Tohoku J. Exp. Med., 2024, **263**, 185-193

Germacrone Protects against NF-κB-Mediated Inflammatory Signaling, Apoptosis, and Retinal Ganglion Cell Survival in a Rat Glaucoma Model

Yu Yuan[1] and Linhai Shao[2]

[1]Department of Ophthalmology, Hanzhong Central Hospital, Hanzhong, Shaanxi, China
[2]Department of Urology, Hanzhong Central Hospital, Hanzhong, Shaanxi, China

Retinal ischemia-reperfusion (I/R) is a pathological phenomenon that causes cellular destruction in several ocular disorders, so there is a need for novel possible neuroprotective drugs. Researchers have reported numerous neuroprotective effects of Germacrone (GM). Therefore, this study aimed to elucidate the underlying processes of GM that may contribute to glaucoma development. 40 healthy rats underwent retinal ischemia-reperfusion (I/R) damage. The animals were divided into control, I/R-induced, GM-1d, and GM-7d. After 7 days of I/R, mice were sacrificed and retinal tissue removed. An enzyme-linked immunosorbent assay (ELISA) was used to assess retinal Malondialdehyde (MDA) and 8-OHdG levels after oxidative injury. The Fluro-Gold (FG) labelling assay counted retinal ganglion cells (RGC) before and after labelling. DNA from retinal tissue RNA was amplified. Western blotting and real-time qRT-PCR were utilised to assess Bax, Casapses-3, Bcl-2, retinal NF-kB, and COX-2 expression. Retinal cell apoptotic mediator expression was measured by a TUNEL assay. Retinal I/R damage increases ganglion cell death. Long-term GM treatment (GM-7d) reduced NF-κB activation and raised COX-2 expression, which suggests antioxidant potential. TUNEL-positive apoptotic cells were reduced in long-term GM-treated rats. In GM-treated retinas, the Bax-Bcl-2 ratio was identical to the control group and significantly different from the I/R group. GM reduces I/R-induced retinal cell damage by inhibiting RG cell death. Seven days after GM therapy, histology showed retinal tissue loss. NF-κB signaling and intrinsic mitochondrial apoptosis are possible mechanisms that may be attenuated by GM and are attributed to a retinal protective effect.

Keywords: Germacrone; glaucoma; intraocular; retina
Tohoku J. Exp. Med., 2024 July, **263** (3), 185-193.
doi: 10.1620/tjem.2024.J028

Introduction

Induction of retinal ischemia/reperfusion (I/R) damage in experimental animals is a frequently used approach to replicate various vision-threatening diseases, including the pathogenic nature of acute glaucoma, hypertensive or diabetic retinopathy, and retinal vascular occlusion (Hsu et al. 2020). Glaucoma is characterized by a group of abnormalities that cause irreversible vision defects, primarily the excessive degeneration of retinal ganglion cells (RGCs) and their axons. The Progression of RGCs and their inability to regenerate or repair them can produce irreversible visual impairment (Vernazza et al. 2021). Glaucoma is the leading cause of visual impairment after cataracts, and it is estimated that more than eighty million individuals worldwide will be afflicted by glaucoma by 2020 (Allison et al. 2020).

Following ageing, certain abnormal increases in IOP are the most appropriate risk factors for inflammation, oxidative stress, gliosis, excitotoxicity, and ischemia (Sun et al. 2022). A transient increase in intraocular pressure (IOP) to promote retinal I/R damage is the most studied animal model for retinal-linked abnormalities. Interruption of retinal blood flow causes several metabolic changes that promote retinal cell damage via the formation of free radicals, thereby increasing the expression of inflammatory cytokines. Retinal I/R damage should ideally produce RGC degeneration and induce loss of the inner nuclear layer, resulting in extensive inner retinal cell apoptosis (Hsu et al.

Received January 20, 2024; revised and accepted April 21, 2024; J-STAGE Advance online publication May 2, 2024
Correspondence: Linhai Shao, Department of Urology, Hanzhong Central Hospital, No.557 Middle Section of Laodong West Road, Hantai District, Hanzhong, Shaanxi 723000, China.
e-mail: linhaishao3@gmail.com or 1352057334@qq.com

2020). The pathological changes observed in patients diagnosed with acute glaucoma or retinal ischemic insults match the animal model of retinal I/R insults. Glaucoma, RGC degeneration, and cellular damage are caused by apoptosis via the cell death pathway. Furthermore, several investigations have provided supporting evidence that the mitochondrial-mediated intrinsic pathway is involved in glaucoma (Allison et al. 2020; Vernazza et al. 2021). This activation then triggers the expression of caspase-3, ultimately leading to the mortality of RGCs. Multiple studies have shown that Bax, a Bax-pro-apoptotic mediator identified as a critical regulator of the intrinsic mitochondrial apoptotic pathway as well as protecting RGCs from apoptosis, is an ideal therapeutic target (Luo et al. 2018).

Therefore, several studies have attempted to explore potential neuroprotective agents that prevent glaucoma, including antioxidants such as flavonoids and astaxanthin. Garcia-Medina et al. (2020) have combined these agents with IOP-lowering therapy to achieve significant protection against glaucoma. Despite promising experimental outcomes through intensive efforts, these agents did not produce a significant protective effect against glaucoma progression in clinical measurements. No neuroprotective agents are available to treat glaucoma-related abnormalities (Van de Velde et al. 2015). To prevent or slow down the loss of RGCs, preserve RGCs, and restore function through the entire mechanism ideal to treat glaucoma, it is necessary to identify potential and clinically efficacious neuroprotective agents (Vishwaraj et al. 2022).

Germacrone (GM) is a potential bioactive sesquiterpene metabolite largely obtained from the Zingiberaceae family of medicinal plants. GM has been reported to have numerous pharmacological applications, including antioxidant, anti-inflammatory, anticancer, and neuroprotective effects. Several studies have shown that GM has significant protective effects on various cancer cells, including breast (Alam et al. 2022), liver (Sharifi-Rad et al. 2020), prostate (Hashem et al. 2022), brain, and esophageal cancer cells, which typically induce apoptosis through cell cycle arrest and modulation of various signaling molecules or pathways involved in cancer cell proliferation and progression (Riaz et al. 2020). In addition, GM treatment of chemo-resistant breast cancer cells combined with adriamycin could suppress bcl-2 upregulation, which indicates an anti-apoptotic condition, and upregulate the expression of the pro-apoptotic proteins p53 and Bax. Additionally, it was observed that GM organisms have the ability to suppress the MDR1 promoter, consequently inhibiting the expression of P-glycoprotein. This discovery provides insights into the role of GM in the restoration of drug resistance in breast cancer cells (Xu et al. 2019). In addition, GM is highly efficient in inhibiting glioma and inducing apoptosis of glioma cells through overexpression of p53 and Bax and controlled expression of bcl2 (Kaloni et al. 2023). The cytotoxic effect of GM on retinoblastoma cells indicates the probable anti-proliferative effect of GM on cancer cell proliferation (Hashem et al. 2022).

Numerous studies have investigated the ability of GM to reduce inflammation under various pharmacological and experimental conditions. GM treatment of neonatal rats with acute lung injury restored pro-inflammatory cytokine expression and induced anti-inflammatory mediator expression. Furthermore, lipopolysaccharide-induced inflammatory tissue damage is significantly lowered by GM, which exhibits anti-inflammatory potential (Tang et al. 2018). Nevertheless, the potential anti-inflammatory effects of GM in other circumstances, especially in the examination of retinal I/R in rodents, have not been explored. Oxidative stress and inflammation are the key factors in the pathogenesis of retinal I/R damage. Hence, it is suggested that GM has the potential to enhance oxidative stress and inflammation, thereby protecting retinal ganglion cells against I/R damage. Additionally, the efficiency of GM in the regulation of NF-kB was identified, and the regulation of retinal inflammation signaling in an animal model of rapid intraocular pressure-I/R damage was assessed.

Materials and Methods

Study area

The research was carried out in Hanzhong Central Hospital, China, between July and September 2023.

Chemicals and reagents

In the present study, Germacrone (GM) (≥ 98%) was purchased from Nanjing Puyi Biological Technologies. 8oHdG and MDA test kits were obtained from the Nanjing Jiancheng Bioengineering Institute in Nanjing, China. Rabbit antibodies targeting proteins associated with apoptosis (Bcl-2, Bax, cleaved caspase-3, NF-κB, and COX-2) were purchased from Abcam (Cambridge, UK and Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Rabbit tubulin and goat anti-rabbit IgG for peroxidase were acquired from Santa Cruz Biotechnology.

I/R damage model

Forty Sprague-Dawley (SD) (250-300 g; 8-10 weeks old) were used. All animals were supplied ad libitum with food and water in a controlled temperature and humidity-maintained chamber with a 12-hour light/dark cycle. Retinal I/R insult surgery was performed under aseptic conditions with pentobarbital sodium anesthesia. Every possible effort was undertaken and adhered to lessen the suffering of animals; 0.5% proparacaine hydrochloride solution was used for corneal analgesia, and 0.5% tropicamide with phenylephrine was used to maintain pupillary dilatation. To maintain body temperature (37°C), the animals were placed on a heated table with temperature control.

After analgesia application and dilatation of the pupil, a 30-gauge infusion needle was cannulated in the left eye of the anterior chamber, and the infusion needle was con-

nected to a glass container with normal saline placed 150 cm above the eye. This accelerates saline flow; thus, the IOP was raised and maintained at 130 mmHg for a 60-minute time slot. Before and after I/R surgery, Tobramycin, an Ophthalmic gel, was used as an antibiotic to prevent bacterial infection of the eyes. Restoration of retinal blood flow and white colour in the fundus area indicate successful ischemia/reperfusion of the retina. In all experiments, the left eye alone was used (Chai et al. 2018; Alarcon-Martinez et al. 2019). The investigational measures adhered to the strategies outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and Hanzhong Central Hospital's Ethics Committee on Animal Experiments approved the animal study protocol. (Reg No. 4367/2022/GM/I/R/2022).

Animals and treatment group

All animals were divided into four groups (n = 10) according to the treatment measures.

Group 1: Surgical procedures to initiate I/R damage were not performed and were considered the control group (Control group).

Group 2: Retinal ischemia/reperfusion damage induced through acute glaucoma, followed by the procedure described above. Furthermore, rats were administered a solitary I.P. administration of normal saline (I/R group).

Group 3: Animals received a single-dose I.P. injection of GM (200 mg/kg bw) before and after 24 h of I/R damage (GM+1d) GM-1d.

Group 4: After 1 hour of I/R, animals received intraperitoneal injections of GM (200 mg/kg bw) for up to 7 days (GM+7d) GM-7d.

After 7 days of I/R, all the animals were sacrificed and Retinal tissue from each group of animals was collected and stored for further analysis.

Measurement of retinal oxidative damage markers

To measure oxidative damage-related parameters, levels of MDA and 8-OHdG in the retina were estimated using ELISA. The experiments were performed according to the manufacturer's instructions. The variation in light absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Richmond, CA, USA).

Counting and labeling retinal ganglion cells

Retrograde tagging of RGCs was conducted 7 days before the introduction of I/R in rats, as reported in previous studies (Pang et al. 2020; Fu et al. 2021). A 5% solution of Fluoro-Gold (FG) (Sigma-Aldrich, St. Louis, MO, USA) was used for RGC labeling. Briefly, 2 mL of FG was introduced into the superior colliculi of animals under anesthesia assisted by a stereotaxic apparatus to ensure zero moments. After 7 days of FG labeling, a retinal I/R insult was induced following the procedures mentioned earlier (Guruvaiah et al. 2018; Wu et al. 2019).

After the study, the eyeballs of each animal were enucleated after euthanasia and preserved for 1 h at ambient temperature in 4% paraformaldehyde. Retinal tissue samples were meticulously sliced and flat-mounted on glass slides using 10% glycerol in PBS (Sigma-Aldrich Corp., Beijing, China) after washing with PBS. Images of FG-labeled RGCs were captured using a fluorescence microscope (Olympus Optical, Tokyo, Japan). RGCs were calculated in four microscopic fields using Image Pro Plus (version 6.0). RGCs in the retinal tissue were counted in each animal group, and the counted area in RGCs per square millimetre (RGCs/mm^2).

qRT-PCR

Retinal tissue was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) to obtain whole RNA. Reverse transcription reactions were performed using the HiScript® II 1st Strand Complementary DNA Synthesis Kit (Vazyme Biotech Co., Ltd., Nanjing, Jiangsu, China). The cDNA product was processed by PCR using specific primers, and DNA amplification was carried out according to the methodology described by Guruvaiah et al. (2018). The data were normalized to rat β-actin, and the densitometric values of each mRNA were calculated.

Western blot assessment

The animals were euthanized 24 h after the loss of GM treatment. The total protein content derived from the tissue was acquired by lysing the tissue specimen in RIPA buffer, followed by treatment with protease inhibitors. The isolated proteins were assessed using a BCA protein assay kit (Beyotime, Beijing, China). Protein segregation was performed using a 10% SDS-PAGE gel (40 g per lane). Subsequently, the separated proteins were transferred onto poly-vinylidene difluoride (PVDF) membranes obtained from Millipore (Bedford, MA, USA). Tris-buffered saline with a 1% Tween 20 (TBST) mixture and 5% skimmed milk was used as a blocking solution. The nitrocellulose membrane was then incubated with anti-iNOS, TNF-α, IL-1, p53, Bax, Bcl-2, phosphorylated p38, and anti-β-actin primary antibodies at 4°C overnight. The membrane was then exposed to a diluted secondary antibody coupled with horseradish peroxidase (HRP) in TBS for 60 min at ambient temperature. The absorbance of the band was determined using DAB, and the band intensities were estimated using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

TUNEL -Apoptosis assay

To assess the efficacy of GM in I/R-induced apoptosis, retinal cross-sections were marked using a TdT-mediated dUTP nick-end labeling technique. This approach was used to identify the DNA fragmentation pattern resulting from the apoptotic signaling mechanism, where the terminal end was labeled. To investigate the DNA fragmentation pattern, the TUNEL test is carried out using retinal cryosections at the instructions of Millipore Corp., USA.

Histopathology

Histochemical analysis of the retinas from both the control and experimental groups was performed on day 7 after 1 h of treatment. Next, 4% paraformaldehyde in PBS was used for the tissue fixation. Hematoxylin and eosin (H&E), which penetrates the head of the optic nerve, was used to stain the vertical meridian slices of each eye that were 1 mm in length. Furthermore, we measured the thickness of various layers to identify ischemic reperfusion-induced damage and GM-mediated changes in the eye after 7-day treatment. The outer nuclear (ONL) and inner nuclear (INL) plexiform layers (IPL) were measured from the inner limiting membrane to the epithelial pigment as the total retinal area. In each sample, the same region of the retina was observed to identify changes in the thickness of various retinal layers, possibly 1 mm ahead of the optic nerve head.

Statistical assessment

The Statistical assessment was provided as mean ± SD with $P < 0.05$, indicating significance. ANOVA and GraphPad Prism's Student's t-test were used to assess the data from the tested groups.

Results

GM restored I/R induced retinal tissue loss and RGCs

In the present study, Hematoxylin and eosin (H&E) staining was used to investigate the histological modifications of the retina after GM treatment in rats to assess the beneficial effects of GM on the reorganization of retinal ganglion cells after retinal I/R damage (Fig. 1). The control group exhibited a retinal thickness of 4.3 μm, which was within the normal range. However, the animals in the I/R group showed noticeable tissue loss and a reduced retinal thickness of 1.9 μm. In contrast, the I/R+GM-7d group showed a significant reduction in retinal damage induced by I/R. Additionally, the retinal thickness in this group (3.9 μm) was also close to normal levels. However, animals treated with a single dose of GM before I/R showed some degree of tissue loss (2.1 μm). The administration of long-term GM therapy before and after I/R has been shown to have a substantial effect on reducing the extent of retinal structural damage and preserving retinal integrity, compared to the use of GM in conjunction with I/R alone.

GM inhibits retinal I/R damage and promotes apoptosis of RGCs

To evaluate the potential of GM to mitigate apoptosis in retinal I/R damage, the TUNEL test was performed 24 h after the induction of I/R damage and subsequent administration of GM. The retinal slices of animals subjected to I/R damage demonstrated a reduced survival rate of 22% (RGCs) in positively stained animals (Fig. 2A). This reduction in RGC survival was observed across multiple layers of the retina, including the outer and inner nuclear layers as well as the ganglion cell layer. In contrast, the control group exhibited a 100% survival rate of RGCs. However, changes related to apoptosis were significantly mitigated by prolonged administration of GM. Specifically, GM-1d exhibited a 28% increase in RGC survival, whereas GM-7d showed a 52% increase in RGC survival. Fig. 2B illustrates a visual representation of the data and the concept being discussed.

GM inhibits I/R injury and declines the retinal cells' apoptosis

To further examine the impact of GM on the retina, TUNEL labeling was used to assess the potential effects of GM on apoptosis. Compared with the control group, the I/R group exhibited a significant increase (39%) in the num-

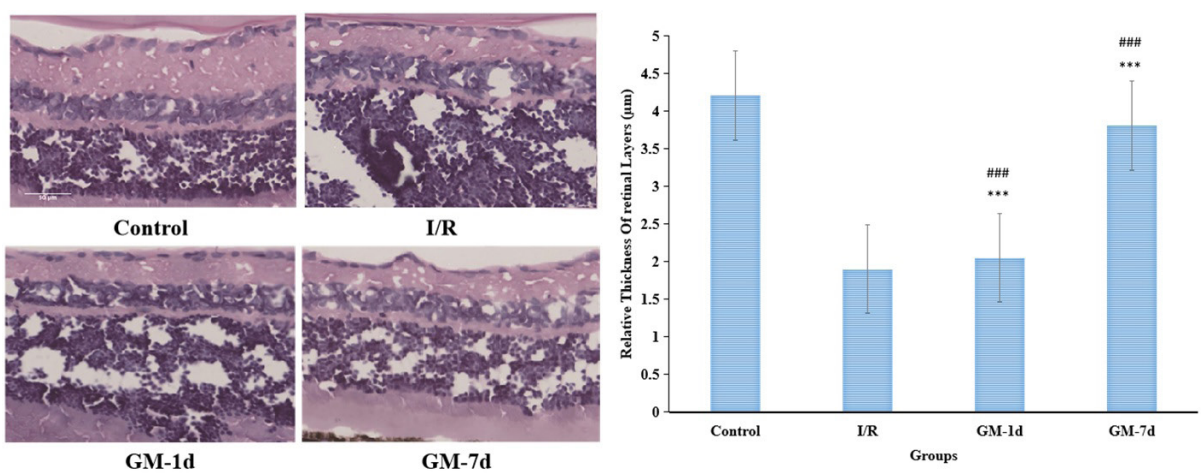

Fig. 1. GM treatment restored the tissue loss made by I/R injury.
Retinal cross-sections showed tissue degeneration after I/R injury, which was significantly restored after 7 days of GM treatment. Representative H&E staining images from the control, I/R, single-dose germacrene-treated, and 7-day GM treatment groups following I/R. Images were obtained at 20 × magnification. Data are presented as mean ± SEM (n = 10, ***$P < 0.05$ vs. Control; $^{###}P < 0.05$, vs. I/R).

ber of TUNEL-positive cells. A significant fluorescent signal of TUNEL-positive cells was detected in GM-1d, with a prevalence of 36%, and in GM-7d, with a prevalence of 17.8% (Fig. 3A, B).

GM treatment suppressed apoptosis mediator molecules

Furthermore, the expression patterns of the apoptosis-associated genes Bax, Bcl-2, and cleaved caspases, both in the form of mRNA and protein, were examined. The mRNA expression data and western blotting demonstrated that retinal I/R damage dramatically increased Bax expression (1.7) 24 h after I/R ($P < 0.05$), which was considerably recovered after lengthy GM therapy (Fig. 4A) at GM-1d (1.5) and GM-7d (1.2) ($P < 0.05$). While I/R damage had a low impact on the Bcl-2 expression pattern (0.5) of I/R+GM-1d, neither was the Bcl-2 level markedly altered ($P < 0.05$) by GM treatment after 7 days of I/R damage (1.05) (Fig. 4B). The activation of caspase-3 is crucial for apoptotic cell death. Western blotting and mRNA expression analysis of cleaved caspase-3 showed that I/R damage had an impact level of 1.5. However, GM-d expressed a reduction in the impact level (1.35) of caspase-3, and GM-d7 expressed an impact level of 0.95 (Fig. 4C). The expression pattern of caspase-3 activation observed by western blotting was highly similar to the mRNA expression pattern observed by qRT-PCR. Together, these results demonstrate that GM could control the apoptosis of RGCs induced by I/R damage, possibly by altering the expression pattern of apoptosis-related molecules.

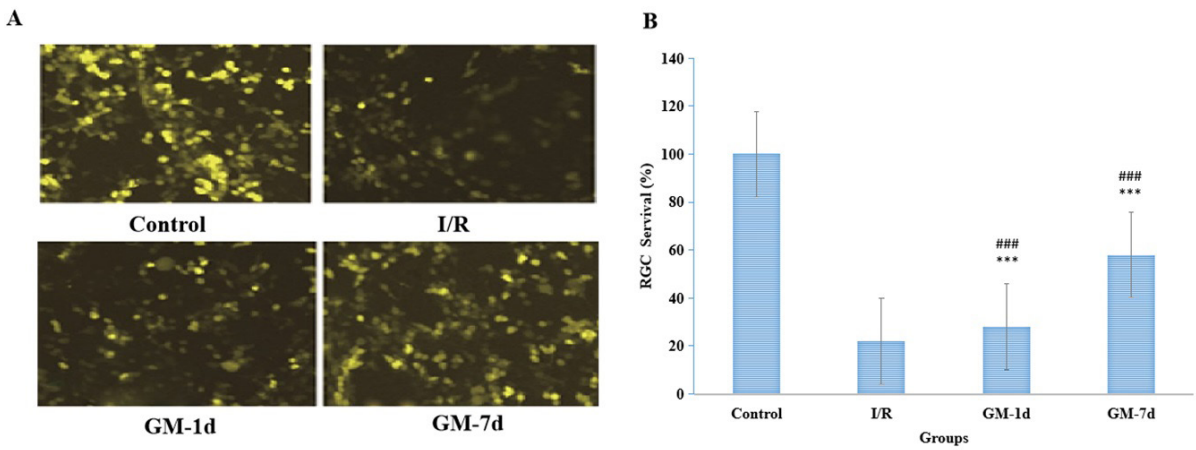

Fig 2. The effect of GM on RGC apoptosis was measured by retrograde labeling of RCGs by Fluro Gold. Seven days before retinal I/R injury.
(A) 14 days after the I/R injury FG labeled cells in flat-mounted retinas were observed under fluorescence microscopy and magnification of 20 ×. (B) The FG-labeled cells were quantified and expressed in terms of the percentage of surviving cells and data presented as Mean ± SEM (***$P < 0.05$ vs. Control. $^{###}P < 0.05$ vs. I/R).

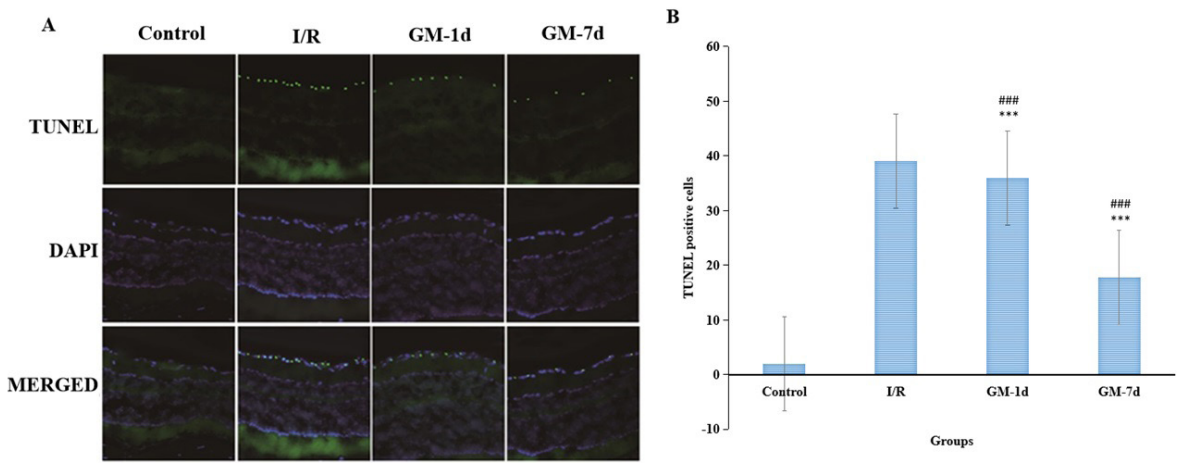

Fig. 3. Seven days of GM administration following retina I/R injury declines the retinal cells apoptosis.
Localization of positive cells is shown in green, and 4′,6-diamidino-2-phenylindole (nuclei, *blue*). (B) Data are presented as the mean ± SEM obtained from three triplicate values. (***$P < 0.05$ vs. control, $^{###}P < 0.05$ vs. I/R).

GM treatment reduced the retinal I/R damage associated with oxidative stress and inflammation

Several reports have shown that I/R damage is linked to the production of excessive oxidative stress markers and the activation of inflammatory mediators, which in turn promote cell death in the retina. ELISA analysis of oxidative stress markers showed that post-I/R damage abruptly elevated the levels of MDA and 8-OHdG (15) (Fig. 5A, B) markers ($P < 0.05$) compared to the control group. However, the changes in oxidative stress markers were significantly mitigated by prolonged administration of GM. Furthermore, the effect of GM on oxidative stress-induced inflammatory events in retinal I/R damage was assessed. Western blotting analysis of inflammatory signaling molecules such as NF-κB and COX-2 (Fig. 5C) showed that in the I/R damage group, a considerable elevation in the expression of these molecules was observed, which was largely restored after long-term GM treatment. Consistent with I/R damage and inflammatory mediators, consistent results were observed in both the ELISA analysis of oxidative stress markers and the expression of inflammatory signaling molecules after GM treatment. The findings of these trials indicate that GM therapy may restore I/R damage-induced inflammation in the retina.

Discussion

Several in vivo investigations have shown that GM organisms possess noteworthy neuroprotective capabilities in mitigating pathophysiological alterations induced by cerebral ischemia (Wu et al. 2019; Kaloni et al. 2023). A wide variety of signaling cascades are involved in the pathophysiology of retinal glaucoma, including oxidative stress (Chen et al. 2015), inflammation (Levkovitch-Verbin 2015), glia activation (Fan Gaskin et al. 2021), ER stress (Ha et al. 2015), glutamate excitotoxicity (Basavarajappa et al. 2023), mitochondrial dysfunction (Garcia-Medina et al. 2020), ischemia (Van de Velde et al. 2015), and ischemia. Numerous pharmacological studies have provided evidence that GM exhibits a diverse range of beneficial effects and has considerable promise in inhibiting the development of different cancer cells while avoiding hazardous repercussions (Sharifi-Rad et al. 2020; Alam et al. 2022; Hashem et al. 2022). However, in situ studies on GM have not explored the effects of GM on acute glaucoma-induced retinal ganglion cell degeneration and related abnormalities (Van de Velde et al. 2015).

Therefore, this study aimed to assess the beneficial effects of GM against RGC degeneration in an animal model and gain insights into the association of basic molecular mechanisms with acute glaucoma-induced ischemia/reperfusion insults. In animals, I/R damage was established by the free flow of saline into the anterior region through cannulation of the left side of the eye, apparently elevating the IOP to 130 mmHg for the 60 min time plan. Retinal I/R damage insults are highly similar to acute glaucoma conditions, which are well-established animal

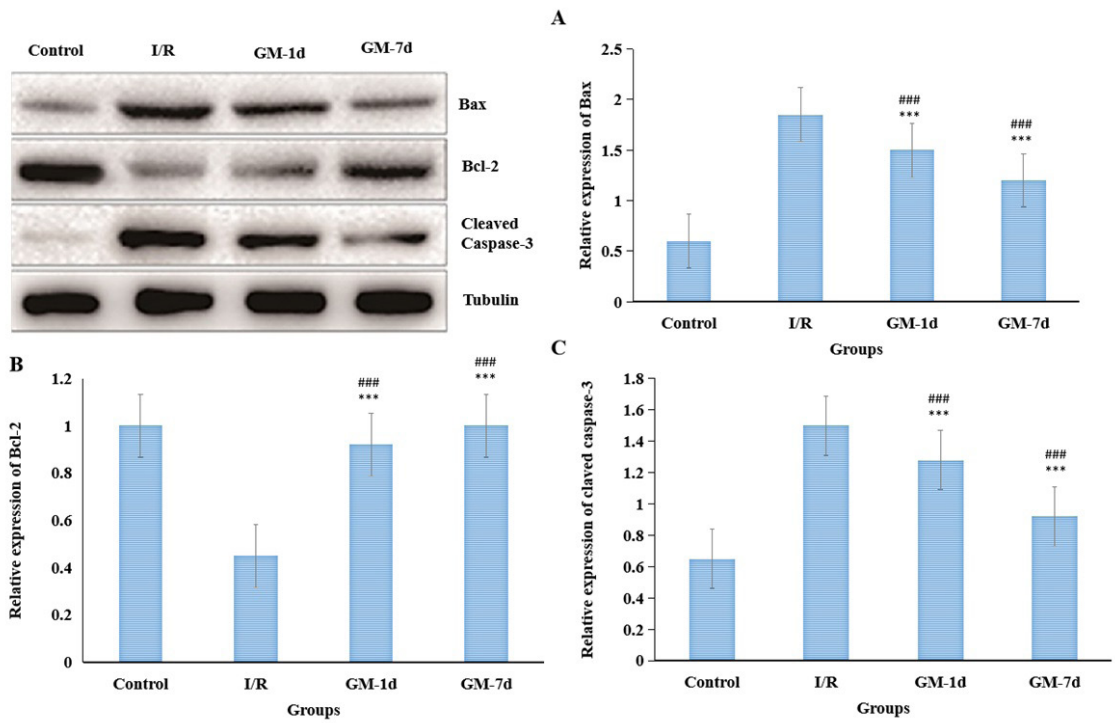

Fig. 4. Effect of GM on apoptosis-related proteins expression pattern in SD rats after retina I/R injury.
Seven days after GM administration following I/R, mRNA expression patterns of pro-apoptotic protein Bax (A), anti-apoptosis related Bcl-2 (B), and cleaved caspase-3 (C). Values are expressed as the mean ± SEM, (n = 10, $^{***}P < 0.05$ vs. Control, $^{\#\#\#}P < 0.05$ vs. I/R).

models of retinal ganglion cell damage (Vishwaraj et al. 2022).

In our study, the quantification of oxidative stress markers demonstrated that the accumulation of 8OHdG and lipid peroxidation resulted in MDA returning to near normalcy after 7 days of GM treatment following I/R injury. Gliosis is another mechanism linked to glaucoma, and activation of reactive gilia in the retina is associated with berm promoting the expression of pro-inflammatory mediators, resulting in the apoptosis of RGCs (García-Bermúdez et al. 2021; Alam et al. 2022). In addition, the phosphorylation of NF-κB stimulates its nuclear translocation, which makes it transcriptionally active. This activation plays a key role and may involve a wide variety of biological processes linked to oxidative, excitotoxic, and inflammatory events (Singh and Singh 2020). Previous studies have suggested that astrocytes, one of the reactive glial cells exposed to various stimuli, can rapidly increase the pro-inflammatory molecule COX-2, which in turn causes secondary damage to RGCs (Padovani-Claudio et al. 2023).

In our study, the expression patterns of the inflammatory mediator molecules NF-κB and COX-2 were examined by western blotting. Western blotting revealed that 7 days of GM treatment following retinal I/R damage noticeably inhibited the expression of inflammatory mediators. Therefore, it was confirmed that GM could normalize the oxidative stress caused by I/R, which was confirmed by ELISA analysis of the respective marker, subsequently suppressing the inflammatory activation of NF-κB and causing an obvious downregulation of pro-inflammatory mediators, as evidenced by blotting analysis.

In this study, FG labeling-assisted quantification of RGCs was considered a key index for assessing the therapeutic impact of GM following a retinal I/R insult. A retrograde labeling procedure was used to count RGCs that survived I/R. However, retinal I/R impedes axonal transport and encourages the loss of RGCs (Vernazza et al. 2021). In our study, 50-60% of RGCs were also preserved by long-term GM, and this effect was confirmed by retrograde labeling of RGCs using the Fluoro-Gold (FG) labeling assay. Histopathological analysis revealed that I/R-induced retinal

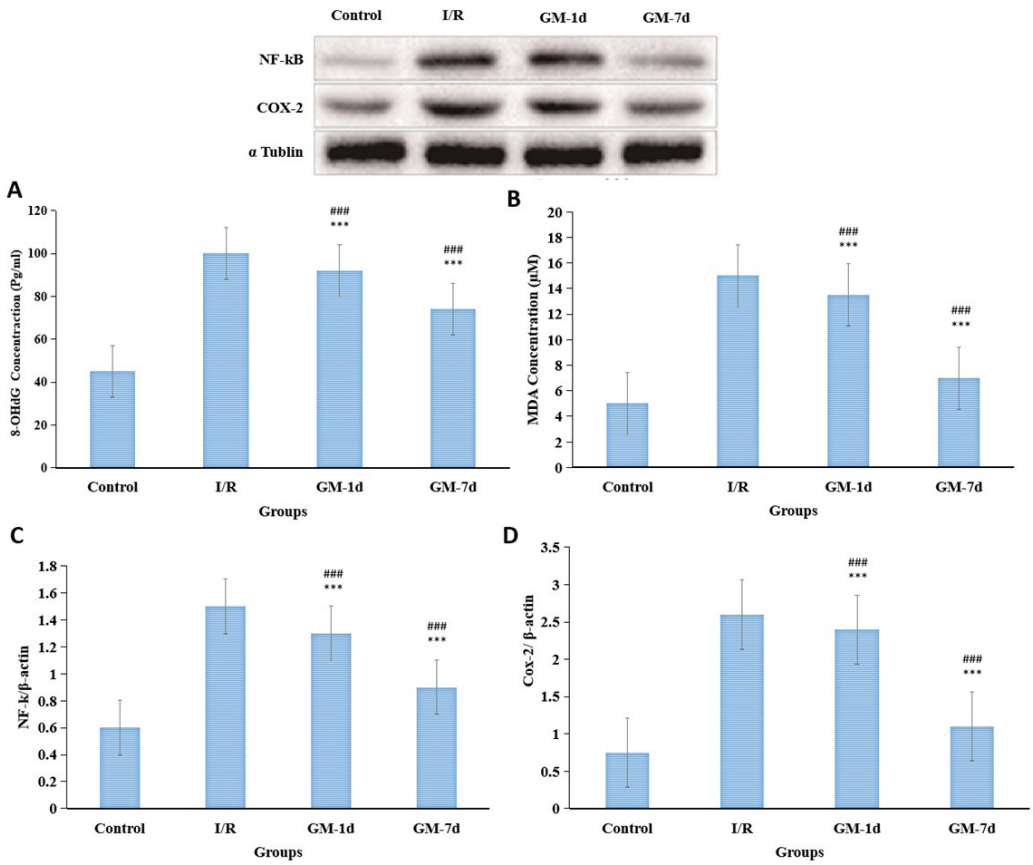

Fig. 5. Long-term Germacrone administration alleviates oxidative stress in RGCs and also suppresses the inflammatory mediators following retinal I/R injury.
The 8OHdG (A) and lipoperoxidation product (MDA) (B) resulting from oxidative stress were efficiently attenuated by 7 days of GM treatment compared to a single dose of GM after I/R injury. Further, western blotting analysis of inflammatory mediator molecules NF-κB (C) and (D) COX-2 showed that GM-7d significantly preserved inflammatory cascade activation.
Values are expressed as the mean ± SEM, (n = 10, $^{***}P < 0.05$ vs. Control, $^{###}P < 0.05$ vs. I/R)

tissue loss and structural integrity were restored, and the thickness was restored to normal in long-term GM (7-day)-treated animals.

In glaucomatous conditions, the programmed cell death pathway was reportedly involved in the death of RGCs and the mammalian system; apoptosis can be activated whether extrinsically or intrinsically (Maes et al. 2017). A crucial event connected to the stimulation of the intrinsic pathway, which in turn triggers the activation of an irreversible apoptotic cascade, is the transport of cytochrome C from the mitochondria to the cytoplasm. This process is frequently involved in apoptotic signaling-mediated mitochondrial dysfunction (Luo et al. 2018).

According to previous studies, the pro-apoptotic Bax molecule mediates the initiation of the intrinsic apoptotic process, which controls the mitochondrial process. Bcl-2 may obstruct the release of cytochrome c into the nucleus, which interferes with the action of Bax and prevents intrinsic apoptosis (Luo et al. 2018). The caspase-3 molecule, an essential apoptosis mediator, may also play a role in both the internal and extrinsic cell death pathways. Consequently, key molecules in apoptosis-related research may include caspase-3, the anti-apoptotic molecule Bcl-2, and the pro-apoptotic protein Bax (Luo et al. 2018). In our study, both qRT-PCR of mRNA expression analysis and western blotting of protein expression analysis of apoptosis-related molecules appeared consistent and in previous studies. The I/R-induced downregulation of Bax and Caspases-3 was markedly downregulated by long-term GM treatment.

Furthermore, compared to the I/R group, the expression of anti-apoptosis-linked Bcl-2 was considerably elevated in the GM-7d group. Furthermore, TUNEL analysis of the apoptosis marker area showed a considerable decrease in apoptotic cells in the GM-7d group compared to that in the I/R group. TUNEL staining also strongly agreed with western blotting and mRNA expression analysis of apoptotic mediators. Previous investigations have revealed that GM has neuroprotective properties in animal cerebral I/R; further, this effect is related to anti-oxidative and anti-apoptotic characteristics. Furthermore, GM treatment significantly restored Bax and Caspaes-3 expression, while the level of Bcl-2 was significantly reduced, and the mechanism of the anti-apoptotic effect was consistent with the results of our studies (Wu et al. 2019; Kaloni et al. 2023). A single-dose *i.p.* injection of GM was administered to animals (Group 3) to assess the effects of GM before and after I/R damage. Our results indicate that after I/R damage, 7 days of long-term GM (GM-7d) treatment could exert better retinal protection than preventive and single-dose GM treatment.

Based on our findings, GM has potent anti-inflammatory and antioxidant characteristics, which may potentially safeguard retinal ganglion cells (RGCs) against inflammatory issues. GM therapy in an experimental rat model of retinal I/R injury has shown many beneficial benefits, such as its ability to reduce inflammation, counteract oxidative stress, and prevent cell death. Our experiment confirms the potential of GM as an effective chemical to protect against retinal damage resulting from acute glaucoma-induced ischemia/reperfusion (I/R).

Conclusion

The results of this study show that GM may be able to protect RGCs from inflammatory problems because it has strong anti-inflammatory and antioxidant properties. In an experimental rat model of retinal I/R damage, GM treatment demonstrated many favorable effects, including anti-inflammatory, antioxidant, and anti-apoptotic capabilities. The results of our investigation provide support for the notion that GM may possess the capacity to serve as a promising compound for safeguarding against retinal damage caused by acute glaucoma-induced I/R.

Acknowledgments

The authors would like to thank the higher authorities at the facilities.

Author Contributions

Yu Yuan carried out the research activities and was involved in study design and manuscript preparation. Linhai Shao supervised the experiments, data analysis and manuscript revision.

Conflict of Interest

The authors declare no conflict of interest.

References

Alam, M., Ahmed, S., Elasbali, A.M., Adnan, M., Alam, S., Hassan, M.I. & Pasupuleti, V.R. (2022) Therapeutic Implications of Caffeic Acid in Cancer and Neurological Diseases. *Front. Oncol.*, **12**, 860508.

Alarcon-Martinez, L., Yilmaz-Ozcan, S., Yemisci, M., Schallek, J., Kilic, K., Villafranca-Baughman, D., Can, A., Di Polo, A. & Dalkara, T. (2019) Retinal ischemia induces alpha-SMA-mediated capillary pericyte contraction coincident with perivascular glycogen depletion. *Acta Neuropathol. Commun.*, **7**, 134.

Allison, K., Patel, D. & Alabi, O. (2020) Epidemiology of Glaucoma: The Past, Present, and Predictions for the Future. *Cureus*, **12**, e11686.

Basavarajappa, D., Gupta, V., Chitranshi, N., Viswanathan, D., Gupta, V., Vander Wall, R., Palanivel, V., Mirzaei, M., You, Y., Klistorner, A. & Graham, S.L. (2023) Anti-inflammatory Effects of Siponimod in a Mouse Model of Excitotoxicity-Induced Retinal Injury. *Mol. Neurobiol.*, **60**, 7222-7237.

Chai, F., Du, S., Zhao, X. & Wang, R. (2018) Reperfusion of occluded branch retinal arteries by transluminal Nd:YAG laser embolysis combined with intravenous thrombolysis of urokinase. *Biosci. Rep.*, **38**, BSR20170930.

Chen, Y.J., Huang, Y.S., Chen, J.T., Chen, Y.H., Tai, M.C., Chen, C.L. & Liang, C.M. (2015) Protective effects of glucosamine on oxidative-stress and ischemia/reperfusion-induced retinal injury. *Invest. Ophthalmol. Vis. Sci.*, **56**, 1506-1516.

Fan Gaskin, J.C., Shah, M.H. & Chan, E.C. (2021) Oxidative Stress and the Role of NADPH Oxidase in Glaucoma. *Antioxidants* (*Basel*), **10**, 238.

Fu, Y., Hu, T., Zhang, Q., Meng, S., Lu, Y., Xiang, A., Yin, Y., Li,

Y., Song, J. & Wen, D. (2021) Transneuronal Degeneration in the Visual Pathway of Rats following Acute Retinal Ischemia/Reperfusion. *Dis. Markers*, **2021**, 2629150.

García-Bermúdez, M.Y., Freude, K.K., Mouhammad, Z.A., van Wijngaarden, P., Martin, K.K. & Kolko, M. (2021) Glial Cells in Glaucoma: Friends, Foes, and Potential Therapeutic Targets. *Front. Neurol.*, **12**, 624983.

Garcia-Medina, J.J., Rubio-Velazquez, E., Lopez-Bernal, M.D., Cobo-Martinez, A., Zanon-Moreno, V., Pinazo-Duran, M.D. & Del-Rio-Vellosillo, M. (2020) Glaucoma and Antioxidants: Review and Update. *Antioxidants* (*Basel*), **9**, 1031.

Guruvaiah, P., Guo, H., Li, D. & Xie, Z. (2018) Preventive Effect of Flavonol Derivatives Abundant Sanglan Tea on Long-Term High-Fat-Diet-Induced Obesity Complications in C57BL/6 Mice. *Nutrients*, **10**, 1276.

Ha, Y., Liu, H., Xu, Z., Yokota, H., Narayanan, S.P., Lemtalsi, T., Smith, S.B., Caldwell, R.W., Caldwell, R.B. & Zhang, W. (2015) Endoplasmic reticulum stress-regulated CXCR3 pathway mediates inflammation and neuronal injury in acute glaucoma. *Cell Death Dis.*, **6**, e1900.

Hashem, S., Ali, T.A., Akhtar, S., Nisar, S., Sageena, G., Ali, S., Al-Mannai, S., Therachiyil, L., Mir, R., Elfaki, I., Mir, M.M., Jamal, F., Masoodi, T., Uddin, S., Singh, M., et al. (2022) Targeting cancer signaling pathways by natural products: Exploring promising anti-cancer agents. *Biomed. Pharmacother.*, **150**, 113054.

Hsu, S.M., Yang, C.H., Tsai, H.Y., Lin, C.J., Fang, Y.H., Shieh, C.C. & Chen, S.H. (2020) Chitosan Oligosaccharides Suppress Nuclear Factor-Kappa B Activation and Ameliorate Experimental Autoimmune Uveoretinitis in Mice. *Int. J. Mol. Sci.*, **21**, 8326.

Kaloni, D., Diepstraten, S.T., Strasser, A. & Kelly, G.L. (2023) BCL-2 protein family: attractive targets for cancer therapy. *Apoptosis*, **28**, 20-38.

Levkovitch-Verbin, H. (2015) Retinal ganglion cell apoptotic pathway in glaucoma: Initiating and downstream mechanisms. *Prog. Brain Res.*, **220**, 37-57.

Luo, H., Zhuang, J., Hu, P., Ye, W., Chen, S., Pang, Y., Li, N., Deng, C. & Zhang, X. (2018) Resveratrol Delays Retinal Ganglion Cell Loss and Attenuates Gliosis-Related Inflammation From Ischemia-Reperfusion Injury. *Invest. Ophthalmol. Vis. Sci.*, **59**, 3879-3888.

Maes, M.E., Schlamp, C.L. & Nickells, R.W. (2017) BAX to basics: How the BCL2 gene family controls the death of retinal ganglion cells. *Prog. Retin. Eye Res.*, **57**, 1-25.

Padovani-Claudio, D.A., Ramos, C.J., Capozzi, M.E. & Penn, J.S. (2023) Elucidating glial responses to products of diabetes-associated systemic dyshomeostasis. *Prog. Retin. Eye Res.*, **94**, 101151.

Pang, Y., Qin, M., Hu, P., Ji, K., Xiao, R., Sun, N., Pan, X. & Zhang, X. (2020) Resveratrol protects retinal ganglion cells against ischemia induced damage by increasing Opa1 expression. *Int. J. Mol. Med.*, **46**, 1707-1720.

Riaz, A., Rasul, A., Kanwal, N., Hussain, G., Shah, M.A., Sarfraz, I., Ishfaq, R., Batool, R., Rukhsar, F. & Adem, S. (2020) Germacrone: A Potent Secondary Metabolite with Therapeutic Potential in Metabolic Diseases, Cancer and Viral Infections. *Curr. Drug Metab.*, **21**, 1079-1090.

Sharifi-Rad, J., Rayess, Y.E., Rizk, A.A., Sadaka, C., Zgheib, R., Zam, W., Sestito, S., Rapposelli, S., Neffe-Skocinska, K., Zielinska, D., Salehi, B., Setzer, W.N., Dosoky, N.S., Taheri, Y., El Beyrouthy, M., et al. (2020) Turmeric and Its Major Compound Curcumin on Health: Bioactive Effects and Safety Profiles for Food, Pharmaceutical, Biotechnological and Medicinal Applications. *Front. Pharmacol.*, **11**, 01021.

Singh, S. & Singh, T.G. (2020) Role of Nuclear Factor Kappa B (NF-κB) Signalling in Neurodegenerative Diseases: An Mechanistic Approach. *Curr. Neuropharmacol.*, **18**, 918-935.

Sun, Y., Chen, A., Zou, M., Zhang, Y., Jin, L., Li, Y., Zheng, D., Jin, G. & Congdon, N. (2022) Time trends, associations and prevalence of blindness and vision loss due to glaucoma: an analysis of observational data from the Global Burden of Disease Study 2017. *BMJ Open*, **12**, e053805.

Tang, M., Yang, Y., Yu, J., Qiu, J., Chen, P., Wu, Y., Wang, Q., Xu, Z., Ge, J., Yu, K. & Zhuang, J. (2018) Tetramethylpyrazine in a Murine Alkali-Burn Model Blocks NFkappaB/NRF-1/CXCR4-Signaling-Induced Corneal Neovascularization. *Invest. Ophthalmol. Vis. Sci.*, **59**, 2133-2141.

Van de Velde, S., De Groef, L., Stalmans, I., Moons, L. & Van Hove, I. (2015) Towards axonal regeneration and neuroprotection in glaucoma: Rho kinase inhibitors as promising therapeutics. *Prog. Neurobiol.*, **131**, 105-119.

Vernazza, S., Oddone, F., Tirendi, S. & Bassi, A.M. (2021) Risk Factors for Retinal Ganglion Cell Distress in Glaucoma and Neuroprotective Potential Intervention. *Int. J. Mol. Sci.*, **22**, 7994.

Vishwaraj, C.R., Kavitha, S., Venkatesh, R., Shukla, A.G., Chandran, P. & Tripathi, S. (2022) Neuroprotection in glaucoma. *Indian J. Ophthalmol.*, **70**, 380-385.

Wu, T., Yin, F., Kong, H. & Peng, J. (2019) Germacrone attenuates cerebral ischemia/reperfusion injury in rats via antioxidative and antiapoptotic mechanisms. *J. Cell. Biochem.*, **120**, 18901-18909.

Xu, J.C., Zhou, X.P., Wang, X.A., Xu, M.D., Chen, T., Chen, T.Y., Zhou, P.H. & Zhang, Y.Q. (2019) Cordycepin Induces Apoptosis and G2/M Phase Arrest through the ERK Pathways in Esophageal Cancer Cells. *J. Cancer*, **10**, 2415-2424.