Development and evaluation of a novel neuroprotective herbal combination for the treatment of glaucoma

Ph.D. thesis

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1. Introduction

Glaucoma is a group of neurodegenerative disorders characterized by the slow and irreversible loss of retinal ganglion cell axons, and represents one of the leading causes of irreversible vision loss worldwide [1–3]. The two major categories are primary angle-closure glaucoma and, in approximately 70% of cases, primary open-angle glaucoma [1,4]. In 2020, an estimated 80 million individuals were diagnosed with glaucoma globally, and this number is projected to rise to approximately 120 million by 2040 [1–3]. The principal objectively measurable risk factor for the disease is elevated intraocular pressure (IOP > 21 mmHg [2,3,5]), the complications of which include progressive optic neuropathy that, if left untreated, leads to irreversible visual impairment in a significant proportion of cases [2,3]. During the disease process, inflammation induced by elevated levels of proinflammatory cytokines [3,6] and increased free radical production [3,7] can be observed in most ocular tissues [3,8]. According to current knowledge, glaucoma is not curable [9]; however, early diagnosis and appropriate treatment can slow disease progression and preserve vision in a substantial number of patients [9]. Current standard glaucoma therapies primarily aim to reduce IOP through pharmacological, laser-based, and/or surgical interventions [2,3]. In recent years, neuroprotection has emerged as an important aspect of complementary glaucoma therapy, with the goal of slowing the apoptosis and functional deterioration of retinal ganglion cells [3].

There is currently no evidence-based recommendation regarding the efficacy of neuroprotective herbal therapies applicable in glaucoma care [2]. Although the number of available herbal products is relatively low, both the increasing availability of such products and the rising interest among patients are predictable trends [10]. In 2010, 13.6% of surveyed Canadian glaucoma patients reported using complementary therapies in addition to their antiglaucoma eye drops—34.5% used herbal therapies, 22.7% dietary interventions, and 18.8% nutritional supplements—yet 62.5% did not inform their treating ophthalmologist about these practices [10,11]. A 2014 survey revealed that younger Canadian ophthalmologists (26% of respondents) were more likely to inquire about the use of complementary therapies among their patients [12]. These findings collectively underscore the growing demand for non-conventional therapeutic approaches from both clinicians and glaucoma patients [10–12]. The literature describes investigations of more than 30 nutritional supplements aimed at enhancing neuroprotection and improving retinal circulation; however, the results remain inconsistent and cannot be elevated to evidence-based recommendations [3,13–16].

The herbal combination presented in this dissertation is currently the subject of a patent application (application number: P2500037). The combined herbal ingredients proposed for use were selected based on their chemical constituents and documented effects relevant to ocular diseases:

Rosmarinus officinalis L. (Lamiaceae) is a widely used culinary and medicinal herb. Its major active compound, rosmarinic acid, modulates multiple signaling pathways, including inhibition of vascular endothelial growth factor (VEGF) and acetylcholinesterase (AChE) [17–19], thereby potentially contributing to the prevention of hypoxia-induced angiogenesis and its complications. It also inhibits proapoptotic and proinflammatory mediators such as transforming growth factor-beta (TGF-β), interleukin (IL)-6, and tumor necrosis factor-alpha (TNF-α), partly through the NFκB/p65 pathway [17–19]. Rosmarinic acid has been used in rabbit models during experimental trabeculectomy due to its antiangiogenic and antifibrotic effects, and has also been incorporated into intravitreal implants [19,20]. However, its potential effects on intraocular pressure reduction and glaucoma progression have not yet been investigated. In animal studies, rosmarinic acid demonstrated no retinal toxicity, even at high doses [17–20]. Additional bioactive constituents, including carnosic acid and carnosol, may further contribute to its antioxidant and therapeutic effects [21,22].

A *Foeniculum vulgare* Mill. (Apiaceae) is another non-toxic plant widely used in the food and pharmaceutical industries. In a glaucomatous animal model, its aqueous extract was shown to reduce intraocular pressure, presumably through AChE inhibition [23]. Its major active constituent, trans-anethole, exhibits anti-inflammatory and antioxidant (aldose reductase inhibitory) properties [23–25]. Notably, rosmarinic acid is also present among the plant's organic acids in considerable amounts. Rodent models have demonstrated neuroprotective effects of fennel, mediated by inhibition of microglial activity, modulation of astrocyte activation, and AChE- and GABA (γ-aminobutyric acid)-ergic mechanisms [24,25]; however, its ophthalmic neuroprotective effects have not yet been explored.

A *Helichrysum italicum* (Roth) G. Don fil. (Asteraceae) contains flavonoids such as rutin and chlorogenic acid, which exhibit anti-inflammatory and antioxidant properties. These compounds inhibit the NFκB, IL-1β, TNF-α, IL-6, IL-8, and prostaglandin-E2 signaling pathways, and promote tissue regeneration [26,27]. In addition, they influence the inflammatory pathways of 5-lipoxygenase and cyclooxygenase (COX-1 and COX-2) [28]. Although these characteristics suggest a potential neuroprotective role in glaucoma, *H. italicum* is not traditionally used in folk medicine for ocular conditions, and its effects on ophthalmic diseases have not yet been investigated [26–28].

2. Background and objective

Glaucoma is a group of neurodegenerative disorders and one of the leading causes of irreversible blindness worldwide. Although the disease is multifactorial, elevated IOP remains one of its most significant risk factors. Current therapeutic approaches, including topical medications, laser procedures, and surgical interventions, primarily aim to normalize IOP. However, it is important to note that these treatments cannot reverse optic nerve fiber damage that has already occurred. The effectiveness of the treatments is greatly influenced by the potential side effects of the antiglaucoma medications used, as well as by patient compliance. The therapeutic response to the applied treatments may also vary considerably between individuals. Neuroprotective treatments, which aim to protect the optic nerve from further damage, are not yet part of standard glaucoma care. This is largely due to the fact that their mechanisms of action, clinical efficacy, and long-term safety profiles remain insufficiently elucidated. Among the ophthalmic solutions currently available on the market, some evidence exists regarding the neuroprotective effect of brimonidine; however, further studies are required to clarify its underlying mechanisms of action. In parallel, there is growing interest in natural, plant-derived therapeutic strategies, despite the fact that the use of phytotherapeutic supplements and herbal formulations in glaucoma remains limited. Increasing research efforts are being directed toward exploring the IOP-lowering, antioxidant, and neuroprotective potential of botanical compounds, which may open promising avenues for future glaucoma treatment strategies.

Objective

The aim of our research was to investigate whether the developed eye drops formulation, containing a combination of three herbal extracts, can mitigate glaucomatous damage. Within this framework, we formulated the following hypotheses, the experimental investigation of which constituted the primary aim of our research:

I. We hypothesize that the combined herbal eye drops containing active compounds from *Rosmarinus officinalis, Foeniculum vulgare* and *Helichrysum italicum* significantly reduces IOP in the microbead-induced glaucoma rat model.

II. We hypothesize that the anti-inflammatory and antioxidant properties of the combined herbal formulation attenuate the progression of optic neuropathy, as reflected by improvements in structural (OCT), functional (ERG) and molecular (Western blot, immunohistochemistry) retinal markers.

III. We hypothesize that the combination of the three herbal extracts is more effective than the individual extracts alone, resulting in synergistic IOP-lowering and neuroprotective effects.

3. Materials and methods

3.1. Animals

The pharmacological and toxicological evaluation of the herbal active compounds was conducted in male Sprague–Dawley rats (300–500 g, 2 months old; Charles River Laboratories). The experiment was conducted in accordance with the provisions of *Act XXVIII of 1998 (Hungary)* and with the approval of the Institutional Animal Welfare Committee of the University of Pécs (MÁB, approval numbers: KA-3637; BA02/2000-68/2022). Animal housing was provided by the Animal Facility of the Department of Anatomy, Medical School, University of Pécs (facility license: BAHU0104L07).

The animals were assigned to the following experimental groups:

- **(1) Absolute control group** (PBS injection into the anterior chamber (10 µl) + *Hyabak* artificial tears® eye drops) (n = 11); see later in the *Results* section under the abbreviation **PBS + P** for this group.
- **(2) Active-compound–treated control group** (PBS injection into the anterior chamber (10 µl) + herbal active compounds administered as eye drops) (combined formulation (X): *R. officinalis*; *F. vulgare*; *H. italicum* (*n* = 7); single-compound evaluation: (I) *R. officinalis* (*n* = 5), (II) *F. vulgare* (n = 5), (III) *H. italicum* (*n* = 5); see later in the *Results* section under the abbreviation **PBS + H** for this group.
- **(3) Placebo-treated glaucomatous group** (microbead injection into the anterior chamber (10 µm bead diameter; 10 µl; 7.2×10^6 beads/µl) + *Hyabak* artificial tears® eye drops) (*n* = 22); see later in the Results section under the abbreviation **Bead + P** for this group.
- **(4) Active-compound–treated glaucomatous group** (microbead injection into the anterior chamber (10 µm bead diameter; 10 µl; 7.2×10^6 beads/µl) + herbal active compounds administered as eye drops) (combined formulation (X): *R. officinalis*; *F. vulgare*; *H. italicum* (*n* = 16); single-compound evaluation: (I) *R. officinalis* (*n* = 5), (II) *F. vulgare* (*n* = 5), (III) *H. italicum* (*n* = 5); see later in the Results section under the abbreviation **Bead + H** for this group.

The sample sizes were estimated based on the experiments of Szabó et al. (2021), which employed a similar methodology [29], as well as on national and international literature data. For precise determination, an a priori power analysis was performed using G*Power. According to the test for the four experimental groups ($\alpha = 0.05$), a total of 345 animals would be required to achieve 90% statistical power (effect size: 0.204; partial $\eta^2 = 0.04$). Based on these calculations, the planned sample size was as follows: $n = 4$ animals (8 eyes) for the allergy test, $n = 42$ animals (84 eyes) for the combined herbal-compound study, and $n = 40$ animals (80 eyes) for the single-compound evaluations, resulting in a total of $n = 86$ animals (172 eyes). The exclusion criterion was the presence of any ophthalmic abnormality detected on day 0 (e.g., corneal scarring, abnormal retina).

3.1.1. Estabilishment of glaucoma model

The experimental animals were anesthetized with an intraperitoneal combination of ketamine (90 mg/kg) and xylazine (10 mg/kg) [29,30]. The periocular region was disinfected with Braunol solution. In Groups 3 and 4, glaucoma was induced in both eyes by injecting fluorescent polystyrene microbeads (10 µm; 3.6×10^6 beads/ml; 10 µl/eye) into the anterior chamber using a Hamilton syringe with a 33G needle. In Groups 1 and 2, PBS solution (10 µl/eye) was injected as a control, creating a sham-operated model. The injection procedure was repeated two weeks later [29].

3.1.2. Pain monitoring and euthanasia of animals

Pain-inducing procedures (with the exception of eye drops administration and intraocular pressure measurements) were performed under ketamine–xylazine anesthesia [29]. The depth of anesthesia was assessed using reflex testing. After each procedure, the animals were transferred to a monitoring room, where they were observed until full recovery, followed by analgesic treatment (Algopyrin™, 200 mg/kg). Euthanasia was performed under isoflurane–oxygen anesthesia, followed by decapitation, a method that minimizes ischemic damage to ocular tissues [29,31,32].

3.1.3. Topical eye-drop treatment

For glaucoma treatment, we developed a water-based, isotonic, sterile, and preserved ophthalmic formulation suitable for twice-daily topical administration, ensuring safe and targeted delivery. The formulation offers the advantages of rapid tissue penetration, the

non-toxicity of its natural active constituents, and enhanced stability and bioavailability. The three herbal compounds were evaluated both individually and in a combined formulation.

The combined preparation consisted of 1% sodium hyaluronate–based artificial tears containing rosmarinic acid (≥98% ex *Rosmarinus officinalis*, Sigma-Aldrich, St. Louis, MO, USA), *Foeniculum vulgare* essential oil diluendum (Formulae Normales *diluendum foeniculi sine alcoholo ex aetheroleo Foeniculum vulgare*, Adrienne Feller Cosmetics Zrt., Jászfényszaru, Hungary), and *Helichrysum italicum* hydrolate (Adrienne Feller Cosmetics Zrt., Jászfényszaru, Hungary). The control group received *Hyabak*® artificial tears as placebo treatment. Therapy was initiated on the day following microbead injection and administered twice daily (morning and afternoon) to both eyes.

3.1.4. Eye drops allergy and distress-monitoring

To assess pain and distress, we employed the Rat Grimace Scale and the Rat Facial Activity Pain Coding system [33–35]. Potential irritative effects of the eye drops were evaluated using the Schreiber corneal opacity scale and the conjunctival hyperemia scoring system [36,37].

3.2. Stability testing of the active compounds

The stability of the ophthalmic formulation was evaluated at the Institute of Medical Chemistry, University of Szeged, using RP-HPLC and LC-MS methodologies. Rosmarinic acid (≥98%, Sigma-Aldrich), used as the marker compound, was dissolved in a sodium-hyaluronate–based vehicle. Samples were stored at +4 °C and at room temperature, and analyzed after 3, 6, 8, 11, and 14 days. Measurements were performed using an Agilent 1200 HPLC system coupled with a Waters SQ Detector mass spectrometer [38].

3.3. IOP measurement

The IOP was measured weekly in both eyes (n = 83) using a non-invasive rebound tonometer (TonoLab, iCare, Finland) to assess the effects of the herbal active compounds on ocular pressure. During each measurement session, six repeated readings were obtained between 7:00 and 9:00 a.m., and the values were averaged for each eye. Corneregel® ophthalmic gel was applied to protect the cornea during the procedure.

3.4. Electroretinography (ERG)

To document baseline visual function, scotopic ERG recordings were performed in all animals (n = 40) on day 0, and subsequently repeated at weeks 4 and 8 following microbead injection. Animals were dark-adapted for more than 12 hours and then anesthetized using a ketamine–xylazine combination. After pharmacological pupil dilation, active electrodes were placed on the corneal surface under red light (632 nm), while reference and ground electrodes were positioned subcutaneously. Retinal responses were elicited using 50 green LED light flashes (503 nm, 5 cd·s/m², 0.25 Hz), and the signals were pre-amplified, amplified, and digitized for analysis.

3.5. Optical Coherence Tomography (OCT)

In vivo imaging of the eyes was performed using a spectral-domain OCT system (Bioptigen, USA; InVivoVue Clinic software, Leica Microsystems) (n = 69) to monitor retinal structural changes during disease progression. The first scan was obtained on day 0, immediately after ERG recording, in anesthetized animals with pharmacologically dilated pupils. The examination was repeated at weeks 4 and 8. To protect the cornea during imaging, preservative-free artificial tears (Systane®) were applied. A 6-mm retinal lens was used, covering a 3.2 × 3.2 mm area (1000 A-scans × 100 B-scans × 3).

3.6. Analysis of retinal ganglion cell changes in Whole-Mount preparation

Eyes (n = 28) were fixed in 4% paraformaldehyde, after which the retinas were isolated and blocked using donkey and bovine serum. Following incubation with mouse anti-Brn3a primary antibodies, retinal ganglion cells were labeled with Alexa Fluor-594–conjugated secondary antibodies. Preparations were mounted using Fluoroshield and examined with a Nikon Eclipse 80i epifluorescence microscope. Cell counting was performed using ImageJ software in eight predefined retinal regions (4 central, 4 peripheral), each covering an area of 500 × 500 μm^2.

3.7. Analysis of retinal blood vessels using Isolectin-B4 staining in retina Whole-Mount preparations

Fixed retinas were labeled with biotinylated *Griffonia simplicifolia* (DC.) Baill. (Fabaceae) isolectin-B4 (Alexa Fluor 568). Preparations were imaged using a Nikon Eclipse 80i microscope (4× magnification, ~2500 × 2500 µm, 150 µm/pixel). Following contrast

enhancement, images were analyzed in ImageJ software to quantify vessel area fraction, vessel diameters, and vascular density based on multichannel images.

3.8. Western blot protein analysis

For Western blot analysis, homogenates were prepared from four retinas per group (n = 54). Samples were stored at –80 °C, then homogenized using an Ultra-Turrax and Potter homogenizer in lysis buffer (50 mM Tris, 50 mM EDTA, 0.5% protease and phosphatase inhibitors). Protein concentrations were determined using the DC™ Protein Assay kit. Samples were diluted in Laemmli buffer, boiled, centrifuged, and separated on SDS-PAGE gels (20 µg/lane), followed by transfer to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk and incubated with primary antibodies (e.g., anti-GAPDH, anti-NFκB, anti-GFAP, anti-Bax, anti-BDNF, anti-CREB, anti-HIF1α, anti-p38-MAPK). After incubation with horseradish-peroxidase–conjugated secondary antibodies, protein bands were visualized using ECL substrate. Experiments were performed in triplicate. Quantification was conducted using ImageJ software, with band intensities normalized to GAPDH.

3.9. Statistical analysis

Data were subjected first to normality testing, followed by two-way ANOVA for variance analysis, with Fisher's post hoc test (Origin2018 64-bit and R Software). For ERG data, two-sample t-tests with Welch correction for F-tests were applied (Origin2018 64-bit and Microsoft Excel). Graphical representations were generated using GraphPad Prism 5 and R Software. Data are presented as mean ± SEM, and differences were considered statistically significant at $p < 0.05$.

4. Results

4.1. Properties of herbal eye drops

Throughout the 8-week treatment period, the administration of the eye drops caused no allergic reactions, ocular surface damage, or other adverse effects in either the examined combined herbal formulation or the placebo (Hyabak®) group. In the case of the *H. italicum*–containing eye drops, mild corneal edema and conjunctival hyperemia were observed in five eyes, likely due to insufficient IOP reduction. Rat Grimace Scale and Rat Facial Activity Pain Coding scores were 0 in all groups, indicating that analgesic intervention was not required. The stability of the formulation was assessed using RP-HPLC and LC-MS in collaboration with the

Institute of Medical Chemistry, University of Szeged (Dr. Gábor Tóth and colleagues). The rosmarinic acid content decreased by 5% after 10 days and by 20% after 14 days at room temperature, indicating adequate stability. When stored under refrigerated conditions (4 °C), the formulation preserved its bioactivity for up to 8 weeks. Among all tested formulations, the combined preparation proved to be the most optimal in terms of minimizing adverse effects and maintaining chemical stability.

4.2. Effect of the herbal eye drops on IOP

Microbead implantation successfully induced anterior chamber angle obstruction and a consequent elevation of IOP. In the placebo-treated glaucomatous group (Bead + P), IOP increased significantly, whereas in the glaucomatous group treated with the combined herbal formulation (Bead + $H_{(X)}$), a significant reduction in IOP was observed from week 1 onward ($p < 0.001$), which persisted throughout the entire 8-week period. Supporting the efficacy of the combined preparation, IOP values remained at levels comparable to the healthy control group (PBS + P), and the formulation effectively prevented microbead-induced IOP spikes. Eye drops containing the individual herbal components (Bead + $H_{(I)}$, $H_{(II)}$, $H_{(III)}$) also reduced IOP, but to a lesser extent than the combined preparation. *R. officinalis* (rosmarinic acid) monotherapy resulted in a significant but moderate decrease in IOP, which was insufficient to prevent glaucomatous progression. *F. vulgare* extract similarly lowered IOP, but did not demonstrate neuroprotective effects. *H. italicum* extract produced a significant reduction in IOP by week 8, yet failed to efficiently suppress post-injection IOP spikes. Overall, the combined herbal-compound eye drops proved to be the most effective in controlling IOP and exhibited the most favorable therapeutic profile in the glaucomatous model.

4.3. Effect of herbal eye drops on visual function

In the scotopic ERG recordings, the a- and b-wave amplitudes did not differ significantly between the control groups (PBS + P and PBS + $H_{(X)}$) ($p > 0.05$), indicating that the combined herbal formulation alone does not affect retinal function. In contrast, the glaucomatous placebo group (Bead + P) exhibited a significant reduction in both a-wave ($p = 0.01$) and b-wave amplitudes ($p = 0.02$) relative to controls, reflecting functional impairment of the retina. In the glaucomatous group treated with the combined herbal formulation (Bead + $H_{(X)}$), ERG waveforms remained comparable to those of the control groups, indicating preserved retinal function. No significant differences were observed in oscillatory potentials

between the Bead + P and PBS + P groups ($p = 0.26$); however, full waveform analysis demonstrated that the combined formulation effectively maintained retinal electrical activity despite the glaucomatous condition.

4.4. Effect of the herbal eye drops on retinal structure (OCT and native histology)

Based on OCT imaging, the glaucomatous placebo group (Bead + P) exhibited a significant reduction in retinal thickness at weeks 4 and 8, particularly in the OPL-ONL and RNFL-INL layers ($p = 0.01$). In contrast, the glaucomatous group treated with the combined herbal formulation (Bead + $H_{(X)}$) maintained retinal layer thicknesses comparable to those of the control groups, indicating a clear neuroprotective effect. Among the three individual herbal extract treatments, *H. italicum* (Bead + $H_{(III)}$) did not show significant structural deviation from controls, whereas the *R. officinalis* (Bead + $H_{(I)}$) and *F. vulgare* (Bead + $H_{(II)}$) groups exhibited significant thinning of the RNFL-INL layers ($p < 0.01$), suggesting a weaker neuroprotective potential. Overall, the combined formulation effectively preserved retinal structural integrity under glaucomatous conditions, while the individual components were less effective when administered separately.

4.5. Effect of the herbal eye drops on retinal ganglion cell survival assessed by Brn3a Whole-Mount immunohistochemistry

Brn3a immunolabeling revealed no significant changes in retinal ganglion cell numbers in the control groups (PBS + P, PBS + $H_{(X)}$). In contrast, the glaucomatous placebo group (Bead + P) demonstrated a significant loss of ganglion cells. In the glaucomatous animals treated with the combined herbal formulation (Bead + $H_{(X)}$), ganglion cell counts remained comparable to control levels in both central and peripheral retinal regions ($p < 0.001$), demonstrating robust neuroprotection. Quantitative analysis showed that ganglion cell survival in the Bead + $H_{(X)}$ group was 16% higher centrally and 27% higher peripherally compared with the glaucomatous placebo group. When evaluating the individual components, neither the *F. vulgare* nor the *R. officinalis* extracts produced significant protection relative to the glaucomatous placebo group, whereas *H. italicum* provided significant ganglion cell preservation in the peripheral retina ($p = 0.02$). Overall, only the combined herbal formulation provided comprehensive protection of retinal ganglion cells, whereas the individual components alone failed to deliver sufficient neuroprotective efficacy.

4.6. Effect of the herbal eye drops on the retinal vasculature assessed by Isolectin-B4 immunolabeling

Isolectin-B4 immunostaining demonstrated preserved retinal vascular morphology in the control groups (PBS + P, PBS + $H_{(X)}$). In contrast, the glaucomatous placebo group (Bead + P) exhibited microaneurysms and microangiopathic alterations, indicative of ischemic retinal damage. Conversely, the glaucomatous group treated with the combined herbal formulation (Bead + $H_{(X)}$) showed retinal vascular morphology comparable to that of the control groups. Quantitative analysis of retinal vascular density revealed a significant decrease in the Bead + P group (58.73 ± 1.69%) relative to controls (PBS + P: 66.55 ± 2.36%; $p = 0.02$). The values in the combined treatment group (Bead + $H_{(X)}$) (60.94 ± 1.47%) did not differ significantly from the glaucomatous placebo group ($p = 0.48$), yet approached control levels ($p = 0.07$), suggesting a partial endothelial-protective effect. The glaucomatous groups treated with the individual herbal constituents (Bead + $H_{(I)}$, $H_{(II)}$, $H_{(III)}$) also showed significantly reduced vascular density compared with controls ($p < 0.05$), but did not differ from the Bead + P group. Efficiency analysis performed in R software confirmed that the combined formulation produced significantly superior outcomes compared with the individual components, reinforcing the superiority of the combination in protecting retinal microcirculation.

4.7. Biochemical properties of the herbal eye drops assessed by Western Blot protein analysis

The Western blot experiments aimed to elucidate the biochemical mechanisms underlying the effects of the combined herbal formulation, focusing on biomarkers of apoptosis, inflammation, stress response, and neovascularization. GAPDH was uniformly expressed across all samples as an internal control. In the glaucomatous placebo group (Bead + P), expression levels of NFκB ($p = 0.01$), GFAP ($p < 0.001$), Bax ($p = 0.001$), BDNF ($p = 0.007$), CREB ($p = 0.006$), and HIF1-α ($p = 0.01$) were significantly elevated, indicating stress-induced cellular injury, glial activation, apoptotic signaling, and hypoxia. Although p38-MAPK expression was increased, the change did not reach statistical significance ($p = 0.16$). In contrast, the glaucomatous group treated with the combined herbal formulation (Bead + $H_{(X)}$) exhibited significantly lower expression of NFκB, GFAP, Bax, BDNF, CREB, and HIF1-α compared with the Bead + P group, with values approaching those of the control group (PBS + P). These findings indicate pronounced neuroprotective, anti-inflammatory, and anti-hypoxic effects. The reduction in p38-MAPK levels further suggests a potential protective effect on axonal transport in retinal ganglion cells.

5. Discussion

5.1. Achieved results

Our findings clearly demonstrated that the developed combined herbal eye drops formulation, containing three plant-derived active compounds, exerts significant IOP-lowering, anti-inflammatory, antioxidant and neuroprotective effects in the microbead-induced glaucoma rat model.

Our experiments showed that the combined formulation effectively attenuated the elevation of IOP from the first week onward, preserved both the functional and structural integrity of the retina (ERG, OCT), enhanced retinal ganglion cell survival, and reduced the activation of inflammatory, apoptotic and hypoxia-related signalling pathways (Western blot, immunohistochemistry). Overall, the combined formulation demonstrated superior efficacy compared with the individual components used alone, indicating a synergistic interaction among the herbal constituents.

5.2. Effect of the herbal eye drops on IOP

The IOP-lowering effect of *F. vulgare* extract had previously been described in a rabbit model in which only transient IOP elevation was induced, and whose methodological reliability was therefore limited [23]. In our study, *F. vulgare* likewise reduced IOP, but did not demonstrate neuroprotective activity. We hypothesize that *F. vulgare* may possess an inherent IOP-lowering effect, potentially via modulation of non-pigmented ciliary body epithelial function (similar to β-blockers) or enhancement of uveoscleral outflow (analogous to prostaglandin analogues), although its exact mechanism of action remains unknown. *H. italicum* extract also reduced IOP but induced corneal haze and conjunctival hyperemia, rendering it unsuitable as monotherapy. The mechanism of action of the combined formulation may partially rely on inhibition of inflammatory mediators (NFκB, IL-6, TNFα) [17–19], similarly to topical corticosteroids used in acute angle-closure glaucoma [5,39,40], yet without their long-term IOP-elevating adverse effects. Through synergism, not only did the therapeutic efficacy increase, but potential side effects were also reduced. This approach may be particularly advantageous for steroid-sensitive (responder) patients. The combined formulation effectively prevented IOP spikes at weeks 1 and 4, whereas the individual components did not. Although its pharmacological mechanism is not yet fully elucidated, previous literature suggests the involvement of anti-inflammatory and antioxidant pathways (NFκB, IL-1β, TNFα, IL-6, IL-8, and PGE2) [3,28]. Overall, the combined herbal eye drops represents a safe,

effective, and well-tolerated alternative for glaucoma management, particularly in cases where the side-effect profile of synthetic agents limits therapeutic options.

5.3. Effect of the herbal eye drops on visual function

In the scotopic ERG recordings used to monitor visual function, the glaucomatous placebo group (Bead + P) exhibited a significant reduction in a- and b-wave amplitudes, whereas the waveforms in the group treated with the combined herbal formulation (Bead + $H_{(X)}$) remained comparable to those of the control groups, demonstrating functional neuroprotection. Although the literature commonly identifies the b-wave as the electrophysiological indicator most sensitive to changes in IOP [41], this was not observed in our study. According to previous reports, the a-wave may be more sensitive to early photoreceptor impairment than the b-wave [42,43], a finding that our results corroborate. Thus, the protective effect of the combined formulation is supported at both structural and functional levels.

5.4. Effect of the herbal eye drops on retinal structure (OCT and native histology)

According to the literature, glaucomatous progression affects not only retinal ganglion cells but the entire vertical retinal pathway, including photoreceptors and the retinal pigment epithelium (RPE) [29,42–44]. Our findings support these observations. The combined formulation exerted protective effects across all retinal layers, whereas the individual components did not provide full-spectrum structural preservation when administered alone.

5.5. Effect of the herbal eye drops on retinal ganglion cell numbers assessed by Brn3a Whole-Mount immunolabeling

Despite its IOP-lowering properties [23], *F. vulgare* extract did not exhibit neuroprotective effects on retinal ganglion cells in our study, reinforcing the concept that glaucoma progression is not solely IOP-dependent. Our findings align with those of Szabó et al., who demonstrated significant ganglion-cell protection in a glaucomatous rat model following PACAP1-38 treatment [29].

5.6. Effect of the herbal eye drops on the retinal vasculature assessed by Isolectin-B4 immunolabeling

According to the literature, human retinal vascular density typically ranges between 53–66% [45,46], yet quantitative assessment in animal models remains challenging, and results are

often inconsistent. Patkó et al. reported approximately a 20% reduction in vascular density in glaucomatous rats, which was mitigated by PACAP1-38 treatment [47]. Our results are consistent with these findings, demonstrating that the combined formulation exerts a protective effect on the retinal microvasculature.

5.7. Biochemical properties of the herbal eye drops assessed by Western Blot protein analysis

GAPDH was used as the internal reference protein for quantitative evaluation of protein expression levels [48,49].

NFκB expression was significantly elevated in the glaucomatous placebo group (Bead + P), whereas it was reduced in the group treated with the combined formulation (Bead + $H_{(X)}$), confirming the NFκB-inhibitory effects of rosmarinic acid and *H. italicum* reported previously [17–19,28]. This observation is consistent with the findings of Amato et al., who demonstrated similar effects using *Mentha spicata* extract [15].

The elevation of GFAP in the glaucomatous condition reflected glial activation, which was normalized by the combined treatment. This agrees with the results of Szabó et al., who observed similar glial modulation following PACAP1-38 therapy [29].

Expression of Bax, a key pro-apoptotic marker, was significantly increased in the glaucomatous placebo group, whereas it was reduced in the Bead + $H_{(X)}$ group, indicating a cytoprotective effect of the combined formulation [50,51].

Analysis of BDNF and CREB expression revealed that the combined formulation promoted neuroplasticity and cell survival, whereas these proteins were abnormally elevated in the glaucomatous placebo group, reflecting activation of stress-response pathways [3,52,53]. Consistent with our findings, Amato et al. demonstrated anti-apoptotic and neuroprotective effects of orally administered *Mentha spicata* extract in a glaucomatous rat model via BDNF induction [15].

HIF1-α levels were significantly elevated in the glaucomatous placebo group, while the combined formulation markedly reduced its expression, suggesting inhibition of hypoxia-induced neovascularization. Immunohistochemical studies have similarly shown increased HIF1-α expression in post-mortem glaucomatous retinal tissue, reflecting hypoxic stress [3,47]. In our study, this effect is likely attributable to the well-established VEGF-inhibitory properties of rosmarinic acid [17–19].

Activation of the p38-MAPK pathway—commonly associated with neurodegenerative conditions—was also observed in glaucomatous animals [54]. The combined treatment

attenuated this activation, potentially contributing to the preservation of axonal transport in retinal ganglion cells.

The inhibitory effects of rosmarinic acid, as well as linalool and geraniol (present in *H. italicum* extracts), on NFκB and p38-MAPK signaling have also been demonstrated by Pandur et al. in a Parkinson's disease model [55], highlighting shared pathogenic mechanisms across neurodegenerative disorders. These components are absent from *F. vulgare* extract, which may explain its weaker neuroprotective profile.

6. Conclusion

Glaucoma is no longer regarded solely as an ocular disease characterized by elevated IOP, but rather as a chronic inflammatory and neurodegenerative disorder. Disease progression involves retinal ganglion cell loss, accumulation of inflammatory mediators, oxidative stress, and disturbances in microcirculation. The aim of our study was to develop a natural, combined herbal eye drops that, beyond reducing IOP, also exhibits anti-inflammatory, antioxidant, and neuroprotective properties.

The eye drops formulated from the combination of three medicinal plants (*Rosmarinus officinalis*, *Foeniculum vulgare*, *Helichrysum italicum*) demonstrated adequate stability and caused no toxic or allergic reactions during the 8-week experimental period. In the microbead-induced glaucomatous rat model, the formulation significantly reduced IOP and maintained values comparable to those of the control groups.

Based on OCT and histological examinations, the combined treatment preserved the structural integrity of all retinal layers, whereas marked degeneration was observed in the placebo group. Isolectin-B4 immunolabeling showed that retinal vascularization remained intact with the combined treatment, while the glaucomatous placebo group exhibited reduced vascular density and signs of ischemia. ERG analyses further confirmed functional preservation under combined therapy.

Western blot analysis revealed that the combined formulation reduced the expression of inflammatory (NFκB, GFAP), apoptotic (Bax), hypoxia- and neovascularization-related (HIF1α) markers, while enhancing the levels of neuroprotective proteins (BDNF, CREB). Attenuation of the p38-MAPK pathway also supported its anti-neurodegenerative profile.

The combination of the three herbal extracts proved significantly more effective than the individual components alone. Owing to its natural origin, favorable tolerability, and

multimodal mechanism of action, the formulation may represent a promising complementary therapeutic approach for human glaucoma and potentially other neurodegenerative diseases (e.g., Parkinson's disease). As the application of phytotherapy in ophthalmology continues to grow, further research is warranted to more precisely elucidate the role and therapeutic potential of natural bioactive compounds.

7. References

1. Artero-Castro, A.; Rodriguez-Jimenez, F.J.; Jendelova, P.; VanderWall, K.B.; Meyer, J.S.; Erceg, S. Glaucoma as a Neurodegenerative Disease Caused by Intrinsic Vulnerability Factors. *Prog. Neurobiol.* **2020**, *193*, 101817, doi:10.1016/j.pneurobio.2020.101817.
2. Ige, M.; Liu, J. Focus: Plant-Based Medicine and Pharmacology: Herbal Medicines in Glaucoma Treatment. *Yale J. Biol. Med.* **2020**, *93*, 347.
3. Kovács-Valasek, A.; Rák, T.; Pöstyéni, E.; Csutak, A.; Gábriel, R. Three Major Causes of Metabolic Retinal Degenerations and Three Ways to Avoid Them. *Int. J. Mol. Sci.* **2023**, *24*, 8728, doi:10.3390/IJMS24108728.
4. Kapetanakis, V. V.; Chan, M.P.Y.; Foster, P.J.; Cook, D.G.; Owen, C.G.; Rudnicka, A.R. Global Variations and Time Trends in the Prevalence of Primary Open Angle Glaucoma (POAG): A Systematic Review and Meta-Analysis. *Br. J. Ophthalmol.* **2016**, *100*, 86–93, doi:10.1136/bjophthalmol-2015-307223.
5. Spaeth, G.L. European Glaucoma Society Terminology and Guidelines for Glaucoma, 5th Edition. *Br. J. Ophthalmol.* **2021**, *105*, 1–169, doi:10.1136/BJOPHTHALMOL-2021-EGSGUIDELINES.
6. Chi, W.; Li, F.; Chen, H.; Wang, Y.; Zhu, Y.; Yang, X.; Zhu, J.; Wu, F.; Ouyang, H.; Ge, J.; et al. Caspase-8 Promotes NLRP1/NLRP3 Inflammasome Activation and IL-1β Production in Acute Glaucoma. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 11181–11186, doi:10.1073/PNAS.1402819111.
7. Radomska-Leśniewska, D.M.; Osiecka-Iwan, A.; Hyc, A.; Góźdź, A.; Dąbrowska, A.M.; Skopiński, P. Therapeutic Potential of Curcumin in Eye Diseases. *Cent. Eur. J. Immunol.* **2019**, *44*, 181–189, doi:10.5114/ceji.2019.87070.
8. Cela, D.; Brignole-Baudouin, F.; Labbé, A.; Baudouin, C. The Trabecular Meshwork in Glaucoma: An Inflammatory Trabeculopathy? *J. Fr. Ophtalmol.* **2021**, *44*, e497–e517, doi:10.1016/J.JFO.2021.09.001.
9. Ayub, J.; Ahmad, J.; Muhammad, J.; Aziz, L.; Ayub, S.; Akram, U.; Basit, I. Glaucoma Detection through Optic Disc and Cup Segmentation Using K-Mean Clustering. *2016 Int. Conf. Comput. Electron. Electr. Eng. ICE Cube 2016 - Proc.* **2016**, 143–147, doi:10.1109/ICECUBE.2016.7495212.
10. Rák, T.; Csutak, A. Complementary Practices in Pharmacy and Their Relation to Glaucoma—Classification, Definitions, and Limitations. *Sci. Pharm.* **2024**, *92*, 16, doi:10.3390/SCIPHARM92010016.
11. Wan, M.J.; Daniel, S.; Kassam, F.; Mutti, G.; Butty, Z.; Kasner, O.; Trope, G.E.; Buys, Y.M. Survey of Complementary and Alternative Medicine Use in Glaucoma Patients. *J. Glaucoma* **2010**, *21*, 79–82, doi:10.1097/IJG.0b013e3182027c0c.
12. Bower, T.N.; Muhsen, S.; Overbury, O.; Birt, C.; Kasner, O. Canadian Ophthalmologists' Opinions Concerning Complementary and Alternative Medicine (CAM) Use in Glaucoma. *J. Glaucoma* **2014**, *23*,

430–434, doi:10.1097/IJG.0B013E31827B139D.

13. Hong, C.T.; Yang, Y.L.; Chen, C.C.; Huang, Y.S.; Chen, C.; Chen, F.A. Intraocular Pressure-Lowering Effect of Cordyceps Cicadae Mycelia Extract in a Glaucoma Rat Model. *Int. J. Med. Sci.* **2021**, *18*, 1007, doi:10.7150/IJMS.47912.
14. Chaudhry, S.; Dunn, H.; Carnt, N.; White, A. Nutritional Supplementation in the Prevention and Treatment of Glaucoma. *Surv. Ophthalmol.* **2022**, *67*, 1081–1098, doi:10.1016/J.SURVOPHTHAL.2021.12.001.
15. Amato, R.; Canovai, A.; Melecchi, A.; Maci, S.; Quintela, F.; Fonseca, B.A.; Cammalleri, M.; Dal Monte, M. Efficacy of a Spearmint (Mentha Spicata L.) Extract as Nutritional Support in a Rat Model of Hypertensive Glaucoma. *Transl. Vis. Sci. Technol.* **2023**, *12*, doi:10.1167/TVST.12.11.6.
16. Karhanová, M.; Eliášová, M.; Kuběna, T.; Pešková, H.; Mlčák, P.; Fryšák, Z.; Marešová, K.; Zapletalová, J. ProVens® in the Therapy of Glaucoma and Ocular Hypertension. [ProVens® v Léčbě Glaukomu a Oční Hypertenze]. *Ces. Slov Oftalmol.* **2015**, *71*, 288–292.
17. Nematolahi, P.; Mehrabani, M.; Karami-Mohajeri, S.; Dabaghzadeh, F. Effects of Rosmarinus Officinalis L. on Memory Performance, Anxiety, Depression, and Sleep Quality in University Students: A Randomized Clinical Trial. *Complement. Ther. Clin. Pract.* **2018**, *30*, 24–28, doi:10.1016/J.CTCP.2017.11.004.
18. Neves, J.A.; Neves, J.A.; Oliveira, R. de C.M. Pharmacological and Biotechnological Advances with Rosmarinus Officinalis L. **2018**, *28*, 399–413, doi:10.1080/13543776.2018.1459570.
19. Vieira, L.C.; Moreira, C.P.D.S.; Castro, B.F.M.; Cotta, O.A.L.; Silva, L.M.; Fulgêncio, G.D.O.; Silva-Cunha, A.; Fialho, S.L. Rosmarinic Acid Intravitreal Implants: A New Therapeutic Approach for Ocular Neovascularization. *Planta Med.* **2020**, *86*, 1286–1297, doi:10.1055/A-1223-2525.
20. Ferreira, J.D.L.M.; Chahud, F.; Ramalho, L.N.; Modulo, C.M.; Vieira, L.C.; Reinach, P.S.; Rodrigues, M.D.L.V.; Cunha, A.S.; Paula, J.S. Rosmarinic Acid Suppresses Subconjunctival Neovascularization in Experimental Glaucoma Surgery. *Curr. Eye Res.* **2014**, *40*, 1134–1140, doi:10.3109/02713683.2014.980911.
21. Lindheimer, J.B.; Loy, B.D.; O'Connor, P.J. Short-Term Effects of Black Pepper (Piper Nigrum) and Rosemary (Rosmarinus Officinalis and Rosmarinus Eriocalyx) on Sustained Attention and on Energy and Fatigue Mood States in Young Adults with Low Energy. *J. Med. Food* **2013**, *16*, 765–771, doi:10.1089/JMF.2012.0216.
22. Younes, M.; Aggett, P.; Aguilar, F.; Crebelli, R.; Dusemund, B.; Filipič, M.; Frutos, M.J.; Galtier, P.; Gott, D.; Gundert-Remy, U.; et al. Refined Exposure Assessment of Extracts of Rosemary (E 392) from Its Use as Food Additive. *EFSA J.* **2018**, *16*, doi:10.2903/J.EFSA.2018.5373.
23. Agarwal, R.; Gupta, S.K.; Agrawal, S.S.; Srivastava, S.; Saxena, R. Oculohypotensive Effects of Foeniculum Vulgare in Experimental Models of Glaucoma. *Indian J. Physiol. Pharmacol.* **2008**, *52*, 77–83.
24. Dongare, V.; Kulkarni, C.; Kondawar, M.; Magdum, C.; Haldavnekar, V.; Arvindekar, A. Inhibition of Aldose Reductase and Anti-Cataract Action of Trans-Anethole Isolated from Foeniculum Vulgare Mill. Fruits. *Food Chem.* **2012**, *132*, 385–390, doi:10.1016/j.foodchem.2011.11.005.
25. Imran, A.; Xiao, L.; Ahmad, W.; Anwar, H.; Rasul, A.; Imran, M.; Aziz, N.; Razzaq, A.; Arshad, M.U.; Shabbir, A.; et al. Foeniculum Vulgare (Fennel) Promotes Functional Recovery and Ameliorates Oxidative

Stress Following a Lesion to the Sciatic Nerve in Mouse Model. *J. Food Biochem.* **2019**, *43*, doi:10.1111/jfbc.12983.

26. Maksimovic, S.; Tadic, V.; Skala, D.; Zizovic, I. Separation of Phytochemicals from Helichrysum Italicum: An Analysis of Different Isolation Techniques and Biological Activity of Prepared Extracts. *Phytochemistry* **2017**, *138*, 9–28, doi:10.1016/j.phytochem.2017.01.001.
27. Sala, A.; Recio, M. del C.; Giner, R.M.; Máñez, S.; Tournier, H.; Schinella, G.; Ríos, J.-L. Anti-Inflammatory and Antioxidant Properties of Helichrysum Italicum . *J. Pharm. Pharmacol.* **2002**, *54*, 365–371, doi:10.1211/0022357021778600.
28. Antunes Viegas, D.; Palmeira-De-Oliveira, A.; Salgueiro, L.; Martinez-De-Oliveira, J.; Palmeira-De-Oliveira, R. Helichrysum Italicum: From Traditional Use to Scientific Data. *J. Ethnopharmacol.* **2014**, *151*, 54–65, doi:10.1016/j.jep.2013.11.005.
29. Szabo, E.; Patko, E.; Vaczy, A.; Molitor, D.; Csutak, A.; Toth, G.; Reglodi, D.; Atlasz, T. Retinoprotective Effects of PACAP Eye Drops in Microbead-Induced Glaucoma Model in Rats. *Int. J. Mol. Sci.* **2021**, *22*, doi:10.3390/IJMS22168825.
30. Sappington, R.M.; Carlson, B.J.; Crish, S.D.; Calkins, D.J. The Microbead Occlusion Model: A Paradigm for Induced Ocular Hypertension in Rats and Mice. *Invest. Ophthalmol. Vis. Sci.* **2010**, *51*, 207–216, doi:10.1167/IOVS.09-3947.
31. Kovacs, K.; Vaczy, A.; Fekete, K.; Kovari, P.; Atlasz, T.; Reglodi, D.; Gabriel, R.; Gallyas, F.; Sumegi, B. PARP Inhibitor Protects Against Chronic Hypoxia/Reoxygenation-Induced Retinal Injury by Regulation of MAPKs, HIF1α, Nrf2, and NFκB. *Invest. Ophthalmol. Vis. Sci.* **2019**, *60*, 1478–1490, doi:10.1167/IOVS.18-25936.
32. Kvarik, T.; Mammel, B.; Reglodi, D.; Kovacs, K.; Werling, D.; Bede, B.; Vaczy, A.; Fabian, E.; Toth, G.; Kiss, P.; et al. PACAP Is Protective in a Rat Model of Retinopathy of Prematurity. *J. Mol. Neurosci.* **2016**, *60*, 179–185, doi:10.1007/S12031-016-0797-5.
33. Sotocinal, S.G.; Sorge, R.E.; Zaloum, A.; Tuttle, A.H.; Martin, L.J.; Wieskopf, J.S.; Mapplebeck, J.C.S.; Wei, P.; Zhan, S.; Zhang, S.; et al. The Rat Grimace Scale: A Partially Automated Method for Quantifying Pain in the Laboratory Rat via Facial Expressions. *Mol. Pain* **2011**, *7*, 55, doi:10.1186/1744-8069-7-55.
34. Silva, B.; Gonçalves, L.M.; Braz, B.S.; Delgado, E. Topical Administration of a Nanoformulation of Chitosan-Hyaluronic Acid-Epoetin Beta in a Rat Model of Glaucoma. *Pharm.* **2023**, *16*, 164, doi:10.3390/PH16020164.
35. Hegarty, D.M.; Carroll, J.R.; Nguyen, D.; Halls, V.S.; Robbins, D.I.; Price, T.J.; Dussor, G.; Aicher, S.A. Resveratrol Increases Tear Production and Spontaneous Ocular Pain after Corneal Abrasion in Male, but Not Female, Rats Using a Preclinical Model of Photorefractive Keratectomy (PRK). *bioRxiv* **2022**, doi:10.1101/2022.01.25.477730.
36. Schreiber, W.; Olbrisch, A.; Vorwerk, C.K.; König, W.; Behrens-Baumann, W. Combined Topical Fluconazole and Corticosteroid Treatment for Experimental Candida Albicans Keratomycosis. *Invest. Ophthalmol. Vis. Sci.* **2003**, *44*, 2634–2643, doi:10.1167/IOVS.02-1135.
37. Leal, A.F.G.; Leite, M.C.; Medeiros, C.S.Q.; Cavalcanti, I.M.F.; Wanderley, A.G.; Santos Magalhães, N.S.; Neves, R.P. Antifungal Activity of a Liposomal Itraconazole Formulation in Experimental Aspergillus Flavus Keratitis with Endophthalmitis. *Mycopathologia* **2015**, *179*, 225–229,

doi:10.1007/S11046-014-9837-2.

38. Kovacs, A.; Atlasz, T.; Werling, D.; Szabo, E.; Reglodi, D.; Toth, G.K. Stability Test of PACAP in Eye Drops. *J. Mol. Neurosci.* **2021**, *71*, 1567, doi:10.1007/S12031-020-01532-9.
39. Sato, K.; Nishiguchi, K.M.; Maruyama, K.; Moritoh, S.; Fujita, K.; Ikuta, Y.; Kasai, H.; Nakazawa, T. Topical Ocular Dexamethasone Decreases Intraocular Pressure and Body Weight in Rats. *J. Negat. Results Biomed.* **2016**, *15*, 1–6, doi:10.1186/S12952-016-0048-X
40. Huang, W.; Li, X.; Gao, K.; Zhang, X. Combined Subconjunctival Injection of Dexamethasone for the Management of Acute Primary Angle Closure: A Randomised Controlled Trial. *Br. J. Ophthalmol.* **2020**, *104*, 87–91, doi:10.1136/BJOPHTHALMOL-2018-313473.
41. Bui, B. V.; He, Z.; Vingrys, A.J.; Nguyen, C.T.O.; Wong, V.H.Y.; Fortune, B. Using the Electroretinogram to Understand How Intraocular Pressure Elevation Affects the Rat Retina. *J. Ophthalmol.* **2013**, 15, doi:10.1155/2013/262467.
42. Bayer, A.U.; Danias, J.; Brodie, S.; Chen, B.; Shen, F.; Podos, S.M.; Mittag, T.W.; Bayer, A.U.; Maag, K.P. Electroretinographic Abnormalities in a Rat Glaucoma Model with Chronic Elevated Intraocular Pressure. *Exp. Eye Res.* **2001**, *72*, 667–677, doi:10.1006/EXER.2001.1004.
43. Guo, L.; Normando, E.M.; Nizari, S.; Lara, D.; Francesca Cordeiro, M. Tracking Longitudinal Retinal Changes in Experimental Ocular Hypertension Using the CSLO and Spectral Domain-OCT. *Invest. Ophthalmol. Vis. Sci.* **2010**, *51*, 6504, doi:10.1167/IOVS.10-5551.
44. Mangan, B.G.; Al-Yahya, K.; Chen, C. Te; Gionfriddo, J.R.; Powell, C.C.; Dubielzig, R.R.; Ehrhart, E.J.; Madl, J.E. Retinal Pigment Epithelial Damage, Breakdown of the Blood-Retinal Barrier, and Retinal Inflammation in Dogs with Primary Glaucoma. *Vet. Ophthalmol.* **2007**, *10 Suppl 1*, 117–124, doi:10.1111/J.1463-5224.2007.00585.X.
45. Liu, G.; Keyal, K.; Wang, F. Interocular Symmetry of Vascular Density and Association with Central Macular Thickness of Healthy Adults by Optical Coherence Tomography Angiography. *Sci. Reports* **2017**, *7*, 1–9, doi:10.1038/s41598-017-16675-w.
46. You, Q.S.; Chan, J.C.H.; Ng, A.L.K.; Choy, B.K.N.; Shih, K.C.; Cheung, J.J.C.; Wong, J.K.W.; Shum, J.W.H.; Ni, M.Y.; Lai, J.S.M.; et al. Macular Vessel Density Measured With Optical Coherence Tomography Angiography and Its Associations in a Large Population-Based Study. *Invest. Ophthalmol. Vis. Sci.* **2019**, *60*, 4830–4837, doi:10.1167/IOVS.19-28137.
47. Patko, E.; Szabo, E.; Vaczy, A.; Molitor, D.; Tari, E.; Li, L.; Csutak, A.; Toth, G.; Reglodi, D.; Atlasz, T. Protective Effects of Pituitary Adenylate-Cyclase-Activating Polypeptide on Retinal Vasculature and Molecular Responses in a Rat Model of Moderate Glaucoma. *Int. J. Mol. Sci.* **2023**, *24*, 13256, doi:10.3390/IJMS241713256.
48. Nie, X.; Li, C.; Hu, S.; Xue, F.; Kang, Y.J.; Zhang, W. An Appropriate Loading Control for Western Blot Analysis in Animal Models of Myocardial Ischemic Infarction. *Biochem. Biophys. Reports* **2017**, *12*, 108, doi:10.1016/J.BBREP.2017.09.001.
49. Ganapathy-Kanniappan, S. Analysis of GAPDH – Native Protein. *Adv. GAPDH Protein Anal. A Funct. Biochem. Approach* **2017**, 17–26, doi:10.1007/978-981-10-7342-7_3.
50. Shen, Y.; Zhao, H.; Wang, Z.; Guan, W.; Kang, X.; Tai, X.; Sun, Y. Silibinin Declines Blue Light-Induced Apoptosis and Inflammation through MEK/ERK/CREB of Retinal Ganglion Cells. **2019**, *47*, 4059–4065,

doi:10.1080/21691401.2019.1671430.

51. Risner, M.L.; Pasini, S.; McGrady, N.R.; Calkins, D.J. Bax Contributes to Retinal Ganglion Cell Dendritic Degeneration During Glaucoma. *Mol. Neurobiol.* **2022**, *59*, 1366–1380, doi:10.1007/S12035-021-02675-5

52. Wang, H.; Xu, J.; Lazarovici, P.; Quirion, R.; Zheng, W. CAMP Response Element-Binding Protein (CREB): A Possible Signaling Molecule Link in the Pathophysiology of Schizophrenia. *Front. Mol. Neurosci.* **2018**, *11*, 255, doi:10.3389/FNMOL.2018.00255.

53. Miranda, M.; Morici, J.F.; Zanoni, M.B.; Bekinschtein, P. Brain-Derived Neurotrophic Factor: A Key Molecule for Memory in the Healthy and the Pathological Brain. *Front. Cell. Neurosci.* **2019**, *13*, 363, doi:10.3389/FNCEL.2019.00363

54. Lambert, W.S.; Pasini, S.; Collyer, J.W.; Formichella, C.R.; Ghose, P.; Carlson, B.J.; Calkins, D.J. Of Mice and Monkeys: Neuroprotective Efficacy of the P38 Inhibitor BIRB 796 Depends on Model Duration in Experimental Glaucoma. *Sci. Rep.* **2020**, *10*, doi:10.1038/S41598-020-65374-6.

55. Pandur, E.; Major, B.; Rák, T.; Sipos, K.; Csutak, A.; Horváth, G. Linalool and Geraniol Defend Neurons from Oxidative Stress, Inflammation, and Iron Accumulation in In Vitro Parkinson's Models. *Antioxidants* **2024**, *13*, 917, doi:10.3390/ANTIOX13080917

8. List of publications

Publications forming the basis of the dissertation:

1. **Rák, T**.; Patkó, E.; Szabó, E.; Váczy, A.; Molitor, D.; Reglődi, D.; Csutak, A.; Atlasz, T. *Combined Herbal Eye Drops Exhibit Neuroprotective and Intraocular Pressure-Reducing Effects in a Glaucoma Rat Model*. *Antioxidants* **2025**, *14*, 549, doi:10.3390/ANTIOX14050549. (IF: 6,6)
2. **Rák, T**.; Csutak, A. Complementary Practices in Pharmacy and Their Relation to Glaucoma—Classification, Definitions, and Limitations. *Sci. Pharm.* **2024**, *92*, 16, doi:10.3390/SCIPHARM92010016. (IF: 2,5)

Other scientific publications:

3. Werling, D.; **Rák, T**; Kanász, G; Göbl, A.; Borbély, G.; Csutak, A. *Exploring hyperbaric oxygen therapy for central retinal artery occlusion beyond 24 h: case report*. Ther Adv Ophthalmol. 2026, doi: 10.1177/25158414251405382. (IF: 2,3)
4. **Rák, T.**; Hegedűs, A.; Molnár, E.; Csutak, A. *Translation and Cultural Adaptation of the I-CAM-Q: The First Hungarian Version for Assessing Complementary and Alternative Medicine Use.* BMC Complement. Med. Ther. 2025, doi:10.1186/S12906-025-05220-2. (IF: 3,4)
5. **Rák, T**.; Hargitai, R.; Sonnevend, Á.; Csutak, A.; Szalai, E. *Beauveria Bassiana Keratitis - the First Hungarian Case Report.* BMC Ophthalmol. 2025, *25*, doi:10.1186/S12886-025-04493-Y. (IF: 1,7)
6. **Rák, T**., Csutak, A. *Centella asiatica* kivonat szerepe a szemhéj plazma szublimáció utókezelésében. Farmakognóziai Hírek, 2025, 20(74), 19–23. (IF nem elérhető)
7. Pandur, E.; Heilmann, L.; Szilágyi-Utczás, M.; **Rák, T**.; Sipos, K.; Csutak, A.; Horváth, G. *Sweet Orange Essential Oil and (+)-Limonene Prevent Oxidative Stress, Reduce Inflammation, and Apoptosis in Differentiated SH-SY5Y Neuroblastoma/BV-2 Microglia Co-Culture Neurodegeneration Models*. BMC Complement. Med. Ther. 2025, *26*, 14, doi:10.1186/S12906-025-05215-Z. (IF: 3,4)
8. **Rák, T**.; Csutak, A. *Reevaluating the Safety of Chamomile Poultices in Ophthalmic Care*. Front. Pharmacol. 2025, *16*, PMID: 1580586, doi:10.3389/fphar.2025.1580586 (IF: 4,8)
9. **Rák, T**.; Kovács-Valasek, A.; Pöstyéni, E.; Gábriel, R.; Csutak, A. *Low Vision Rehabilitation and Eye Exercises: A Comprehensive Guide to Tertiary Prevention of Diabetic Retinopathy*. Life 2025, *15*, 857. https://doi.org/10.3390/life15060857 (IF: 3,4)
10. Pandur, E.; Major, B.; **Rák, T.**; Sipos, K.; Csutak, A.; Horváth, Gy. *Linalool and Geraniol Defend Neurons from Oxidative Stress, Inflammation, and Iron Accumulation in In Vitro Parkinson's Models.* Antioxidants 2024, 13, 917. https://doi.org/10.3390/antiox13080917 (IF: 6,6)
11. **Rák T.**, Csutak, A. *Exploring novel pharmacological trends: Natural compounds in dry eye disease management*. Acta Pharm. 2024, 74(3):383-404. doi: 10.2478/acph-2024-0028. PMID: 39279530. (IF: 1,4)

12. Csutak, A.; **Rák, T**.; Hámor, A.; Sükösd, A.K.; Kardos, Z.; Halmosi, Á.; Rend, P.; Bátor, Gy. *First Clinical Experiences with PreserFlo^{TM} MicroShunt Implantation. [Első Hazai Klinikai Tapasztalatok PreserFlo^{TM} MicroShunt Implantációval]. Szemeszet* 2024, *161*, 30–37, doi:10.55342/SZEMHUNGARICA.2024.161.1.30. (IF nem elérhető)
13. **Rák, T.**, Csutak, A. *Péczely Ignác öröksége: a colobomától a mesterséges intelligenciáig* [The legacy of Ignác Péczely: from coloboma to artificial intelligence]. Orv Hetil. 2024, 165(22):872-880. doi: 10.1556/650.2024.HO2785. PMID: 38824617. (IF: 0,9)
14. **Rák, T.**, Kovács-Valasek, A., Pöstyéni, E., Csutak, A., Gábriel, R. *Complementary Approaches to Retinal Health Focusing on Diabetic Retinopathy*. Cells. 2023, 12(23):2699. doi: 10.3390/cells12232699. PMID: 38067127; PMCID: PMC10705724. (IF: 5,1)
15. Kovács-Valasek, A., **Rák, T.**, Pöstyéni, E., Csutak, A., Gábriel, R. *Three Major Causes of Metabolic Retinal Degenerations and Three Ways to Avoid Them*. Int J Mol Sci 2023, 24:8728. https://doi.org/10.3390/IJMS24108728 (IF: 4,9)
16. Hámor, A., Markó, R., **Rák, T.**, Csutak, A. *A szteroidterápia hatása az intraocularis nyomásra.* Orv Hetil. 2022, 163(34):1345-1352. doi: 10.1556/650.2022.32548. PMID: 35988086. (IF: 0,6)
17. Tóth, N., Szalai, E., **Rák, T.**, Lillik, V., Nagy, A., Csutak, A. *Reliability and clinical applicability of a novel tear film imaging tool.* Graefes Arch Clin Exp Ophthalmol. 2021, 259(7):1935-1943. doi: 10.1007/s00417-021-05162-8. PMID: 33779800; PMCID: PMC8277647. (IF: 3,535)

Patent applications derived from the dissertation topic:

Rák, T.; Atlasz, T.; Váczy, A.; Csutak, A. *Combinational formulation for therapeutic application* – Patent application, Filing date: 04 February 2025, Application number: P2500037, Hungarian Intellectual Property Office.

Rák, T.; Atlasz, T.; Váczy, A.; Csutak, A. *Combinational formulation for therapeutic application* – International patent application, Filing date: 30 January 2026, Application number: PCT/HU2026/050006, Hungarian Intellectual Property Office.

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H-index: **6**

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