

# Monoterpene $\alpha$ -Pinene Alleviates Diabetic Retinopathy in Rodent Model via Attenuating Hyperglycemia Induced VEGF Signaling

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## ABSTRACT

**Background:** Diabetic Retinopathy (DR) has emerged as a significant global health issue that requires urgent and collaborative action to tackle. As the leading causes of preventable blindness, DR predominantly impacts adults in their working years, posing a significant threat to both individual quality of life and global economic productivity. This research work was aimed at assessing the potency of  $\alpha$ -Pinene; a miracle compound on ameliorating hyperglycemia induced diabetic retinopathy in rodent model. **Materials and Methods:** Male Wistar rats were administered with single shot intraperitoneal 60 mg/kg STZ injection to induce diabetes. The rats exhibited glucose levels 250 mg/dL or higher were classified as diabetic rats and were treated with  $\alpha$ -Pinene and a group of diabetic rats were treated with standard anti-hyperglycemic drug metformin. The body weight, feed intake was monitored and the HbA1C, blood glucose levels were quantified to analyse the antihyperglycemic effect of  $\alpha$ -Pinene. **Results:** The lipid profile and atherogenic index were quantified to assess the anti-hypercholesteremic effect of  $\alpha$ -Pinene. MDA levels, antioxidants and glutathione levels were measured to examine the antioxidant potency of  $\alpha$ -Pinene in hyperglycemic condition. Inflammatory triggering molecules NF $\kappa$ B, TNF $\alpha$  and interleukins 6, 1 $\beta$  were quantified to analyse the inflammatory attenuating effect of  $\alpha$ -Pinene. The impact of  $\alpha$ -Pinene on VEGF, MMP-9 and MCP-1 were analysed to confirm the attenuating effect of  $\alpha$ -Pinene against diabetic retinopathy development and progression. BAX and BCL2 proteins were quantified to examine  $\alpha$ -Pinene anti-apoptotic potency in hyperglycemic condition. The retinal thickness and the ganglion cell count were measured to confirm the ameliorating potency of  $\alpha$ -Pinene against diabetic retinopathy. Our results demonstrated that  $\alpha$ -Pinene is a powerful antioxidant with significant anti-diabetic, anti-hypercholesterolemic, inflammatory modulation, and apoptosis inhibiting effects in a rodent model of diabetic retinopathy. **Conclusion:** It effectively reduced the initiation and advancement of diabetic induced retinal disorder, as confirmed by histopathological analysis of retinal tissue. In conclusion,  $\alpha$ -Pinene is a promising compound with diverse pharmacological properties that can help mitigate diabetic induced retinal disorder.

**Keywords:** Anti-VEGF drug, Diabetic induced retinal disorder, Inflammation, Oxidative stress, Phytocompound,  $\alpha$ -Pinene.

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## INTRODUCTION

Diabetic Retinal disorder (DR) is a substantial and escalating worldwide health issue, following an alarming increase in Diabetes Mellitus (DM) incidence, which was predictable at 463 million cases in 2019 and projected to reach 700 million by 2045.<sup>1,2</sup> DR, as a frequent disorder of diabetes, ranks among the foremost causes of preventable loss of sight, particularly affecting

the adult working population.<sup>3,4</sup> Research indicates that DR has ranked fifth in causing blindness and drastic vision weakening for people aged 50 and older, with a 14.9% to 18.5% increase in age-standardized global prevalence from 1990 to 2020.<sup>5</sup> It is the only leading cause of blindness with an increasing global prevalence since 1990 (IDF, 2021).<sup>6</sup> At present, there are 537 million adults between the ages of 20 and 79 who are affected by diabetes, with 95% likely to have type 2 diabetes, and a significant number—approximately 240 million—are undiagnosed, which heightens the risk of complications like DR. The rising incidence of this metabolic disorder particularly pronounced in underdeveloped and developing nations, where 75% of affected individuals reside.<sup>7,8</sup>



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DR is a multifaceted condition involving complex interactions between various cellular components, molecular pathways, and pathological processes (Whitehead *et al.*, 2018).<sup>9</sup> It primarily results from damage to the retinal capillaries caused by prolonged increased glucose levels in blood, reported to cause vision impairment through mechanisms such as proliferative retinopathy and maculopathy.<sup>10</sup> The development of DR is driven by intricate biochemical processes, including the accumulation of sorbitol and Advanced Glycation End-products (AGEs), increased oxidative stress, inflammation, and activation of pathways like the renin-angiotensin system and Vascular Endothelial Growth Factor (VEGF).<sup>11,12</sup>

VEGF is a critical molecule in the development of DR, promoting abnormal blood vessel growth and permeability. Inflammation also plays an increasingly significant role, as hyperglycemia induces inflammatory responses that damage the retinal microvasculature.<sup>13</sup> Blood-retinal barrier disruption leads to leakage and contributes to Diabetic Macular Edema (DME). Inadequate management of DR can amplify hyperglycemia and oxidative stress, resulting in mitochondrial dysfunction and further VEGF production, thereby accelerating disease progression.<sup>14,15</sup> Currently, anti-VEGF drugs such as ranibizumab and aflibercept have undergone clinical trials for DR treatment and have received approval from the US FDA.<sup>16,17</sup> However, because these anti-VEGF agents have a decreased longevity, patients typically require regular shots to maintain their effectiveness. This frequent injection schedule may increase the risk of endophthalmitis, a rare but serious complication associated with intravitreal injections.<sup>18</sup> Additionally, corticosteroids have been recognized for their anti-inflammatory and anti-angiogenic properties, making them a viable option for managing DR.<sup>19</sup> Nonetheless, their use is not without risks, as side effects such as cataract formation, glaucoma, and potential infections have been reported.<sup>20</sup>

$\alpha$ -Pinene is a bicyclic monoterpene commonly found in essential oils of coniferous trees, particularly in pine trees.<sup>21</sup> It possesses several pharmacological properties that contribute to its therapeutic potential.<sup>22</sup> Primarily known for its anti-inflammatory and analgesic effects,  $\alpha$ -Pinene can modulate the body's inflammatory response and alleviate pain, making it beneficial in managing conditions like arthritis.<sup>23,24</sup> It is reported to be a potent antioxidant,<sup>25</sup> anticancer<sup>26</sup> and antibacterial agent.<sup>27</sup> In this study, we sought to analyze the  $\alpha$ -Pinene effectiveness in managing hyperglycemia and inhibiting the progression of diabetic retinopathy in a rodent model.

## MATERIALS AND METHODS

### Experimental Subjects

Male Wistar rats with a weight of 220-250 g were utilized in this study. The animals were housed in polypropylene cages bedded

with rice husks. The rats were fed with laboratory rodent standard pellet diet and filtered water. The whole experimental design and the procedures performed on the animals were permitted by the members of the ethical committee.

### Diabetic induction

Rats used for the current research were acclimatized for a week in the animal research laboratory before initiation of the experiment. The rats were fasted for 16 hr before hyperglycemic induction. Animals were administered with single shot intraperitoneal 60 mg/kg STZ injection to induce diabetes. Blood glucose concentration was assessed 48 hr after STZ injection with blood collected from orbital plexus. The rats exhibited glucose levels 250 mg/dL or higher were classified as diabetic rats and were utilized for the further experiments.

### Animal Grouping and Treatment

The experimental animals were grouped into four Group I are the sham group treated with vehicle 0.1M saline and Group II are the diabetic control rats which were induced with diabetes and not treated with any drug. The Group III rats were induced diabetic and subsequently treated with 30 mg/kg of  $\alpha$ -Pinene orally for 9 weeks. Group IV rats were diabetic induced rats treated with standard antidiabetic drug metformin (350 mg/kg) for 60 days. The body weight of experimental animals and the feed intake were measured regularly throughout the experimental period. Upon termination of treatment period the rats were euthanized, blood was collected, and the retinas were excised for the molecular and histopathological analysis.

### Quantification of glucose levels

The blood glucose levels were estimated using the Crystal Chem Rat Glucose Assay Kit and the levels of Glycated Hemoglobin A1c were quantified using the MyBiosource Rat Glycated Hemoglobin A1c ELISA Kit. The reagents, standards and the samples were prepared as per the instructions provided in the Kit. The test was performed in triplicates and the final absorbance was quantified at 505 nm for blood glucose level estimation and 450 nm for HbA<sub>1c</sub> estimation.

### Assessment of Lipid profile

The lipid profile was examined in experimental subjects to analyze the impact of  $\alpha$ -Pinene on regulating cholesterol intensities in diabetic rats. The levels of total cholesterol, triglycerides and HDL cholesterol were quantified with the colorimetric assay kits procured from Cayman, USA. The levels of LDL cholesterol were calculated using the formula described by Friedewald  $LDL=TC-(HDL+TG/5)$ .<sup>28</sup>

Atherogenic index of plasma was measured using the formula  $AI=(TC-HDL)/HDL$ .<sup>29</sup>

### Assessment of oxidative stress markers

Homogenized tissue was prepared with PBS buffer; homogenized tissue was subjected to centrifugation at 2000 g for 15 min at 4°C and antioxidant stress markers was quantified in the supernatant. Malondialdehyde levels were quantified with the protocol of Hermann Esterbauer and Zollner (1989).<sup>30</sup> Antioxidants catalase and superoxide dismutase were quantified using the protocol of Luck (1965)<sup>31</sup> and Paoletti and Mocali (1990).<sup>32</sup>

The glutathione levels were quantified in the tissue homogenate of the experimental animals using the Glutathione GSH/GSSG Assay Kit procured from Sigma Aldrich, USA. The glutathione levels were quantified using Ellman's method and the optical density was measured at 412 nm. The total oxidized and the reduced glutathione levels were quantified to assess the GSH/GSSG ratio. Glutathione peroxidase was quantified via measuring the consumption of NADPH in the coupled enzyme reactions using the Glutathione Peroxidase kit procured from Sigma Aldrich and Glutathione Reductase Activity Assay Kit purchased from Elabscience. The final absorbance was quantified to 340 nm.

### Quantification of inflammatory stimulating cytokines

The cytokines stimulating inflammation were quantified in the experimental rats to assess the efficacy of  $\alpha$ -Pinene attenuating the hyperglycemia induced inflammatory reactions. The levels of NF- $\kappa$ B, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were quantified using the ELISA kit procured from Abcam, USA. The assays were performed in triplicates following the kit manual.

### Assessment of diabetic retinopathy progression

The initiation and advancement of retinal disorder in the hyperglycemia induced rats were assessed via quantifying the levels of Vascular Endothelial Growth Factor (VEGF), Matrix Metalloproteinase-9 (MMP-9), and Monocyte Chemoattractant Protein-1 (MCP-1) which plays significant roles in the diabetic retinopathy pathology. The VEGF, MMP-9 and MCP-1 levels were quantified with ELISA kits purchased from R and D Systems. The assays were performed in triplicates following the kit manual.

### Histopathological analysis of Retinal tissue

Eyes were fixed in a solution of 10% formalin-glacial acetic acid-ethanol for 10 min, followed by overnight fixation in PBS-buffered 4% paraformaldehyde. The posterior eyecups were then dehydrated through a graded ethanol series for 30 min each, cleared in xylene for 10 min twice, and infiltrated with paraffin wax twice for 1 hr each, after which they were embedded in paraffin blocks. Thin sections (4  $\mu$ m) were cut using a microtome and stained with hematoxylin and eosin for histological analysis. Images were captured at 200 $\times$ , the retinal thickness and the GCL number were counted with Image-Pro Plus 6.0 software.

### Assessment of apoptosis in diabetic retinopathy induced rats

The apoptotic protein Bax and the anti-apoptotic Bcl2 levels were measured in the test and  $\alpha$ -Pinene treated hyperglycemia induced rats to analyze the anti-apoptotic efficacy of  $\alpha$ -Pinene. Bax and Bcl2 levels were quantified using the ELISA kits purchased from Abcam. The experiment was performed in triplicates following the protocol of manual provided in the kit.

### Statistics

Data analysis was performed using SPSS 18 software (SPSS, Inc., Chicago, IL). Results are presented as Mean $\pm$ SEM, and statistical comparisons were made using one-way Analysis of Variance (ANOVA), followed by Student's Newman-Keuls test. A  $p$ -value of  $\leq 0.05$  was deemed statistically significant.

## RESULTS

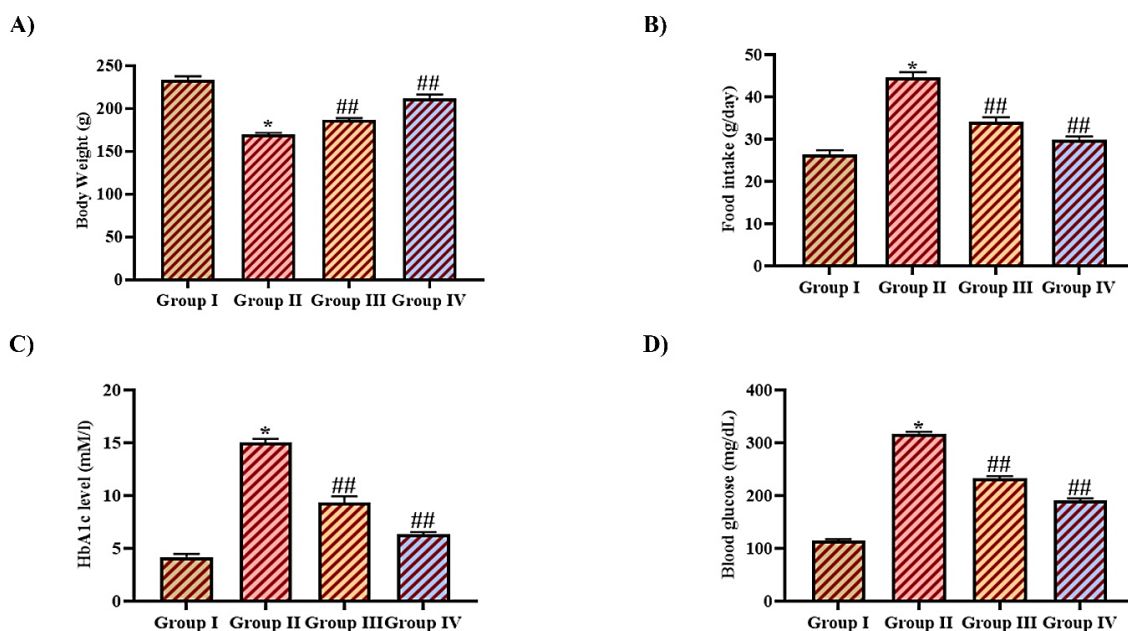
### Monoterpene $\alpha$ -Pinene attenuates hyperglycemia in diabetic retinopathy rodent model

Figure 1 illustrates the impact of  $\alpha$ -Pinene inhibiting hyperglycemia in diabetic retinopathy rodent model. The experimental animal body weight was monitored regularly and the final weight was depicted in Figure 1A. Body weight was reduced in the untreated diabetic subjects compared to the control animals. Treatment with  $\alpha$ -Pinene and standard drug metformin prevented the weight loss in experimental rats. The diabetic rats treated with  $\alpha$ -Pinene and/or metformin shown increased body weight. Feed intake was drastically elevated in the diabetic untreated subjects than the control and drug-treated rats (1B).

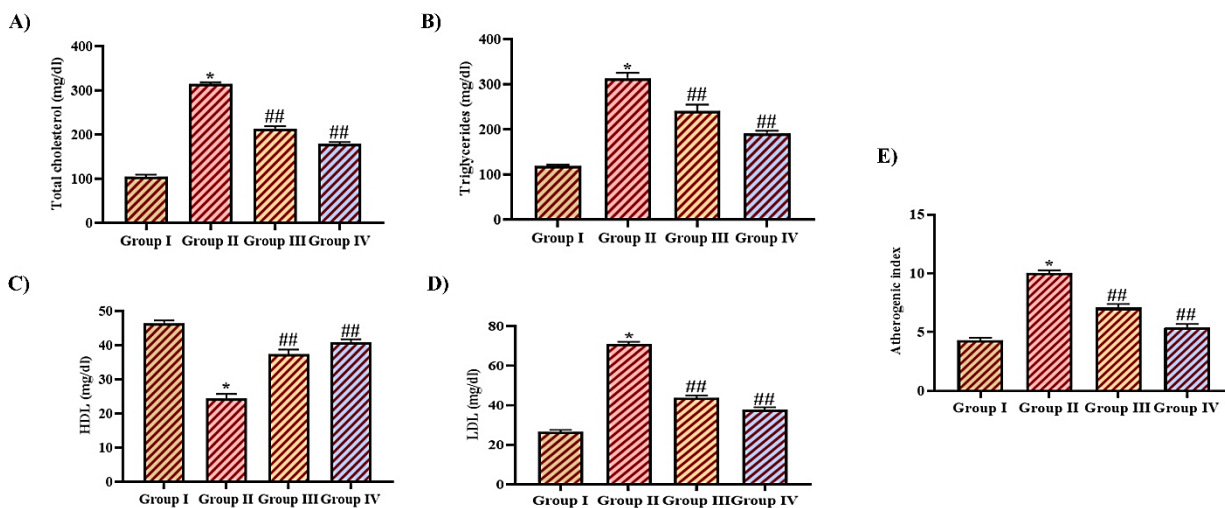
Figure 1C and D depict HbA1C and blood glucose levels in the experimental rats. Diabetic induced rats had shown significantly increased levels of glycated hemoglobin and blood glucose than the control animals. Untreated diabetic rats exhibited elevated levels of HbA1c and blood glucose, whereas treatment with  $\alpha$ -Pinene decreased both HbA1C and blood glucose levels. Treatment with metformin also decreased the HbA1C and blood glucose levels.

### Monoterpene $\alpha$ -Pinene attenuates hypocholesteremia in diabetic retinopathy rodent model

Figure 2 signifies the lipid profile levels quantified in the control, drug treated and untreated diabetic retinopathy induced rats. Diabetic induction in rats had elevated total cholesterol and triglycerides than the control rats. Treatment with  $\alpha$ -Pinene and metformin exhibited decrease in the levels of both total cholesterol and triglycerides. The HDL intensities were significantly enhanced, and LDL levels were reduced in the  $\alpha$ -Pinene and metformin treated rats than the untreated diabetic rats. The atherogenic index was significantly reduced in  $\alpha$ -Pinene and



**Figure 1:** Monoterpene  $\alpha$ -Pinene attenuates hyperglycemia in diabetic retinopathy rodent model. A) Body weight B) Feed intake C) Glycated Haemoglobin D) Blood glucose level of the control and experimental groups. Data were represented as Mean $\pm$ SD value ( $n=6$ ). A  $p$ -value of  $\leq 0.05$  was considered statistically significant. \*Normal control vs Diabetic control, ## Diabetic control vs  $\alpha$ -Pinene, Metformin treated groups.



**Figure 2:** Monoterpene  $\alpha$ -Pinene attenuates hypocholesteremia in diabetic retinopathy rodent model. A) Total cholesterol, B) Triglycerides, C) High Density Lipoprotein D) Low Density Lipoprotein E) Atherogenic index of the control and experimental groups. Data were represented as Mean $\pm$ SD value ( $n=6$ ). A  $p$ -value of  $\leq 0.05$  was considered statistically significant. \*Normal control vs Diabetic control, ## Diabetic control vs  $\alpha$ -Pinene, Metformin treated groups.

metformin treated rats than the diabetic untreated rats. Control rats shown significantly reduced level of atherogenic index.

### Monoterpene $\alpha$ -Pinene scavenged oxidative insult in diabetic retinopathy rodent model

Figure 3 illustrates the efficacy of  $\alpha$ -Pinene scavenging oxidative insult triggered by increased glucose intensities in diabetic retinopathy rodent model. The diabetic rats exhibited increased Malondialdehyde (MDA) and Decreased Antioxidant Superoxide Dismutase (SOD) and Catalase (CAT) levels.  $\alpha$ -Pinene

administration increased the antioxidants SOD, CAT thereby scavenged free radicals and signified decreased lipid peroxidation.

The glutathione system plays a multifaceted role in combating oxidative stress, regulating inflammation, supporting cellular repair, and maintaining vascular health in the retina. Treatment with  $\alpha$ -Pinene increased Glutathione Peroxidase (GPx) and Glutathione Reductase (GR) activity there decreased oxidation of glutathione and increased the glutathione reduction in diabetic rats. The GSH/GSSG was significantly reduced in the diabetic

untreated rats whereas treatment with  $\alpha$ -Pinene and metformin increased the ratio between GSH and GSSG.

### Monoterpene $\alpha$ -Pinene ameliorated inflammatory response in diabetic retinopathy rodent model

Figure 4 illustrates impact of  $\alpha$ -Pinene on cytokines involved in inflammatory response induced due to hyperglycemia in diabetic retinopathy rodent model.  $\alpha$ -Pinene treatment attenuated the inflammatory response in diabetic retinopathy rodent model. NF- $\kappa$ B, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were decreased with both  $\alpha$ -Pinene and metformin treatment in diabetic rats. Diabetic untreated rats exhibited significantly increased levels of interleukins 6 and 1 $\beta$ . The transcription factor NF- $\kappa$ B and TNF- $\alpha$  were also elevated in the diabetic untreated group than the other group experimental rats.

### Monoterpene $\alpha$ -Pinene alleviated progression of retinopathy in diabetic retinopathy rodent model

Figure 5 depicts  $\alpha$ -Pinene potency on attenuating initiation and advancement of diabetic retinopathy. Diabetic untreated rats had shown enhanced VEGF expression, a key regulator of angiogenesis in the retina and MMP-9 which involved in the remodeling of the extracellular matrix and the regulation of angiogenesis in retina. The levels of MCP-1, a chemokine that recruits monocytes to sites of inflammation and is involved in immune response in retina of

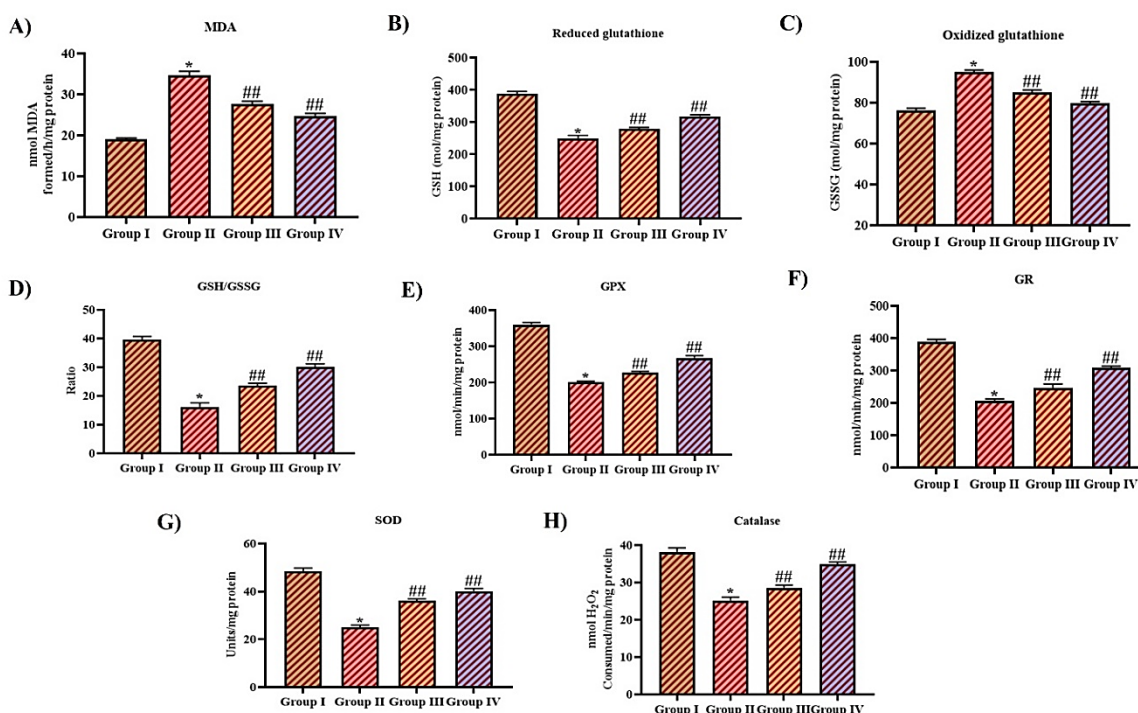
diabetic rats were also increased in the diabetic untreated rats.  $\alpha$ -Pinene treatment had decreased VEGF, MMP-9 and MCP-1 in diabetic rats.

### Monoterpene $\alpha$ -Pinene mitigated retinal inflammation in diabetic retinopathy rodent model

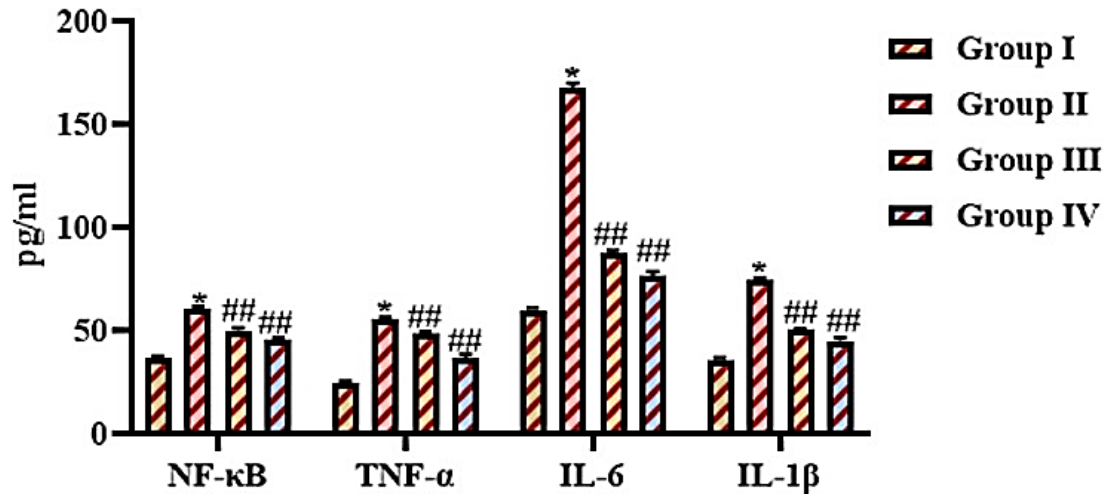
Figure 6 illustrates the impact of  $\alpha$ -Pinene treatment on the retinal thickness and ganglion cell layer count in the retinal tissue of diabetic retinopathy rodent model. Diabetic induction had decreased the retinal thickness and the GCL count when compared with control rats.  $\alpha$ -Pinene and metformin treatments increased the retinal thickness and the GCL count in comparison to the diabetic rats.

### Monoterpene $\alpha$ -Pinene attenuated apoptotic protein in diabetic retinopathy rodent model

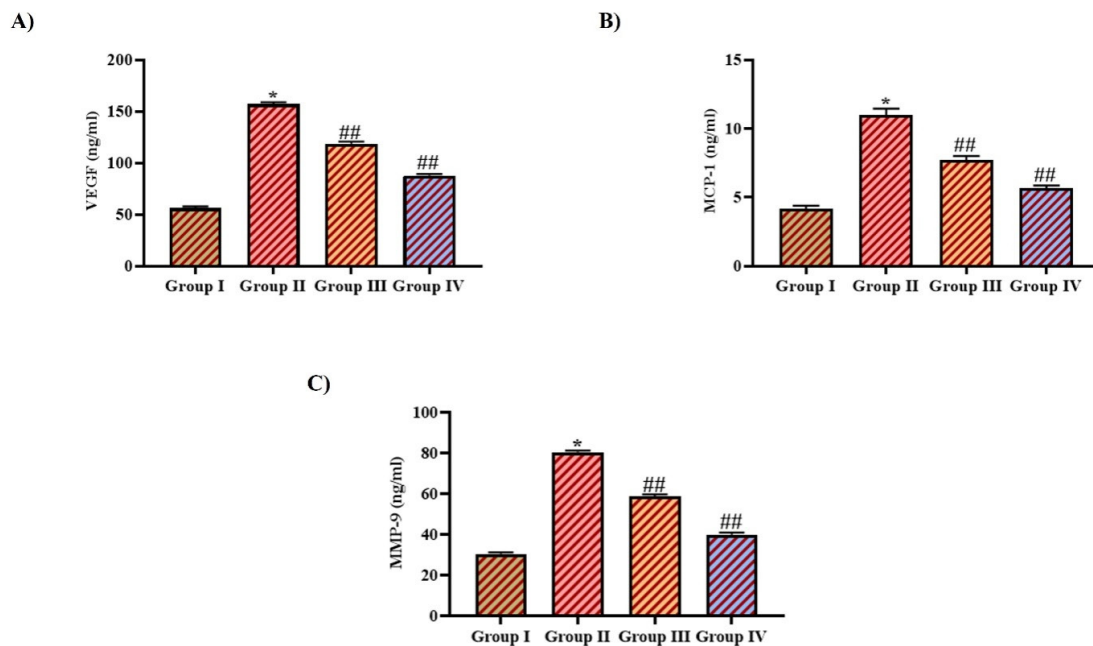
Figure 7 exemplifies the apoptotic protein levels in the control and the experimental rats. Diabetic induction increased the levels of apoptotic protein BAX and decreased the antiapoptotic protein BCL2 level. The BAX/BCL2 ratio was also increased in the diabetic rats.  $\alpha$ -Pinene and metformin treatments decreased the BAX protein level and increased the level of BCL2 protein. Furthermore, the BAX/BCL2 ratio also decreased in both  $\alpha$ -Pinene and metformin-treated rats.



**Figure 3:** Monoterpene  $\alpha$ -Pinene scavenged oxidative insult in diabetic retinopathy rodent model. A) Malondialdehyde, B) Reduced glutathione, C) Oxidised glutathione, D) GSH/GSSG, E) Glutathione Peroxidase, F) Glutathione reductase, G) Superoxide Dismutase, H) Catalase levels of the control and experimental groups. Data were represented as Mean $\pm$ SD value ( $n=6$ ). A  $p$ -value of  $\leq 0.05$  was considered statistically significant. \*Normal control vs Diabetic control, ## Diabetic control vs  $\alpha$ -Pinene, Metformin treated groups.



**Figure 4:** Monoterpene  $\alpha$ -Pinene ameliorated inflammatory response in diabetic retinopathy rodent model. Nuclear Factor Kappa B, Tumor Necrosis Factor Alpha, Interleukin 6, Interleukin 1 beta levels of the control and experimental groups. Data were represented as Mean $\pm$ SD value ( $n=6$ ). A  $p$ -value of  $\leq 0.05$  was considered statistically significant. \*Normal control vs Diabetic control, ## Diabetic control vs  $\alpha$ -Pinene, Metformin treated groups.



**Figure 5:** Monoterpene  $\alpha$ -Pinene alleviated progression of retinopathy in diabetic retinopathy rodent model. A) Vascular endothelial growth factor, B) Matrix metalloproteinase-9, C) Monocyte chemoattractant protein-1 levels of the control and experimental groups. Data were represented as Mean $\pm$ SD value ( $n=6$ ). A  $p$ -value of  $\leq 0.05$  was considered statistically significant. \*Normal control vs Diabetic control, ## Diabetic control vs  $\alpha$ -Pinene, Metformin treated groups.

## DISCUSSION

Diabetic triggered retinal disorder is a prominent microvascular disease associated with diabetes. Persistent, uncontrolled blood sugar levels lead to impairment of retinal capillary endothelial cells.<sup>33</sup> Key factors contributing to Diabetic triggered retinal disorder include modifiable risks, such as poor glycemic control, obesity, dyslipidemia, hypertension, and smoking, as well as non-modifiable risks, including age, genetic predisposition,

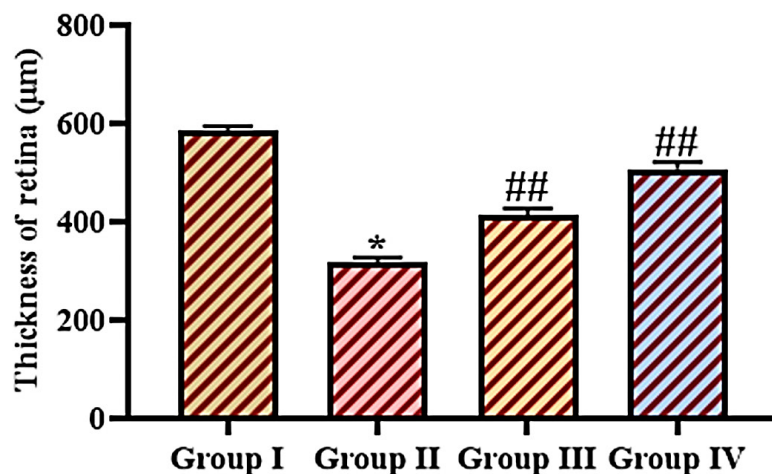
family history, type of diabetes, and duration of the condition. Additional contributing factors include oxidative stress, inflammation, and underlying metabolic disorders.<sup>15</sup> The progression of Diabetic Retinopathy (DR) typically begins with Non-Proliferative Diabetic Retinopathy (NPDR) and advances to Proliferative Diabetic Retinopathy (PDR). NPDR is marked by abnormalities in the retinal blood vessels, including increased vascular permeability and capillary blockage.<sup>34</sup> The resulting ischemia from these vascular changes promotes the onset of

angiogenesis, which is a defining feature of PDR. Abnormal blood vessel growth and subsequent bleeding can lead to DME.<sup>35</sup>

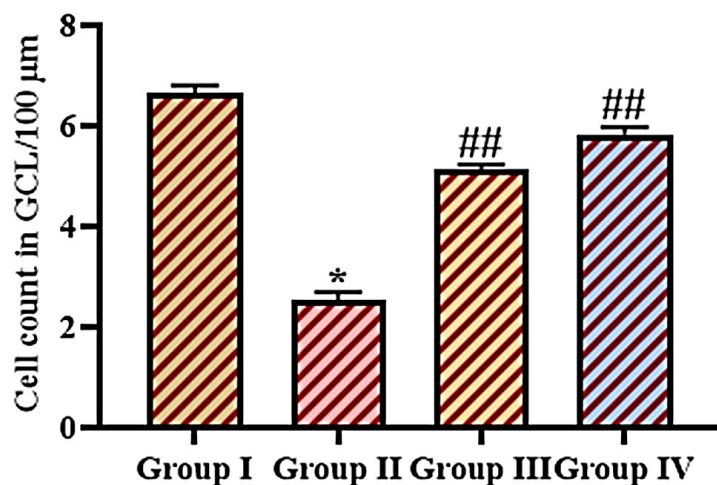
DR initiation is an intricate and multifaceted mechanism, with hyperglycemia triggers onset of microvascular impairment. Elevated blood glucose levels, resulting from inadequate insulin action, lead to non-enzymatic glycation and the production of AGEs.<sup>36,37</sup> Chronic hyperglycemia, is a primary driver of DR, with research showing that both the period of diabetes and glycemic control, as indicated by HbA1c, are critical risk factors.<sup>33</sup> Therefore regulating the hyperglycemic levels is the prime target for preventing and treating diabetic retinopathy. In our analysis treatment with  $\alpha$ -Pinene had effectively reduced the blood glucose and HbA1C levels in diabetic rats proving its anti-hyperglycemic effect. It also regulated the weight gain and feeding intake in diabetic rats.

Dyslipidemias, including elevated cholesterol and triglycerides levels, are prevalent concurrent diseases in individuals with type 2 diabetes and increase microvascular complications in type 2 diabetes.<sup>38</sup> Studies indicate a strong association between elevated triglyceride and cholesterol levels and the onset of DR in patients with type 2 diabetes mellitus, suggesting that reducing serum lipids may benefit these patients.<sup>39</sup> Individuals with DR typically had increased Total Cholesterol (TC), Triglycerides (TG), and Low-Density Lipoprotein Cholesterol (LDL-C) than to newly diagnosed diabetes patients without DR.<sup>40</sup> Researches had proven lipid lowering drugs may provide an effective remedy for DR therefore we analysed the lipid lowering potency of  $\alpha$ -Pinene in diabetic rats.  $\alpha$ -Pinene had effectively regulated the dyslipidemic condition in diabetic rats.

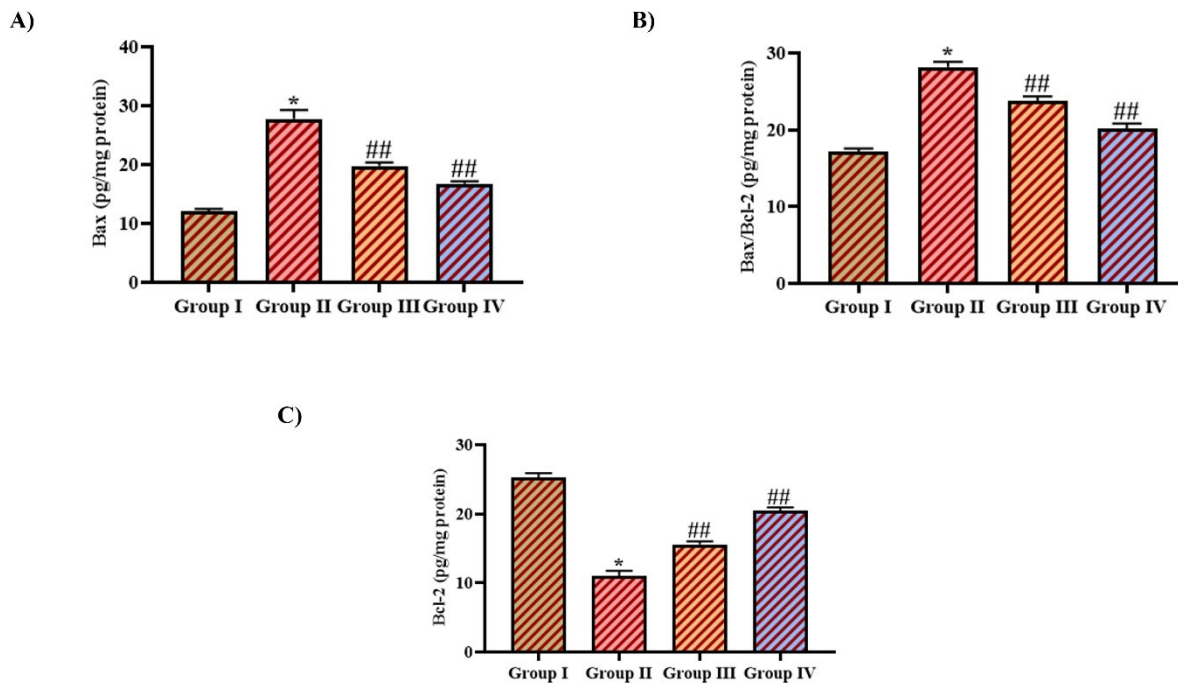
A)



B)



**Figure 6:** Monoterpene  $\alpha$ -Pinene mitigated retinal inflammation in diabetic retinopathy rodent model. A) Retinal thickness B) Ganglion cell count of the control and experimental groups. Data were represented as Mean $\pm$ SD value ( $n=6$ ). A  $p$ -value of  $\leq 0.05$  was considered statistically significant. \*Normal control vs Diabetic control, ## Diabetic control vs  $\alpha$ -Pinene, Metformin treated groups.



**Figure 7:** Monoterpene  $\alpha$ -Pinene attenuated apoptotic protein in diabetic retinopathy rodent model. A) Bcl-2-Associated protein X (BAX), B) B-cell lymphoma 2 (Bcl-2) protein levels of the control and experimental groups. Data were represented as Mean $\pm$ SD value ( $n=6$ ). A  $p$ -value of  $\leq 0.05$  was considered statistically significant. \*Normal control vs Diabetic control, ## Diabetic control vs  $\alpha$ -Pinene, Metformin treated groups.

High glucose levels result in non-enzymatic glycosylation, leading to the formation of AGEs. These substances play a significant role in secondary complications by increasing intracellular reactive oxygen species, which create oxidative stress and harm retinal cells.<sup>41</sup> Furthermore, the buildup of AGEs suppresses the production of pigment epithelium-derived factor, a protective agent for retinal microvascular endothelial cells that also helps regulate inflammation. The reduced PEDF levels activate the NADPH oxidase enzyme complex and the nuclear factor-kappa B pathway, intensifying inflammation and cell damage.<sup>42,43</sup> Chronic hyperglycemia worsens oxidative stress and stimulates various signaling pathways, increasing inflammatory triggering cytokines such as MCP-1, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in the retina.<sup>44,45</sup> These inflammatory mechanisms promote attachment and penetration of leukocytes, leading to cell disruption, capillary blockage, and further release of inflammatory mediators.<sup>46</sup> The impact of inflammation in the progression of DR is evidenced by the effectiveness of corticosteroids in managing DME and DR. However, there is a growing demand for new inflammatory attenuating treatments with lesser side effects than corticosteroids.<sup>47</sup> Although several therapies targeting inflammatory pathways have undergone clinical evaluation, none have been approved for treating DR or DME.<sup>48</sup>

Previous researches had proven  $\alpha$ -Pinene has been shown to enhance antioxidant enzyme activity and reduced the inflammatory factors in the focal ischemia induced brain.<sup>49</sup> It exhibited anti-inflammatory properties by suppressing the MAPK and NF- $\kappa$ B pathways in mouse peritoneal macrophages.<sup>50</sup>

Additionally, alpha-pinene demonstrates pain relieving and free radical scavenging activities.<sup>51,52</sup>  $\alpha$ -Pinene treatment had effectively increased the antioxidant status scavenged oxidative stress thereby attenuated inflammatory mediators NF $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in diabetic rats.

VEGF is critical for the advancement of the condition of PDR and DME by increasing the permeability of retinal capillaries. It achieves this by enhancing the tight junction protein phosphorylation.<sup>53</sup> VEGF activation triggers a cascade that stimulates the mitogen-activated protein pathway, promoting endothelial cells surge. This process is further influenced by the PI3K/Akt pathway following the stimulation of VEGF receptor-2.<sup>54,55</sup> Intravitreal anti-VEGF therapies have appeared as a treatment option for DME. Conversely, the invasive nature of these procedures poses risks, including endophthalmitis and retinal detachment.<sup>56</sup> This is particularly concerning for diabetic patients who often require long-term treatment. Moreover, aside from local side effects, the anti-VEGF agent's usage can cause systemic complications.<sup>57,58</sup>  $\alpha$ -Pinene treatment in diabetic rats reduced VEGF and MMP-9 levels confirming its ameliorative potency against diabetic retinopathy. The decrease in VEGF and MMP-9 levels may be due to the antioxidant, anti-inflammatory properties of  $\alpha$ -Pinene.

Various mechanisms driven by inflammatory mediators, oxidative stress, mitochondrial dysfunction, and enhanced pathways resulting from chronic hyperglycemia result elevated pro-apoptotic signaling over survival signaling.<sup>59</sup> This imbalance is particularly evident in retinal ganglion cells and amacrine

cells, which are among the first neural elements in the retina to undergo apoptosis due to diabetes.<sup>60</sup> The resulting apoptotic processes contribute to a thinning of the inner retinal layers.<sup>61,62</sup> The retinal thickness in diabetic rats were increased with  $\alpha$ -Pinene and it also increased the ganglion cell count. This may be due to the anti-apoptotic effect of  $\alpha$ -Pinene which significantly increased the anti-apoptotic protein BCL2 levels and attenuated the apoptotic protein BAX thereby prevent neuronal apoptosis in retina of diabetic rats.

## CONCLUSION

In summary, this study highlights the efficacy of  $\alpha$ -Pinene as a multifaceted compound in combating hyperglycemia-induced diabetic retinopathy in a rodent model. Our findings indicate that  $\alpha$ -Pinene exhibits powerful antioxidant, anti-diabetic, anti-hypercholesterolemic, anti-inflammatory, and anti-apoptotic properties, contributing to a significant reduction in both the development and progression of diabetic retinopathy. The positive effects observed in retinal thickness and ganglion cell counts, along with supporting histopathological analysis, underscore the potential of  $\alpha$ -Pinene as a therapeutic agent in managing this prevalent condition. Consequently,  $\alpha$ -Pinene appears to be a strong candidate for further research and development to alleviate the effects of diabetic retinopathy and enhance the quality of life for those suffering from this challenging condition.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**DR:** Diabetic retinopathy; **DM:** Diabetes mellitus; **GBD:** Global Burden of Disease; **IDF:** International Diabetes Federation; **AGE:** Advanced Glycation End-products; **VEGF:** Vascular Endothelial Growth Factor; **DME:** Diabetic Macular Edema; **USFDA:** United States Food and Drug Administration; **DRCRN:** Diabetic Retinopathy Clinical Research Network; **STZ:** Streptozotocin; **MDA:** Malondialdehyde; **SOD:** Superoxide Dismutase; **CAT:** Catalase; **GSH:** Reduced Glutathione; **GSSG:** Oxidized glutathione; **GPx:** Glutathione Peroxidase; **GR:** Glutathione Reductase; **NF- $\kappa$ B:** Nuclear Factor Kappa B; **TNF- $\alpha$ :** Tumor Necrosis Factor Alpha; **IL-6:** Interleukin 6; **IL-1 $\beta$ :** Interleukin 1 $\beta$ ; **MMP-9:** Matrix metalloproteinase-9; **MCP-1:** Monocyte chemoattractant Protein-1; **BAX:** Bcl-2-Associated X Protein; **BCL2:** B-Cell Lymphoma Protein 2; **NPDR:** Non-Proliferative Diabetic Retinopathy; **PDR:** Proliferative Diabetic Retinopathy; **PKC:** Protein Kinase C; **ROS:** Reactive Oxygen Species; **PEDF:**

Pigment Epithelium-Derived Factor; **MAPK:** Mitogen-Activated Protein Kinases; **PI3K:** Phosphatidylinositol 3-Kinase.

## ETHICAL STATEMENT

This work was approved by the IEAC OF Xi'an Aier Ancient City Eye Hospital, Xi'an 710014, Shaanxi Province, China.

## SUMMARY

Diabetic Retinopathy (DR) has become a pressing global health challenge that demands immediate and concerted efforts to address.  $\alpha$ -Pinene is a bicyclic monoterpene commonly found in essential oils of coniferous trees, particularly in pine trees.  $\alpha$ -Pinene is a powerful antioxidant with significant anti-diabetic, anti-hypercholesterolemic, anti-inflammatory, and anti-apoptotic effects in a rodent model of diabetic retinopathy.

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