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Prevention of diabetic retinopathy in a rat model by a functional food mix

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Purpose: Diabetic retinopathy (DR), a severe microvascular complication of both type 1 and type 2 diabetes, is one of the leading causes of blindness. Prolonged hyperglycemia leads to vascular endothelial changes, inflammation, neovascularization, and apoptosis through multiple mechanisms, including increased aldose reductase (AR) activity and formation of advanced glycation end products, which contribute to the development of DR. Based on our previous studies with various functional foods that showed AR inhibition and prevented the formation of advanced glycation end products, in this study, a functional food (FF) mix was formulated and investigated its efficacy against DR progression in a rat model.
Methods: An FF mix was prepared with powders of amla pericarp, turmeric rhizome, ginger rhizome, cinnamon bark, and black pepper seeds in a specific proportion. Two-month-old Sprague-Dawley rats were grouped into control (C), streptozotocin-induced diabetes (D), and diabetes fed with FF at two levels (FF1, 0.85 g/100 g diet; FF2, 4.25 g/100 g diet) for 6 months from the induction of diabetes. At the end of the experiment, electroretinography was performed, and the eyes were dissected after the animals were sacrificed. A set of eyes was formalin-fixed for histology and immunohistochemistry examination, and the retina from the remaining eyes was used for immunoblotting analysis.
Results: Supplementation of FF mix in the diet to diabetic rats has improved retinal function (electroretinography), as well as prevented histomorphological changes and loss of photoreceptor cells (rhodopsin), compared to untreated diabetic rats. Further, FF mix ameliorated hyperglycemia-induced angiogenesis (vascular endothelial growth factor, hypoxia-inducible factor 1α) and gliosis (glial fibrillary acidic protein) in the diabetic rats, accompanied by decreased inflammation (phosphorylated nuclear factor κB, tumor necrosis factor α, monocyte chemoattractant protein 1) and apoptosis (Bax, Bcl2, caspase3, and caspase12).
Conclusions: This study illustrates the potential of an FF mix, attributed to the synergistic effects of its components, alleviating the progression of diabetic retinopathy by reducing diabetes-induced hypoxia, gliosis, and inflammation, while also inhibiting apoptosis in retinal cells.

Diabetic retinopathy (DR), a severe microvascular complication of both type 1 and type 2 diabetes, is one of the leading causes of blindness and loss of vision globally [1]. The prevalence of DR is 22%, with an estimated population of 103.2 million [2], and it also accounts for 4.8% of 37 million cases of blindness worldwide [3]. Chronic hyperglycemia disrupts the neural components and vasculature of the retina by altering the retinal blood flow and causing the loss of pericytes, tight junctions, and basement membrane thickening. The formation of microaneurysms further leads to neovascularization, which is a feature of vision-threatening DR [4-6]. In the irreversible proliferative stage, ischemia increases the neovascularization as blood vessels grow into the vitreous, leading to retinal detachment. In DR, multiple mechanisms driven by prolonged exposure to hyperglycemia lead to vascular endothelial changes, inflammation, neovascularization, and apoptosis. Among the many mechanisms, the altered polyol pathway, driven by increased aldose reductase (AR) activity and enhanced formation of advanced glycation end products (AGEs), contributes to the development of DR [7]. In chronic hyperglycemia, blood–retinal barrier dysfunction and sorbitol accumulation through the polyol pathway cause pericyte edema with increased expression of vascular endothelial growth factor (VEGF), leading to angiogenesis and uncontrolled neovascularization [8-10]. Hyperglycemia also leads to increased accumulation of AGEs and activation of the receptor for AGEs (RAGE), which promotes abnormal blood vessel formation through VEGF [11]. AGEs contribute to pericyte loss, impair the function of bipolar cells, and increase glial fibrillary acidic protein (GFAP) levels [12-14].

Inhibition of AR and AGE formation could be an effective approach to prevent or manage DR progression [15,16]. We previously screened and tested AR inhibitors and anti-AGE compounds from common dietary sources and functional foods for targeting the molecular mechanisms involved in the progression of diabetic complications [17-22]. We

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showed that ellagic acid and procyanidin B2, found in many functional foods such as cinnamon, inhibit the accumulation of AGEs and attenuate the AGE-RAGE pathway, thereby improving retinal thickness in diabetes. Additionally, these compounds decreased the expression of GFAP, VEGF, and hypoxia-inducible factor 1α (HIF-1α) and inhibited apoptosis in the retina of diabetic rats [23]. Similarly, administration of curcumin, the active principle present in turmeric, due to its AR inhibitory (ARI) and antiglycating action (AGA), reduced VEGF expression in the retina of streptozotocin (STZ)–induced diabetic animals [24]. We hypothesized that a combination of AGA and ARI could provide a better preventive strategy than a single-target approach in managing diabetic complications. Hence, in this study, we formulated a functional food (FF) mix with amla, turmeric, cinnamon, ginger, and black pepper in a specific composition based on our earlier studies for their ARI and AGA potential [17-23] and tested its efficacy against DR in a diabetic rat model.

METHODS

Streptozotocin, diaminobenzidine, 4′,6-diamidino-2-phenylindole, antimouse, and antirabbit secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibodies for β-actin, tumor necrosis factor α (TNF-α), B-cell lymphoma 2, GFAP, VEGF, rhodopsin, aldose reductase (AKR1B1), and secondary Alexa Fluor antibodies were obtained from Thermo Fisher Scientific (Waltham, MA). Phosphorylated nuclear factor κB (p-NFκB), NFκB, monocyte chemoattractant protein 1 (MCP-1), B-cell lymphoma 2–associated X protein (BAX), caspase 3, and caspase 12 from Cell Signaling Technology (Danvers, MA), and HIF-1α were from Abcam (Cambridge, UK). Other analytical grade reagents and chemicals were procured from Sisco Research Laboratories (Mumbai, India).

Functional food mix: Amla, black pepper, cinnamon, ginger, and turmeric were obtained from local markets of Hyderabad, India. Dried turmeric rhizome, black pepper seeds, and cinnamon bark were powdered. The fresh pericarp of amla and ginger rhizome were freeze-dried before grinding them into powder. Powders of amla (1 g), black pepper (0.5 g), cinnamon (1 g), ginger (1.5 g), and turmeric (0.25 g; combined amount 4.25 g) were mixed in 100 g of AIN-93 diet and given to rats as functional food 2 (FF2) mix. The amounts used in the formulation were based on our earlier studies on these ingredients individually [20,21,25-28]. We have also tested a fivefold lower dosage (FF1; 0.85 g/100 g diet) to understand dose dependency and to examine if the effects of this combination on DR can be achieved even at lower doses due to synergism.

Animal experiment: Two-month-old Sprague-Dawley rats were procured from the Animal Facility, Indian Council of Medical Research (ICMR)–National Institute of Nutrition (NIN), Hyderabad. The animals were housed under controlled conditions with a 12-h light/dark cycle, 50%–60% humidity, and 22–25 °C room temperature, and they had free access to food and water. Animals were acclimatized for 2 weeks before treatment. Diabetes was induced by a single intraperitoneal injection of 39 mg/kg STZ dissolved in citrate buffer (pH 4.5), and control animals were injected with only citrate buffer. Blood glucose from the tail vein prick after overnight fasting was measured using a blood glucometer (ACCU-CHEK; Roche, Basel, Switzerland) on day 7 after the STZ injection to confirm the induction of diabetes. Animals with fasting blood glucose >160 mg/dl were considered diabetic, and they were randomly divided into diabetic (D), diabetic with FF1 (D+FF1), and diabetic with FF2 (D+FF2) treatments. There were seven (n = 7) animals in each of these groups, as well as the control (C) group. Animals were maintained on respective diets for 20 weeks, and daily diet intake, biweekly bodyweight, and fasting blood glucose were monitored. Animal experimentation was performed following the committee for control and supervision of experiments on animals (CCSEA) guidelines, and procedures were approved by the institutional animal ethics committee of ICMR-NIN (IAEC Approval No: ICMR-NIN/IAEC/02/004-A/2020). At the end of the experiment, an electroretinogram was performed, and animals were sacrificed by CO_2 asphyxiation. Three eyeballs per group were collected and placed in 4% neutral paraformaldehyde for histological examination and immunohistochemistry. The retina was separated from the remaining eyeballs (n = 4), snap-frozen, and stored at −80 °C for protein analysis.

Electroretinography: Electroretinography (ERG) was performed to assess the retinal function of the rats. Electroretinogram recording was performed using ERG-JET electrodes (UTAS Visual Diagnostic System; LKC Technologies, Germantown, MD) in a dark room with safe red light as previously described [29]. Animals were weighed, dark-adapted for 12 h with ad libitum access to food and water, and then anesthetized before recording. The JET electrode was placed 1 mm away from the temporal limbus on the cornea-sclera of each eye, while the ground electrode was attached to the tail. Retinal responses were recorded at 0.3–500 Hz at a 10-kHz rate after eliminating 60 Hz noise and were amplified at a 10,000 gain. Scotopic responses were averaged depending on the intensity with a stimulus interval of 0–180 s. Animals were then adapted to a light intensity of 8 dB for 10 min before recording photopic responses. Photopic responses were

Table 1. Details of primary and secondary antibodies and their concentrations used for immunoblot and immunofluorescence experiments.

Primary antibody				
Name	Cat. No	Concentration	Dilution	Application
GFAP	PA3–16727	2 mg/ml	1:4000	Immunofluorescence
Rhodopsin	MA1–722	1 mg/ml	1:1000	Immunofluorescence
AKR1B1	PA5–29718	1.06 mg/ml	1:1000	Immunoblotting
Bax	2772S	112 µg/ml	1:1000	Immunoblotting
Bcl2	MA5–11757	0.2 mg/ml	1:500	Immunoblotting
Caspase12	PA5–19963	1 mg/ ml	1:1000	Immunoblotting
Caspase3	9662S	26 µg/ml	1:1000	Immunoblotting
GAPDH	2118S	42 µg/ml	1:1000	Immunoblotting
HIF1α	ab187524	1.78 mg/ ml	1:1000	Immunoblotting
MCP1	PA1–84244	1 mg/ml	1:1000	Immunoblotting
NF-κB	8242S	139 µg/ml	1:1000	Immunoblotting
pNF-kB	3033S	57 µg/ml	1:1000	Immunoblotting
TNFα	PA5–19810	0.9 mg/ml	1:1000	Immunoblotting
VEGF	MA1–16629	1 mg/ml	1:1000	Immunoblotting
β-Actin	MA1–91399	2.9 mg/ml	1:5000	Immunoblotting
Secondary antibody				
Anti-mouse	A9044	10.5 mg/ml	1:10000	Immunoblotting
Anti-Rabbit	A9169	16.1mg/ml	1:10000	Immunoblotting
Anti-mouse Alexa Flour 555	A31570	2 mg/ml	1:1000	Immunofluorescence
Anti-Rabbit Alexa Flour 568	A10042	2 mg/ml	1:2000	Immunofluorescence

The manufacturer and supplier details of the antibody were mentioned in the material section.

recorded from −10 dB to 8 dB intensity light and averaged with a stimulus interval of 1 s.

Histology: The formaldehyde-fixed eyeball was embedded in paraffin, cut into 4-µm-thick sections, and mounted on glass slides. Sections were stained with hematoxylin and eosin and observed for histological differences under a light microscope (Leica Microsystems, Wetzlar, Germany).

Immunoblotting: Tissue lysate was prepared in Tris lysis homogenization buffer (pH 7.5) containing protease inhibitors, sodium orthovanadate, and sodium fluoride. The homogenate was centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatant was collected, and proteins were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto the nitrocellulose membrane. The membrane was blocked with 5% skimmed milk dissolved in phosphate-buffered saline (PBS, pH 7.5) for 1 h and incubated with the corresponding primary antibodies (Table 1) at 4 °C. After overnight incubation, the membrane was washed and probed with horseradish peroxidase–labeled secondary antibody. The antigen-antibody complex was visualized using an enhanced chemiluminescence substrate (Bio-Rad, Hercules, CA, USA), and images were recorded (G-Box; iChemi XR, Syngene, UK). Images were quantified for band intensity using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Protein expression was normalized with internal control β-actin expression.

Immunohistochemistry: Formaldehyde-fixed eyeballs mounted on chrome alum gelatin-coated slides were initially deparaffinized by incubating slides in xylene for 10 min and repeating this twice. Sections were then rehydrated using a decreased concentration of isopropyl alcohol (90%, 75%, and 50%). Slides were then heated at 0.1 M citrate buffer, pH 6.0, for 10 min to retrieve the antigen. Slides were dipped in 3% H_2O_2 for 30 min to quench the endogenous peroxidase activity. Slides were washed with PBS and incubated with 3% goat serum in PBS for 1 h at room temperature. Later slides

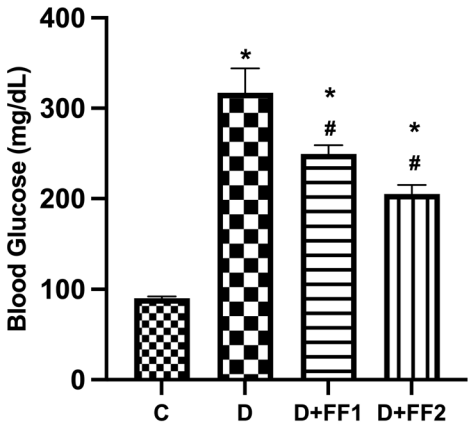

Figure 1. Functional food mix ameliorated fasting blood glucose levels of streptozotocin induced diabetic rats. Data are presented as mean ± SEM, n = 7 per group. Significant differences between control (C) and diabetes (D, D+FF1, D+FF2) groups are indicated by *$p \leq 0.05$. Significant differences between untreated diabetes (D) and treated diabetes (D+FF1, D+FF2) groups are indicated by #$p \leq 0.05$. C, control; D, diabetes (untreated); D+FF1, diabetes treated with FF1; D+FF2, diabetes treated with FF2 group.

were incubated with primary antibody (Table 1) dissolved in 1.5% goat serum at 4 °C overnight. After incubation with the primary antibody, the slides were washed with phosphate-buffered saline with tween 20 (PBST) thrice and incubated with Alexa Fluor–conjugated secondary antibodies (Table 1) at room temperature and mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole as a nuclear counterstain (Sigma-Aldrich). Negative controls were performed to rule out any nonspecific binding of secondary antibody by omitting the primary antibody and incubating the sections with the secondary antibody alone under identical conditions. We observed minimal nonspecific binding of the secondary antibody. Slides were visualized using appropriate filters, and images were captured by a Leica fluorescence microscope (Leica Microsystems).

Statistical analysis: The data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). Data are presented as the mean ± SD or SEM. One-way analysis of variance, along with Tukey's post hoc test, was used to determine statistical differences between groups. The differences between groups were considered statistically significant at $p < 0.05$.

RESULTS

Effect of functional food mix on fasting blood glucose: The STZ-injected diabetic rats showed a persistent increase in fasting blood glucose levels compared to the non-diabetic controls ($p < 0.01$). By the end of the study, dietary supplementation with FF1 and FF2 significantly reduced fasting blood glucose levels in diabetic rats relative to untreated diabetic controls ($p < 0.05$). However, no significant difference was observed between the two supplementation doses ($p > 0.05$). Although the functional food supplementation exerted a glucose-lowering effect, fasting glucose levels in FF1- and FF2-treated rats remained significantly elevated compared to controls ($p < 0.01$, n = 7; Figure 1).

Effect of functional food mix on the retinal function: Retinal function in rats was assessed using electroretinography. Diabetic rats exhibited significantly reduced photoreceptor, bipolar, and Müller cell activity, as evidenced by decreased amplitudes of the scotopic a-wave (Figure 2A-C) and b-wave (Figure 2D-F) compared to control animals ($p < 0.05$ and $p < 0.01$, respectively, n = 7). Similarly, the photopic b-wave amplitude (Figure 3A,B) was significantly diminished in diabetic rats relative to controls ($p < 0.01$). Supplementation with the FF mix at both doses significantly improved retinal function in diabetic rats, as indicated by increased scotopic a-wave and b-wave amplitudes (Figure 2), as well as improved scotopic oscillatory potentials ($p < 0.05$; Figure 3C, D). Additionally, FF supplementation improved photopic b-wave activity and oscillatory potentials, with only the lower dose (FF1) showing a statistically significant effect compared to untreated diabetic rats ($p < 0.05$); no significant difference was observed between the two doses (FF1 vs. FF2; $p > 0.05$). These findings demonstrate that FF supplementation, particularly at the FF1 dose, significantly improved retinal function in diabetic rats, with no observed difference between the two dose levels.

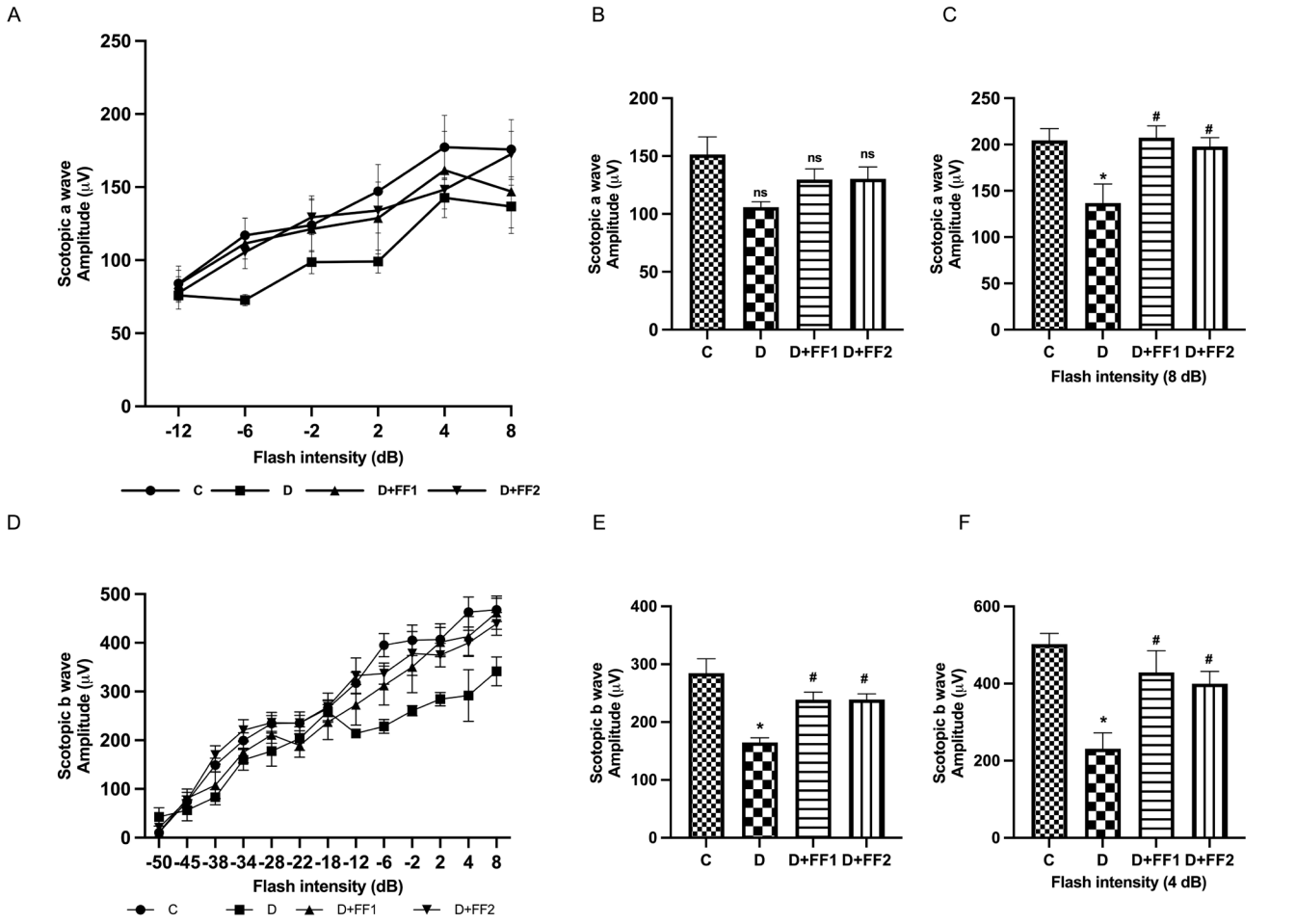

Figure 2. Functional food mix improved scotopic retinal function in streptozotocin induced diabetic rats as assessed by electroretinogram. **A**: Scotopic a-wave amplitudes of electroretinogram of rats. **B**: Mean amplitude of all the a-wave flash intensities together. **C**: Scotopic a-wave amplitude at 8-dB flash intensity. **D**: Scotopic b-wave. **E**. Mean amplitude of all the b-wave flash intensities together. **F**: Scotopic b-wave amplitude at a 4-dB flash intensity. Data are presented as mean ± SEM, n = 7 per group. Significant differences between control (C) and diabetes (D, D+FF1, D+FF2) groups are indicated by *$p \leq 0.05$. Significant differences between untreated diabetes (D) and treated diabetes (D+FF1, D+FF2) groups are indicated by #$p \leq 0.05$. C, control; D, diabetes (untreated); D+FF1, diabetes treated with FF1; D+FF2, diabetes treated with FF2 group.

Effect of functional food mix on retinal histology and thickness: The progression of DR is accompanied by changes in the retinal thickness. Since the FF mix improved retinal function, we examined its effect on retinal morphology. Histological examination after hematoxylin and eosin staining revealed a significant decrease in the retinal thickness. We observed a reduction in the thickness of the plexiform and nuclear layers and loss of photoreceptor cells in diabetic rats compared to the controls. Supplementation with the FF mix at both doses attenuated these changes (Figure 4A). Further, to confirm the effect of the FF mix on scotopic vision in diabetic rats (Figure 2), we measured the expression of the photoreceptor molecule rhodopsin, which mediates phototransduction in rod cells. Immunofluorescence staining showed a significant loss of rhodopsin expression in the retina of diabetic rats, indicating photoreceptor cell death, which was prevented in FF mix-supplemented groups at both doses (Figure 4B). These results demonstrate that the FF mix not only improves retinal function but also preserves the retinal morphology and photoreceptor cell activity.

Effect of functional food mix on retinal angiogenesis: Immunoblotting analysis of retinal tissue demonstrated that diabetic rats exhibited significantly elevated expression levels of HIF-1α and VEGF, key molecular markers of hypoxia and angiogenesis, respectively, compared to nondiabetic control rats ($p < 0.01$; Figure 5A). Dietary supplementation with both

low and high doses of the FF mix significantly attenuated the retinal expression of HIF-1α ($p < 0.05$, n = 4; Figure 5B) and VEGF ($p < 0.01$, n = 4; Figure 5C) in diabetic rats relative to untreated diabetic controls. No statistically significant difference in the expression of these markers was observed between the low- and high-dose treatment groups ($p > 0.05$). As diabetes progresses, Müller cell gliosis is more evident. Immunofluorescence analysis showed that the expression of GFAP was limited to the ganglionic cell layer in control retinal sections, whereas GFAP expression was observed in all retinal layers in the diabetic rats, indicating gliosis. Dietary supplementation with the FF mix at both doses significantly lowered GFAP expression (Figure 5D). These results suggest that the FF mix ameliorated the aberrant angiogenesis and gliosis.

Effect of FF supplementation on retinal aldose reductase and inflammatory markers: Protein expression of AR in the retina was significantly increased in diabetic rats compared to nondiabetic controls ($p < 0.01$, n = 4, Figure 6A). FF supplementation resulted in a reduction in AR expression, with the FF2 group showing a statistically significant decrease compared to untreated diabetic rats ($p < 0.05$). No significant difference in AR expression was observed between the FF1 and FF2 treatment groups ($p > 0.05$; Figure 6B).

Immunoblotting analysis of retinal lysates (Figure 6A) revealed significantly elevated expression of inflammatory markers, including phosphorylated NFκB relative to total NFκB (p-NFκB/NFκB), MCP-1, and TNF-α, in diabetic rats compared to controls ($p < 0.01$ for all, n = 4). Supplementation with both low (FF1) and high (FF2) doses of the FF mix significantly reduced the expression of these inflammatory mediators compared to untreated diabetic rats ($p < 0.05$; Figure 6C-E). While TNF-α expression was more markedly reduced in the FF1 group than in the FF2 group ($p = 0.054$), the difference between the two was not statistically significant (FF1 vs. FF2, $p > 0.05$). These results indicate that FF supplementation attenuates aldose reductase expression and exerts anti-inflammatory effects in the diabetic retina.

Effect of FF supplementation on apoptotic markers in the retina: We assessed the expression of key apoptotic markers in the retina. Immunoblotting analysis revealed significantly elevated levels of proapoptotic proteins Bax, cleaved caspase 3, and caspase 12, along with a marked decrease in the expression of the antiapoptotic protein Bcl-2 in diabetic rats compared to controls ($p < 0.01$; Figure 7). Dietary supplementation with the FF mix significantly reduced the expression of Bax ($p < 0.05$) and caspase 12 ($p < 0.01$), as well as significantly increased Bcl-2 expression ($p < 0.01$) compared to untreated diabetic rats (Figure 7B-F). A significant reduction in cleaved caspase 3 was observed only in the FF1 group. No statistically significant differences were found between the FF1 and FF2 treatment groups for any of the apoptotic markers. These findings indicate that FF supplementation attenuates apoptosis in the diabetic retina by modulating both pro- and antiapoptotic signaling pathways.

DISCUSSION

Exposure to chronic hyperglycemia during diabetes mellitus leads to several microvascular complications, including DR, which is a leading cause of blindness worldwide with an increasing socioeconomic burden, especially in developing countries. The current treatment options for DR include laser-based photocoagulation, injections of anti-VEGF drugs or corticosteroids into the eye, and surgery, in addition

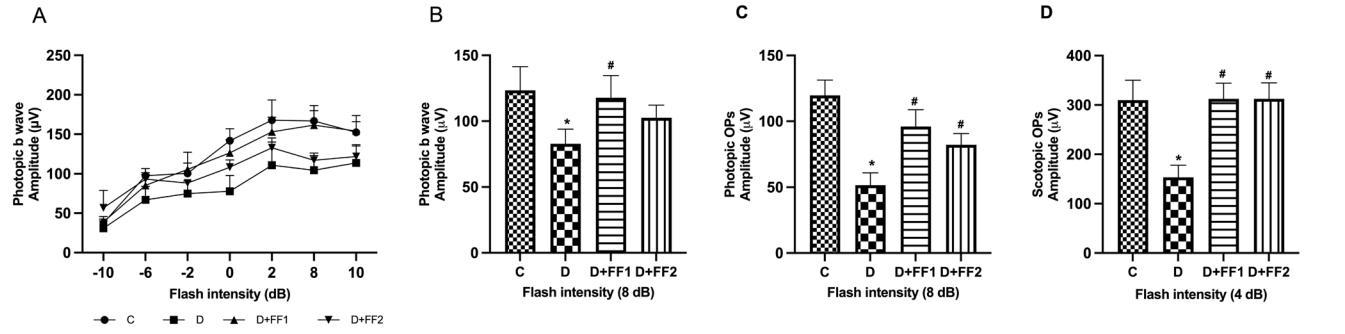

Figure 3. Functional food mix improved photopic retinal function in streptozotocin induced diabetic rats as assessed by electroretinogram. **A**: Photopic b-wave amplitudes of electroretinogram of rats. **B**: Photopic b-wave amplitude at an 8-dB flash intensity. **C**: Amplitude of photopic oscillatory potentials at an 8-dB flash intensity. **D**: Amplitude of scotopic oscillatory potentials at a 4-dB flash intensity. Data are presented as mean ± SEM, n = 7 per group. Significant differences between control (C) and diabetes (D, D+FF1, D+FF2) groups are indicated by *$p \leq 0.05$. Significant differences between untreated diabetes (D) and treated diabetes (D+FF1, D+FF2) groups are indicated by #$p \leq 0.05$. C, control; D, diabetes (untreated); D+FF1, diabetes treated with FF1; D+FF2, diabetes treated with FF2 group.

to antidiabetic medication [30]. While these options are successful in most cases, they are expensive, invasive, and have potential side effects such as loss of peripheral vision, bleeding, and further loss of vision. These limitations emphasize the need for alternative, noninvasive, and cost-effective adjunct therapies. FFs such as amla, black pepper, cinnamon, ginger, and turmeric are commonly available and widely used for culinary purposes and in traditional medicine. The bioactive compounds derived from these sources were previously reported to exhibit various pharmacological effects. Earlier, we reported that amla, ginger, cinnamon, pepper, turmeric, and their active compounds possess antiglycating properties and aldose reductase inhibition. In this study, we developed a dietary FF mix comprising these five ingredients in a specific proportion and evaluated its efficacy against DR in a STZ-induced diabetic rat model.

Our results demonstrated that the FF mix preserves retinal structure and function by modulating multiple pathological mechanisms involved in DR. Although fasting blood glucose levels remained higher than those in nondiabetic controls, both FF1 and FF2 supplementation significantly reduced hyperglycemia in diabetic rats, suggesting a partial glycemic control effect. FF supplementation, in particular FF1, significantly improved the scotopic and photopic

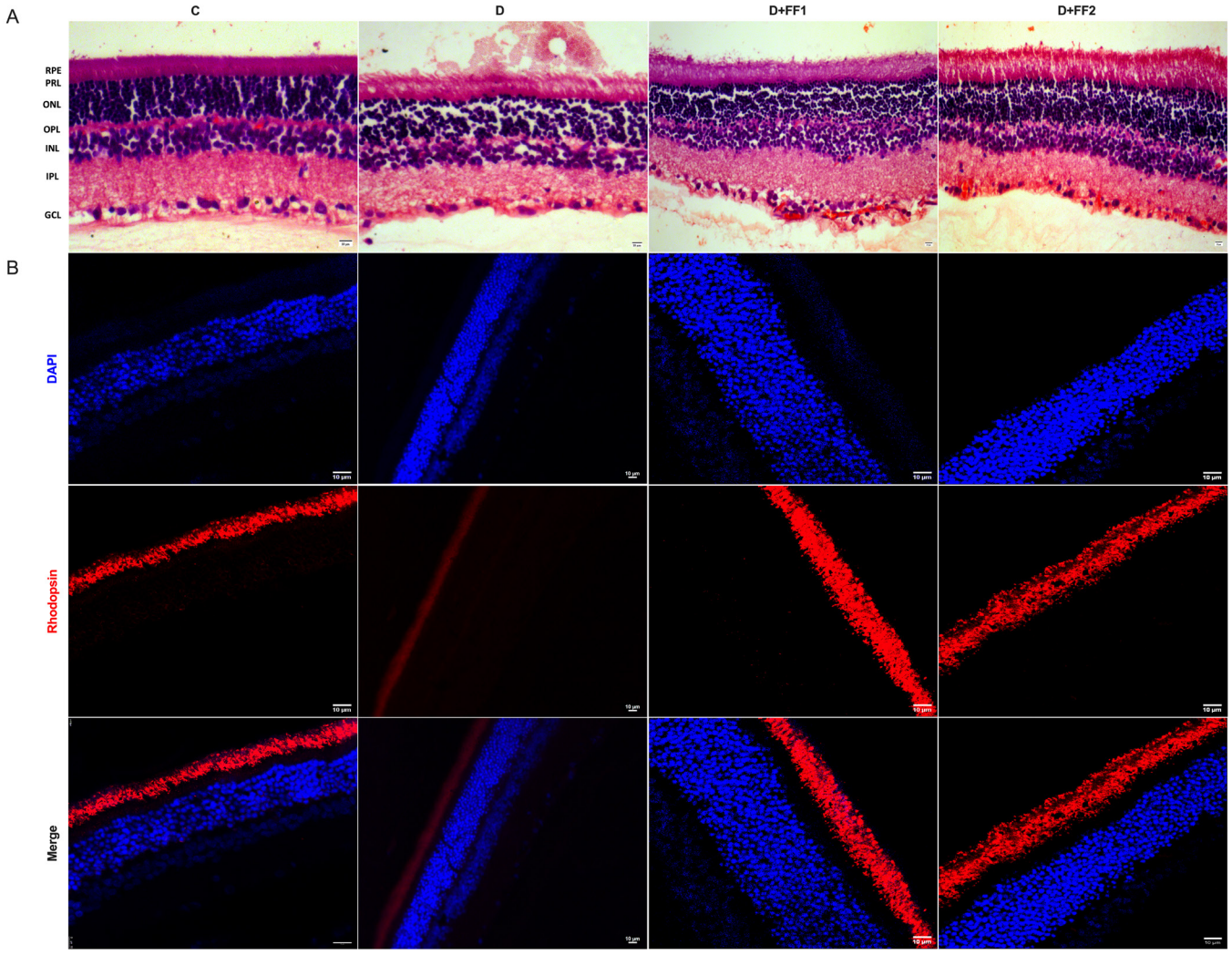

Figure 4. Functional food mix preserved retinal architecture and loss of retinal cells in streptozotocin induced diabetes. **A**: Hematoxylin and eosin staining of rat retinal sections. **B**: Immunofluorescence staining of rhodopsin in the retina. Rhodopsin stain was reduced in the D group and improved with FF supplementation. Blue: Nuclear stain with 4′,6-diamidino-2-phenylindole. Red: Rhodopsin stain. Merged: 4′,6-diamidino-2-phenylindole and rhodopsin. 40× magnification, scale bar = 10 µm, and n = 3 per group. C, control; D, diabetes (untreated); D+FF1, diabetes treated with FF1; D+FF2, diabetes treated with FF2 group; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PRL, photoreceptor layer; RPE, retinal pigment epithelium layer.

responses, and these functional improvements correlated with preservation of retinal structure, as histological analysis also showed reduced retinal thinning and photoreceptor cell loss (rhodopsin expression) in the FF-treated groups. It is evident that hyperglycemia-induced hypoxia and adaptive responses are mediated by HIF-1α and a further increase in VEGF expression [23]. In the present study, the FF mixture significantly ameliorated HIF-1α and VEGF expression in diabetic animals, suggesting potential antiangiogenic effects. Further, we have previously demonstrated that FF supplementation mitigated carboxymethyllysine (CML-AGE) accumulation and modulated the AGE/RAGE/NFκB signaling pathway in the STZ-induced diabetic kidney disease, with a similar dosage used in the current investigation [32], as well as galactose-induced renal injury [31]. AR, the rate-limiting enzyme in the polyol pathway, was significantly upregulated in diabetic rats. FF supplementation, particularly with FF2, reduced AR expression, implicating a role for the FF mix in modulating hyperglycemia-induced metabolic stress, and similar effects were observed in hyperglycemia-induced lens

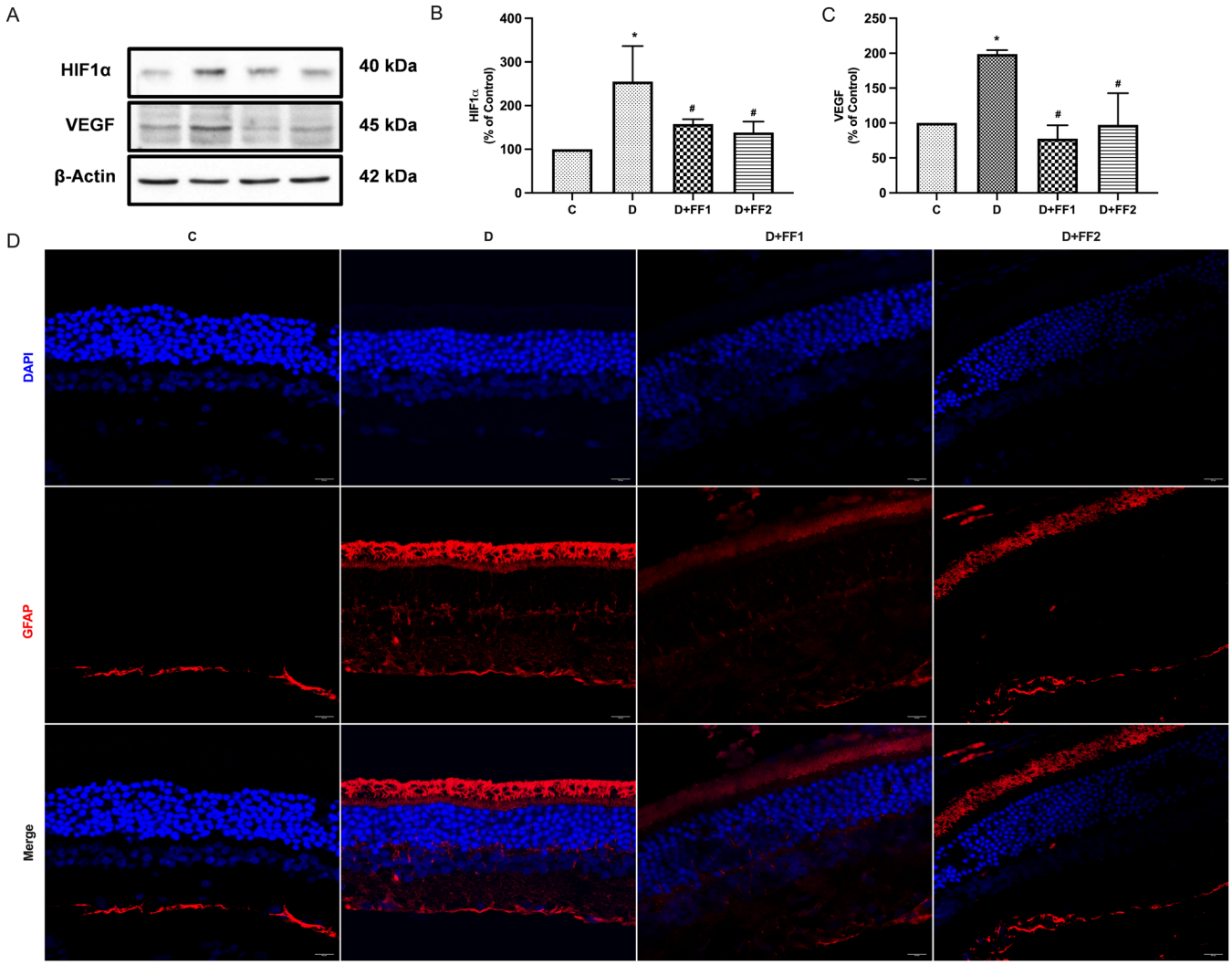

Figure 5. Functional food mix prevented retinal angiogenesis and gliosis in streptozotocin induced diabetes. **A**: Representative immunoblots depicting expression of HIF-1α and VEGF in rat retina. **B**, **C**: Quantitative analysis of HIF-1α and VEGF normalized with β-actin. Data represented as mean ± SD, n = 4 per group. Significant differences between control (C) and diabetes (D, D+FF1, D+FF2) groups are indicated by $*p \leq 0.05$. Significant differences between untreated diabetes (D) and treated diabetes (D+FF1, D+FF2) groups are indicated by $\#p \leq 0.05$. **D**: Immunofluorescence staining of glial fibrillary acidic protein (GFAP) in the retina. GFAP stain was increased in the D group and reduced with FF supplementation. Blue: Nuclear stain with 4′,6-diamidino-2-phenylindole. Red: GFAP stain. Merged: 4′,6-diamidino-2-phenylindole and GFAP. 40× magnification, scale bar = 10 µm, and n = 3 per group. C, control; D, diabetes (untreated); D+FF1, diabetes treated with FF1; D+FF2, diabetes treated with FF2 group.

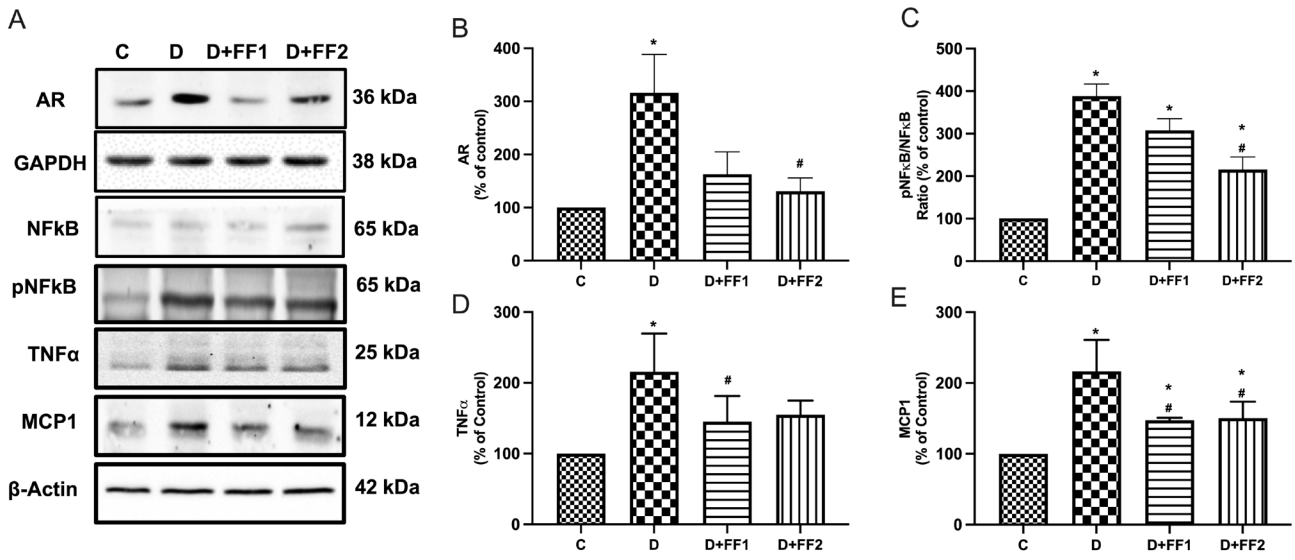

Figure 6. Functional food supplementation reduced aldose reductase and inflammation in streptozotocin induced diabetes. **A**: Representative immunoblots depicting expression of AR, NFκB, pNFκB, TNF-α, and MCP-1 in rat retina. **B**: Quantitative analysis of AR normalized with GAPDH. **C-E**: Quantitative analysis of NFκB, MCP-1, and TNF-α normalized with β-actin. Data are presented as mean ± SD, n = 4 per group. Significant differences between control (C) and diabetes (D, D+FF1, D+FF2) groups are indicated by *$p \leq 0.05$. Significant differences between untreated diabetes (D) and treated diabetes (D+FF1, D+FF2) groups are indicated by #$p \leq 0.05$. AR, aldose reductase; C, control; D, diabetes (untreated); D+FF1, diabetes treated with FF1; D+FF2, diabetes treated with FF2 group; MCP-1, monocyte chemoattractant protein 1; NFκB, nuclear factor κB; TNF-α, tumor necrosis factor α.

opacification [25]. Studies show that the retinal Müller cells, astrocytes, and microglia are greatly affected by STZ-induced diabetes [33]. GFAP expression is confined to ganglionic and nerve fiber cells under normal conditions. In the state of stress, Müller cells also express GFAP, indicating gliosis. GFAP expression is altered by cellular stress from polyol pathway activation, AGE formation, and other inflammatory cytokines and growth factors, including VEGF, MCP-1, intercellular adhesion molecules (ICAMs), and vascular cell adhesion molecule (VCAMs). In addition, FF supplementation attenuated Müller cell gliosis, as shown by reduced GFAP expression, indicating a suppression of glial activation

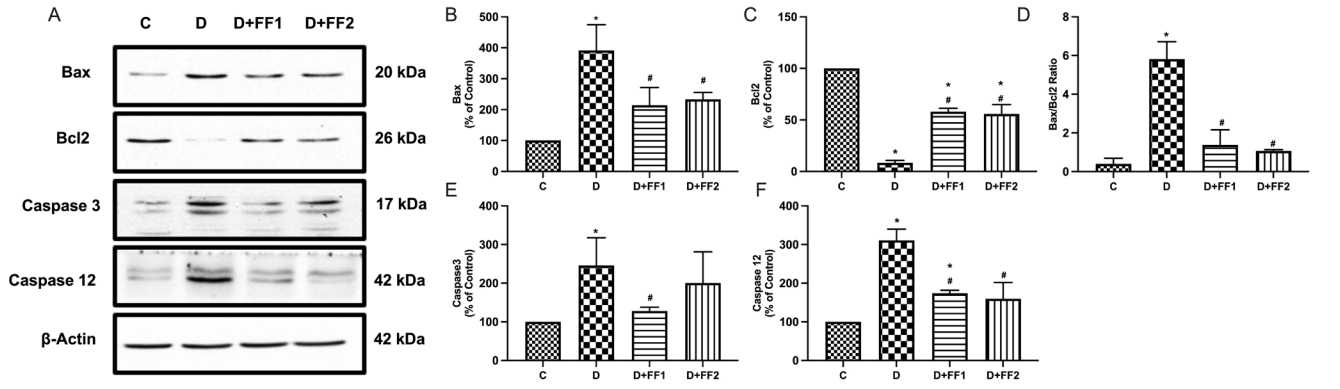

Figure 7. Functional food mix attenuated retinal cell apoptosis in streptozotocin induced diabetes. **A**: Representative immunoblots depicting expression of Bax, Bcl2, cleaved caspase 3, and caspase 12 in rat retina. **B-F**: Quantitative analysis of Bax, Bcl2, cleaved caspase 3, and caspase 12 normalized with β-actin. Data are presented as mean ± SD, n = 4 per group. Significant differences between control (C) and diabetes (D, D+FF1, D+FF2) groups are indicated by *$p \leq 0.05$. Significant differences between untreated diabetes (D) and treated diabetes (D+FF1, D+FF2) groups are indicated by #$p \leq 0.05$. C, control; D, diabetes (untreated); D+FF1, diabetes treated with FF1; D+FF2, diabetes treated with FF2 group.

and associated neuroinflammation. Moreover, inflammation, a major contributor to diabetic retinal damage, was significantly reduced in FF-treated animals. Expression of NFκB, MCP-1, and TNF-α was elevated in diabetic retina, and their attenuation in response to FF supplementation highlights the anti-inflammatory properties of the mix. FF supplementation conferred strong antiapoptotic effects in the diabetic retina. Diabetic rats showed elevated levels of Bax, cleaved caspase 3, and caspase 12, along with decreased Bcl-2 expression, hallmarks of apoptosis, which could have been driven by inflammation in the retina as a result of hyperglycemia and associated pathways, both intrinsic and endoplasmic reticulum stress pathways. Treatment with the FF mix prevented these changes, particularly increasing Bcl-2 and decreasing caspase 12 levels. Interestingly, improvement in photopic ERG and protein expression of TNF-α and cleaved caspase 3 protein were significantly reduced only with FF1, with no significant difference between low and higher doses, which suggests that a lower dose was sufficient for the therapeutic benefit, as we observed no dose-dependent effect in the retina. The lack of a dose-dependent effect in the retina could be due to the bioavailability of these compounds beyond certain levels in the retina, and effects may plateau beyond this level in the type 1 diabetes model.

Limitations of the current study include incomplete characterization of the FF mixture, as well as the lack of detailed identification and quantification of bioactive compounds and their metabolites in both serum and retinal tissue. Additionally, direct assessment of retinal capillary degeneration, such as acellular capillaries or pericyte loss, was not performed, and limited animal numbers precluded retinal trypsin digest or flat-mount analysis. Although we could not report frank neovascularization changes, FF treatment attenuated HIF-1α and VEGF expression, which gets upregulated after 1 month of diabetes induction [34,35], suggesting a potential vascular-protective effect. While FF treatment has demonstrated potential preventive effects against DR, further investigations are required to substantiate these findings, particularly a comprehensive evaluation of retinal microvascular changes.

Conclusion: In summary, in this study, we demonstrate the synergistic effect of an FF mix containing five foods in ameliorating HIF-1α/VEGF/GFAP expression in the retina of a diabetic rat by reducing polyol-mediated inflammatory markers and apoptosis of retinal cells. These results suggest that the FF mix, formulated from a synergistic combination of herbs and spices, holds a significant potential for managing diabetic complications. However, further investigations, including clinical trials, are required to establish its safety and efficacy in humans. Finally, this could play an important role in the prevention and management of diabetic retinopathy, ultimately improving the quality of life for individuals living with diabetes.

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