Saudi Journal of Biological Sciences 28 (2021) 4255–4262

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Nimbolide ameliorates the streptozotocin-induced diabetic retinopathy in rats through the inhibition of TLR4/NF-κB signaling pathway

Xiangwen Shu [a], Yali Hu [b], Chao Huang [c], Ning Wei [c,*]

[a] Second Department of Ophthalmology, Second People's Hospital of Jinan, Jinan 250001, China
[b] Department of Ophthalmology, Binzhou Hospital of Traditional Chinese Medicine, Binzhou 256600, China
[c] Department of Ophthalmology, The Second People's Hospital of Jinan, Jinan 250000, China

ARTICLE INFO

Article history:
Received 21 May 2021
Revised 1 June 2021
Accepted 13 June 2021
Available online 19 June 2021

Keywords:
Diabetic retinopathy
Nimbolide
Inflammation
TLR4/NF-κB pathway

ABSTRACT

Background: Diabetic retinopathy (DR) is a common problem in the diabetic patients due to the high blood glucose level. DR affects more number of diabetic patients worldwide with irreversible vision loss.
Objective: The current investigation was focused to reveal the therapeutic actions of nimbolide against the streptozotocin (STZ)-provoked DR in rats through inhibition of TLR4/NF-κB pathway.
Methodology: DR was provoked to the rats through administering a single dose of STZ (60 mg/kg) intraperitoneally. The DR rats were then supplemented with the 50 mg/kg of nimbolide for 60 days. The bodyweight and blood glucose level was measured using standard methods. The lipid profiles (c-holesterol, TG, LDL, and HDL), inflammatory markers, and antioxidants level was detected using respective kits. The level of MCP-1, VEGF, and MMP-9 was quantified using kits. The morphometric analysis of retinal tissues were done. The mRNA expressions of target genes were studied using RT-PCR assay.
Results: Nimbolide treatment effective decreased the food intake and blood glucose, and improved the bodyweight of STZ-provoked animals. The levels of pro-inflammatory mediators, cholesterol, TG, LDL, and HDL, MCP-1, VEGF, and MMP-9 was remarkably suppressed by the nimbolide treatment. Nimbolide also improved the antioxidants, retinal thickness and cell numbers. The TLR4/NF-κB pathway was appreciably inhibited by the nimbolide.
Conclusion: Overall, our findings demonstrated that the nimbolide attenuated the STZ-provoked DR in rats through inhibiting the TLR4/NF-κB pathway.

1. Introduction

Diabetes mellitus (DM) is a common metabolic disorder categorized by hyperglycemic condition. The occurrence of DM rapidly increased around the world and affecting more children and teenagers in developed and developing countries, standing as an imperative health challenge to world population with several other complications (Park and Roh, 2016). Additionally, the prolonged impediment of DM results in hastened secondary difficulties like diabetic nephropathy, myopathy, and retinopathy through the modification macro and micro-vascular functions (Harding et al., 2019). Diabetic retinopathy (DR) is a persistent problem affecting the diabetic patients because of the unrestrained elevation in the blood glucose level (Klein, 2007). Numerous mechanisms are participated in the development of DR like pro-inflammatory mediators, microvascular injury, and oxidative stress (Guzman et al., 2016).

DR is regarded as a severe problem and latent risk factor in the reduction of the patients life quality. The pathology of DR is takes place in response to the continues injury of retina and changes in the functions of blood-retinal barrier. The initial signs of DR are the appearance of dark strings/spots float in vision; unstable and unclear vision, vision color impairments, empty/dark regions in vision, vision loss in later stages (Cheloni et al., 2019). All these ocular symptoms were occur because of the augmentation in the blood glucose and triglycerides, hypertension, and urinary proteins that results in the progression of hemorrhage and retinal tract detachments, and formation of pre-retinal fibrous tissues (Kusuhara et al., 2018). Moreover, DR also elevates the possibilities

* Corresponding author.
E-mail address: 18678770656@sina.cn (N. Wei).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2021.06.039
1319-562X/

of retinal blood vessel injuries further directs to causes the leakage/bleeding of fluids (Wang and Lo, 2018).

Ocular inflammation was categorized as one of the imperative player of numerous ocular ailments like uveitis, glaucoma, and DR (Perez and Caspi, 2015). Proinflammatory regulators like interleukin (IL)-1β, IL-6, and TNF-α was reported to crosstalk with the insulin signaling and participated in the insulin resistance (Zhu et al., 2015). Hence, the targeting these inflammatory mediators could benefit in the DM associated complication including DR. The NF-κB is a crucial inflammatory transcription factor and its stimulation triggers the excessive accumulation of pro-inflammatory regulators like TNF-α, IL-β and IL-6 (Guillonneau et al., 2017).

Toll-like receptor 4 (TLR4) is a member of the TLR family protein and it is a prime player of pro-inflammatory reactions. The stimulation of TLR4 signaling cascade directs towards the excessive generation of pro-inflammatory regulators via the triggering of numerous transcription factors like NF-κB (O'Neill et al., 2013). It was already highlighted that the elevated expression of TLR4 was found in the DM patients (Xie et al., 2014). The stimulation of TLR4/NF-κB signaling cascades could enhance the DM-activated inflammatory reactions and contribute to the DR pathogenesis (Luo et al., 2015). The existing therapeutic options to treat the DR is less effective in preventing and attenuating the progression of DR. The experts committee organized by the WHO suggested that the conventional folklore and its derivative medicines should be refreshed to prevent and treat the diabetic associated problems like DR due to the less efficacy of synthetic drugs (Modak et al., 2007).

Nimbolide is a terpenoid compound that extensively occurs in the neem plant (*Azadirachta indica*) parts. The more number of studies already unveiled the anticancer potentials of nimbolide against many cancers (Roy et al., 2007; Bodduluru et al., 2014; Lin et al., 2017; Wang et al., 016). Nimbolide was extensively utilized folklore medicinal plant to treat the diverse ailments (Bodduluru et al., 2014). Many preceding investigations has highlighted that the nimbolide has excellent biological actions like anti-inflammatory and antioxidant actions (Sophia et al., 2018; Pooladanda et al., 2019). The therapeutic benefits of nimbolide against the DR was not studied yet. Hence, the current investigation was focused to decode the therapeutic role of nimbolide against the streptozotocin (STZ)-provoked DR in rats through the inhibition of TLR4/NF-κB signaling cascade.

2. Materials and methods

2.1. Chemicals

Nimbolide, STZ, and other reagents were attained from the Sigma-Aldrich, USA. All the respective assay kits for the biochemical examinations were acquired from the Thermo Fisher, MA, USA, Cayman Chemicals, MI, USA, Abcam, Shanghai, China, and R&D Systems, MN, USA, respectively.

2.2. Experimental animals

The male Wistar rats, three months aged, and weighing above 220 ± 50 g was chosen and then acquired from the institutional animal facility. All animals were housed on the well set up laboratory conditions along with temperature 22–26 °C; moisture 40–70%; light/dark series 12-h. Animals permitted to obtain the water and diet freely throughout the investigations. The current experiments were ethically approved by the institutional animal ethics committee. All animals were adapted to laboratory situations for a week before the commencement of examinations. This research was approved by The Second People's Hospital of Jinan animal ethical committee, Approved No. 554699872S.

2.3. Experimental design

All adapted animals were distributed into four groups as group I to IV with six rats in each. Group I animals were control and excluded from all treatments. Group II was DR provoked animals. For this, animals were administered (i.p) with the single dose of STZ (60 mg/kg), which is newly prepared in 0.1 M of citrate buffer. Meantime, control animals received only 0.1 M citrate buffer. After 48 h of STZ challenge, the blood glucose was measured and the animals with above 200 mg/dl or more glucose level was regarded as diabetic. Group III was diabetic animals supplemented with the 50 mg/kg of nimbolide orally for 60 days. Group IV was diabetic animals administered with the standard drug metformin (350 mg/kg) for 60 days. After the completion of study period, the blood was gathered from the *retro*-orbital plexus for the biochemical examinations. The retinal tissues were gathered from the animals and homogenate was prepared using 50 mM of triphosphate buffer (pH-7.4) and centrifuged at 6000 rpm for 10 min at 4 °C. the resultant homogenate was utilized for additional biochemical examinations.

2.4. Bodyweight, food intake, blood glucose and glycosylated haemoglobin (HbA1c) measurement

The food consumption rate and body weight of the animals were carefully checked during the experiments. The blood glucose rate of the animals were detected using the glucometer (Roche, USA). The status of HbA1c in the blood was detected with the aid of commercial assay kit as per the guidelines of manufacturer (Thermo Fisher, MA, USA).

2.5. Measurement of lipid profile and atherogenic index (AI)

The status of lipids like cholesterol (Ch), triglyceride (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) in the blood samples of control and treated animals were examined using respective assay kits as per the protocols given by manufacturer (Cayman Chemicals, MI, USA). The AI was determined as per the equation given below: (Ch − HDL-cholesterol)/HDL-cholesterol.

2.6. Measurement of antioxidants level

The status of superoxide dismutase (SOD), glutathione (GSH), SOD/catalase (CAT), and GSH/oxidised glutathione (GSSG) ratio in the retinal tissues of control and treated animals were studied with the help of commercial assay kits as per the suggestions of manufacturer (Abcam, Shanghai, China).

2.7. Detection of pro-inflammatory markers

The status of IL-1β, IL-6, and TNF-α in the retinal tissues of control and treated animals were quantified with the aid of respective assay kits as the suggestion of manufacturer (R&D Systems, MN, USA).

2.8. Quantification of MCP-1, VEGF, and MMP-9 levels

The levels of MCP-1, VEGF, and MMP-9 in the serum tissues of control and treated animals were quantified with the help of commercial assay kits according to the manufacturer's suggestions (Mybiosource, USA).

2.9. Morphometric study

The morphometric study of retinal tissues of control and treated animals were performed using computer-aided image analysis

approach to detect the retinal thickness and number of cells in the ganglion cell layer (GCL). For this, the Image J software was utilized as described earlier by Jiang et al. (2010).

2.10. RT-PCR analysis

For PCR study, the total RNA was separated from the retinal tissues of both control and treated rats using TRIzol kit as mentioned in the protocols of manufacturer (Thermofisher, USA). Later, the separated RNA was utilized to build the cDNA by reverse-transcription using respective kit by manufacturer's suggestions (Takara, China). The utilized primers for the target genes were listed hereunder: Bax: 5′-AAGAAGCTGAGCGAGTGTCT-3′ (upstream) and 5′-CAAAGATGGTCACTGTCTGC-3′ (downstream); Bcl2: 5′-GTATGATAACCGGGAGATCG-3′ (upstream) and 5′AGCCA GGAGAAATCAAACAG-3′ (downstream); TLR4 5′-TTGAAGACAAGG CATGGCATGC-3′ (upstream) and 5′-TCTCCCAAGATCAACCGATG-3′ (downstream); NF-κB 5′-CCTAGCTTTCTCTGAACTGCAAA-3′ (upstream) and 5′-GGGTCAGAGGCCAATAGAGA-3′ (downstream). GAPDH was utilized as an control and the relative expressions of target genes were resolved using $2 - \Delta\Delta Cq$ method.

2.11. Statistical analysis

GraphPad Prism version 7.0 was utilized for the statistical examinations. Data were portrayed as mean ± SD of triplicates. Data were assayed using one-way ANOVA sequentially Tukey's multiple comparisons test. *P* value<0.05 was regarded as significant.

3. Results

3.1. Effect of nimbolide on the food intake, bodyweight, blood glucose, and HbA1c levels in the STZ-provoked DR animals

As illustrated in the Fig. 1, the average food consumption, blood glucose, and HbA1c status was found augmented in the STZ-challenged DR animals when compared with control. The STZ-challenge also reduced the bodyweight of the animals. The treatment with 50 mg/kg of nimbolide substantially suppressed the food consumption, blood glucose, and HbA1c status in the STZ-provoked animals. Nimbolide also elevated the average bodyweight of the STZ-challenged animals (Fig. 1). The metformin administration also improved the bodyweight and diminished the food consumption, blood glucose, and HbA1c status of STZ-challenged DR animals, which is more similar to the nimbolide treatment.

3.2. Effect of nimbolide on the lipid profiles and AI of STZ-provoked DR animals

The STZ-challenged DR animals displayed the drastic elevation of Ch, LDL, TG, and AI status in the serum of animals when compared with control. The HDL status was found decreased in the STZ animals (Fig. 2). However, the same was effectively modulated by the nimbolide treatment. The administration of 50 mg/kg of nimbolide to the STZ-provoked DR animals displayed the appreciable reduction in the Ch, LDL, TG, and AI status and also improved the HDL level in the serum of STZ-provoked animals. The metformin treatment also modulated the changes lipid profiles and the outcomes of nimbolide and metformin was found similar with each other (Fig. 2).

3.3. Effect of nimbolide on the antioxidants level in the STZ-provoked DR animals

Fig. 3 demonstrated that the status of antioxidants like SOD, GST, SOD/CAT ratio, and GSH/GSSG ratio was drastically diminished in the retinal tissues of STZ-provoked DR animals when compared with control. Substantially, the 50 mg/kg of nimbolide supplementation appreciably improved the SOD, GST, SOD/CAT ratio, and GSH/GSSG ratio in the retinal tissues of STZ-challenged animals (Fig. 3). The treatment with the metformin also improved these antioxidants status in the STZ-provoked DR animals.

3.4. Effect of nimbolide on the pro-inflammatory markers in the STZ-provoked DR animals

The status of IL-1β, IL-6, and TNF-α in the retinal tissues of control and treated animals were presented in the Fig. 4. The

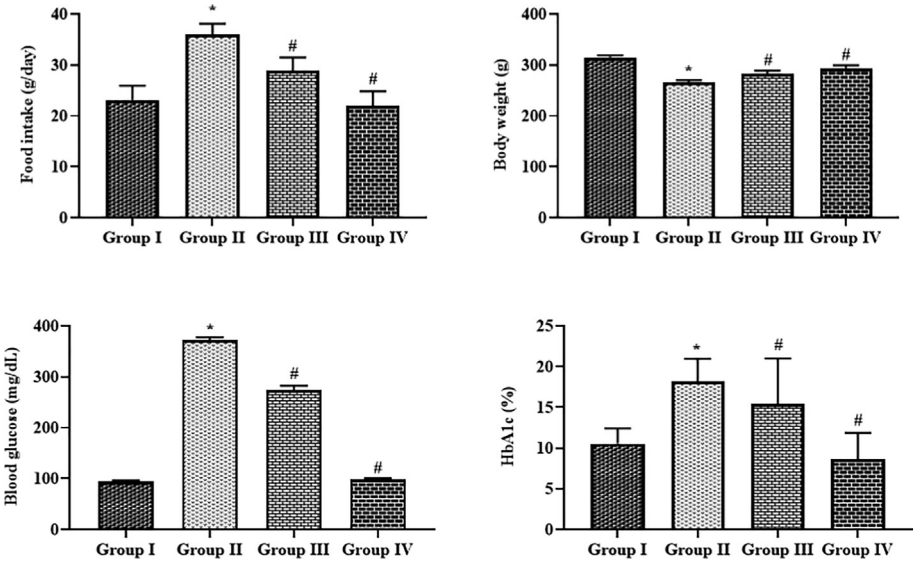

Fig. 1. Effect of nimbolide on the food intake, bodyweight, blood glucose, and HbA1c levels in the STZ-provoked DR animals Results were represented as mean ± SD of triplicates. Data were inspected by one-way ANOVA consecutively Tukey's post hoc test. Note: '*' $p < 0.05$ compared with control '#' $p < 0.01$ compared with DR-provoked animals. **Group I:** control animals, **Group II:** DR provoked animals by administering STZ (60 mg/kg). **Group III:** DR animals supplemented with the 50 mg/kg of nimbolide. **Group IV:** DR animals administered with the standard drug metformin (350 mg/kg).

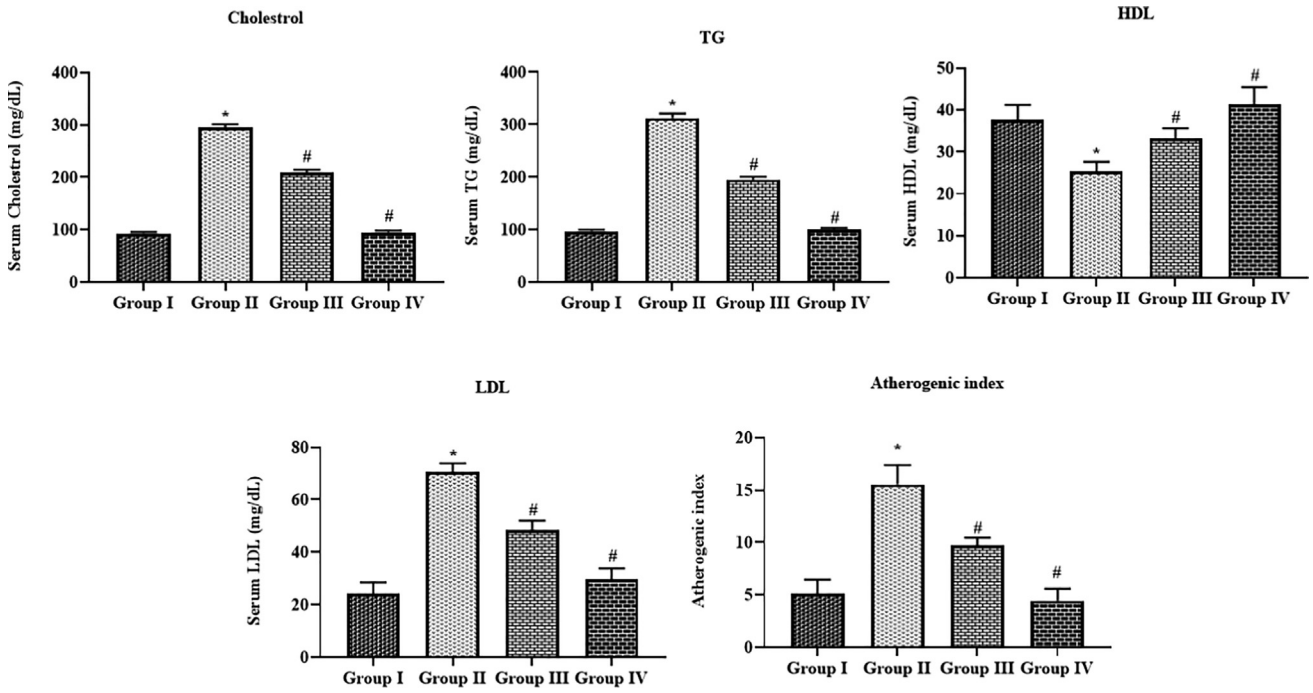

Fig. 2. Effect of nimbolide on the lipid profiles and AI of STZ-provoked DR animals Results were represented as mean ± SD of triplicates. Data were inspected by one-way ANOVA consecutively Tukey's post hoc test. Note: '*' $p < 0.05$ compared with control '#' $p < 0.01$ compared with DR-provoked animals. **Group I:** control animals, **Group II:** DR provoked animals by administering STZ (60 mg/kg). **Group III:** DR animals supplemented with the 50 mg/kg of nimbolide. **Group IV:** DR animals administered with the standard drug metformin (350 mg/kg).

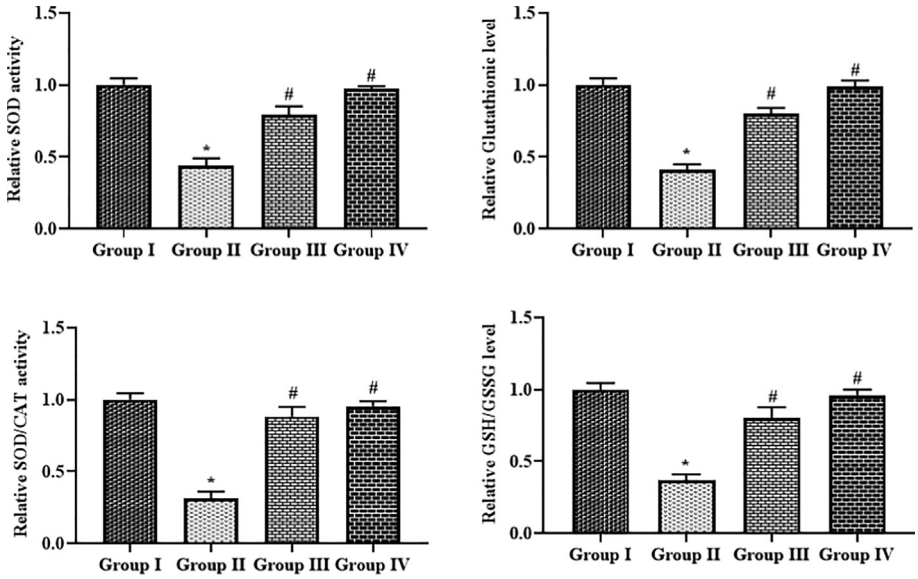

Fig. 3. Effect of nimbolide on the antioxidants level in the STZ-provoked DR animals Results were represented as mean ± SD of triplicates. Data were inspected by one-way ANOVA consecutively Tukey's post hoc test. Note: '*' $p < 0.05$ compared with control '#' $p < 0.01$ compared with DR-provoked animals. **Group I:** control animals, **Group II:** DR provoked animals by administering STZ (60 mg/kg). **Group III:** DR animals supplemented with the 50 mg/kg of nimbolide. **Group IV:** DR animals administered with the standard drug metformin (350 mg/kg).

STZ-provoked DR animals demonstrated the severe elevation in the status of IL-1β, IL-6, and TNF-α in the retinal tissues when matched with control. Though, the supplementation of 50 mg/kg of nimbolide to the STZ-provoked animals displayed the substantial reduction in the IL-1β, IL-6, and TNF-α status (Fig. 4). The metformin treatment also diminished these markers status in the retinal tissues of STZ-provoked DR animals.

3.5. Effect of nimbolide on the MCP-1, VEGF, and MMP-9 levels in the STZ-provoked DR animals

The MCP-1, VEGF, and MMP-9 levels in the control treated animals were displayed in the Fig. 5. The status of MCP-1, VEGF, and MMP-9 was severely elevated in the STZ-provoked DR animals when compared with control. Interestingly, the supplementation

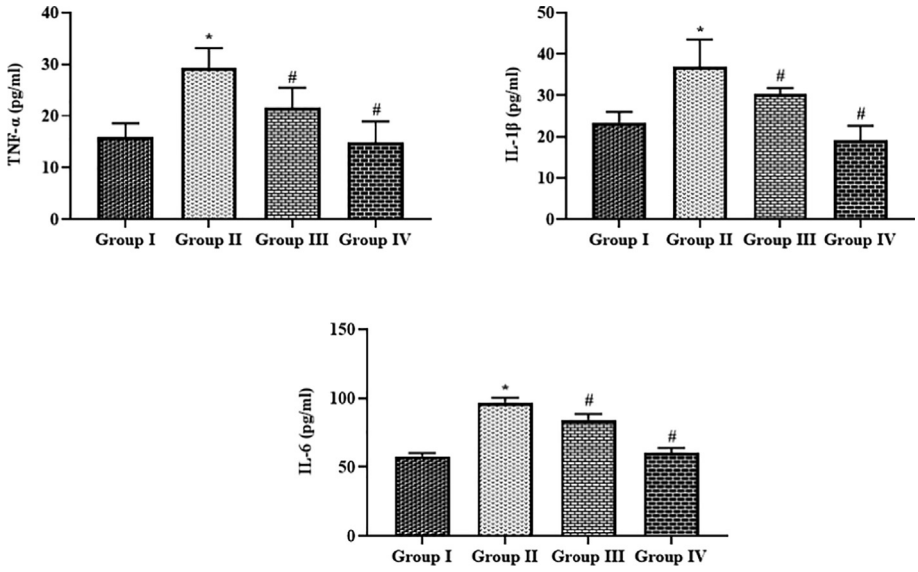

Fig. 4. Effect of nimbolide on the pro-inflammatory markers in the STZ-provoked DR animals Results were represented as mean ± SD of triplicates. Data were inspected by one-way ANOVA consecutively Tukey's post hoc test. Note: '*' $p < 0.05$ compared with control '#' $p < 0.01$ compared with DR-provoked animals. **Group I:** control animals, **Group II:** DR provoked animals by administering STZ (60 mg/kg). **Group III:** DR animals supplemented with the 50 mg/kg of nimbolide. **Group IV:** DR animals administered with the standard drug metformin (350 mg/kg).

of the 50 mg/kg of nimbolide was appreciably suppressed these markers level in the STZ-provoked DR animals (Fig. 5). The standard drug metformin treatment also suppressed the MCP-1, VEGF, and MMP-9 levels in the DR animals, which is similar to the nimbolide treatment.

3.6. Effect of nimbolide on the morphometric analysis of retina in the STZ-provoked DR animals

The outcomes of morphometric analysis of retinal tissues were depicted in the Fig. 6. It revealed that the retinal thickness and the total cell numbers in the GCL was found decreased when compared with control. Conversely, the 50 mg/kg of nimbolide supplementation to the STZ-challenged animals exhibited the remarkable improvement in the thickness and the total cells in the GCL, which is in contrast to the STZ alone treatment (Fig. 6). Similarly, the metformin treatment also improved the thickness and the total cells in the GCL, which is similar to nimbolide.

3.7. Effect of nimbolide on the Bax and Bcl-2 expression in the STZ-provoked DR animals

Fig. 7 demonstrating the mRNA expressions of Bax, Bcl-2, and Bax/Bcl-2 in the retinal tissues of control and treated animals. The mRNA expressions Bax and Bax/Bcl-2 was found up-regulated, whereas the Bcl-2 expression was suppressed in the

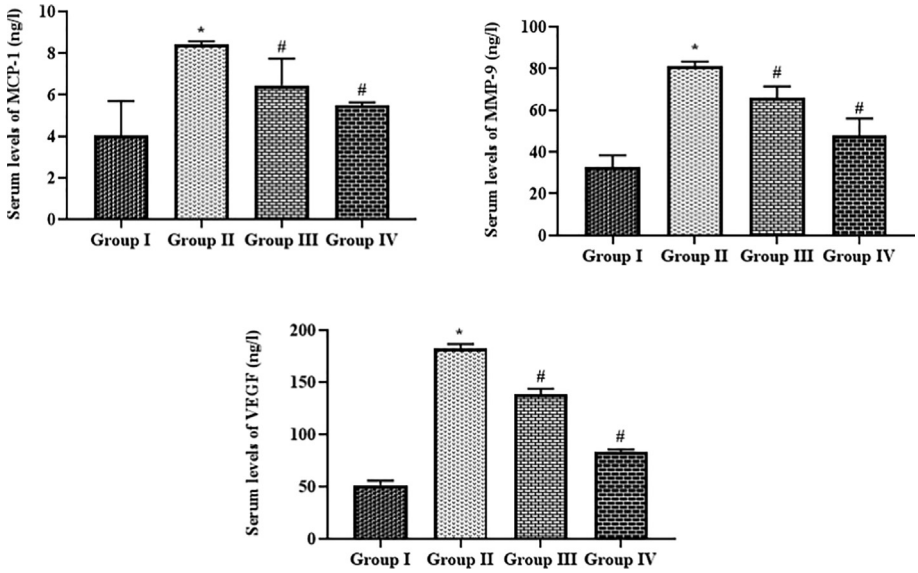

Fig. 5. Effect of nimbolide on the MCP-1, VEGF, and MMP-9 levels in the STZ-provoked DR animals Results were represented as mean ± SD of triplicates. Data were inspected by one-way ANOVA consecutively Tukey's post hoc test. Note: '*' $p < 0.05$ compared with control '#' $p < 0.01$ compared with DR-provoked animals. **Group I:** control animals, **Group II:** DR provoked animals by administering STZ (60 mg/kg). **Group III:** DR animals supplemented with the 50 mg/kg of nimbolide. **Group IV:** DR animals administered with the standard drug metformin (350 mg/kg).

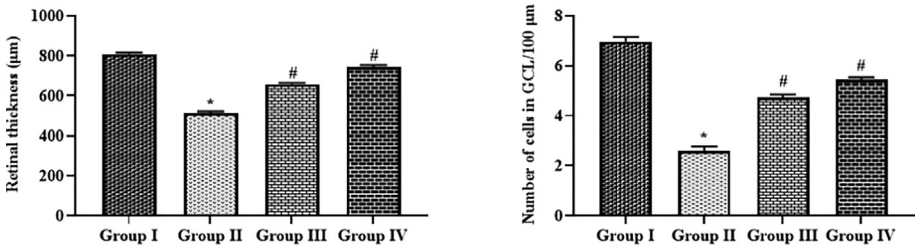

Fig. 6. Effect of nimbolide on the morphometric analysis of retina in the STZ-provoked DR animals Results were represented as mean ± SD of triplicates. Data were inspected by one-way ANOVA consecutively Tukey's post hoc test. Note: '*' $p < 0.05$ compared with control '#' $p < 0.01$ compared with DR-provoked animals. **Group I:** control animals, **Group II:** DR provoked animals by administering STZ (60 mg/kg). **Group III:** DR animals supplemented with the 50 mg/kg of nimbolide. **Group IV:** DR animals administered with the standard drug metformin (350 mg/kg).

STZ-provoked DR animals when compared with control. Interestingly, the supplementation of the 50 mg/kg of nimbolide exhibited the remarkable suppression in the Bax and Bax/Bcl-2 expressions and improved the Bcl-2 expression in the retinal tissues of STZ-provoked DR animals (Fig. 7). The metformin treatment also appreciably modulated the expressions of Bax, Bcl-2, and Bax/Bcl-2 ratio in the retinal tissues of DR animals.

3.8. Effect of nimbolide on the TLR4/NF-κB pathway in the STZ-provoked DR animals

The mRNA expression of TLR4 and NF-κB in the control treated animals depicted in the Fig. 8. The STZ-challenged DR animals demonstrated the up-regulated expressions of TLR4 and NF-κB in the retinal tissues when matched with control. however, the treatment with the 50 mg/kg of nimbolide exhibited the appreciable reduction in the mRNA expression of TLR4 and NF-κB in the retinal tissues of STZ-provoked DR animals (Fig. 8). The metformin treatment also remarkably suppressed the TLR4 and NF-κB expressions, which is similar to the nimbolide treatment.

4. Discussion

DR is a common difficulty in DM with multifaceted pathology and affects nearly 80% of diabetic victims around the world (Barsegian et al., 2017). Chronic hyperglycemia triggers the alternative cascades for over glucose metabolism that participates in the pathological progression of DR (Brownlee, 2005). DR is characterized as one of the microvasculature difficulty in DM that displays sight intimidating effects to the eyes (Beli et al., 2018). It is mostly recognized that DR is the prime most cause of DM-related ocular injury or loss among aged and young populations worldwide (Wong et al., 2016). The microvascular damages in the retina like blood barrier collapse, capillary dropout, and microaneurysms was said to be the imperative characteristics of DR (Chawla et al., 2016).

The inflammatory mediators are the vital players of progression of both early and late stages of DR. The activated NF-κB was often participated in the enhancement of proinflammatory regulators like TNF-α, IL-6, and IL-1β that eventually speed up the DR progression (Yang et al., 2006). Additionally, it was also highlighted that the elevated oxidative stress stimulates the NF-κB cascade. The over accumulation of TNF-α and IL-6 in the vitreous fluid of the

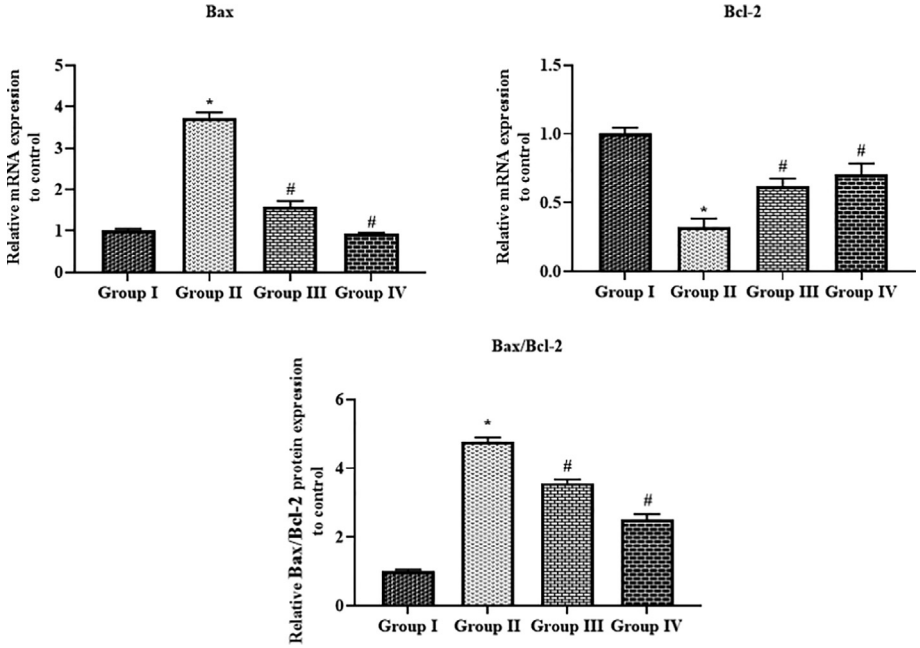

Fig. 7. Effect of nimbolide on the Bax and Bcl-2 expression in the STZ-provoked DR animals Results were represented as mean ± SD of triplicates. Data were inspected by one-way ANOVA consecutively Tukey's post hoc test. Note: '*' $p < 0.05$ compared with control '#' $p < 0.01$ compared with DR-provoked animals. **Group I:** control animals, **Group II:** DR provoked animals by administering STZ (60 mg/kg). **Group III:** DR animals supplemented with the 50 mg/kg of nimbolide. **Group IV:** DR animals administered with the standard drug metformin (350 mg/kg).

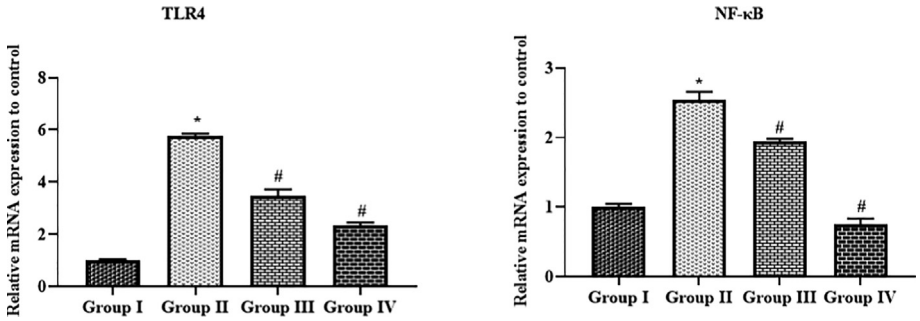

Fig. 8. Effect of nimbolide on the TLR4/NF-κB pathway in the STZ-provoked DR animals Results were represented as mean ± SD of triplicates. Data were inspected by one-way ANOVA consecutively Tukey's post hoc test. Note: '*' $p < 0.05$ compared with control '#' $p < 0.01$ compared with DR-provoked animals. **Group I:** control animals, **Group II:** DR provoked animals by administering STZ (60 mg/kg). **Group III:** DR animals supplemented with the 50 mg/kg of nimbolide. **Group IV:** DR animals administered with the standard drug metformin (350 mg/kg).

DR patients were already reported (Sato et al., 2009). TNF-α is a renowned inflammatory regulator that actively participates in the aggravation of inflammatory reactions (Gao et al., 2007). It was suggested that the TNF-α upregulation could activate the leukocytes adhesion to retina and collapsing of retina-blood barrier (Huang et al., 2011). The elevated pro-inflammatory regulators was said to be connected with the DM. Also these factors could cross-talk with insulin resistance (Jager et al., 2007). Here, we identified that the STZ-provoked DR animals demonstrated the severe elevation in IL-1β, IL-6, and TNF-α levels in the retinal tissues. Interestingly, the nimbolide supplementation to the STZ-provoked animals substantially suppressed the IL-1β, IL-6, and TNF-α status.

VEGF plays a crucial function in the DR instigation and it is reported the VEGF in the development of early stage DR (Treins et al., 2001). Chronic hyperglycemia directs to tissue hypoxia leading to up-regulation of VEGF. It elevates the permeability and dilation of blood vessels that participating in the progression of DR (Kota et al., 2012). It was already reported that the intra-ocular administration of VEGF in non-diabetic animal models produced severe aberrations in the retina that resembling DR, which recommends the role of VEGF in the DR development (Bressler et al., 2020). Oxidative stress triggers the inflammatory regulators that eventually participates in the retinal cell injury and successively DR progression. Additionally, oxidative stress can also modify the various growth factor expressions like VEGF and stimulate the other metabolic cascades implicated in the DR development (Son et al., 2013; Keshari et al., 2013). The anti-VEGF therapy is said to be a possible therapeutic option for the DR (Titchenell and Antonetti, 2013). In this exploration, we disclosed that the VEGF level was severely elevated in the STZ-provoked DR animals, which effectively reduced by the nimbolide treatment.

The MMP-9 belongs to the proteinase family that could destroy the most components of extracellular matrix and performs a critical functions in numerous normal and pathological events (Nagase et al., 1999). Many preceding investigations has unveiled that the MMP-9 actively participates in the commencement of DR (Kowluru et al., 2012). It was already highlighted that the MMP-9 activity was augmented in several phases of DR in human (Kowluru et al., 2016). We also noted that the MMP-9 level was drastically elevated in the STZ-provoked DR animals and the same was appreciably reduced by the nimbolide treatment.

The retinal tissues often experiences the numerous metabolic disorders and alterations seem in gene expressions. The diabetic condition could lead to the retinal capillary cell necrosis and histological modifications. The activated NF-κB in the DR could augment the capillary cell apoptosis that was imperative histological feature of DR (Antzelevitch, 2018; Jiang et al., 2017). NF-κB is known as a vital player of inflammatory reactions and a suppressor of antioxidants. It was said that the NF-κB recruits proapoptotic events because of high glucose mediated pressure in retina (Romeo et al., 2002). We also noted that the status of SOD, GST, SOD/CAT ratio was severely suppressed in the retinal tissues of STZ-provoked DR animals. Interestingly, the nimbolide treatment appreciably improved the antioxidants level in the retinal tissues of STZ-challenged animals.

The Bax, a apoptosis-encouraging factor performs a crucial functions in the retinal neuronal cell death via its apoptosis triggering efficacy. The Bcl-2 elevates the cell survival via its capacity to block the cell apoptosis. Meanwhile, Bax/Bcl-2 ratio is vital for the stimulation of cell apoptosis, the raised Bax/Bcl-2 ratio is participated in the retinal cell death (Sharpe et al., 2004). In this exploration, we noted that the mRNA expressions Bax and Bax/Bcl-2 ratio was up-regulated and the Bcl-2 expression was suppressed in the STZ-provoked DR animals. Interestingly, the nimbolide treatment substantially diminished the Bax expression and improved the Bcl-2 expression in the retinal tissues of STZ-provoked DR animals.

The stimulation of inflammatory cascades in the retina was explicated to participate to the pathogenesis of DR. TLR4/NF-κB cascade was known to be stimulated during the expansion of DR in STZ-challenged animals. (Wang et al., 2016a,b). Moreover, it was well-known that the TLR4/NF-κB cascade could speed up the DR progression (Wang et al., 2015). The inflammatory regulators is triggered by the stimulation of TLR4/NF-κB cascade during the systemic inflammatory conditions (Chu et al., 2014). It was highlighted that the suppression of inflammatory reactions via the hindering of TLR4/NF-κB cascade could benefit for the treatment of DR (Zhu et al., 2017). TLR4 was reported to have the vital molecular actions in DR. The improved TLR4 expressions were often noted in the DM (Feng et al., 2016). It was reported that the diabetic animals has unusual stimulation of NF-κB pathway that enhances the accumulation of inflammatory mediators and further results in the retinal neovascularization, hemorrhage, and speed up the DR progression (Luo et al., 2015; Yin et al., 2017). Additionally, the retinal degeneration in animals has highlighted to be related with the stimulation of TLR4 (Huang et al., 2018). Prominently, the TLR4 cascade was explicated to be enhanced during the DR progression in rats (Wang et al., 2019). Similarly, we also noted that the TLR4 and NF-κB expressions were up-regulated in the retinal tissues, which is appreciably suppressed by the nimbolide treatment.

5. Conclusion

Our findings from this study disclosed that the nimbolide attenuated the STZ-provoked DR in rats through the reduction of inflammatory mediators, oxidative stress, improved antioxidants, and inhibition of TLR4/NF-κB signaling cascade. These findings supported the recommendation that the nimbolide could be a hopeful remedy to treat the DR. However, the additional investigations still

needed in the future to make concrete evidence about the ameliorative effects of nimbolide against DR.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Antzelevitch, C., 2018. Genetic, molecular and cellular mechanisms underlying the J wave syndromes. Cardiac. Electrophysiol. Cell. Bedside. 76 (5), 483–493.

Barsegian, A., Kotlyar, B., Lee, J., Salifu, M.O., McFarlane, S.I., 2017. Diabetic retinopathy: focus on minority populations. Int. J. Clin. Endocrinol. Metab. 3 (1), 34–45.

Beli, E., Yan, Y., Moldovan, L., Vieira, C.P., Gao, R., Duan, Y., Prasad, R., Bhatwadekar, A., et al., 2018. Grant, Restructuring of the gut microbiome by intermittent fasting prevents retinopathy and prolongs survival in db/db mice. Diabetes. 67, 1867–1879.

Bodduluru, L.N., Kasala, E.R., Thota, N., Barua, C.C., Sistla, R., 2014. Chemopreventive and therapeutic effects of nimbolide in cancer: the underlying mechanisms. Toxicol. In. Vitro. 28, 1026–1035.

Bressler, N.M., Beaulieu, W.T., Bressler, S.B., Glassman, A.R., Melia, B.M., Jampol, L. M., Jhaveri, C.D., Salehi-Had, H., Velez, G., Sun, J.K., DRCR Retina Network., 2020. Anti-vascular endothelial growth factor therapy and risk of traction retinal detachment in eyes with proliferative diabetic retinopathy: pooled analysis of five DRCR retina network randomized clinical trials. Retina. 40(6), 1021-1028.

Brownlee, M., 2005. The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 54 (6), 1615–1625.

Chawla, A., Chawla, R., Jaggi, S., 2016. Microvascular and macrovascular complications in diabetes mellitus: distinct or continuum? Indian J Endocrinol Metab. 20 (4), 546–551.

Cheloni, R., Gandolfi, S.A., Signorelli, C., Odone, A., 2019. Global prevalence of diabetic retinopathy: protocol for a systematic review and meta-analysis. BMJ Open. 9, e022188.

Chu, M., Ding, R., Chu, Z.Y., Zhang, M.B., Liu, X.Y., Xie, S.H., et al., 2014. Role of berberine in anti-bacterial as a high-affinity LPS antagonist binding to TLR4/MD-2 receptor. BMC Complement. Altern. Med. 14, 89.

Feng, H., Su, R., Song, Y., et al., 2016. Positive correlation between enhanced expression of TLR4/MyD88/NF-kappaB with insulin resistance in placentae of gestational diabetes mellitus. PLoS One. 11, e0157185.

Gao, X., Belmadani, S., Picchi, A., Xu, X., Potter, B.J., Tewari-Singh, N., Capobianco, S., Chilian, W.M., Zhang, C., 2007. Tumor necrosis factor-alpha induces endothelial dysfunction in Lepr(db) mice. Circulation. 115 (2), 245–254.

Guillonneau, X., Eandi, C.M., Paques, M., Sahel, J.A., Sapieha, P., Sennlaub, F., 2017. On phagocytes and macular degeneration. Prog. Retin. Eye Res. 61, 98–128.

Guzman, D.C., Olguín, H.J., García, E.H., Peraza, A.V., de la Cruz, D.Z., Soto, M.P., 2016. Mechanisms involved in the development of diabetic retinopathy induced by oxidative stress. Redox. Rep. 22 (1), 1–7.

Harding, J.L., Pavkov, M.E., Magliano, D.J., Shaw, J.E., Gregg, E.W., 2019. Global trends in diabetes complications: a review of current evidence. Diabetologia. 62, 3–16.

Huang, H., Gandhi, J.K., Zhong, X., Wei, Y., Gong, J., Duh, E.J., Vinores, S.A., 2011. TNF alpha Is Required for Late BRB Breakdown in Diabetic Retinopathy, and Its Inhibition Prevents Leukostasis and Protects Vessels and Neurons from Apoptosis. Invest. Ophthalmol. Vis. Sci. 52 (3), 1336–1344.

Huang, Z., Zhou, T., Sun, X., Zheng, Y., Cheng, B., Li, M., Liu, X., He, C., 2018. Necroptosis in microglia contributes to neuroinflammation and retinal degeneration through TLR4 activation. Cell Death Differ. 25, 180–189.

Jager, J., Gremeaux, T., Cormont, M., et al., 2007. Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. Endocrinology. 148, 241–251.

Jiang, T., Guan, L.Y., Ye, Y.S., Liu, H.Y., Li, R., 2017. MiR-874 inhibits metastasis and epithelial-mesenchymal transition in hepatocellular carcinoma by targeting SOX12. American. J. Cancer. Res. 7 (6), 1310–1321.

Jiang, Y., Walker, R.J., Kern, T.S., Steinle, J.J., 2010. Application of isoproterenol inhibits diabetic-like changes in the rat retina. Exp. Eye. Res. 9, 171–179.

Keshari, R.S., Verma, A., Barthwal, M.K., Dikshit, M., 2013. Reactive oxygen species-induced activation of ERK and p38 MAPK mediates PMA-induced NETs release from human neutrophils. J. Cell. Biochem. 114 (3), 532–540.

Klein, B.E., 2007. Overview of epidemiologic studies of diabetic retinopathy. Ophthalmic Epidemiol. 14, 179–183.

Kota, S.K., Meher, L.K., Jammula, S., Kota, S.K., Krishna, S.V., et al., 2012. Aberrant angiogenesis: the gateway to diabetic complications. Ind. J. Endo. Metabol. 16 (6), 918.

Kowluru, R.A., Shan, Y., Mishra, M., 2016. Dynamic DNA methylation of matrix metalloproteinase-9 in the development of diabetic retinopathy. Lab. Invest. 96 (10), 1040–1049.

Kowluru, R.A., Zhong, Q., Santos, J.M., 2012. Matrix metalloproteinases in diabetic retinopathy: potential role of MMP-9. Expert. Opin. Investig. Drugs. 21, 797–805.

Kusuhara, S., Fukushima, Y., Ogura, S., Inoue, N., Uemura, A., 2018. Pathophysiology of diabetic retinopathy: the old and the new. Diabetes. Metab. J. 42, 364–376.

Lin, H., Qiu, S., Xie, L., Liu, C., Sun, S., 2017. Nimbolide suppresses non-small cell lung cancer cell invasion and migration via manipulation of DUSP4 expression and ERK1/2 signaling. Biomed. Pharmacother. 92, 340–346.

Luo, D.W., Zheng, Z., Wang, H., Fan, Y., Chen, F., Sun, Y., et al., 2015. UPP mediated Diabetic Retinopathy via ROS/PARP and NF-kappaB inflammatory factor pathways. Current molecular medicine. 15 (8), 790–799.

Modak, M., Dixit, P., Londhe, J., Ghaskadbi, S., Devasagayam, T., 2007. Indian herbs and herbal drugs used for the treatment of diabetes. J Clin Biochem Nutr 40 (3), 163–173.

Nagase, H., Woessner, J.J.F., 1999. Matrix metalloproteinases. J Biol Chem 274, 21491–21494.

O'Neill, L.A., Golenbock, D., Bowie, A.G., 2013. The history of toll-like receptors - redefining innate immunity. Nat Rev Immunol. 13, 453–460.

Park, Y.G., Roh, Y.J., 2016. New diagnostic and therapeutic approaches for preventing the progression of diabetic retinopathy. J. Diabetes Res. 2016, 1753584.

Perez, V.L., Caspi, R.R., 2015. Immune mechanisms in inflammatory and degenerative eye disease. Trends Immunol. 36, 354–363.

Pooladanda, V., Thatikonda, S., Bale, S., Pattnaik, B., Sigalapalli, D.K., Bathini, N.B., Singh, S.B., Godugu, C., 2019. Nimbolide protects against endotoxin-induced acute respiratory distress syndrome by inhibiting TNF-α mediated NF-κB and HDAC-3 nuclear translocation. Cell Death Dis. 10 (2), 81.

Romeo, G., Liu, W.H., Asnaghi, V., Kern, T.S., Lorenzi, M., 2002. Activation of nuclear factor-kappaB induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes. Diabetes 51, 2241–2248.

Roy, M.K., Kobori, M., Takenaka, M., Nakahara, K., Shinmoto, H., Isobe, S., Tsushida, T., 2007. Antiproliferative effect on human cancer cell lines after treatment with nimbolide extracted from an edible part of the neem tree (Azadirachta indica). Phytother. Res. 21, 245–250.

Sato, T., Kusaka, S., Shimojo, H., Fujikado, T., 2009. Simultaneous analyses of vitreous levels of 27 cytokines in eyes with retinopathy of prematurity. Ophthalmol. 116 (11), 2165–2169.

Sharpe, J.C., Arnoult, D., Youle, R.J., 2004. Control of mitochondrial permeability by Bcl-2 family members. Biochim. Biophys. Acta 1644, 107–113.

Son, Y., Kim, S., Chung, H.T., Pae, H.O., 2013. Reactive oxygen species in the activation of MAP kinases. Methods in enzymology. 528, 27–48.

Sophia, J., Kowshik, J., Dwivedi, A., Bhutia, S.K., Manavathi, B., Mishra, R., Nagini, S., 2018. Nimbolide, a neem limonoid inhibits cytoprotective autophagy to activate apoptosis via modulation of the PI3K/Akt/GSK-3β signalling pathway in oral cancer. Cell Death Dis. 9 (11), 1087.

Titchenell, P.M., Antonetti, D.A., 2013. Using the past to inform the future: anti-VEGF therapy as a road map to develop novel therapies for diabetic retinopathy. Diabetes 62, 1808–1815.

Treins, C., Giorgetti-Peraldi, S., Murdaca, J., Van Obberghen, E., 2001. Regulation of vascular endothelial growth factor expression by advanced glycation end products. J. Biol. Chem. 276, 43836–43841.

Wang, W., Lo, A.C.Y., 2018. Diabetic retinopathy: pathophysiology and treatments. Int. J. Mol, Sci, p. 19.

Wang, L., Phan, D.D., Zhang, J., Ong, P.S., Thuya, W.L., Soo, R., Wong, A.L., Yong, W.P., Lee, S.C., et al., 2016a. Anticancer properties of nimbolide and pharmacokinetic considerations to accelerate its development. Oncotarget 7, 44790–44802.

Wang, L., Wang, J., Fang, J., Zhou, H., Liu, X., Su, S.B., 2015. High glucose induces and activates Toll-like receptor 4 in endothelial cells of diabetic retinopathy. Diabetol. Metab. Syndrome 7, 89.

Wang, Q., Navitskaya, S., Chakravarthy, H., Huang, C., Kady, N., Lydic, T.A., Chen, Y.E., Yin, K.J., Powell, F.L., Martin, P.M., Grant, M.B., Busik, J.V., 2016b. Dual anti-inflammatory and anti-angiogenic action of miR-15a in diabetic retinopathy. EBioMedicine 11, 138–150.

Wang, Y., Tao, J., Jiang, M., Yao, Y., 2019. Apocynin ameliorates diabetic retinopathy in rats: involvement of TLR4/NF-kappaB signaling pathway. Int. Immunopharm. 73, 49–56.

Wong, T.Y., Cheung, C.M., Larsen, M., Sharma, S., Simo, R., 2016. Diabetic retinopathy. Nat Rev Dis Primers 2, 16012.

Xie BG, Jin S, ZhuWJ. Expression of toll-like receptor 4 in maternal monocytes of patients with gestational diabetes mellitus. Exp Ther Med. 2014;7:236–40.

Yang, S.R., Chida, A.S., Bauter, M.R., Shafiq, N., Seweryniak, K., Maggirwar, S.B., Kilty, I., Rahman, I., 2006. Cigarette smoke induces proinflammatory cytokine release by activation of NF-kappaB and posttranslational modifications of histone deacetylase in macrophages. Am. J. Physiol. Lung. Cell. Mol. Physiol. 291 (1), L46–L57.

Yin, Y., Chen, F., Wang, W., Wang, H., Zhang, X., 2017. Resolvin D1 inhibits inflammatory response in STZ-induced diabetic retinopathy rats: Possible involvement of NLRP3 inflammasome and NF-kappaB signalling pathway. Molecular vision. 23, 242–250.

Zhu, C., Yang, H., Geng, Q., et al., 2015. Association of oxidative stress biomarkers with gestational diabetes mellitus in pregnant women: a case-control study. PLoS One. 10, e0126490.

Zhu, S.H., Liu, B.Q., Hao, M.J., Fan, Y.X., Qian, C., Teng, P., Zhou, X.W., Hu, L., Liu, W.T., Yuan, Z.L., Li, Q.P., 2017. Paeoniflorin suppressed high glucose-induced retinal microglia MMP-9 expression and inflammatory response via inhibition of TLR4/NFkappaB pathway through upregulation of SOCS3 in diabetic retinopathy. Inflammation 40, 1475–1486.