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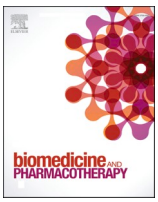

Nrf-2-dependent antioxidant and anti-inflammatory effects underlie the protective effect of esculeoside A against retinal damage in streptozotocin-induced diabetic rats

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ABSTRACT

Esculeoside A (ESA) is a tomato-derived glycoside with antioxidant and anti-inflammatory properties. The protective effect of ESA against diabetic retinopathy is not well-investigated and was the core objective of this study. In addition, we tested if such protection involves the activation of Nrf2 signaling. Type 1 diabetes mellitus (T1DM) was induced in adult Wistar male rats by an intraperitoneal injection of streptozotocin (65 mg/kg). Non-diabetic and T1DM rats were divided into two subgroup groups given either the vehicle or ESA (100 mg)/kg. An additional T1DM group was given ESA (100 mg/kg) and an Nrf2 inhibitor (2 mg/kg) (n=8 rats/group). Treatments continued for 12 weeks. In this study, according to the histological features, ESA improved the structure of ganglionic cells and increased the number of cells of the inner nuclear and plexiform layers in the retinas of T1DM rats. Concomitantly, it reduced the retina levels of malondialdehyde (lipid peroxides), vascular endothelial growth factor, interleukin-6, tumor necrosis factor-α, Bax, and caspase-3. In the retinas of the control and diabetic rats, ESA boosted the levels of total glutathione, superoxide dismutase, heme-oxygenase-1, and Bcl2, reduced the mRNA levels of REDD1, and enhanced cytoplasmic and nuclear levels of Nrf2. However, ESA failed to alter the mRNA levels of Nrf2 and keap1, protein levels of keap1, plasma glucose, plasma insulin, serum triglycerides, cholesterol, and LDL-c in both the control and T1DM rats. In conclusion, ESA alleviates retinopathy in T1DM rats by suppressing REDD1-associated degradation and inhibiting the Nrf2/antioxidant axis.

1. Introduction

Diabetic retinopathy (DR) is the leading cause of visual problems and blindness in diabetic individuals of both type 1 and type 2 [1]. In those patients, DR could be of both types, either proliferative or not proliferative. However, the non-proliferative diabetic stage remains the most therapeutic phase, where drug intervention is still adequate to avoid blindness [2]. In animals, DR can studied after successful induction of experimental diabetes mellitus (DM). In rodents, the analog of the *N*-acetylglucosamine antibiotic, streptozotocin (STZ), is the best-known drug to induce DM and its complications by destroying the pancreatic beta (β)-cells [3]. Multiple studies have utilized the STZ animal model to understand the short and long-term pathogenesis of DR and to discover suitable treatments [4,5].

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Structural and functional abnormalities in the diabetic retina are major complications associated with DM and are the leading causes of the development and progression of DR [6]. Hyperglycemia in DM is the major cause of DR that not only affects the blood-retinal barrier (BRB) and vasculature of the retina but also induces degeneration in the majority of retina cells including pericytes, endothelial cells, pigmented epithelium, Müller cells, and photoreceptors by promoting oxidative stress, inflammation, and apoptosis [6,7]. In addition, it was confirmed that hyperglycemia-derived ROS in the retina remains the major critical factor and the upstream arm for the development of DR by scavenging antioxidants and promoting lipid peroxidation, oxidative stress, mitochondria damage, and upregulating several markers that are involved in inflammation, angiogenesis, and apoptosis [7,8]. The mechanism by which hyperglycemia induces ROS is mediated by activating several intracellular signaling pathways and has been revised in excellent reports [8–10]. Nonetheless, antioxidant therapy, not only in animals, but also in diabetic humans, alleviated several functional and structural aspects of the retina and improved visual functions by decreasing oxidative stress, inflammation, and apoptosis [10–13].

On the other hand, regulating gene expression is essential to maintain the cellular physiological homeostasis. The nuclear factor E2-related factor 2 (Nrf2) is the most known chief regulator in the cell that can induce antioxidant genes and affords cytoprotection in the tissue of several animal models of chronic or metabolic disease [14,15]. Using Nrf2-null mice, it was shown that the nuclear transactivation of Nrf2 is the most important feature of this factor, which is rapidly induced by oxidative or electrophilic stress [16]. In the nucleus, Nrf2 binds to the antioxidant response element (ARE) to stimulate the transcriptions of several antioxidant genes [14,16]. In addition, Nrf2 can inhibit inflammation by suppressing the nuclear factor-kappa beta (NF-κB) and NRLP3 inflammasome [17]. It can also suppress cell apoptosis by upregulating the anti-apoptotic protein, Bcl2 [18]. However, the activation of Nrf2 is mainly regulated by its cytoplasmic stability which is maintained by an inhibitory protein called the Kelch-like ECH-associated protein 1 (keap1) [19]. This protein acts as a linker between Nrf2 and the E3-ubiquitin ligase complex to ensure Nrf2 ubiquitinates and continuous degradation [16,19]. ROS, electrophilic, and several drugs and chemicals can dissociate Nrf2 from keap1 by two major mechanisms including oxidation of keap1 cysteine residues or increasing p62 that competes with the binding of keap1 to Nrf2 [14, 16; 19]. In addition, Nrf2 can be also positively or negatively regulated at the transcriptional and post-translational levels by the activity of miRNAs and several cytoplasmic proteins such as protein kinase C (PKC), aryl hydrocarbon receptor (AhR), and glycogen synthase kinase-3 beta (GSK-3β] [19].

The emerging role of Nrf2 in preventing diabetic complications and hyperglycemia multi-organ damage is well-documented and was shown to be mediated by restoring insulin expression, decreasing fasting blood glucose and lipid levels, suppressing gluconeogenesis, and attenuating oxidative stress and inflammation [15,20]. Other authors have also reported that the impairment in Nrf2 signaling is a major leading mechanism underlying the hyperglycemia-mediated oxidative and inflammatory damage in DR [20–25]. Indeed, diabetic mice lacking Nrf2 showed more significant oxidative and inflammatory neural damage in their retina than $Nrf2^{+}/^{+}$ diabetic rats [21]. In addition, higher expression levels of Nrf2 that were associated with reduced nuclear activity were seen in the retinas of diabetic rodents and were correlated with the severity of oxidative and inflammatory damage [20,23,24]. On the contrary, Nrf2 activation alleviated, slowed down, and protected against DR by attenuating oxidative stress and other pathological damaging mechanisms [26–30]. Therefore, drugs that can stimulate Nrf2 signaling are seen to provide a novel therapy against DR.

During the last decades, many discoveries have focused on plant-derived phytochemicals to treat DR due to their antioxidant and anti-inflammatory properties [31]. Several phytochemicals such as anthocyanins, eriodictyol, scutellarin, galanin, and myricetin were reported to alleviate DR by activating the Nrf2/antioxidant axis [31,32]. Esculeoside A (ESA) is a newly reported spirosolane steroidal glycoside (C58H95NO29) that is isolated from mini and ripe tomato fruits as a result of the oxidation of tomatine [33,34]. The pharmacological and anti-diabetic potentials of ESA are poorly examined. In one study, ESA showed a hypoglycemic effect mediated by suppressing hepatic gluconeogenesis and increasing insulin sensitivity [35]. Other authors have shown exceptional hypolipidemic effects of ESA in apoE-deficient mice, which underlies its anti-atherogenic potential [33].

Besides, the protective effect of ESA against neuropathy and DR is completely lacking. In very recent studies, AlTamimi et al. [36,37] have shown that ESA could prevent diabetic reproductive toxicity and cardiomyopathy by activating the Nrf2/antioxidant axis [36,37]. This was a stimulus for us to examine the protective effect of ESA against DR in rats. Therefore, in this study, we assumed that chronic treatment with ESA might alleviate retina damage in STZ-induced T1DM rats by attenuating oxidative stress, inflammation, and apoptosis. In addition, we hypothesized that this protection also involves the activation of Nrf2. Furthermore, we shed light on the effect of ESA on e some possible regulators of Nrf2 in the retina of these rats.

2. Materials and methods

2.1. Rats

Adult male Wistar rats (9 weeks/180–200 g) were used in this study. All animals were housed in suitable plastic cages under ambient conditions (21 ± 1℃ & 12/12 h day/light). All protocols conducted in this study were approved by the ethical committee for the use of laboratory animals at King Khalid University.

2.2. Induction of T1DM model

Induction of T1DM in rats was done using STZ (Sigma Aldrich, # 142155) as demonstrated by others [38]. In brief, each rat was i.p. injected with STZ solution (freshly prepared in 0.5 M sodium citrate) as a single dose of 65 mg/kg. Post injection, all rats were immediately fed 0.5% oral glucose solution for the next three days to avoid any sudden death due to hypoglycemia. By the end of day 7, the animals were anesthetized by ketamine/xylazine (80/10 mg/mg) solution, and their blood samples (0.25 ml) were collected by tail tip cut and used to measure glucose levels. Those animals showing blood glucose levels greater than 290 mg/dl were considered hyperglycemic and used for the rest of the experiments.

2.3. Isolation of the ESGA

The isolation and preparation of ESA were performed per the recently described method of AlTamimi et al. [36,37]. In brief, fresh ripe tomato fruits were smashed, filtered, and centrifuged (500 x g/10 min). The isolated supernatants were run into column chromatography with a Diaion HP20 and then eluted with 60% methanol. The pellet containing ESA was concentrated under pressure. ESA structure was identified and confirmed at the Department of Pharmaceutical Chemistry, College of Pharmacy at the College, King Khalid University.

2.4. In vitro DPPH radical scavenging activity

The diphenyl-1-picrylhydrazyl (DPPH) is a widely accepted test to examine the antioxidant potential of plant extract and active ingredients [39]. The principle of this test is based on the ability of antioxidants to donate hydrogen atoms to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and truing its color from violet to yellow which can be measured spectrophotometrically at 517 nm. In brief, the stock solution of DPPH was freshly prepared as 0.001 M DPPH in methanol and covered with aluminum foil to keep it away from light. To prepare the working DPPH reagent, 3 ml of this stock solution was added to 47 ml of

methanol. ESA was dissolved in methanol to prepare different concentrations (10, 50, 100, and 200 μg/ml). Then 0.5 ml of each ESA sample was mixed with 3 ml of the DPPH working solution. The blank solution contained only the working reagents. During the test, all tubes were kept in in dark for 30 min and then the absorbance (ABS) was read at 517 nm. The test was done in triplicate for each sample. The antioxidant capacity of ESA was then calculated using this formula: [% of antioxidant activity $= [(ABS_{blank} - ABS_{sample}) \div ABS_{blank}] \times 100]$.

2.5. Animal experimental design

ESA was dissolved in 0.5% carboxymethylcellulose. This study included 5 animal groups (n=12 rats/each). **1) Control group:** were non-diabetic rats that were only treated with normal 0.5% CMC, orally, as a vehicle, **2) Control + ESA-treated group:** were non-diabetic rats that were orally treated with ESA (100 mg/kg), **3) T1DM-model group:** were diabetic rats that were treated with 0.5% CMC, **4) T1DM + ESA-treated group:** were diabetic rats that were treated with ESA solution (100 mg/kg), and **5) T1DM + ESA + Brusatol-treated group:** were diabetic rats that were treated with ESA solution (100 mg/kg) + Nrf2 inhibitor (Brusatol, 2 mg/kg). All treatments were given orally and daily for a total period of 12 weeks. This period was shown to be required for the development of DR after this dose of STZ injection in rats [40]. Final body weights were monitored weekly.

2.6. Dose selection

The use of Brusatol to block the *in vivo* activity of Nrf2 in multiple tissues was adapted from others [36]. In addition, in our preliminary data, daily brusatol administration to control rats over 4 weeks, did not affect mRNA or total cytoplasmic levels of Nrf2 but only inhibited the nuclear localization of this protein in the retina of rats. The dose of ESA was previously used by others and shown to alleviate diabetic cardiomyopathy and reproductive toxicity in STZ-treated rats by activating the cardiac and testicular Nrf2 axis [36,37].

2.7. Biochemical measurements in the plasma and serum

By the end of week 12, anesthesia was introduced to 12-hour fasting rats of all groups using a ketamine/xylazine mixture (80/10 mg/mg). Blood samples (1 ml) were directly collected from the heart and placed into plain or EDTA tubes (0.5 ml each). The blood tubes were allowed to be set for 30 min at room temperature and then centrifuged at 500 *x g* for 10 min. Plasma and serum were collected and placed at −20 ℃ until analysis. Plasma levels of glucose and insulin were measured using assay and ELISA kits (# 10009582, Cayman Chemicals, CA, USA, and #589501, Ann Arbor, TX, USA, respectively). Plasma levels of hemoglobin A1c (HbA1c) were measured by ELISA (#MBS2033689, MyBiosource, CA, USA). Serum levels of total cholesterol (CHOL), triglycerides (TGs), and low-density lipoprotein cholesterol (LDL-c) were also measured using ELISA kits (# EK720559, # EK720636, #EK720763, AFG scientific, IL, USA, respectively). All used kits were specific to rats and the analysis was performed for n=8 samples/group and as per each kit's instructions.

2.8. Collection of the retina and preparation of tissues

After blood collection, all animals were euthanized by cervical dislocation. The right and left eyes of each rat were dissected on the ice, and their retinas were removed under a compound microscope while maintaining them on ice. Parts of the right retina were placed in 10% buffered formalin and sent to the pathology laboratory at the College of Medicine for further histological processing. The remaining parts of the right retina and the retina of the left eye of each rat were quickly placed in liquid nitrogen and kept at −80℃ until further processing. Later, some parts of these retinas were homogenized in neutral phosphate-buffered saline and centrifuged (10000*g*/10 min/4℃) to collect tissue homogenates (supernatants). Other parts were used to extract the nuclear and cytoplasmic fractions using a commercial nuclear/cytoplasmic separation kit (# NT-032; Invent Biotechnologies, MN, USA).

2.9. Biochemical analysis in the retina homogenates

The levels of malondialdehyde (MDA), total glutathione (GSH), superoxide dismutase (SOD), heme-oxygenase-1 (HO-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were measured by ELISA kits (# EK720889, # EK720816, # EK720188, # EK720658, #EK720267, and # EK720127, AFG scientific, IL, USA, respectively). The cytoplasmic and nuclear levels of Nrf2 were assayed using special ELISA kits for rodents (# 50296, Active Motif, CA, USA). The cytoplasmic total levels of keap1 were measured by ELISA (# MBS7218529, Mybiosource, CA, USA). The total homogenate levels of Bcl2, Bax, caspase-3, and advanced glycation end products (AGEs) were measured using ELISA kits purchased from LSBIO, MA, USA Bcl2 (# LS-F4135, #LS-F5064, # LS-F4138, and #LS-F39268 respectively). The levels of vascular endothelial growth factor (VGEF) in the retina homogenates were measured using an ELISA kit (# MBS724516, Mybiosource, CA, USA). All kits were rat's specific and all measurements were performed for n=8 samples/group per each kit manufacturer's instructions.

2.10. Real-time PCR (qPCR)

The levels of transcription were determined using qPCR. Briefly, total RNA was isolated from frozen retina tissues using the TRIZOL reagent. The first strand of DNA was prepared using a commercial kit (# ab 286905, Abcam, Cambridge, UK). Primer sequences for Nrf2, keap1, and β-actin were adopted from of study of AlTamimi et al. [37]. The primer sequence for REDD1 was adopted from the study of Wang et al. [41]. All these primers were validated on the NIH primer design tool and were synthesized and provided by ThermoFisher and are shown in Table 1. All amplification reactions were conducted using a real-time PCR machine (Model number CFX96, BioRad) using the Ssofast Evergreen Supermix kit and as per instructions. The levels of expression of each target were presented relative to the mRNA levels of β-actin using the 2ΔΔCT method. Analysis was done for 6 samples/group.

2.11. Statistical analysis

Data of all parameters were collected and analyzed with the help of GraphPad Prism analysis software (version 8, USA). Analysis was done by the 2-way ANOVA test, and Tukey's test was post hoc. Correlations were determined using the Pearson correlation coefficient. Data were considered significantly different at $p < 0.05$ and were presented or graphed as means ± standard deviation (SD).

3. Results

3.1. Free radical scavenging potential of ESA

Results of the free radical scavenging activity as assayed by the DPPH test are shown in Table 2. Different concentration of ESA was used in the assay (10, 50, 100, 200 μg/ml). In accordance, ESA showed a dose-response increase in antioxidant potential where the antioxidant potential was 35.2% with the highest ESA concentration (200 μg/ml).

3.2. Survival rate in all groups of rats

Survival rates in all groups of rats are depicted in Fig. 1**A&B**. No death was observed in the control or ESA-treated control rats. Four rats died (on weeks 4, 6, 7, and 9) in the T1DM yielding a survival rate of 66.3%. However, the survival rate was increased to 90.9% in the T1DM rats as only one rat died on week 11. The T1DM + ESA + brusatol

Table 1
Primer pairs used in the real-time polymerase chain reaction.

		Froward primer (5' → 3')	Reserve primer (5' –3')	Product size (bp)
Nrf2	NM_031789.2	AAAATCATTAACCTCCCTGTTGAT	CGGCGACTTTATTCTTACCTCTC	118
Keap1	NM_057152.2	CTTCGGGGAGGAGGAGTTCT	CGTTCAGATCATCGCGGCTG	140
REDD1	NM_080906.2	TAGTGCCCACCTTTCAGTT	GTCAGGGACTGGCTGTAACC	121
β-actin	NM_031144.3	CGAGTACAACCTTCTTGCAGC	CCTTCTGACCCATACCCACC	209

Table 2
The antioxidant activity of ESA as measured in vitro by the DPPH scavenging activity.

Sample	ABS 517	% of antioxidant
Blank	0.915 ± 0.008	
ESA (10 μg/ml)	0.841 ± 0.016	8.15 ± 1.04%
ESA (50 μg/ml)	0.746 ± 0.014	18.4 ± 2.3%***
ESA (100 μg/ml)	0.652 ± 0.009	28.4 ± 1.09%***###
ESA (200 μg/ml)	0.593 ± 0.005	35.2 ± 0.64%***###$$$

Data were given as means ± SD for 3 samples/test. ***: significantly different vs. 50 μg/ml ($p<0.0001$) ###: significantly different vs. 100 μg/ml ($p<0.0001$). $$$: significantly different vs. 100 μg/ml ($p<0.0001$).

showed the highest number of deaths where 5 deaths yielding a survival rate of 58.3%. In this group of rats, 1 rat died on week 3, 2 rats on week 5, 1 rat on week 8, and 1 rat on week 9. These data indicate that ESA not only reduces the death rate but also increases the longevity of rats.

3.3. Effect of ESA on diabetic markers and lipid profile

The alterations in final body weights, plasma levels of glucose, insulin, and HbA1c, as well as in serum fasting levels of triglycerides, cholesterol, LDL-c, and FFAs in all groups of rats are shown in Table 3. No significant changes in the levels of all these parameters were observed between the control rats that were treated with the vehicle or ESA ($P>0.05$). The final body weights and the fasting levels of glucose, HbA1c, triglycerides, cholesterol, LDL-c, and FFAs were significantly higher ($p<0.0001$) but fasting insulin levels were significantly less in the diabetic rats that received the vehicle (T1DM), ESA (T1DM + ESA), and ESA + Bursatol (T1DM + ESA + Brusatol) as compared to control or ESA groups. However, no changes in the levels of all these markers were observed between T1DM, T1DM + ESA, and T1DM + ESA + Bursatol-treated groups of rats ($p>0.05$).

3.4. Effect of ESA on retina histopathology

Retina sections that were collected from the control (Fig. 2A) or ESA-treated control rats (Fig. 2B) showed normal structure of all layers including the nerve fiber layer (NFL), ganglionic cell layer (GC) inner plexiform layer (IPL), inner nuclear layer (INL), outer nuclear layer (ONL), and external limiting membrane (ELM), and innermost photoreceptor layer (rods and cones). The number of cells forming the GC layer, INL, and ONL were not significantly different between the control and control + ESA-treated rats (Figure F). Retinas from T1DM model rats (Fig. 2C) showed various pathological alterations, including a detachment of the NFL layer, an increased number of pyknotic GCs, partial damage of cells of the IPL, and a reduction in the number of cells INL. Within this view, the number of cells forming the GC layer and ONL were not significantly different but there was a significant reduction in the number of cells forming the INL in T1DM rats as compared to the control or ESA-treated rats (Fig. 2F). This group of rats also showed few RBCs in their GC layer. On the contrary, the retinas from T1DM + ESA-treated rats (Fig. 2D) exhibited intact NFL and most GC cells and had the normal appearance of all other layers forming it. Yet, detached NFL with few pyknotic GCs and little loss in photoreceptor cells were observed in this group of rats. However, they showed a significant increase in the number of cells forming the INL as compared with T1DM rats (Fig. 2F). The retinas of the T1DM + ESA + brustaol-treated rats (Fig. 2E) showed almost similar pathological changes as those observed in the retinas of T1DM model rats. These included an increased number of pyknotic GCs, partial loss of cells forming the IPL, severe loss of the cells of the INL, increased loss of photoreceptors, and the presence of some RBCs in the GC layer and OPL. However, no detachment of NFL was observed in this

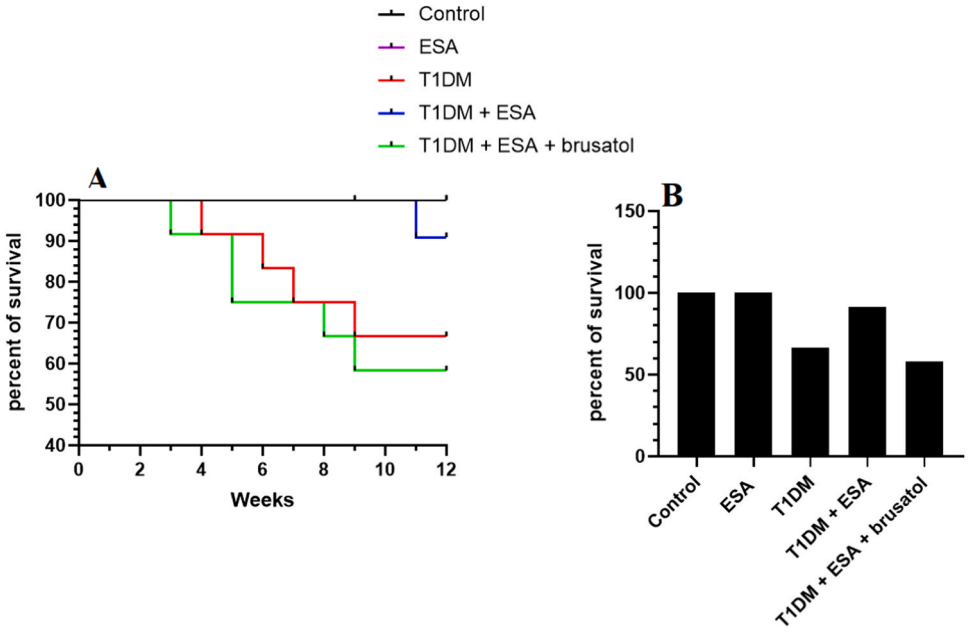


Fig. 1. : Survival percent in all groups of rats over the total 12 weeks treatment period.

Table 3
Alterations in final body weights, plasma levels of glucose, insulin, hemoglobin A1c (HbA1c), and serum lipid profile in all groups of rats.

	Control	ESA	T1DM	T1DM + ESA	T1DM + ESA + brusatol
Final body weight (g)	450.2 ± 39.4	455.7 ± 45.9	355.8 ± 32.2$^{***\#\#\#}$	335.7 ± 31.9$^{***\#\#\#}$	356.4 ± 27.6$^{***\#\#\#}$
Plasma					
Fasting glucose (mg/dl)	99.4 ± 6.81	97.2 ± 8.36	305.1 ± 22.4$^{***\#\#\#}$	292.3 ± 32.5$^{***\#\#\#}$	309.9 ± 26.4$^{***\#\#\#}$
fasting insulin (μU/ml)	4.08 ± 0.54	4.17 ± 0.60	1.61 ± 0.26$^{***\#\#\#}$	1.57 ± 0.36$^{***\#\#\#}$	1.76 ± 0.35$^{***\#\#\#}$
HbA1c (%)	2.96 ± 0.44	2.80 ± 0.36	7.53 ± 0.47$^{***\#\#\#}$	7.4 ± 0.59$^{***\#\#\#}$	7.62± 0.74$^{***\#\#\#}$
Serum					
Triglycerids (mg/dl)	78.4 ± 7.7	75.7 ± 7.2	165.7 ± 12.2$^{***\#\#\#}$	1.66.8 ± 14.5$^{***\#\#\#}$	168.3 ± 16.7$^{***\#\#\#}$
Cholesterol (mg/dl)	83.5 ± 6.4	81.7 ± 7.6	185.6 ± 18.8$^{***\#\#\#}$	183.4 ± 18.7$^{***\#\#\#}$	180.4 ± 13.4^{ab}
LDL-c (mg/dl)	48.7 ± 5.7	45.4 ± 6.2	89.5 ± 7.5$^{***\#\#\#}$	90.66 ± 7.9$^{***\#\#\#}$	89.4 ± 7.4$^{***\#\#\#}$
FFAs (mmol/ml)	0.74 ± 0.09	0.79 ± 0.9	1.75 ± 0.11^{ab}	1.84 ± 0.16^{ab}	0.171 ± 0.13$^{***\#\#\#}$

Analysis was conducted using the 2-way ANOVA test, and Tukey's test was post hoc. Data were presented as means ± SD for n = 8 samples/group. ***: significantly different as compared to control ($p<0.0001$); $^{\#\#\#}$: significantly different as compared to non-diabetic rats that received ESA (ESA group) ($p<0.0001$).

group of rats. Statistically, the number of cells forming the INL was significantly lower than those counted in the control, ESA, and T1DM + ESA-treated rats and not significantly different as compared to T1DM rats (Fig. 2F).

3.5. Effect of ESA on retinal levels of the antioxidants and selected markers of oxidative stress

Retinas from the control non-diabetic rats which were treated with ESA showed a significant increase ($p<0.001$) in the total levels of (Fig. 3A), SOD (Fig. 3B), and HO-1 (Fig. 3C) and a significant reduction in the levels of MDA (Fig. 3D) as compared to control rats. No significant alterations ($p>0.5$) in the levels of AGEs were observed between these two control groups of rats (Fig. 4A). Opposing this, the retinas obtained from T1DM rats showed a significant reduction in their levels of GSH (Fig. 3A), SOD (Fig. 3B), and HO-1 (Fig. 3C) that were concomitant with significant increments ($p<0.0001$) in the levels of MDA (Fig. 3D) and AGEs (Fig. 4A) as compared to the control or ESA rats (Fig. 3A). On the other hand, the T1DM + ESA-treated group of rats revealed significantly higher levels of GSH, HO-1, and SOD ($p<0.0001$) with significantly lower levels of MDA and AGEs ($p<0.0001$) in their retinas when compared to T1DM rats. This was significantly and completely reversed in T1DM + ESA + brustaol-treated rats. In the retinas of T1DM + ESA + brustaol-treated rats, the levels of GSH, HO-1, and SOD were significantly lower ($p<0.0001$) but the levels of MDA and AGEs were significantly higher ($p<0.0001$) as compared to control, ESA, and T1DM + ESA groups. The levels of all these biochemical endpoints were not statistically varied between T1DM rats and T1DM + ESA + brustaol-treated rats ($p>0.05$).

3.6. Effect of ESA on retinal levels of VEGF and selected inflammatory cytokines

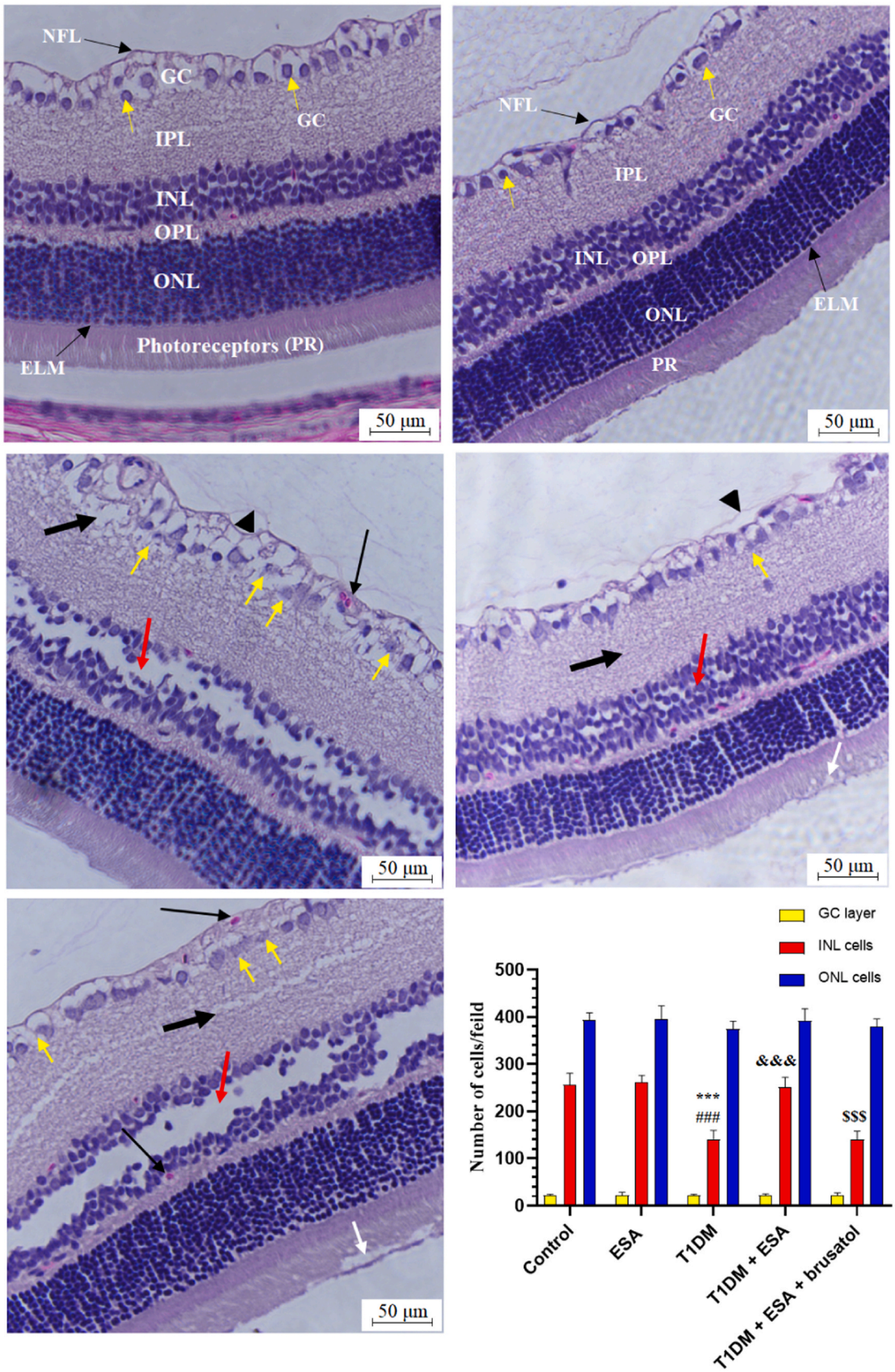
The retinal levels of VEGF, TNF-α, and IL-6 are depicted in Fig. 4**B-C**, respectively. No changes ($p>0.05$) in the levels of VGEF, TNF-α, and IL-6 were observed between the control and ESA non-diabetic groups. The levels of VGEF (Fig. 4B), TNF-α (Fig. 4C), and IL-6 (Fig. 4D) were significantly higher ($p<0.0001$) in the retinas of T1DM rats as compared either to control or ESA-treated groups. The measured levels of all these markers were significantly reduced ($p<0.0001$) in the retinas of T1DM + ESA-treated rats when compared to T1DM rats. On the other hand, T1DM + ESA + brustaol-treated rats showed significantly higher ($p<0.0001$) retinal levels of VGEF, TNF-α, and IL-6 as compared to control, ESA, and T1DM + ESA-treated rats, levels which were not significantly varied ($p>0.05$) with the T1DM rats.

3.7. Effect of ESA on retina selected markers of intrinsic cell apoptosis

Treating the non-diabetic control rats with ESA (ESA group) significantly enhanced the total levels of Bcl2 (Fig. 5A) with no alterations in the total levels of Bax (Fig. 5B) or caspase-3 (Fig. 5C) as compared to control rats. As a result, the retinal of ESA-treated rats showed significantly higher ratios ($p<0.0001$) of Bcl2/Bax (Fig. 5D). A significant reduction ($P<0.0001$) in the total levels of Bcl2 (Fig. 5A) that is concomitant with a significant increase ($p<0.0001$) in the total levels of Bax (Fig. 5C) and caspase-3 (Fig. 5D), as well as lower ratios of Bcl2/Bax (Fig. 5D) ($P<0.0001$) were observed in the retinas of T1DM rats as compared to control and ESA-treated rats. The total levels of Bax and caspase-3 were significantly lower ($p<0.0001$) but the levels of Bcl2, as well as the ratios of Bcl2/Bax, were significantly higher ($p<0.0001$) in the retinas of T1DM + ESA-treated rats as compared to T1DM rats. Interestingly, the retinas of T1DM + ESA + brusatol demonstrated significantly reduced levels of Bcl2, higher levels of Bax and caspase-3, and lower ratios of Bcl2/Bax ($p<0.0001$) when compared to the control, ESA, and T1DM + ESA treated rats. In addition, no significant alterations ($p>0.05$) in the levels of all these apoptotic/anti-apoptotic markers were observed when the retinas of T1DM rats were compared to those collected from T1DM + ESA + brusatol-treated rats.

3.8. Effect of ESA on retina keap1/Nrf2 axis

Retinas from T1DM rats revealed significantly higher mRNA levels ($p<0.0001$) and protein levels of keap1 as compared to control rats (Fig. 6A & B, respectively). They also showed significantly higher mRNA of Nrf2 (Fig. 6A) but significantly reduced total cytoplasmic levels of Nrf2 (Fig. 5B) ($p<0.0001$). As a result, the retina of this group of rats showed significantly ($p<0.0001$) lower total cytoplasmic Nrf2/keap1 protein ratio (Fig. 6C) and nuclear protein levels of Nrf2 ($p<0.0001$) (Fig. 6D). However, retinas from ESA-treated rats or T1DM + ESA-treated rats showed no significant changes ($p>0.05$) in the mRNA levels of keap1 and Nrf2 (Fig. 6A) neither in the total protein levels of keap1 (Fig. 6B) but had significantly higher ($p<0.0001$) total cytoplasmic protein levels of Nrf2 (Fig. 6B) as compared to their controls (control or T1DM model rats, respectively). In accordance, control + ESA-treated rats showed significantly higher cytoplasmic protein ratio of Nrf2/keap1 ($p<0.0001$) and nuclear protein levels of Nrf2 ($p<0.0001$) as compared to control rats. Similar significant increments in the cytoplasmic protein ratio of Nrf2/keap1 and nuclear protein levels **Nrf2** were also observed in the retinas of T1DM + ESA-treated rats when compared to T1DM rats. Treatment with brusatol didn't affect the mRNA levels of both Nrf2 and keap1 nor the total keap1 protein levels in the retinal T1DM + ESA + brustaol-treated rats and the levels of all these markers were not significantly different when this group of rats was compared with the T1DM rats or with the T1DM + ESA-treated rats. On the contrary, retinas from T1DM + ESA + brusatol showed significantly lower levels of total cytoplasmic and nuclear levels of Nrf2 and had lower cytoplasmic protein ratio of Nrf2/keap1 as compared to T1DM + ESA-treated rats. When compared to each other, the cytoplasmic protein ratios of Nrf2/keap1, as well as the nuclear levels of Nrf2 were not significantly different ($p>0.05$) between the retinas of T1DM rats and T1DM + ESA + brusatol-treated rats. These data indicate that brusatol suppressed Nrf2 nuclear translocation independent of modulating the expression of Nrf2 or keap1 and not through the translation of keap1 but most likely by increasing the cytoplasmic degradation of Nrf2.



(caption on next page)

Fig. 2. : Photomicrograph of retinas from sample rats of each experimental group as routinely stained with hematoxylin and eosin (H & E) staining. **Photomicrograph A:** represents a retina section from a control non-diabetic. **Photomicrograph B:** represents a retina section from an Esculeoside A (ESA) (100 mg/kg)-treated rat. Both photomicrographs indicate normal histological features of the retina with intact layers (NFL: nerve fiber layer; GC: ganglionic cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; ELM: external limiting membrane (OLM); PR: photoreceptors). Note an abundant number of cells of each layer in both retinas. **Photomicrograph C:** represents a retina from a T1DM rat and shows obvious detachment of the NFL (arrowhead), pyknotic GC (yellow arrow), increased loss of cells forming the INL (red arrow), partial loss of cells forming IPL (thick black arrow), and presence of some RBCs in GC layer (yellow arrow) (thin long black arrow). **Photomicrograph D:** represents a retina section of a T1DM + ESA (100 mg/kg)-treated rat and shows much improvement in terms of the structures of GCs, IPL, and INL layers. The IPL and INL layers appeared normal with no damage or cell loss (thick black and red arrows, respectively). However, the detachment of NFL, the presence of very few pyknotic GCs (yellow arrow), and the slight loss of photoreceptors (white arrow) are seen. **Photomicrograph E:** represents a retina section of a T1DM + ESA (100 mg/kg) + Brusatol-treated rat and shows similar pathological alteration to those observed in T1DM. These include an increased number of pyknotic GCs (yellow arrow), damage and loss of cells of forming IPL(thick black arrow) and INL (red arrow). However, while an intact NFL layer was observed, some red blood cells in the OPL and GC layer (thin long black arrow) along with partial loss of PR cells (white arrow) were observed in this group of rats. (200X). Image **F:** shows the count of cells in the IPL, INL, and ONL layers in the retinas of all groups of rats. In F: Analysis was conducted using the 2-way ANOVA test, and Tukey's test was post hoc. Data were presented as means ± SD for n = 8 samples/group. ***: significantly different as compared to control ($p<0.0001$). $^{\#\#\#}$: significantly different as compared to non-diabetic rats who received ESA (ESA group) ($p<0.0001$).). $^{\&\&\&}$: significantly different as compared to T1DM ($p<0.0001$). $^{\$\$\$}$: significantly different as compared to T1DM + ESA ($p<0.0001$).

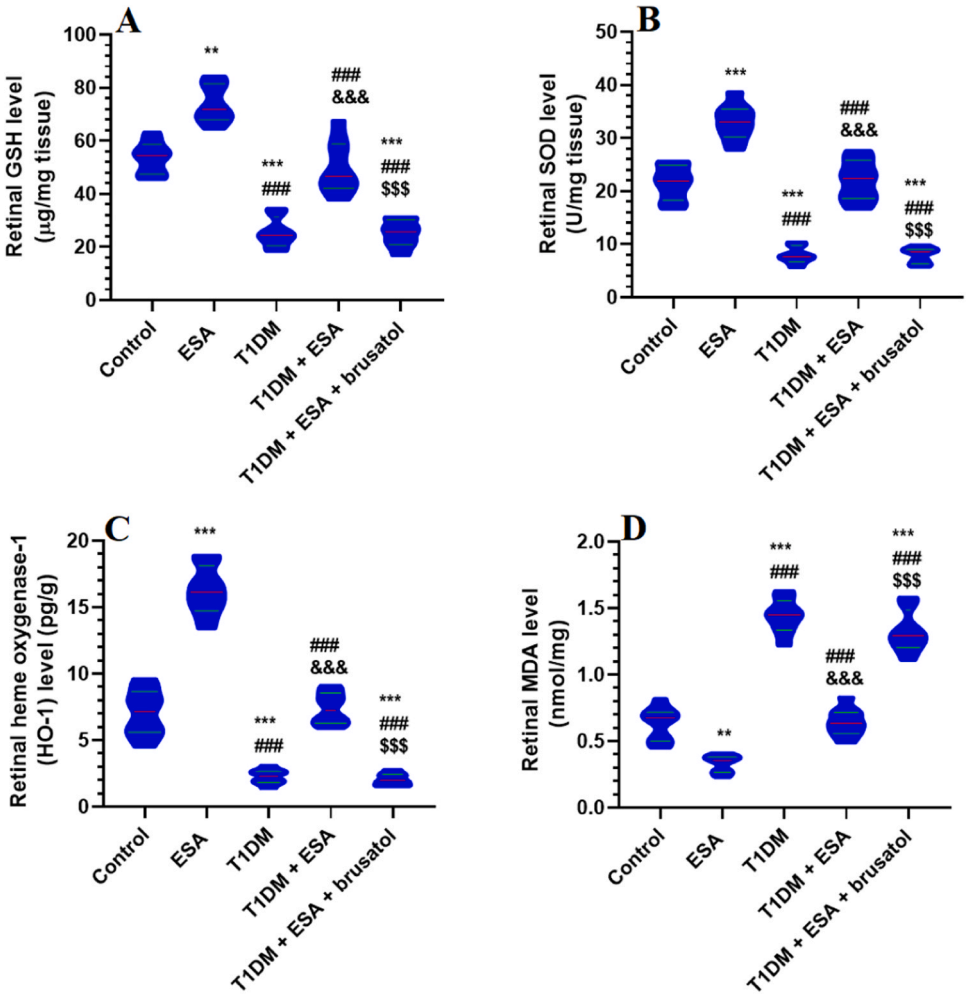


Fig. 3. : Esculeoside A (ESA) boosts levels of total glutathione (GSH, A), superoxide dismutase (SOD, B), hemeoxygenase-1 (HO-1, C), and reduced levels of malondialdehyde (MDA, D) in the retina of the control and diabetic rats in an Nrf2-dependent manner. Analysis was conducted using the 2-way ANOVA test, and Tukey's test was post hoc. Data were presented as means ± SD for n = 8 samples/group. **,***: significantly different compared to control ($p<0.0001$). $^{\#\#\#}$: significantly different as compared to non-diabetic rats which received ESA (ESA group) ($p<0.001$ and <0.0001). $^{\&\&\&}$: significantly different as compared to T1DM ($p<0.0001$). $^{\$\$\$}$: significantly different as compared to T1DM + ESA ($p<0.0001$).

3.9. Effect of ESA on retina transcription of REDD1

The mRNA levels of REDD1 were significantly increased ($p<0.001$) in the retina of T1DM rats as compared to the control or ESA-treated groups (Fig. 7A). The retinas of the ESA-treated rats and T1DM + ESA-treated rats showed a significant decline in the levels of REDD1 as compared to the control or T1DM rats, respectively (Fig. 7A). Interestingly, the retinal mRNA levels of REDD1 were not significantly different between T1DM + ESA rats and T1DM + ESA + brusatol-treated rats. These data suggest that the regulation of ESA on REDD1 is Nrf2 independent, which means not regulated by suppressing oxidative stress. When correlated with mRNA levels, cytoplasmic levels, and protein levels of Nrf2 REDD1 showed a weak positive interaction with mRNA levels of Nrf2 (R2 = 0.3964) (Fig. 7B) but exhibited strong

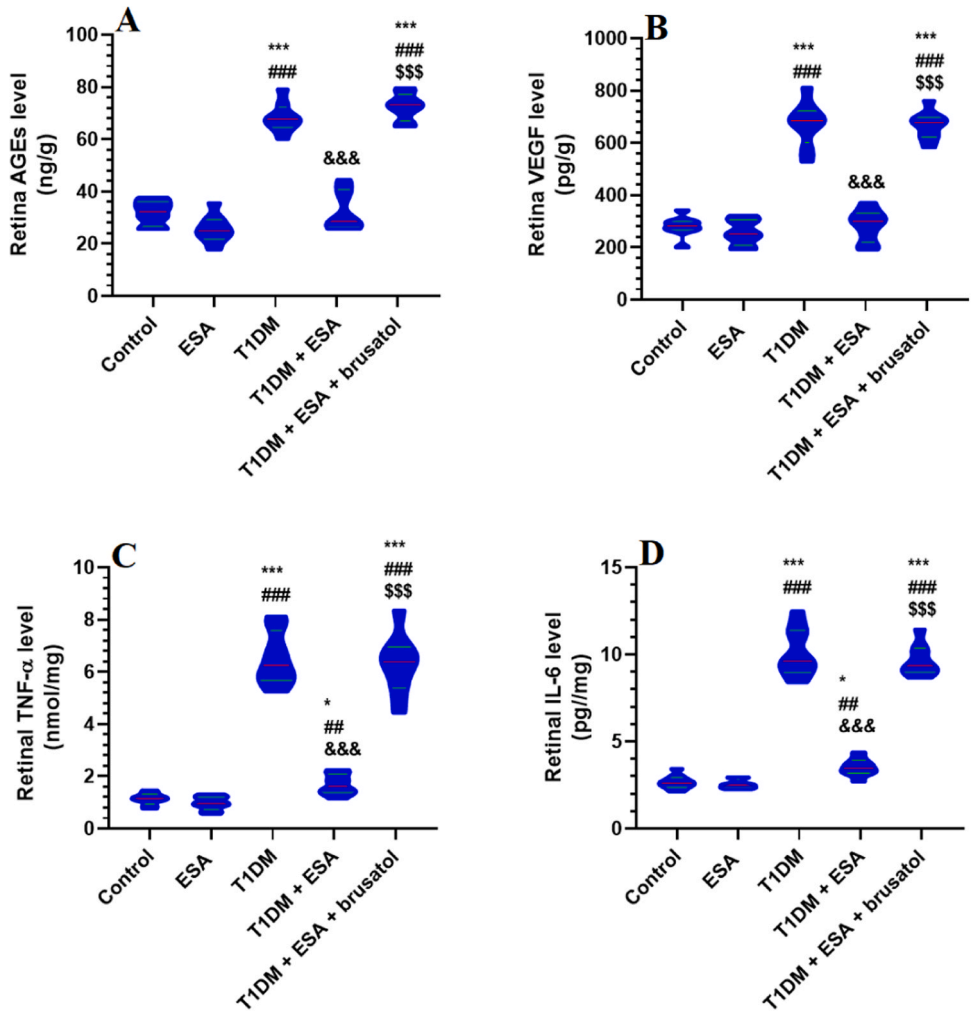


Fig. 4. : Esculeoside A (ESA) lowers the levels of advanced glycation end products (AGEs, A), vascular endothelial growth factor (VEGF, B), Tumor Necrosis Factor-α (TNF-α, C), and interleukine-6 (IL-6, D) only in the retina of diabetic rats in an Nrf2-dependent manner. Analysis was conducted using the 2-way ANOVA test, and Tukey's test was post hoc. Data were presented as means ± SD for n = 8 samples/group. *,***: significantly different as compared to control ($p<0.01$ and $p<0.0001$). $^{###}$: significantly different as compared to non-diabetic rats who received ESA (ESA group) ($p<0.0001$). $^{\&\&\&}$: significantly different as compared to T1DM ($p<0.0001$). $^{\$\$\$}$: significantly different as compared to T1DM + ESA ($p<0.0001$).

negative correlations with the cytoplasmic (Fig. 7C) and nuclear protein levels (Fig. 7D) of Nrf2 (R2=0.8189 and 0.7286, respectively).

4. Discussion

This study examined the potential of ESA, a major glycoside of ripe tomatoes, to prevent DR in STZ-induced T1DM rats. The findings support our hypothesis and further suggest possible mechanisms of action. In this context, our data suggest that the protective effect of ESA against T1DM-mediated retinal damage is a local ocular effect that includes potent antioxidant, anti-inflammatory, and anti-apoptotic effects. Besides, these effects are not mediated by modulating T1DM-associated hyperglycemia or hyperlipidemia but rather through transactivation of Nrf2. In addition, ESA seems to activate Nrf2 in a keep-1-independent mechanism that involves the downregulation of REDD1, a common regulator of Nrf2 signaling. Supporting this, co-treatment with brusatol, an inhibitor of Nrf2, completely diminished all the protective effects of ESA on the retina and enhanced oxidative and inflammatory damage. A graphical abstract summarizing these events is shown in the provided graphical abstract.

ROS is a driving force that damages the retina during DM by triggering all other pathological pathways [10,13]. The retina is one of the organs that is vulnerable to such oxidative damage due to its high metabolic rate and content of polyunsaturated fatty acids (PUFA) [44]. Hyperglycemia and hyperlipidemia are two major sources of ROS and the damage in DR and controlling either factor by diet or drugs not only prevented the onset of the disease but its progression [43–45]. On one hand, hyperglycemia generates a massive amount of ROS in the retina by autoxidation of glucose, impairing mitochondria electron transport (ETC) chain, and over-activation of NADPH oxidase (NOX) and other metabolic pathways (i.e. polyol, PKC, AGEs, & hexosamine) [10; 13]. On the other hand, hypertriglyceridemia and hypercholesterolemia enhance ROS generation in this tissue by damaging the BRB, activating nitric oxide synthase (NOS), increasing the production and the accumulation of amyloid-beta (Aβ), and activating/upregulating 7-ketocholesterol (7-KCh) [45]. Other sources of ROS in the diabetic retina could be also derived from the reduction and scavenging of antioxidants (e.g. vitamin E and GSH), and the impairment in the activity of antioxidants [42].

In this study, We first evaluated the effect of ESA on the survival rate of diabetic rats. We have shown a significant reduction in the mortality of diabetic rats that were administered ESA which was increased with the use of Nrf2. This could be explained by the ESA-induced systemic protective effect against the systemic toxicity of STZ which seems to be Nrf2-dependent. Also, hypoinsulinemia, hyperglycemia, hyperlipidemia, high HbA1c levels, weight loss (through lack of insulin and

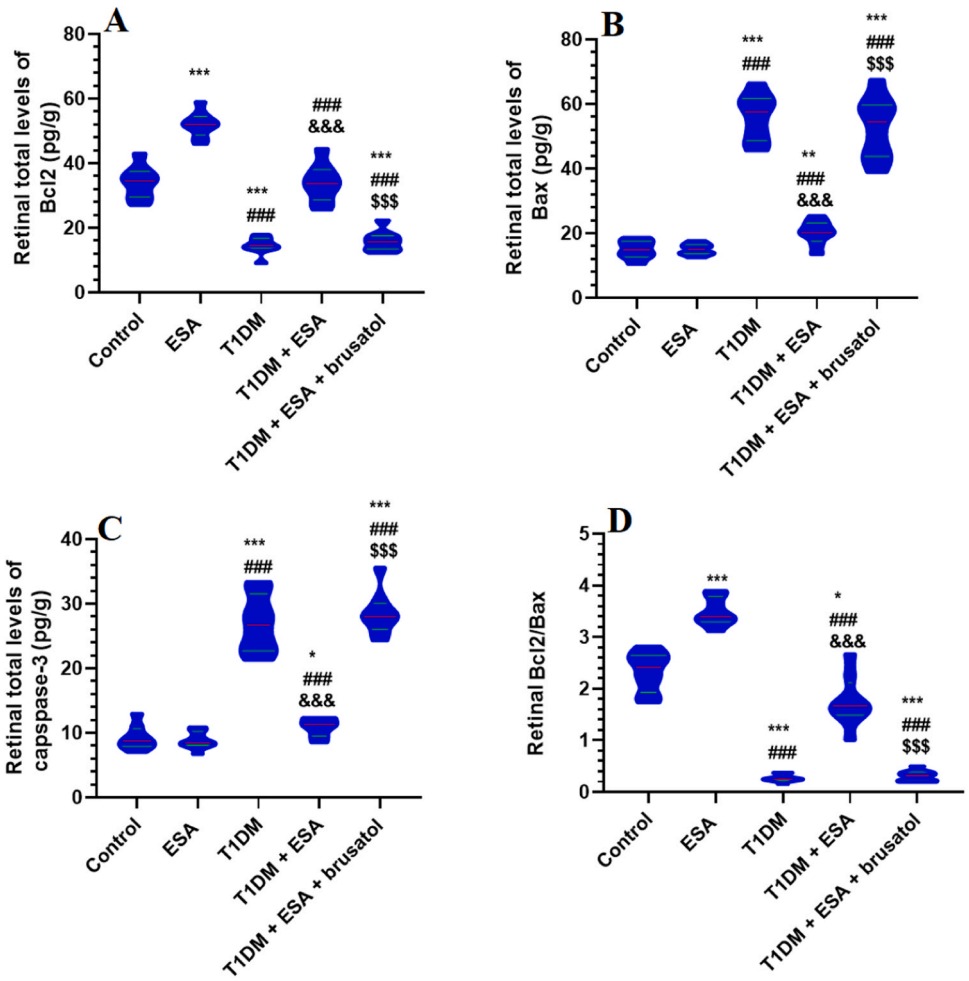


Fig. 5. : Esculeoside A (ESA) enhances total levels of Bcl2 anti-apoptotic marker levels (A) and lowers total levels of Bax (B), Bcl2/Bax ratio (C), and caspase-3 (D) only in the retina of diabetic rats in an Nrf2-dependent manner. Analysis was conducted using the 2-way ANOVA test, and Tukey's test was post hoc. Data were presented as means ± SD for n = 8 samples/group. *,**,***: significantly different as compared to control ($p<0.01$, $p<0.001$, and $p<0.0001$). $^{###}$: significantly different as compared to non-diabetic rats who received ESA (ESA group) ($p<0.0001$). $^{\&\&\&}$: significantly different as compared to T1DM ($p<0.0001$). $^{\$\$\$}$: significantly different as compared to T1DM + ESA ($p<0.0001$).

increased lipolysis), and retinal damage were evident in our diabetic rats which are following multiple previous studies [46–49]. However, the failure of ESA to control hyperglycemia as hyperlipidemia, as well as to increase insulin levels in the control and diabetic rat dissipates its metabolic effect from its ocular protective effect. On one hand, these findings support some other authors who have shown no effect of ESA on fasting glucose and insulin levels in STZ-induced diabetic rats, despite its testicular and cardioprotective effects [36,37]. On the other hand, our data contradict Yang et al. [35] who demonstrated an AMPK-dependent insulin-sensitizing and hypoglycemic effect of ESA in *db/db* mice which could be explained by the variations in animal models (i.e. T1DM vs T2DM). It also opposes the results of AL Tamimi et al. [37] who showed a hypolipidemic effect of ESA in in STZ-diabetic rats. Such contradiction between these studies and ours could be explained by the variation in the species used, treatment period, and sample collection and analysis.

In the diabetic retina, ROS, as an upstream pathway, damages the retina by promoting lipid peroxidation, angiogenesis, inflammation, and apoptosis [6–13]. The VGEF is the major molecule that induces angiogenesis in the DR and anti-VGEF therapy is currently examined to treat rubeosis and glaucoma in diabetic patients [50]. However, intrinsic cell apoptosis is the most common cell modality in diabetic eyes [51]. In the retina, ROS can directly upregulate TNF-α, IL-6, and VGEF or indirectly through activating the nuclear factor kappa beta (NF-κB) [42,52,53]. ROS also promotes retina mitochondria-mediated apoptosis (intrinsic cell death) through activating upregulating p53/Bax axis, down-regulating Bcl2, stimulating cytochrome-c release, and upregulation of caspases [13,51,52]. Like ROS, TNF-α and NF-κB can independently promote retinal cell caspase-3 mediated apoptosis by activating the metalloproteases/caspase pathway [7,5 4]. The activation of all these damaging pathways was also evident in the retinas of T1DM rats of this study given the higher levels of MDA, TNF-α, IL-6, VEGF, Bax, and caspaspe-3. However, an impairment in the level of antioxidants (i.e. GSH, SOD, and HO-1) with a significant reduction in the levels of the anti-apoptotic Bcl2 protein was also committed. This supports many previous studies that have also shown similar biochemical alterations in the levels of these markers in the retinas of diabetic mice and rats [53–58].

On the opposite, the ability of ESA to reduce retina damage in association with attenuating lipid peroxidation, as well as reducing markers of oxidative stress, inflammation, and apoptosis in the diabetic retinas of T1DM rats of this study supports its previously documented antioxidant and anti-inflammatory effects [36,37]. In addition, ESA was able to stimulate Bcl2, GSH, and antioxidant genes in the retinas of both the control and diabetic rats, which indicates potent antioxidant and anti-apoptotic effects under basal and diabetic conditions. Similar stimulatory effects of ESA on Bcl2 and antioxidant genes were also observed in the diabetic hearts and testes of control or STZ rats [36,37]. We have further documented a free radical scavenging activity of ESA

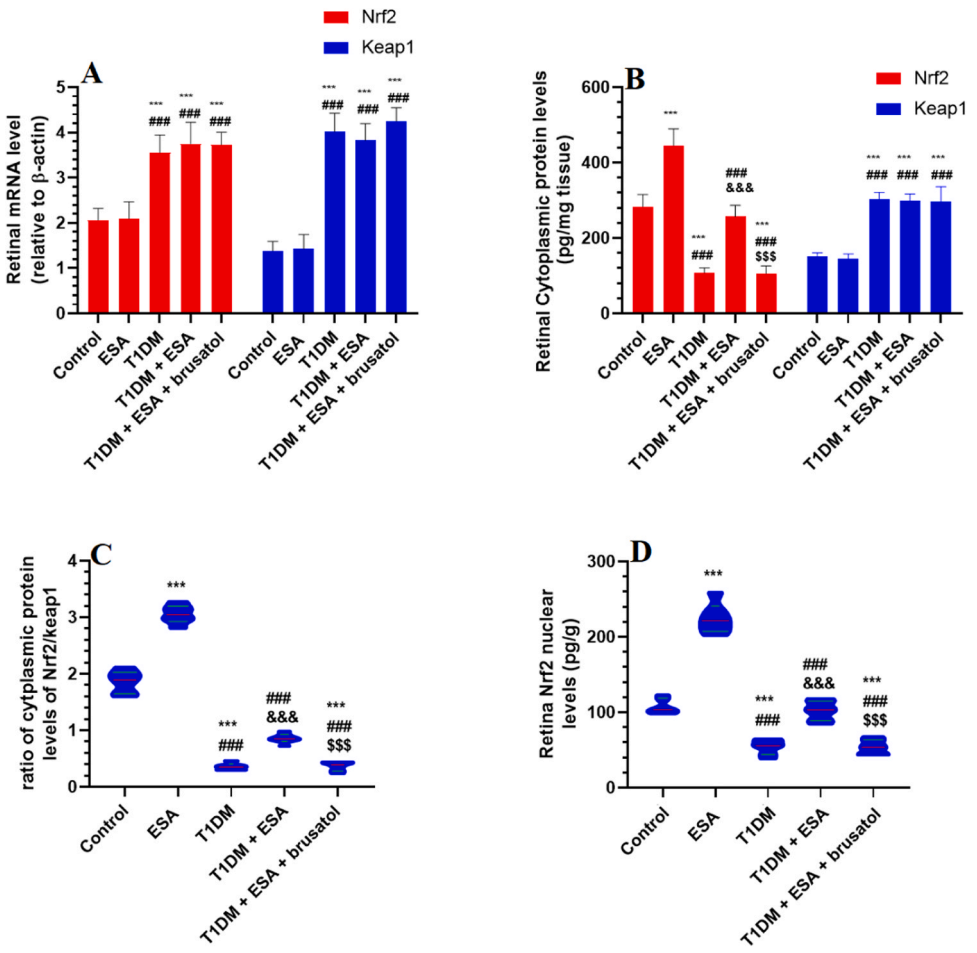


Fig. 6. : Esculeoside A (ESA) stimulates the cytoplasmic and nuclear levels of Nrf2 in the retinas of rats. A: mRNA levels of keap1 and Nrf2, B: the cytoplasmic protein levels of Nrf2 and keap1, C: the ratio of cytoplasmic levels of Nrf2/keap1, and D: the nuclear protein levels of Nrf2 Analysis was conducted using the 2-way ANOVA test, and Tukey's test was post hoc. Data were presented as means ± SD for 6 samples for mRNA determination and n = 8 samples/group for other markers. ***: significantly different as compared to control ($p<0.0001$). $^{\#\#\#}$: significantly different as compared to non-diabetic rats who received ESA (ESA group) ($p<0.0001$). $^{\&\&\&}$: significantly different as compared to T1DM ($p<0.0001$). $^{\$\$\$}$: significantly different as compared to T1DM + ESA ($p<0.0001$).

against DPPH *in vitro*. These data indicate an exceptional ability of ESA to stimulate the retina's antioxidant capacity either by upregulating antioxidants and/or scavenging ROS under normal or diabetic conditions. However, the failure of ESA to interfere with the levels of inflammatory cytokines, Bax, caspase-3, and VEGF in the retinas of the control rats confirms that the anti-inflammatory, angiogenesis, and anti-apoptotic effects of ESA in these T1DM rats are secondary to its antioxidant effects thus supporting the above-mentioned reports for the key role of oxidative stress in the pathogenies of DR [10,13].

Nrf2 is the most-known transcription factor that attenuates oxidative stress by upregulating antioxidants in the cells [24]. Nrf2 transactivation is regulated by the cytoplasmic stability, as well as the levels of transcription and post-translation modification [19]. The cytoplasmic stability of Nrf2 is maintained by several factors. In the cytoplasm, the Kelch-like ECH-associated protein 1 (keap1), is the most known inhibitor of nrf2 (also called INr2) [19,24]. Under normal conditions, Keap1 serves as a linker between Nrf2 and the E3-ubiquitin ligase complex to ensure Nrf2 ubiquitination and continuous degradation [19,21,24]. However, this dissociation can be broken down by electrophilic and oxidative stressors, as well as by drugs, which induce keap1 cysteine oxidation [14, 16; 19]. Other factors, rather than ROS, can also stabilize Nrf2 by decreasing its cytoplasmic interaction with keap1. For example, the activation of PKC rapidly induces phosphorylate Nrf2 at serine 40 which leads to its dissociation from Keap1 [59]. Likewise, cellular factors such as partner and localizer of BRCA2 (BALB2), p21 (a cyclin-dependent kinase inhibitor), tumor suppressor BRCA1, p62 (a ubiquitin-binding scaffold protein), DJ-1 (protein deglycase) can protect against neural and cellular damage by stabilizing Nrf2 and decreasing its association with keap1 [19,60–63].

We next aimed to search for the possible mechanisms, which underlie the antioxidant protective effect of ESA and tried to understand how ESA regulates Nrf2 signaling under basal and diabetic conditions. For this reason, we have targeted the Nrf2 signaling pathway given its crucial role in regulating antioxidants and cytoprotection [24]. In addition, Nrf2 activity is significantly depleted in the diabetic retina and the activation of this pathway always conferred protection [18–25]. Within this view, it was shown that hyperglycemia could directly induce methylation of the ARE4 region, thus inhibiting the binding of Nrf2 to this region [64]. Also, hyperglycemia promotes epigenetic modification-induced upregulation of keap1, which strengthens the interaction between Nrf2 and keap1, thus reducing the stability of Nrf2 and stimulating its cytoplasmic degradation [25,65].

The role of hyperglycemia and ROS in suppressing Nrf2 in the retina of diabetic rats was also confirmed in this study higher mRNA and cytoplasmic levels of keap1, as well as an increase in mRNA levels of Nrf2, were observed in the retina of diabetic rats. Such increments in the transcription of Nrf2 are most likely due to hyperglycemia-mediated increase in the levels of ROS. However, the retinal reduced stability of Nrf2 could be explained by the induction of keap1. These findings also support many other previous reports [21–29]. On the other side, ESA

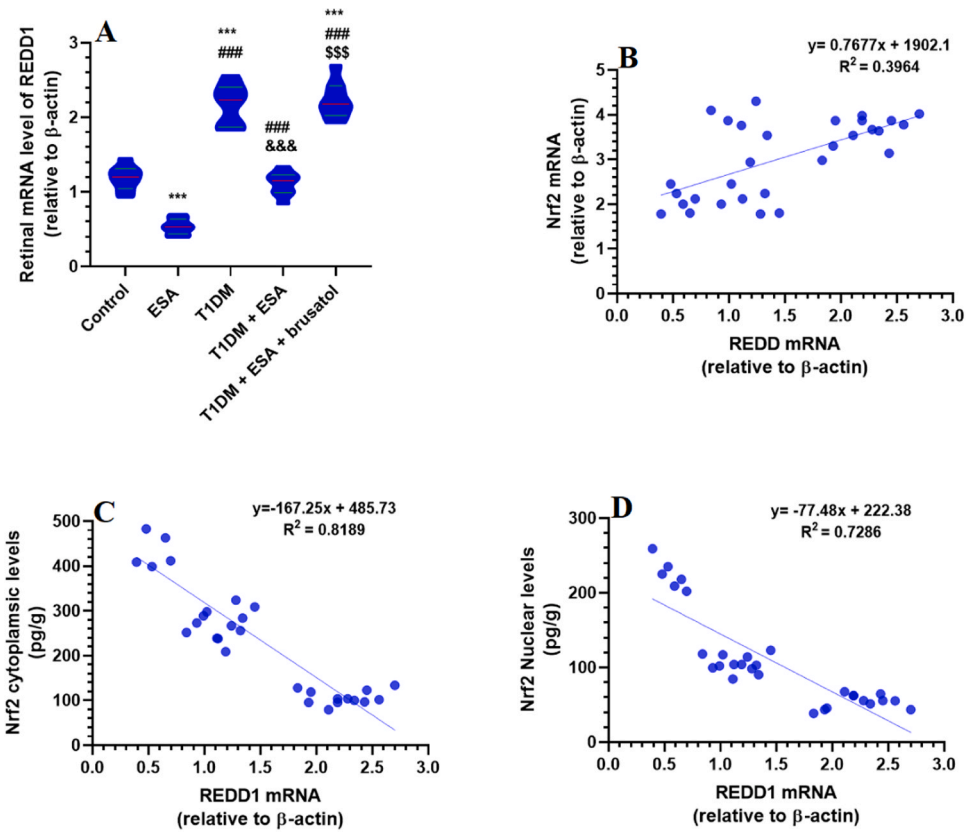


Fig. 7. : Esculeoside A (ESA) increases the transcription of REDD1 in the retinas of all groups of rats. B-D: correlation between mRNA levels of REDD1 and mRNA levels of Nrf2 (B), cytoplasmic levels of Nrf2 (C), and nuclear levels of Nrf2 (D). In A: Data were presented as means ± SD for n = 6 samples/group. ***: significantly different as compared to control ($p<0.0001$). $^{\#\#\#}$: significantly different as compared to non-diabetic rats who received ESA (ESA group) ($p<0.0001$). $^{\&\&\&}$: significantly different as compared to T1DM ($p<0.0001$). $^{\$\$\$}$: significantly different as compared to T1DM + ESA ($p<0.0001$).

stimulated the cytoplasmic and nuclear levels of Nrf2 not only in the retinas of the T1DM rats but also in the retinas of diabetic rats, indicating the role of this drug in the transactivation of Nrf2. This could explain the previously discussed increments in the levels of SOD, GSH, HO-1, and Bcl2 in the retinas of these treated groups of rats. Of note, brusatol, a potent Nrf2, inhibitor, Also prevented the nuclear activation of Nrf2 in the retinas of diabetic rats and abolished all the retina antioxidant, anti-inflammatory, and anti-apoptotic effects of ESA. Even though brusatol reduced the cytoplasmic levels of Nrf2, it did not affect the mRNA levels of keap1 or the mRNA levels of Nrf2. This also suggests that brusatol alters the activation of Nrf2 in a keep-1-independent mechanism. Indeed, Olayanju et al. [66] have shown that brusatol stimulated Nrf2 degradation and repressed its nuclear translocation at the post-translational levels and independent of keap1-proteasome or autophagy-mediated mechanisms. Based on these data, we become more confident that ESA protects against DR by enhancing the translocations of Nrf2.

To understand the regulation of ESA on the transactivation of Nrf2, we have first targeted the major inhibitor of Nrf2, keap1. Interestingly, we have also observed no effect of ESA on the transcription or total cytoplasmic levels of keap1 in the retinas of the control or diabetic animals, which indicates that the stabilization of Nrf2 post-ESA treatment is keap1-independent. This could be most probably due to disturbing the interaction between the two proteins. Indeed, several keap-1 independent Nrf2 regulators have been identified. The glycogen synthase kinase-3beta (GSK-3β) enzymes and the 3-hydroxy-3-methylglutaryl reductase degradation-1 (Hrd1) enzyme are the best-known negative regulators on Nrf2 in the cytoplasm [19]. Within this view, Hrd1 is an E3 ligase that is induced in response to endoplasmic reticulum stress and exaggerates oxidative stress by stimulating direct proteasome degradation of Nrf2 [67,68]. In the same boat, GSK-3β phosphorylates Nrf2 at serine residues, thus increasing its nuclear exclusion and the recognition by the beta-transducing repeat-containing protein (β-TrCP), which in turn marks it, for ubiquitination proteasome-mediated degradation [69,70]. However, activation of PI3K/Akt pathway stimulates Nrf2 by phosphorylating and inhibiting GSK-3β [71]. Hyperglycemia-mediated damage of the diabetic retina also involved suppression of the PI3K/Akt/Nrf2 axis [72]. In the retina of diabetic rodents, emerging recent data have characterized a unique role responsive protein regulated in development and DNA damage 1 (REDD1) as an essential contributor to oxidative stress and inflammatory response [73–76]. Further, they have also confirmed that activation of GSK-3β/β-TrCP is essential for the onset of these pathological mechanisms by stimulating the degradation of Nrf2 [76]. Among all non-keap1 regulators, and as this protein was retina-specific and is induced by DM, we have decided to study the effect of ESA on this protein to further enrich our understanding of how ESA regulates Nrf2.

As expected, and aligned with the above-mentioned studies, we have also observed higher mRNA levels of REDD1 in the retinas of T1DM rats. On the contrary, the mRNA levels of REDD1 were silenced in the retinas of both the control and T1DM rats that were co-treated with ESA. In addition, the mRNA levels of REDD1 exhibited a very strong negative correlation with the cytoplasmic and nuclear levels of Nrf2 but not with the mRNA levels of this protein. This might support the findings of Miller et al. [75,76] and confirmed the negative regulation of Nrf2 by REDD1. Therefore, it could be possible that ESA activates Nrf2 by repressing REDD1 and possibly through regulating the PI3K/Akt/ GSK-3β axis. Yet, this remains a piece of highlight evidence and more advanced studies are needed to confirm such hypothetical regulatory effect of ESA on REDD1. In addition, even if it is not within the scope of this article, we observed

that brusatol does not affect the transcription of REDD1. Such findings reconfirm those reported by Olayanju et al. [66] that the inhibitory effect of brusatol on Nrf2 is not mediated by a degradative mechanism.

In conclusion, the overall effect of ESA in this study confirms the emerging role of ESA as a potent ocular protective agent against hyperglycemia-mediated DR in STZ-treated animals. The evidence between our hands suggests that the mechanisms of protection are mediated by the Nrf2/antioxidant axis, which simultaneously acts to suppress neuroinflammation and neurodegeneration of diabetic retinas. In addition, our study establishes a hypothesis that the downregulation of REDD1 is linked to ESA-mediated activation of Nrf2 which still needs further validation.

5. Study limitations

The effect of ESA on Nrf2 activation remains observational and needs further advanced molecular studies such as repeating the experiments in animals that are deficient with Nrf2. In addition, the mechanisms by which ESA could inhibit REDD1 remain a matter that cannot be easily drawn from the current data and requires further investigation. In fact, the regulation of REDD1 in the cell is a very complicated process that involves multiple stressors, hormones, and signaling molecules [77]. Hyperglycemia and hyperinsulinemia are the most known stimulators for REDD1 [78–80]. Based on our data and due to the lack of effect of ESA on these parameters under basal and diabetic conditions, we could dissipate these mechanisms from the regulatory role of ESA on REDD1. On the other hand, ROS can directly upregulate REDD1 thus creating a positive feedback of activation [75]. Hence, and given the observed *in vitro* free scavenging effect and antioxidant potential of ESA in both the control and diabetic retinas, it could be possible that ESA suppresses REDD1 by these antioxidant mechanisms. Other cellular factors that can induce the transcription of REDD1 include growth factors, cortisol, IGF-1, p53, catecholamine, corticosteroids, estrogen, aldosterone, testosterone, melatonin, and vitamin D [77]. In addition, numerous transcription factors such as hypoxia-inducible factor (HIF), hepatic nuclear factor-1 (HNF-1), NF-κB, p53, and CEBP stimulate the transcription of REDD1, as they are located on the REDD1 promoter [77]. Moreover, energy depletion (AMPK activation), and ER-stress-induced activation of PERK/eIF-2α/ATF4 are sufficient to promote REDD1 transcription [81–84]. All these targets should be considered as possible targets of ESA in future experimental or pre-clinical studies. Finally, if proven further to be correct, the activation of Nrf2 by ESA should be carefully monitored and a suitable therapeutic dose of the drug must be established. Even Nrf2 is normally cytoprotective; sustained activation of Nrf2 is associated with cancer and other chronic disorders through altering metabolism and promoting reductive stress [85,86].

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CRediT authorship contribution statement

Waqas Sami: Writing – original draft, Software, Formal analysis, Data curation. **Badr Alkhalaf:** Writing – review & editing, Writing – original draft, Supervision. **Mahmoud A. Alkhateeb:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Formal analysis, Conceptualization. **Hisham Alshaikhli:** Writing – review & editing, Writing – original draft, Validation. **Nasser AlSabaani:** Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Sami Eleawa:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Data curation. **Kawther Amawi:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Formal analysis. **Walid Aldhaban:** Writing – review & editing, Writing – original draft, Software, Resources, Project administration, Investigation, Data curation. **Wisam Nabeel Ibrahim:** Writing – review & editing, Writing – original draft, Project administration, Formal analysis. **Ahmad Alaraj:** Writing – review & editing, Writing – original draft, Visualization, Resources, Investigation, Conceptualization.

Declaration of Competing Interest

all authors declare no conflict of interest

Data Availability

Data will be made available on request.

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