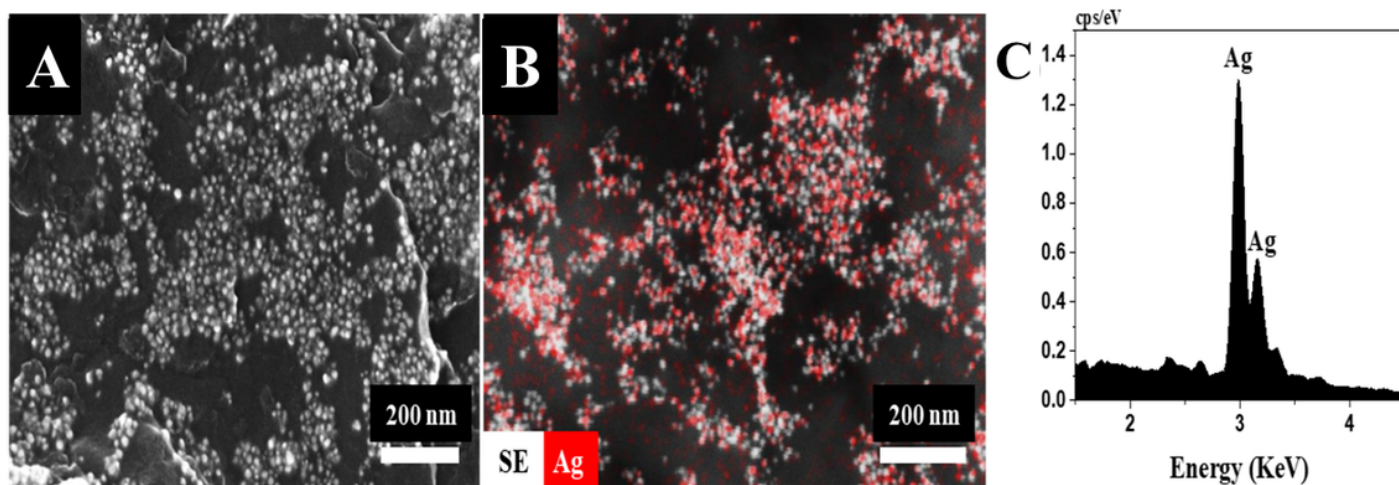


Figure 1

Morphology, size, and chemical composition of NPs. (A), (B) SEM micrograph. (C) EDS analysis.

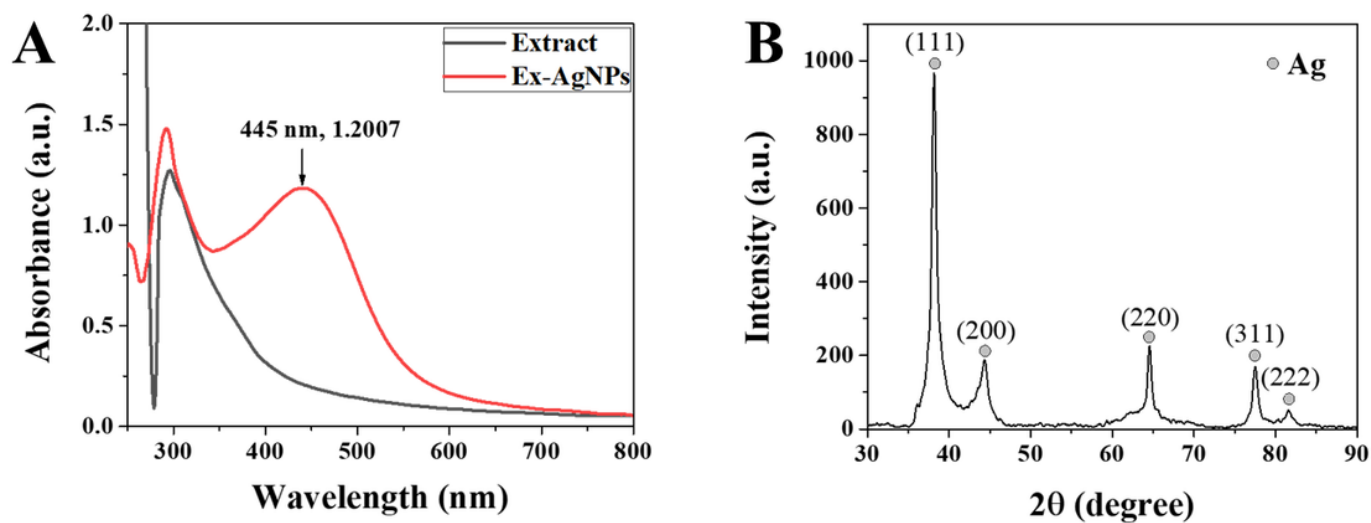


Figure 2

Plasmon resonance and crystal structure of AgNP. (A) UV-Vis absorption spectrum of the extract and AgNPs. (B) XRD pattern of the reaction products.

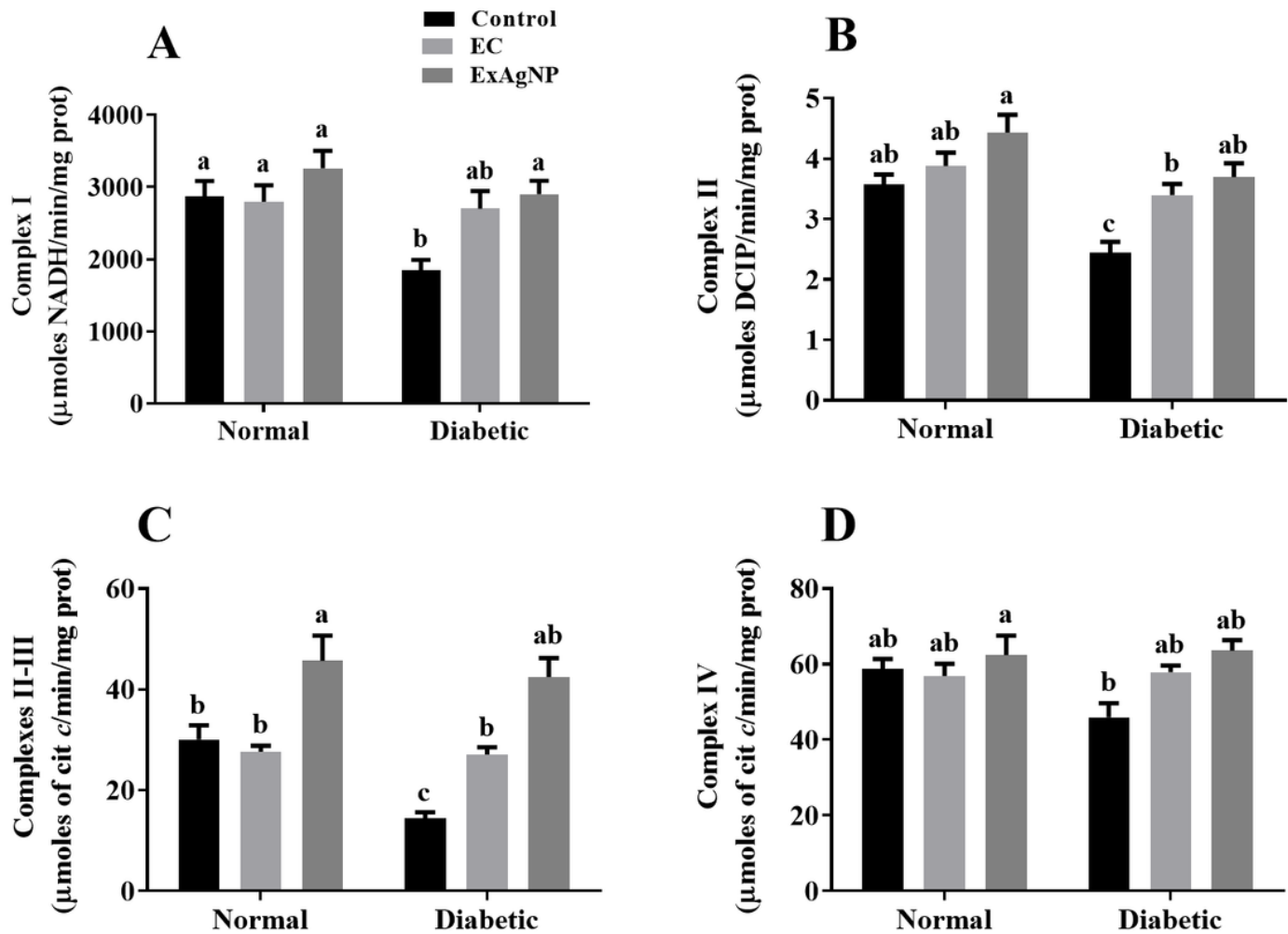


Figure 3

Effect of administration of extract and extract-AgNP combination on respiratory chain complexes activities. (A) Complex I activity, (B) complex II activity, (C) complexes II-III activity, (D) complex IV activity. The results were expressed as the mean $\pm$ SE, $n=6$. Different letters indicate significant differences between groups (p value, $p \leq 0.05$), two-way ANOVA, Tukey's post-hoc test. NEC, normoglycemic plus extract; NExAgNP, normoglycemic plus extract-AgNP combination; DEC, diabetic plus extract; DExAgNP, diabetic plus extract-AgNP combination; Normal, normoglycemic groups; Diabetic, diabetic groups.

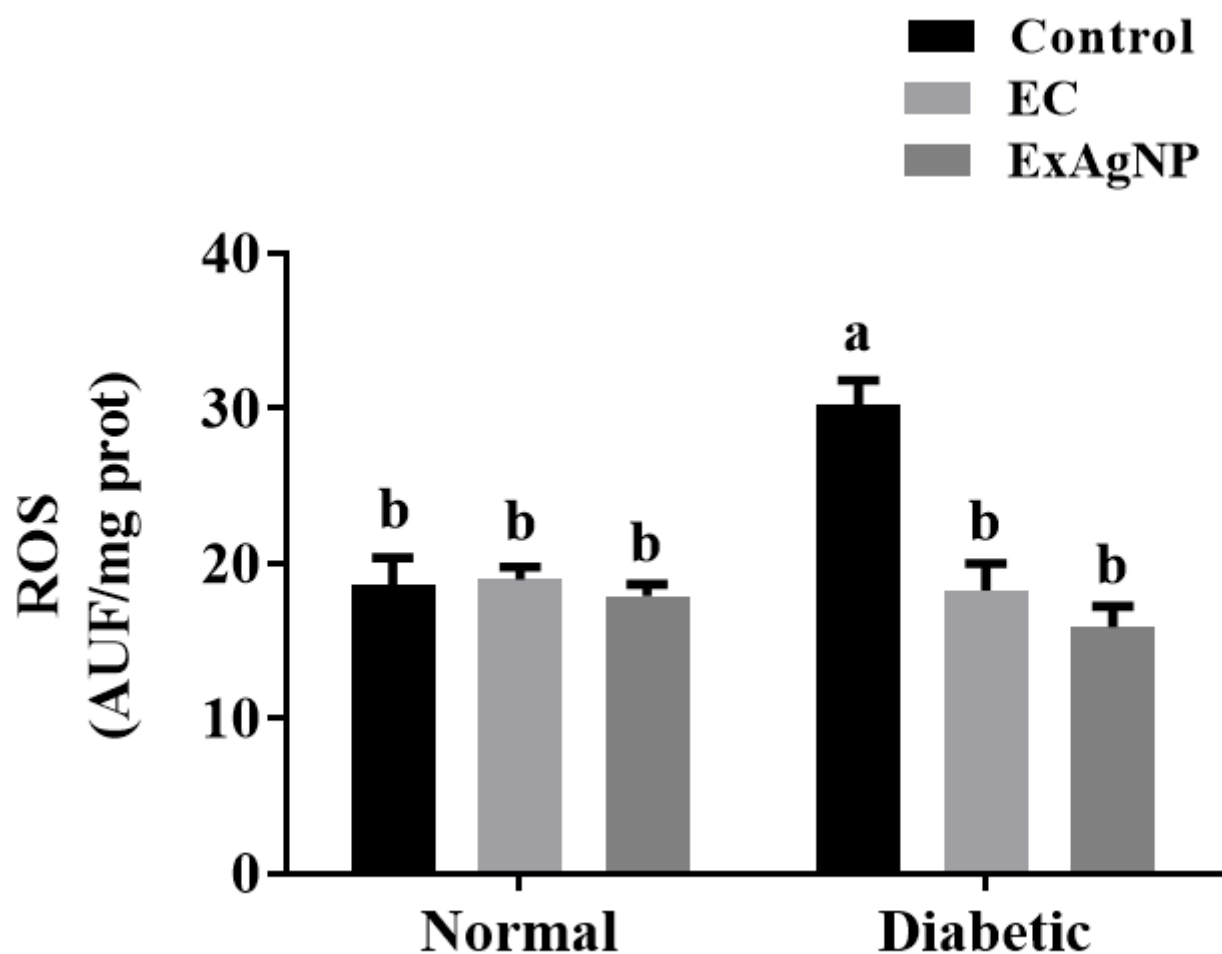


Figure 4

Effect of the administration of the extract and extract-AgNP combination on ROS production. The results were expressed as the mean $\pm$ SE, n=6. Different letters indicate significant differences between groups (p value, $p \leq 0.05$), two-way ANOVA, Tukey's post-hoc test. NEC, normoglycemic plus extract; NExAgNP, normoglycemic plus extract-AgNP combination; DEC, diabetic plus extract; DExAgNP, diabetic plus extract-AgNP combination; Normal, normoglycemic groups; Diabetic, diabetic groups.

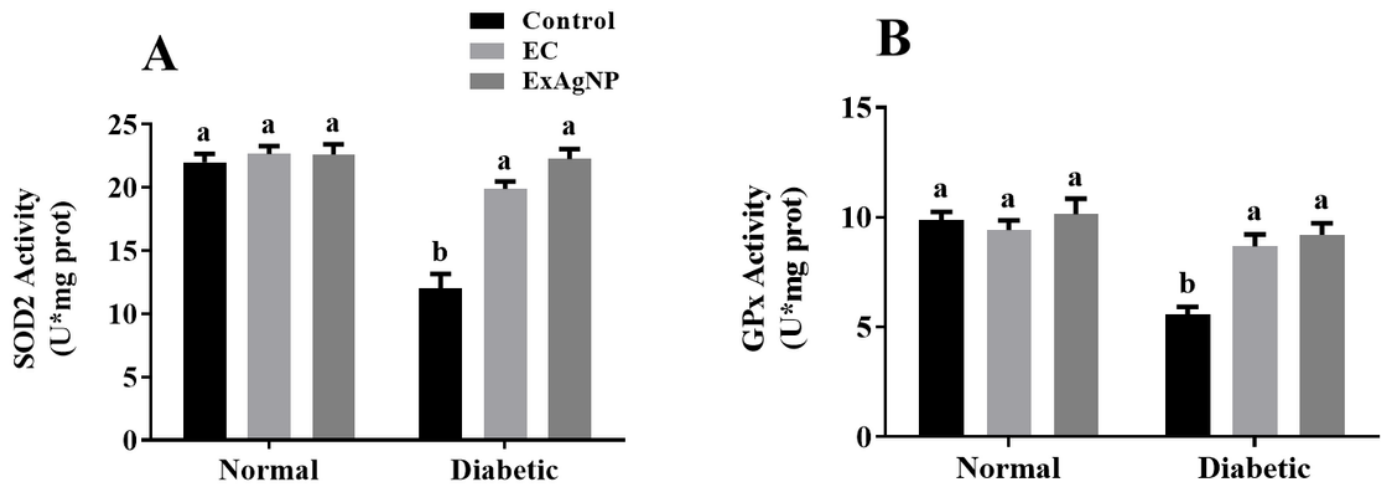


Figure 5

Effect of the extract and extract-AgNP administration on antioxidant enzymes activity. **(A)** SOD2 activity, **(B)** GPx activity. The results were expressed as the mean $\pm$ SE, n=6. Different letters indicate significant differences between groups (p value, $p \leq 0.05$), two-way ANOVA, Tukey's post-hoc test. NEC, normoglycemic plus extract; NExAgNP, normoglycemic plus extract-AgNP combination; DEC, diabetic plus extract; DExAgNP, diabetic plus extract-AgNP combination; Normal, normoglycemic groups; Diabetic, diabetic groups.

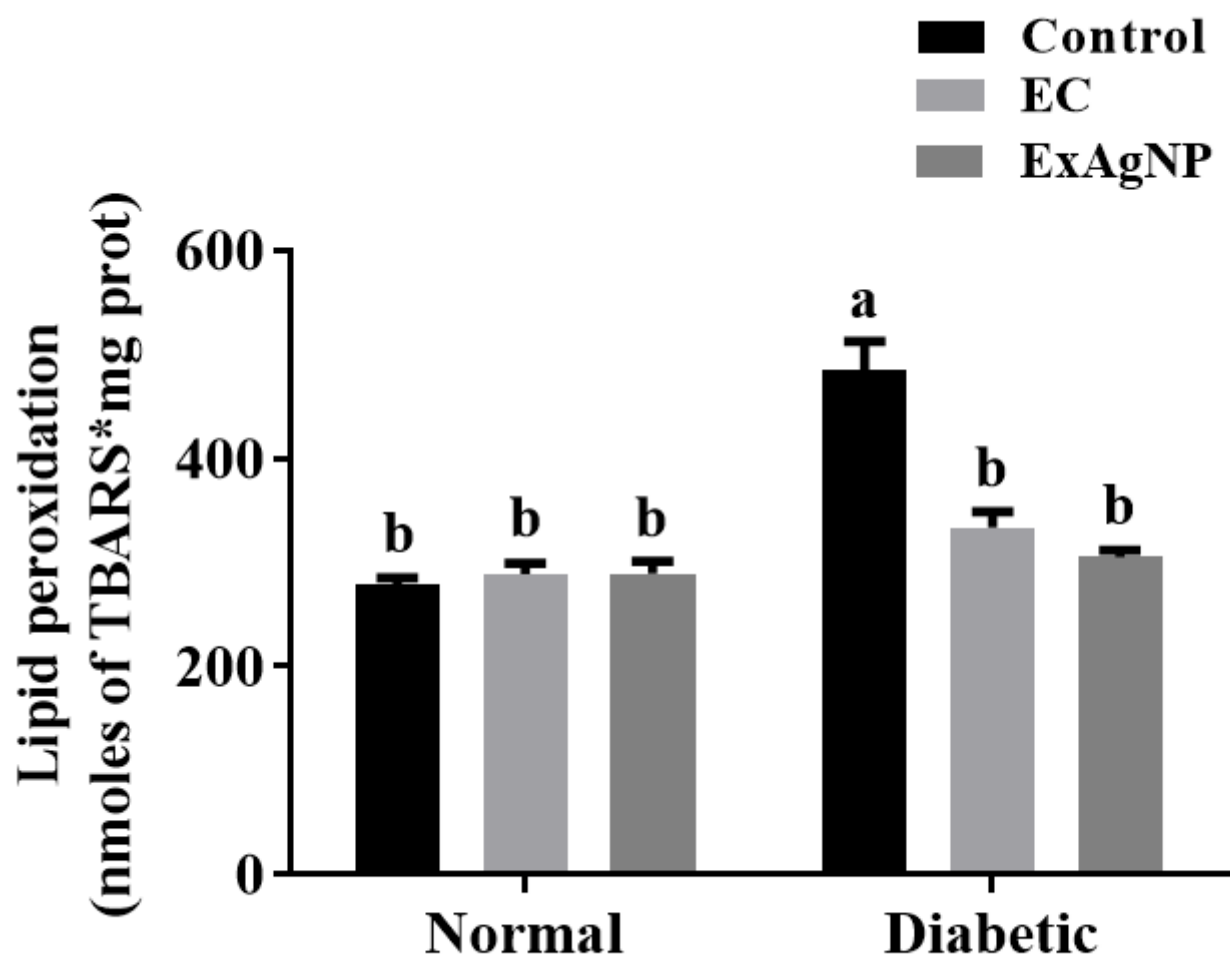


Figure 6

Effect of the extract and extract-AgNP combination administration on lipid peroxidation. The results were expressed as the mean $\pm$ SE, n=6. Different letters indicate significant differences between groups (p value, $p \leq 0.05$), two-way ANOVA, Tukey's post-hoc test. NEC, normoglycemic plus extract; NExAgNP, normoglycemic plus extract-AgNP combination; DEC, diabetic plus extract; DExAgNP, diabetic plus extract-AgNP combination; Normal, normoglycemic groups; Diabetic, diabetic groups.