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Original Article

Cardamonin Lowers Intraocular Pressure and Inhibits Glaucoma Development in Steroid-Induced Rats by Activation of Nrf-2/HO-1 Pathway

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ABSTRACT

Background: Glaucoma, a progressive optic neuropathy characterized by optic nerve injury, is a pivotal cause of vision loss globally. **Objectives:** The current work focuses on assessing the therapeutic role of cardamonin against experimentally-induced glaucoma in rats. **Materials and Methods:** The experimental glaucoma in rats was induced by betamethasone, and then cardamonin was treated at 25 and 50 mg/kg concentrations. The Intraocular Pressure (IOP) level was assessed on a weekly basis. The glutamate and glutathione levels and the oxidative stress biomarker levels, including 8-Hydroxydeoxyguanosine (8-OH-dG), 4-Hydroxynonenal (4-HNE), and Malondialdehyde (MDA), were investigated using the commercial kits. The inflammatory markers and Nuclear factor erythroid 2-related factor (Nrf2) transcription factor/Hemoxygenase 1 (HO-1) levels were assessed using the commercial kits. **Results:** The present findings of this study highlighted that cardamonin successfully reduced the IOP levels and consequently increased the RGCs level in the glaucoma-induced rats. The inflammatory cytokines and oxidative biomarkers (MDA, 8-OH-dG, and 4-HNE) levels were successfully reduced by the cardamonin treatment in the glaucoma-induced rats. Furthermore, the cardamonin effectively activated the Nrf-2/HO-1 cascade in the glaucoma-induced rats, thereby mitigating the progression of glaucoma development. **Conclusion:** The current findings showed that cardamonin treatment successfully reduced the development of steroid-induced glaucoma in rats. The findings of this work highlight that cardamonin could be a potentially effective option for the management of glaucoma.

Keywords: Glaucoma, Retinal Ganglial Cells, Nrf-2/HO-1 Pathway, Inflammation, 4-Hydroxynonenal.

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INTRODUCTION

Glaucoma is a complex and multifaceted eye disorder characterized by optic nerve injury and vision loss, often connected with increased Intraocular Pressure (IOP). This neurodegenerative condition is the major cause of irreversible vision loss worldwide, affecting over 70 million individuals globally.[1] The primary pathological phenomenon of glaucoma is Retinal Ganglion Cell (RGC) degeneration. This degeneration leads to characteristic optic disc changes, known as cupping, and ultimately leads to visual field defects and blindness.[2] The exact mechanisms by which higher IOP leads to RG cell death and optic nerve injury are not fully elucidated. It was reported that mitochondrial dysfunction may play a central role in the progression of glaucoma.[3] Furthermore, aging, genetic factors, vascular dysregulation, oxidative stress, and other environmental and systemic factors have contributed to the development of glaucoma.[4]

The development of effective treatments for glaucoma has been hindered by the lack of reliable animal models that accurately replicate the complex pathophysiology of the disease. Researchers have long sought to establish an experimental model of glaucoma in rodents, particularly rats, as they provide a cost-effective and readily available platform for investigating the progression of the disease and evaluating potential therapies.[5] One promising approach to modeling glaucoma in rats involves the induction of increased IOP. This experimentally-induced glaucoma model has been widely utilized to investigate the degenerative cascade associated with glaucomatous optic neuropathy and to explore neuroprotective strategies that may rescue RGCs from secondary degeneration.[6] The use of experimentally-induced glaucoma in rats has offered valuable insights into the underlying

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pathophysiology of the disease and has enabled the evaluation of novel neuroprotective strategies.[7]

One of the primary treatments for glaucoma is the use of topical anti-glaucoma medications, which aim to reduce IOP, the primary cause of the development of glaucoma. Nonetheless, the long-term utilization of these medications can lead to several adverse effects, like drug intolerance, poor patient compliance, and the development of drug resistance, which can ultimately reduce the effectiveness of the treatment. Another challenge in the management of glaucoma is the effect of the condition on quality of life. Glaucoma-related vision impairment can significantly affect the patient's performance and increase the risk of depression and other mental health issues.[8] To effectively combat glaucoma and prevent vision loss, a multifaceted approach is required, involving the strengthening of healthcare systems, the development of innovative treatment options, and the implementation of comprehensive patient-centered care strategies.

One potential approach to the management of glaucoma is the use of plant-derived bioactive compounds. Plant-based medicines have garnered increasing interest in the field of ophthalmology due to their potential for providing neuroprotective, anti-inflammatory, and IOP-lowering effects.[9] Ongoing research in this field may result in the exploration of novel, plant-based therapeutic interventions for the prevention and treatment of this sight-threatening condition. Cardamonin is a member of the chalcones, which are well known for their many biological activities, including antioxidant, cytotoxic, and antitumor effects. Cardamonin is a major bioactive compound present in significant amounts in the seeds of Alpinia katsumadai. It possesses various beneficial features, including antinociceptive (pain-relieving), anti-inflammatory, antiprotozoal (against protozoan parasites), antiulcer, antihistaminic (against histamine effects), anti-tumor, nephroprotective, and neuroprotective activities.[10-14] Whereas, its therapeutic roles against glaucoma have not been studied yet. Therefore, the current work focuses on assessing the therapeutic roles of cardamonin against experimentally-induced glaucoma in rats.

MATERIALS AND METHODS

Chemicals

The cardamonin, betamethasone, moxifloxacin, and other chemicals are attained from Sigma Aldrich, USA. All the test kits were procured from Elabscience, Abcam, and Cusabio, USA.

Experimental rats

The male Wistar rats were acquired from the institutional animal facility. The rats, weighing between 180-210 g, were kept in controlled environments with typical atmospheric conditions. These circumstances included 12 hr of light and dark sequence at a temperature of 25±0.5 °C and a humidity of 60±5%. The animals were provided unrestricted access to clean water and a meal specifically formulated for rodents.

Experimental glaucoma model in rats

One-week-acclimated rats were categorized into five groups, each having six rats: a normal group, a glaucoma-induced group, a glaucoma+25 mg/kg of cardamonin-treated group, a glaucoma+50 mg/kg of cardamonin-treated group, and a glaucoma+5 mg/kg of acetazolamide (the standard drug)-treated group. All the rats except the normal group were administered betamethasone (10 mg/mL, 0.03 mL) through the subconjunctival route to generate high IOP. The IOP levels were evaluated using an AccuPen portable tonometer. To avoid ocular infection, 1-2 drops of a 0.15% moxifloxacin hydrochloride antibiotic solution were supplied after the injection of steroids. After approximately 7 days, the increased IOP fluctuated between 30 and 40 mmHg in the experimental Sprague-Dawley rats, which suggests the presence of glaucoma. The experimental groups received cardamonin through oral administration using a gastric gavage method. The IOP level was thereafter assessed on a weekly basis. After four weeks of completing the treatments, all the rats were sacrificed, and eye tissue samples were then gathered and utilized for further assessments. The total number of RGCs in the retina of the experimental rats was investigated using Image Pro Plus (Media Cybernetics, USA) software. The IOP changes (percentage) were assessed using the formula:

$$\text{Changes in IOP } (\%) = (\text{IOPt-IOP0}/\text{IOP0}) \times 100$$

Where: IOPt: the ocular tension at various time periods after steroid (betamethasone) treatment; IOP0: the ocular tension before steroid (betamethasone) administration (time zero).

Analysis of glutamate levels

The glutamate level in the vitreous humor was measured using a commercially available assay kit (Abcam, USA). In summary, the sterile tube was used to collect vitreous humor, and the vitreous bodies were subjected to sonication using perchloric acid (0.2 M). First, the homogenate was gathered and subjected to centrifugation at 15,000 g under cold conditions for 5 min. Subsequently, the liquid portion was gathered to estimate the concentration of glutamate. Assays were executed as per the guidelines given in the kit and then transferred using a pipette into a 96-well plate, and absorbance was taken at 405 nm. This value was recorded as the final absorbance.

Analysis of glutathione levels

The concentration of glutathione in the aqueous humor was measured using a commercially available assay kit (Abcam, USA) following the provided instructions. The anterior chamber was penetrated with a 30-gauge needle following the euthanization of the rats. The collected aqueous humor from all the eyes and placed it in a sterile tube. Assays were performed as per the

guidelines given with the kit. The final absorbance was measured at a wavelength of 405 nm using a microplate reader.

Analysis of oxidative stress markers

The 8-Hydroxydeoxyguanosine (8-OH-dG), 4-Hydroxynonenal (4-HNE), and Malondialdehyde (MDA) levels in the retinal tissue samples of the rats were investigated using commercial kits (Elabscience, USA).

Analysis of inflammatory cytokine levels

The inflammatory cytokines, including NF-κB, IL-1β, IL-10, and IL-6 levels, in the retinal tissues of both the control and treatment rats were quantified using commercial kits. The measurements were conducted as per the instructions given by the manufacturer (Abcam, USA).

Analysis of Nrf2/HO-1 levels

The Nrf2 and HO-1 levels in the retinal tissues of the rats were quantified using commercial kits. The experiments were executed as per the instructions provided by the manufacturer (Cusabio, USA).

Statistical analysis

The values were studied using the SPSS program. The data are represented as the mean±SD of three repeated assays. The one-way ANOVA and Tukey's *post hoc* analysis were performed to measure the variations between groups, with $p<0.05$ as significant.

RESULTS

Effects of cardamonin on the IOP level in the experimental rats

The impact of cardaminin on the IOP levels in the glaucoma-induced rats is illustrated in Figure 1. The findings demonstrated that the rats treated with steroids exhibited a significant rise in IOP following the administration of steroids, in comparison to the normal group. This indicates the successful development of glaucoma in the rats. The IOP levels following the oral administration of cardamonin indicated a substantial decrease in the high IOP level caused by steroids. Significantly, the IOP levels returned to their normal level in the cardamonin-treated group.

Effect of cardamonin on the glutathione and glutamate levels

Figure 2 illustrates the levels of glutathione and glutamate in both control and treatment rats. The rats with glaucoma experienced a significant increase in glutamate in their vitreous humor and a decrease in glutathione levels in their aqueous humor in comparison to the control. The cardamonin (25 and 50 mg/kg) treatment revealed a considerable decrease in glutamate and an increase in glutathione levels. The standard drug acetazolamide administration also led to a reduction in the glutamate level and an elevation in the glutathione levels in rats with glaucoma.

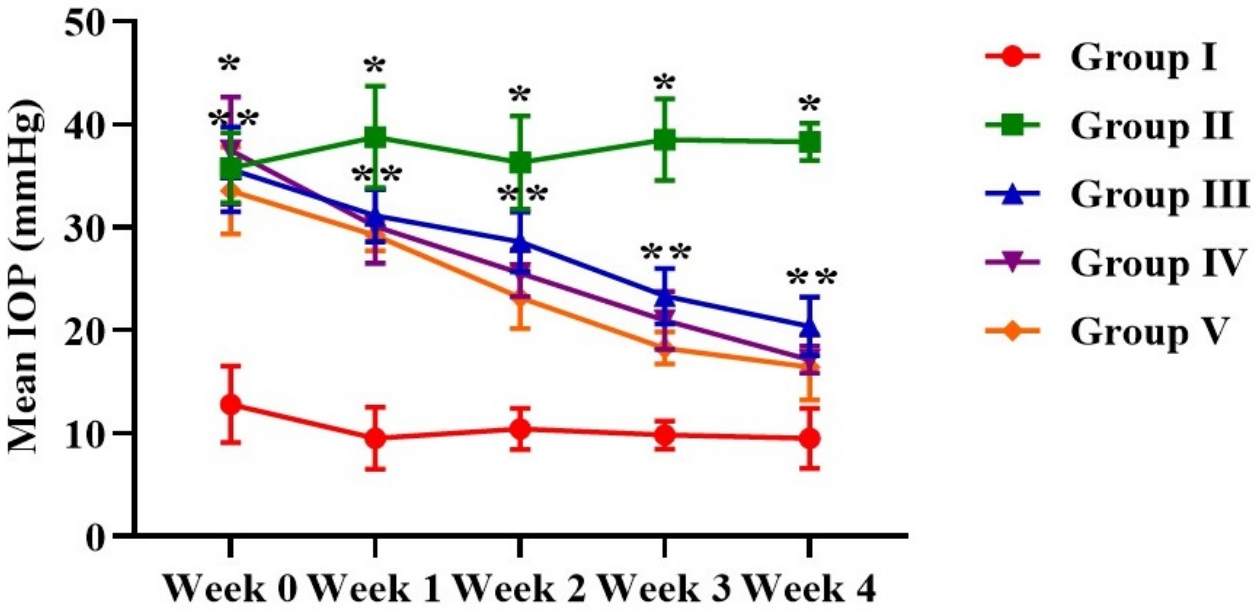

Figure 1: Effects of cardamonin on the IOP level in the experimental rats.

The values are presented as the mean±SD of three repeated experiments. A one-way ANOVA and Tukey's *post hoc* analysis were performed to measure the differences between groups using SPSS software. Note: '*' reveals that data are significantly differ at $p<0.01$ from the control group; '**' reveals that data are significantly differ at $p<0.05$ from glaucoma-induced group.

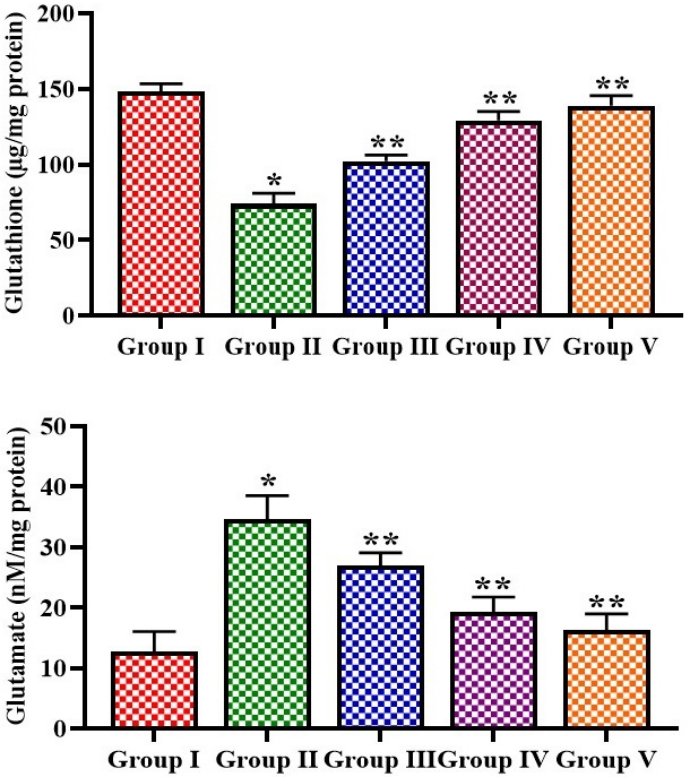

Figure 2: Effect of cardamonin on the glutathione and glutamate levels in the experimental rats.

The values are presented as the mean±SD of three repeated experiments. A one-way ANOVA and Tukey's *post hoc* analysis were performed to measure the differences between groups using SPSS software. Note: '*' reveals that data are significantly differ at $p<0.01$ from the control group; '**' reveals that data are significantly differ at $p<0.05$ from glaucoma-induced group.

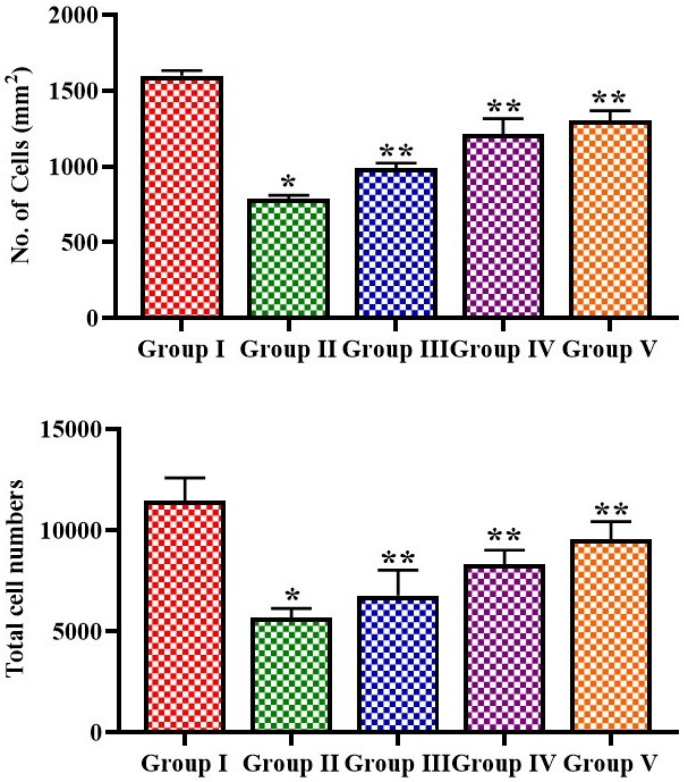

Figure 3: Effect of cardamonin on the RGCs level in the retinal tissues of the experimental rats.

The values are presented as the mean±SD of three repeated experiments. A one-way ANOVA and Tukey's *post hoc* analysis were performed to measure the differences between groups using SPSS software. Note: '*' reveals that data are significantly differ at $p<0.01$ from the control group; '**' reveals that data are significantly differ at $p<0.05$ from glaucoma-induced group..

Effect of cardamonin on the RGCs level in the retinal tissues

Figure 3 shows the RGCs level in the retina of the experimental rats. The rats with glaucoma exhibited reduced levels of RGCs when compared to the control. Notably, the cardamonin at 25 and 50 mg/kg concentrations effectively elevated the levels of RGCs in the retina of rats with glaucoma. Comparable results were also observed in the rats treated with the standard drug acetazolamide, providing more evidence for the effectiveness of cardamonin.

Effect of cardamonin on the oxidative stress biomarker levels

Figure 4 presents the effect of cardamonin on the MDA, 8-OH-dG, and 4-HNE levels in the retina of the experimental rats. The rats with glaucoma showed a significant elevation in the MDA, 8-OH-dG, and 4-HNE levels in their retina. Remarkably, the 25 and 50 mg/kg of cardamonin resulted in a considerable reduction in these marker levels. Furthermore, the treatment of acetazolamide also reduced these biomarker levels in the rats with glaucoma (Figure 4).

Effect of cardamonin on the inflammatory cytokines

The glaucoma-induced rats exhibited a considerable elevation in NF-κB, IL-1β, and IL-6, whereas IL-10 was diminished. In contrast, the treatment of 25 and 50 mg/kg of cardamonin significantly reduced the NF-κB, IL-1β, and IL-6 levels in the glaucoma rats, while simultaneously boosting the level of IL-10 (Figure 5). Furthermore, acetazolamide effectively modulated these cytokine levels in the rats with glaucoma, thus emphasizing the anti-inflammatory activities of cardamonin.

Effect of cardamonin on the Nrf-2/HO-1 pathway

The effect of cardamonin on the Nrf-2/HO-1 signaling protein expressions was analyzed, and the results are presented in Figure 6. Nrf-2 and HO-1 expressions were drastically diminished in the retinal tissues of the glaucoma-induced rats. Whereas, the treatment of 25 and 50 mg/kg of cardamonin leads to a considerable elevation in the Nrf-2 and HO-1 levels in the retina of the glaucoma-induced rats. Furthermore, the treatment with acetazolamide effectively elevated the Nrf-2/HO-1 expression levels in the glaucoma rats.

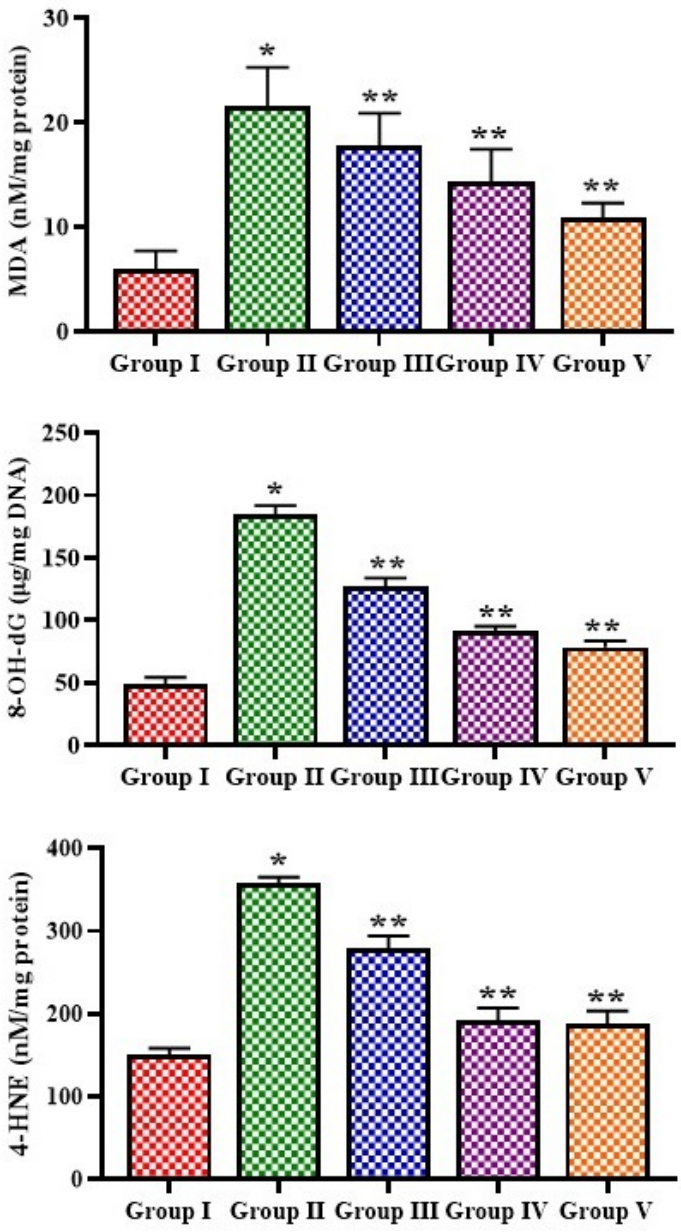

Figure 4: Effect of cardamonin on the oxidative stress biomarker levels in the experimental rats.

The values are presented as the mean±SD of three repeated experiments. A one-way ANOVA and Tukey's *post hoc* analysis were performed to measure the differences between groups using SPSS software. Note: '*' reveals that data are significantly differ at $p<0.01$ from the control group; '**' reveals that data are significantly differ at $p<0.05$ from glaucoma-induced group.

DISCUSSION

The prevalence of glaucoma is significant, with over 60 million people affected worldwide, and the global burden is predicted to increase with the aging population. Despite ongoing research and advancements in medical interventions, the management of glaucoma remains a significant challenge, with various treatment options and their associated limitations.[15] The glaucoma pathophysiology is not fully described, but it is thought to involve several causes, including increased IOP, optic nerve injury, and progressive RGC loss. The biological basis for this pressure-induced damage is not entirely clear, but it is thought to involve numerous mechanisms, including mechanical stress on the optic nerve, impaired blood flow, and inflammatory processes. Degeneration of the RGCs is a hallmark of glaucomatous damage. This degeneration results in characteristic changes to the optic disc, known as cupping, and ultimately leads to visual field loss.[16]

Understanding the association between IOP and the progression of glaucoma is crucial for effective management of this debilitating disease. IOP is a key factor in glaucoma development, and lowering IOP is the primary therapeutic approach for managing the condition. Numerous studies have demonstrated a strong connection between augmented IOP and the development of visual complications, which are a hallmark of glaucomatous disease. Furthermore, the connection between IOP and visual field loss is complicated, as some patients may experience significant visual impairment despite relatively normal IOP, while others may have augmented IOP. The analysis of IOP is a critical component of glaucoma management, but it must be considered in the context of a comprehensive clinical assessment, including evaluation of the optic nerve and visual fields.[17] The outcomes of this work highlighted that the glaucoma-induced rats exhibited a considerable elevation in IOP. Interestingly, the IOP levels following the treatment of cardamonin demonstrated a substantial decrease in IOP levels in the glaucoma-induced rats.

In addition to the pressure-dependent mechanisms, there is growing evidence that pressure-independent mechanisms may also participate in glaucoma progression. These pressure-independent mechanisms include oxidative stress, inflammation, and impaired neurotrophic support, all of which can result in the degeneration of RGCs and optic nerve fibers.[18] Understanding the complex etiology of glaucoma is essential for the development of new therapeutic strategies. Recent efforts have focused on the development of neuroprotective approaches that aim to rescue RGCs from secondary degeneration and preserve visual function.[19,20] The loss of RGCs is a hallmark of glaucoma, and unveiling the underlying mechanisms of this degeneration is essential for developing potential therapeutic strategies. Moreover, researchers have also explored the potential of neuroprotective strategies to rescue the RGCs. This therapeutic approach, known as neuroprotection, aims to target the degenerative mechanisms involved in glaucomatous injury, with the ultimate goal of preserving visual function in patients with glaucoma.[21] The present work demonstrated a significant reduction in the RGCs in the retina of rats with glaucoma. Remarkably, the cardamonin successfully elevated the RGCs level in the retina of glaucoma rats.

The dysregulation of glutathione and glutamate homeostasis is one proposed mechanism that can contribute to the degeneration of RGCs.[22] Glutamate, a neurotransmitter essential for neuronal function, can become excitotoxic when present in excess. High

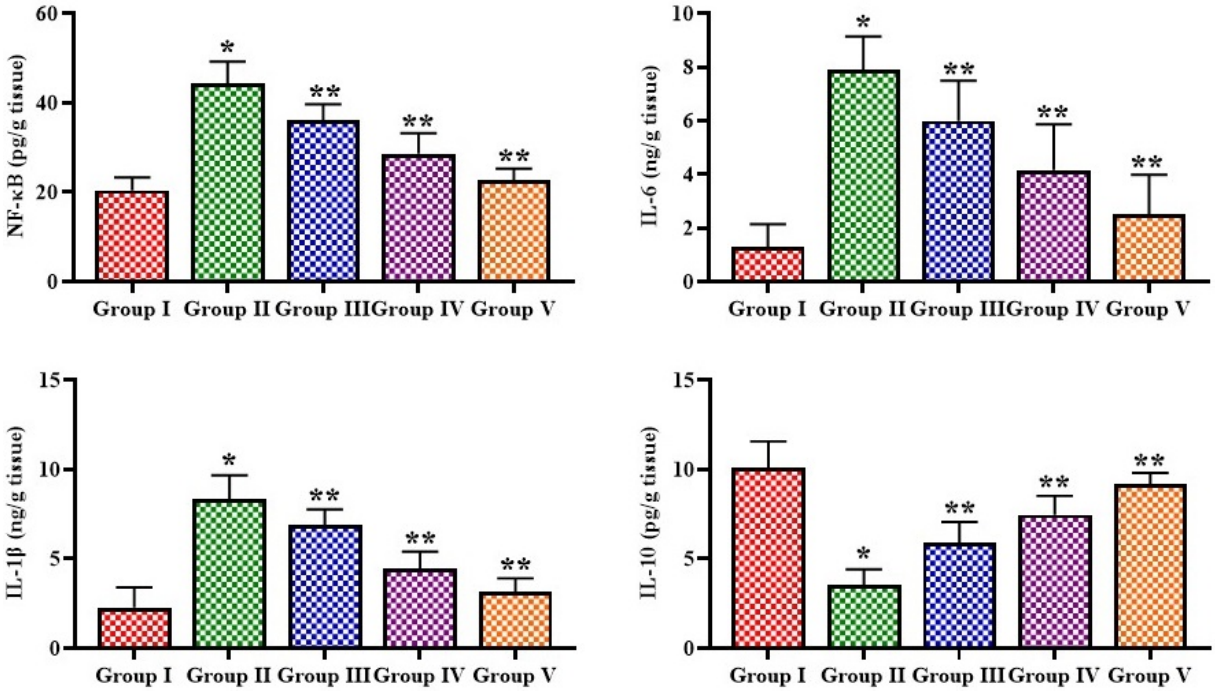

Figure 5: Effect of cardamonin on the inflammatory cytokine levels in the experimental rats.

The values are presented as the mean±SD of three repeated experiments. A one-way ANOVA and Tukey's *post hoc* analysis were performed to measure the differences between groups using SPSS software. Note: '*' reveals that data are significantly differ at $p<0.01$ from the control group; '**' reveals that data are significantly differ at $p<0.05$ from glaucoma-induced group.

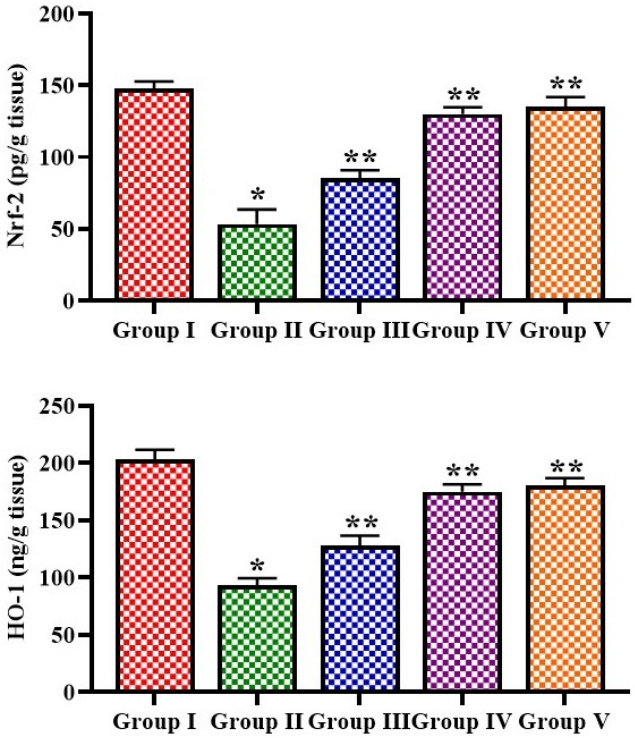

Figure 6: Effect of cardamonin on the Nrf-2/HO-1 signaling pathway in the experimental rats.

The values are presented as the mean±SD of three repeated experiments. A one-way ANOVA and Tukey's *post hoc* analysis were performed to measure the differences between groups using SPSS software. Note: '*' reveals that data are significantly differ at $p<0.01$ from the control group; '**' reveals that data are significantly differ at $p<0.05$ from glaucoma-induced group.

concentrations of glutamate can induce calcium overload and oxidative stress, thereby resulting in neuronal apoptosis. This excitotoxicity has contributed to the onset of numerous neurodegenerative ailments, including glaucoma.[23] Glutathione, a critical antioxidant, plays a central role in the cellular redox balance. Imbalances in glutathione homeostasis have been observed in glaucoma patients, with diminished glutathione levels. This disruption in glutathione levels can contribute to the oxidative stress and inflammation associated with glaucoma, further exacerbating the degeneration of RGCs.[24] Neuroprotective strategies that target the modulation of glutamate and glutathione homeostasis have been explored as potential therapeutic approaches for glaucoma. For example, compounds that can reduce excitotoxicity by modulating glutamate receptor activity or enhancing glutathione levels have shown promising results in preclinical studies.[25] The present findings exhibited that the rats with glaucoma demonstrated increased glutamate in their vitreous humor and a decrease in glutathione in their aqueous humor. Interestingly, the cardamonin treatment highlighted a significant diminution in the glutamate and an elevation in the glutathione levels.

Oxidative stress plays an essential role in the progression of glaucoma. MDA, 8-OH-dG, and 4-HNE are well-established markers of oxidative stress that have been involved in the pathophysiology of glaucoma. MDA, a byproduct of lipid peroxidation, is an extensively utilized marker of oxidative stress. Numerous studies have reported higher MDA levels in the aqueous humor, blood, and tissue samples of glaucoma patients, indicating increased oxidative damage in the eyes of these individuals.[26] Similarly, 8-OH-dG, a marker of DNA oxidation, was found to be elevated in the glaucoma patients. The accumulation of 8-OH-dG suggests that oxidative DNA damage is a key feature of glaucoma pathogenesis.[27] 4-HNE, another lipid peroxidation product, has also participated in the development of glaucoma. The increased 4-HNE level was observed in the retina of glaucoma patients, and this marker has been connected with the activation of inflammatory pathways, which can further exacerbate the disease process.[28] In addition to their role as biomarkers, these oxidative stress markers may also have therapeutic implications. Strategies aimed at reducing oxidative stress, either through pharmacological interventions or dietary modifications, have shown promise in inhibiting glaucoma development and maintaining visual function.[29] The present findings proved that the cardamonin treatment successfully reduced the levels of MDA, 8-OH-dG, and 4-HNE in the retinal tissues of the glaucoma rats, which proves its antioxidant properties.

Increasing evidence highlights that neuroinflammation plays an essential role in the progression of glaucoma. The stimulation of key inflammatory mediators, including NF-κB, IL-1β, IL-6, and IL-10, has been involved in the pathophysiology of glaucoma.[30,31] NF-κB controls the expression of various genes and participates in inflammation, apoptosis, and oxidative stress. In glaucoma, NF-κB is believed to be activated in response to numerous stimuli, including increased IOP, oxidative stress, and neuronal damage. When activated, NF-κB can trigger inflammatory cytokine expressions like IL-1β and IL-6, further propagating the inflammatory cascade.[32] IL-1β is a major cytokine that plays a central role in the pathogenesis of glaucoma. IL-1β is augmented in the vitreous fluid and retinal tissue of glaucoma patients, and its overexpression can lead to retinal cell apoptosis, vascular dysfunction, and neuroinflammation.[33] IL-6, another pro-inflammatory cytokine, has also been implicated in glaucoma pathogenesis.[34] In contrast, IL-10 is an anti-inflammatory marker that may have a protective role in glaucoma. IL-10 is thought to counteract the pro-inflammatory effects of pro-inflammatory cytokines, potentially mitigating the damaging consequences of neuroinflammation in the glaucomatous eye.[35,36] The current findings clearly proved that the cardamonin treatment significantly reduced the NF-κB, IL-1β, and IL-6 levels in the glaucoma rats while simultaneously increasing the IL-10 level, which indicates the anti-inflammatory properties of the cardamonin.

One potential target for neuroprotective therapies is the Nrf-2/HO-1 cascade, which plays an essential role in the body's antioxidant mechanisms. Nrf-2/HO-1 signaling is a key factor in the cell's response to oxidative stress and inflammation, which are two of the primary mechanisms of glaucoma development.[37] The Nrf-2/HO-1 pathway represents a promising therapeutic target for glaucoma, as it can modulate both oxidative stress and inflammation. Nrf-2 is a transcription factor that upregulates cytoprotective and antioxidant gene expressions, including HO-1. HO-1 is an enzyme that promotes the breakdown of heme, biliverdin, and free iron, all of which have antioxidant properties. By activating the Nrf-2/HO-1 signaling, it may be possible to counteract the deleterious effects of oxidative stress and inflammation in the optic nerve and retina, thereby slowing glaucoma development and preserving vision.[38] Emerging evidence suggests that compounds that can modulate the Nrf-2/HO-1 pathway may have therapeutic potential for glaucoma.[39-41] In this study, the present outcomes proved that the cardamonin treatment demonstrated a considerable increase in the Nrf-2 and HO-1 expressions in the retinal tissues of the glaucoma-induced rats. These outcomes highlighted that the cardamonin activated the Nrf-2/HO-1 signaling, thereby mitigating the glaucoma progression in the rats.

CONCLUSION

The findings of this work showed that cardamonin successfully reduced the development of steroid-induced glaucoma in rats, as demonstrated by the decrease in IOP levels, increase in RGC levels, decreased inflammatory and oxidative stress biomarkers, and Nrf-2/HO-1 signaling activation in the retina of

the glaucoma-induced rats. Overall, the induction of glaucoma in rats by steroids serves as a valuable method for assessing the therapeutic efficacy of cardamonin in the treatment of glaucoma. The findings of this work highlight that cardamonin could be a potentially effective option for the management of glaucoma and therefore should be further examined in clinical trials.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS APPROVAL

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

ABBREVIATIONS

IOP: Intraocular pressure; **8-OH-dG:** 8-Hydroxydeoxyguanosine; **4-HNE:** 4-Hydroxynonenal; **MDA:** Malondialdehyde; **Nrf2:** Nuclear factor erythroid 2-related factor; **HO-1**: Transcription factor/Hemoxygenase 1.

SUMMARY

Glaucoma is a complex and multifaceted eye disorder characterized by optic nerve injury and vision loss, often connected with increased intraocular pressure. The development of effective treatments for glaucoma has been hindered by the lack of reliable animal models that accurately replicate the complex pathophysiology of the disease. Cardamonin is a member of the chalcones, which are well known for their many biological activities, including antioxidant, cytotoxic, and antitumor effects. Cardamonin successfully reduced the IOP levels and consequently increased the RGCs level in the glaucoma-induced rats. The inflammatory cytokines and oxidative biomarkers (MDA, 8-OH-dG, and 4-HNE) levels were successfully reduced by the cardamonin treatment in the glaucoma-induced rats.

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