Tropical Journal of Pharmaceutical Research December 2021; 20 (12): 2489-2495
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v20i12.5

Original Research Article

Arbutin mitigates chronic hypertension-induced optic nerve damage in rats via regulation of inflammatory cytokine levels and oxidative stress

Zongshan Fan, Fengqiong Zhao, Hai Huang, Xuewen Huang*
Department of Ophthalmology, Nanchong Central Hospital, The Second Clinical College of North Sichuan Medical College, Nanchong, Sichuan 637000, China

For correspondence:* ***Email:* *gxrjrx@163.com;* ***Tel/Fax:*** *0086-18281701631*

Sent for review: 14 March 2020 *Revised accepted: 2 December 2021*

Abstract

Purpose: *To investigate the effect of arbutin on chronic hypertension-induced optic nerve damage in rats, and the mechanism(s) of action involved.*
Methods: *Adult Sprague Dawley rats (n = 32) of both sexes weighing 200 - 230 g (mean weight = 215 ± 15 g) were randomly assigned to 4 groups (8 rats/group): control group, chronic hypertension glaucoma (CHG) group, 50 mg arbutin/kg body weight group, and 100 mg arbutin/kg group. Intraocular hypertension was produced in the right eye of each rat via ischemia/reperfusion. Intraocular pressure (IOP), retinal ganglion cell (RGC) survival and histopathological changes were determined. The levels of interleukin (IL)-1β, IL-6, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and nitric oxide (NO) were assayed using their respective kits. Messenger RNA (mRNA) and protein expressions of pro- and anti-apoptotic factors in retinal tissue were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting, respectively.*
Results: *Intraocular pressure (IOP) was significantly higher in CHG group than in control group, but was significantly and time-dependently reduced by arbutin ($p < 0.05$). Arbutin treatment significantly enhanced the survival of RGCs in retinal tissues of CHG rats ($p < 0.05$. It significantly and dose-dependently lowered the levels of circulating IL-1β, IL-6, NF-kB and NO ($p < 0.05$). Moreover, arbutin significantly and dose-dependently downregulated the mRNA expressions of caspase-3 and caspase-8, and bax and Akt, but upregulated bcl-2 mRNA expression ($p < 0.05$). Similarly, it significantly and dose-dependently downregulated the protein expressions of caspase-3, bax, p-NF-kB, inducible nitric oxide synthase (iNOS) and Akt, while it upregulated bcl-2 protein expression ($p < 0.05$).*
Conclusion: *These results show that arbutin mitigates chronic hypertension-induced optic nerve damage in rats via the regulation of inflammatory cytokine levels and oxidative stress, and therefore can potentially be used for the management of glaucoma.*

Keywords: *Apoptosis, Arbutin, Glaucoma, Intraocular pressure, Neuroprotection*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Glaucoma is a group of eye conditions caused by damage to the optic nerve. This damage is often due to abnormally high pressure in the eye. Glaucoma is a neurodegenerative disease and one of the leading causes of blindness for people over the age of 60 [1]. A study has

predicted that by 2020, approximately 79.7 million people would suffer from glaucoma-induced blindness [2]. This disease is characterized by optic nerve degeneration and loss of RGCs.

The predominant and only modifiable risk factor for glaucoma is elevated IOP [3]. Studies suggest that activation of neuroglial cells causes neuro-inflammation involving an interplay of signaling pathways [4]. In glaucoma, optic nerve injury is caused by increased release of inflammatory cytokines which in turn cause inflammation [5]. It is speculated that the activation of NF-kB pathway in turn activates neuroglial cells and results in the death of RGCs [6]. However, the precise molecular mechanism responsible for the loss of vision in glaucoma is yet to be fully understood. In addition, conventional drugs used for the treatment of glaucoma have limited effectiveness.

Alternative medicine has shown great promise in the treatment of chronic disorders. Arbutin is a glycosylated hydroquinone isolated from bearberry plant which belongs to in the genus *Arctostaphylos* [7]. Arbutin has been reported to possess alpha-amylase and alpha-glucosidase inhibitory properties, as well as gastroprotective, antioxidant, antihyperlipidemic, antihyperglycemic and anti-inflammatory effects [8-11]. It protects against osteoporosis via mechanisms involving the inhibition of osteoclast proliferation and regulation of RANKL/NF-kB pathway [12]. The aim of this study was to investigate the effect of arbutin on chronic hypertension-induced optic nerve damage, and the mechanism(s) involved.

EXPERIMENTAL

Rats

Adult Sprague Dawley rats (n = 32) of both sexes weighing 200 - 230 g (mean weight = 215 ± 15 g) were used in this study. The rats were housed in metal cages under standard conditions and allowed free access to standard feed and water. Prior to commencement of study, the rats were acclimatized to the laboratory environment for 3 days. Thereafter, they were exposed to 12-h light/12 h dark cycle, and maintained at an average temperature of 24 ± 3 °C, and 55 – 65 % humidity. The study protocol was approved by the Institutional Animal Care and Use Committee of The Second Clinical College of North Sichuan Medical College, China (no. IAEC/SCC-NSMC/2017/05). The procedures used were carried out according to the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) [13].

Establishment of rat model of intraocular hypertension

The rats were randomly assigned to 4 groups (8 rats/group): control group, CHG group, 50 mg arbutin/kg bwt group, and 100 mg arbutin/kg bwt group. Intraocular hypertension was produced in the right eye of each rat via ischemia/reperfusion as described in previous studies [14]. Normal rats served as control. The rats were anesthetized with pentobarbitone (100 mg/kg bwt, i.p.) and topical application of 0.5 % alcaine eye drop. Tropicamide (1 %) was used to dilate the pupils. The anterior chamber of the right eye was cannulated using 30-gauge needle. This procedure was performed by maintaining IOP of 70 mmHg for 1 h. The IOP was normalized after needle withdrawal. The IOP was measured after vein cauterization using Tonopen XL tonometer. Rats in the treatment group received arbutin at doses of 50 and 100 mg/kg bwt subcutaneously for a period of 5 weeks, and IOP was measured on weekly basis.

Determination of RGC survival

Retina was isolated from each rat and washed with phosphate-buffered saline (PBS) containing 30 % sucrose. Topographic analysis of Rbpms immunolabeling was performed on retinal wholemounts. Polyclonal goat anti-brain-specific homeo box/POU domain protein 3a (Brn3a) antibody was incubated with retinal tissue at 4 °C for 3 days. The retina tissues were thereafter washed with PBS and incubated for 4 h with secondary antibody. A fluorescence microscope was used to examine retinal images to determine loss of RGCs. The surviving RGCs were counted at periphery, middle and center of each tissue sample.

Nitric oxide (NO) assay

The level of NO in retinal tissue was determined using standard method [15]. Retinal tissue homogenate (20 %) was prepared in isotonic KCl solution using a mechanical homogenizer. The homogenate was centrifuged at 12,000 rpm for 10 min to obtain supernatant which was then reacted with 1 % sulfanilamide and 1 % N-(1-napthylethylenediamine dihydrochloride) for 10 min at room temperature. The absorbance of the solution was read at 550 nm in a microplate reader, and the corresponding NO level was extrapolated from standard NO calibration curve.

Histopathological examination of retinal tissue

Portions of the retina were serially sectioned and fixed in Davidson's solution containing 12.5 % acetic acid, 9.3 % paraformaldehyde and 37.5 % ethanol for 24 h. The specimen was then dehydrated through graded series of alcohol and cleared in three changes of xylene before being embedded in paraffin. Serial sections, each 4-µm thick, were cut using a microtome, and the sections were stained with hematoxylin and eosin (H&E) according to standard procedures. The sections were examined under the light microscope and photographed. The retinal ganglion cells were counted using an image analyzer (Image Proplus 3.0).

Determination of levels of inflammatory cytokines

The levels of IL-1β, IL-6 and NF-kB were determined using their respective commercial enzyme-linked immunosorbent assay (ELISA) kits.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The mRNA expressions of caspases-3, caspase-8, bax, bcl-2 and Akt were determined using qRT-PCR. Trizol RNA extraction reagent was used to extract total RNA from cell suspension resulting from the trypsinization of retinal tissue, while cDNA synthesis kit was used to perform cDNA synthesis reaction according to the instructions of the manufacturer. Light Cycler 1536 RT-PCR detection system was used for the estimation of the expression levels of the genes. Variation in the cDNA content was normalized using β-actin. The qRT-PCR reaction conditions were: pre-denaturation at 95 °C for 5 min, PCR reaction at 95 °C for 5 sec and 60 °C for 30 sec, and a total of 40 cycles. The PCR reaction mixture (20 µL) consisted of 6.4 µL of dH_2O, 1.6 µL of gene-specific primer (10 µM), 2 µL of synthesized cDNA, and 10 µL of SYBR Premix Ex Taq™ II. The Ct value of U6 was taken as the internal parameter, and $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression levels of the genes.

Western blotting

Cell suspension resulting from trypsinization of lung tissues was washed twice with phosphate-buffered saline (PBS) and lysed with NP40 protein lysis buffer containing protease and phosphatase inhibitors at a volume ratio of 1:5. The resultant lysate was centrifuged at 12,000 rpm for 10 min at 4 °C, and the protein concentration of the supernatant was determined using detergent compactible (DC) protein assay kit. A portion of total cell protein (30 µg) from each sample was separated on a 10 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min.

Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added to the membrane, with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Thereafter, the blots were incubated overnight at 4 °C with primary antibodies (rabbit polyclonal anti- caspase-3, bax, bcl-2, Akt, iNOS, pNF-kB, NF-kB and β-actin, each at a dilution of 1 to 1000). Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 2 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using enhanced chemiluminescence (ECL). The various protein expression levels were normalized to that of β-actin which was used as a standard.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism (6.1). Groups were compared using Student's *t*-test. Statistical significance was assumed at $p < 0.05$.

RESULTS

Effect of arbutin on IOP in rats

Intraocular pressure (IOP) of the CHG group was significantly higher than that of control group, but was significantly and time-dependently reduced after arbutin treatment ($p < 0.05$; Figure 1).

Effect of arbutin on the survival of RGCs

As shown in Figure 2, arbutin significantly enhanced the survival of RGCs in retinal tissues of CHG rats ($p < 0.05$).

Pathological changes in retinal tissues of CHG rats

Rats in control group exhibited normal retina morphology (normal structure and thickness of retinal tissue) with high number of cells. The retinal tissues of CHG rats had a few number of

cells and reduced layer thickness. However, treatment with arbutin significantly reversed the altered structure, thickness and number of cells in retinal tissues of CHG rats (Figure 3 A). Cell number was significantly reduced in CHG group, relative to control group, but was significantly increased after treatment with arbutin ($p < 0.05$; Figure 3 B).

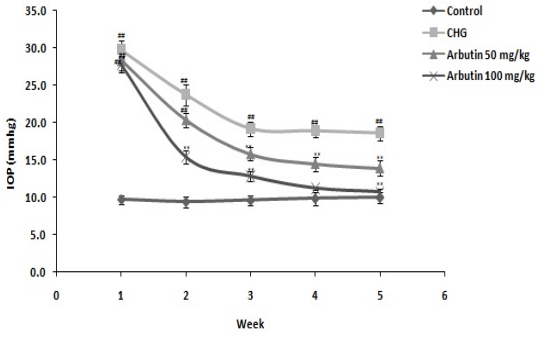

Figure 1: Effect of arbutin on IOP of CHG rats. $^{\#\#}P < 0.05$, compared with control group; $^{**}p < 0.01$, compared with CHG group

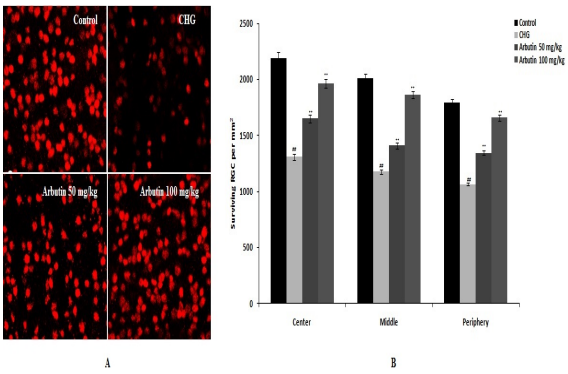

Figure 2: Effect of arbutin on the survival of RGCs in retinal tissues of CHG rats. **(A):** Retinal tissue labeled with Brn3a; **(B):** Surviving RGCs per mm^2. $^{\#\#}P < 0.05$, compared with control group; $^{**}p < 0.01$, compared with CHG group

Effect of arbutin on levels of inflammatory cytokines

Treatment of CHG rats with arbutin led to significant and dose-dependent reductions in the levels of circulating IL-1β, IL-6 and NF-kB ($p < 0.05$; Figure 4).

Effect of arbutin on NO level in retinal tissues of CHG rats

As shown in Figure 5, arbutin treatment significantly and dose-dependently reduced the levels of NO in retinal tissues of CHG rats ($p < 0.05$).

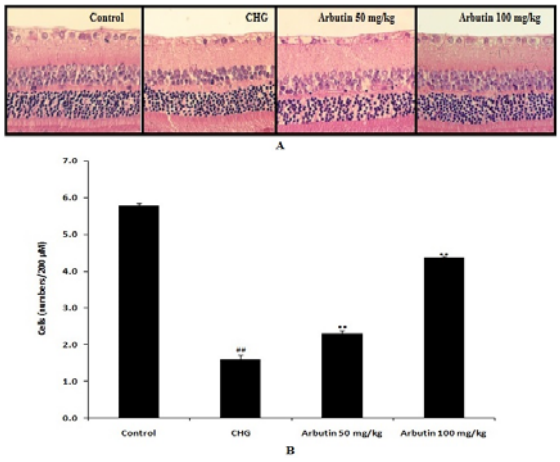

Figure 3: Pathological changes in retinal tissues of CHG rats. **(A):** Hematoxylin and eosin (H & E)-stained retinal tissues. **(B):** Number of cells present in the retina. $^{\#\#}P < 0.05$, compared with control group; $^{**}p < 0.01$, compared with CHG group

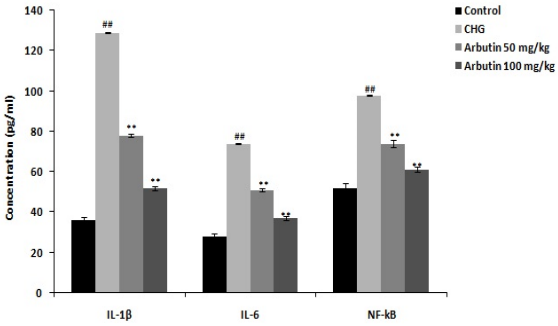

Figure 4: Effect of arbutin on the levels of inflammatory cytokines**.** $^{\#\#}P < 0.05$, compared with control group; $^{**}p < 0.01$, compared with CHG group

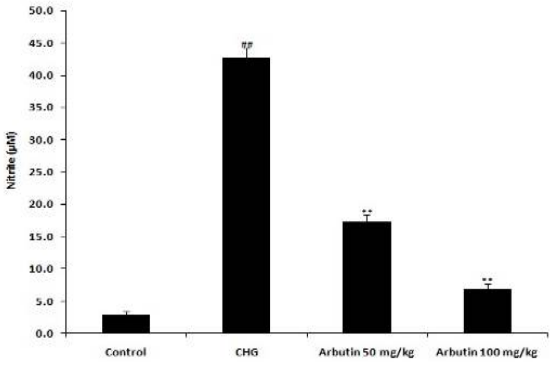

Figure 5: Effect of arbutin on NO level in retinal tissues of CHG rats. $^{\#\#}P < 0.05$, compared with control group; $^{**}p < 0.01$, compared with CHG group

Effect of arbutin on RGC apoptosis

Arbutin treatment significantly and dose-dependently downregulated the mRNA

expressions of caspase-3, caspase-8, bax and Akt, but upregulated bcl-2 mRNA expression ($p < 0.05$; Figure 5A). Moreover, arbutin significantly and dose-dependently downregulated caspase-3 and bax protein expressions, while it significantly upregulated bcl-2 protein expression in a dose-dependent fashion ($p < 0.05$; Figure 5 B and C).

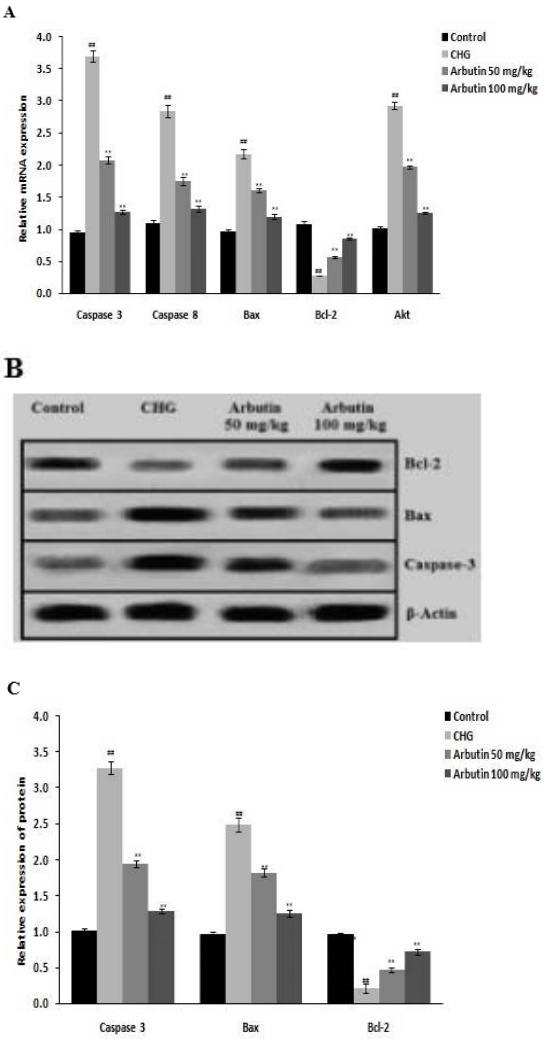

Figure 6: Effect of arbutin on RGC apoptosis. **(A):** Relative mRNA expressions of caspase 3, caspase 8, bax, bcl-2 and Akt in retinal tissue as measured using qRT-PCR; **(B):** Relative expression of caspase 3, bax and bcl-2 proteins as measured using Western blotting. $^{\#\#}p < 0.05$, compared with control group; $^{**}p < 0.01$, compared with CHG group

Effect of arbutin on NF-kB signaling pathway

Treatment of CHG rats with arbutin led to significant and dose-dependent downregulation of the protein expressions of p-NF-kB, iNOS and Akt ($p < 0.05$). These results are shown in Figure 7.

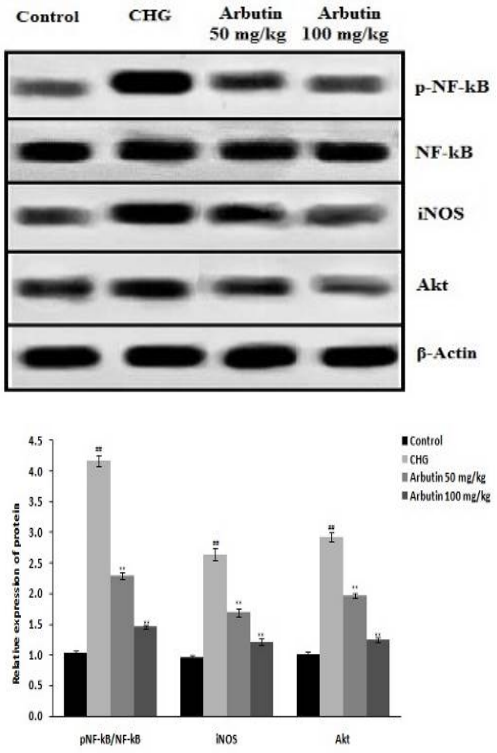

Figure 7: Effect of arbutin on NF-kB signaling pathway. $^{\#\#}P < 0.05$, compared with control group; $^{**}p < 0.01$, compared with CHG group

DISCUSSION

Glaucoma is an ocular disease associated with increased IOP and increased degeneration of RGCs, thereby leading to irreversible loss of vision [16]. Conventional drugs used for the treatment of glaucoma cannot prevent the degeneration of RGCs. A general understanding of the disease with respect to pathophysiology, diagnosis, and treatment may assist primary care physicians in identifying high-risk patients for comprehensive ophthalmologic examination. The reduction of IOP is the only proven method for treating glaucoma. Although treatment is usually initiated with ocular hypotensive drops, laser trabeculoplasty and surgery may also be used to slow down the disease. The present study investigated the effect of arbutin on chronic hypertension-induced optic nerve damage, and the mechanism(s) involved. The results showed that treatment of CHG rats with arbutin significantly and time-dependently reduced their IOP. This suggests that arbutin may confer protection on RGCs.

Apoptosis refers to programmed cell death which that occurs in multicellular organisms. Biochemical events lead to characteristic cell changes and death. These changes include

blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay. Retinal ganglion cell (RGC) apoptosis has been shown to contribute to retina damage in glaucoma due to increased IOP [17]. The increased IOP contributes to the loss of RGCs. In this study, arbutin treatment significantly enhanced the survival of RGCs in retinal tissues of chronic hypertensive glaucoma rats, an indication that the crude drug may possess antioxidant and anti-inflammatory properties. This finding is in agreement with results of previous studies [18].

The concentrations of inflammatory cytokines are elevated in glaucoma [19]. Although the specific triggers for inflammatory responses in glaucoma remain poorly defined, inflammatory processes mediated in part by astrocytes and resident microglia, are clearly implicated in role glaucoma. Inflammatory glaucoma, also known as uveitic glaucoma, is a condition in which ocular inflammation causes persistent or recurrent elevation in IOP, resulting in anatomical and physiological changes characteristic of primary open angle glaucoma. The results of this study showed that treatment of CHG rats with arbutin led to significant and dose-dependent reductions in the levels of circulating IL-1β, IL-6 and NF-kB.

Apoptosis involves an interplay of pro- and anti-apoptotic proteins. The main function of anti-apoptotic bcl-2 proteins is to suppress pro-apoptotic bax and bak, thereby preserving the integrity of the mitochondrial outer membrane. This is achieved by direct binding and sequestration of pro-apoptotic BH3-only proteins which are able to directly or indirectly activate bax/bak. Caspase-3 is a caspase protein that interacts with caspase-8 and caspase-9 [20]. Caspase activation is the hallmark of the mitochondrial pathway of apoptosis which begins with the permeabilization of the mitochondrial outer membrane [21]. The resultant complex promotes the activation of caspase 9, which in turn activates effector caspases that collectively orchestrate the execution of apoptosis [21]. The upregulation of bax and downregulation bcl-2 protein expression also contributes to mitochondrial dysfunction, and ultimately cell apoptosis [22]. In this study, treatment of CHG rats with arbutin significantly reversed the altered levels of pro- and anti-apoptotic factors in rat retinal tissues.

CONCLUSION

The results obtained in this study show that arbutin mitigates chronic hypertension-induced optic nerve damage in rats via the regulation of inflammatory cytokine levels and oxidative stress, and thus may be useful as a therapeutic agent for the management of glaucoma.

DECLARATIONS

Acknowledgement

All the authors are thankful to Nanchong Central Hospital, China for providing necessary facility to conduct this work.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xuewen Huang - conceived and designed the study; Fengqiong Zhao, Hai Huang - collected and analyzed the data; Zongshan Fan wrote the manuscript. All authors read and approved the manuscript for publication.

REFERENCES

1. *Weinreb RN, Aung T, Medeiros FA. The pathophysiology and treatment of glaucoma: a review. JAMA 2014; 311(18): 1901-1911.*
2. *Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. Br J Ophthalmol 2006; 90(3): 262-267.*
3. *Guo L, Moss SE, Alexander RA, Ali RR, Fitzke FW, Cordeiro MF. Retinal ganglion cell apoptosis in glaucoma is related to intraocular pressure and IOP-induced effects on extracellular matrix. Invest Ophthalmol Vis Sci 2005; 46(1): 175-182.*

4. Lian H, Zheng H. Signaling pathways regulating neuron-glia interaction and their implications in Alzheimer's disease. J Neurochem 2016; 136(3): 475-491.
5. Tezel G. Immune regulation toward immunomodulation for neuroprotection in glaucoma. Curr Opin Pharmacol 2013; 13(1): 23-31.
6. Dresselhaus EC, Meffert MK. Cellular Specificity of NF-κB Function in the Nervous System. Front Immunol 2019; 10: 1043.
7. Ştefănescu BE, Szabo K, Mocan A, Crişan G. Phenolic Compounds from Five Ericaceae Species Leaves and Their Related Bioavailability and Health Benefits. Molecules 2019; 24(11): 2046.
8. Shahaboddin ME, Pouramir M, Moghadamnia AA, Parsian H, Lakzaei M, Mir H. Pyrus biossieriana Buhse leaf extract: An antioxidant, anti-hyperglycaemic and antihyperlipidemic agent. Food Chem 2011; 126: 1730-1733.
9. Myagmar B, Shinno E, Ichiba T, Aniya Y. Antioxidant activity of medicinal herb Rhodococcum vitis-idaea on galactosamine-induced liver injury in rats. Phytomed 2004; 11: 416-423.
10. Taha MM, Salga MS, Ali HM, Abdulla MA, Abdelwahab SI, Hadi AH. Gastroprotective activities of Turnera diffusa Willd. ex Schult. revisited: Role of arbutin. J Ethnopharmacol 2012; 141: 273-281.
11. Yousefi F, Mahjoub S, Pouramir M, Khadir F. Hypoglycemic activity of Pyrus biossieriana Buhse leaf extract and arbutin: Inhibitory effects on alpha amylase and alpha glucosidase. Casp J Intern Med 2013; 4: 763-767.
12. Omori A, Yoshimura Y, Deyama Y, Suzuki K. Rosmarinic acid and arbutin suppress osteoclast differentiation by inhibiting superoxide and NFATc1 downregulation in RAW 264.7 cells. Biomed Rep 2015; 3(4): 483-490.
13. Guide for the Care and Use of Laboratory Animals: Eighth Edition Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council. 2010; ISBN: 0-309-15401-4.
14. Varano GP, Parisi V, Adornetto A, Cavaliere F, Amantea D, Nucci C, Corasaniti MT, Morrone LA, Bagetta G, Russo R. Post-ischemic treatment with azithromycin protects ganglion cells against retinal ischemia/reperfusion injury in the rat. Mol Vis 2017; 23: 911-921.
15. Bryan NS, Grisham MB. Methods to detect nitric oxide and its metabolites in biological samples. Free Radic Biol Med. 2007; 43(5): 645-657.
16. Daniel S, Meyer KJ, Clark AF, Anderson MG, McDowell CM. Effect of ocular hypertension on the pattern of retinal ganglion cell subtype loss in a mouse model of early-onset glaucoma. Exp Eye Res 2019; 185: 107703.
17. Rusciano D, Pezzino S, Mutolo MG, Giannotti R, Librando A, Pescosolido N. Neuroprotection in Glaucoma: Old and New Promising Treatments. Advances in Pharmacological Sciences, 2017; 2017: Article ID 4320408.
18. Park BK, Kim YH, Kim YR, Choi JJ, Yang C, Jang IS, Lee MY. Anti-neuroinflammatory and Neuroprotective Effects of Gyejibokryeong-Hwan in Lipopolysaccharide-Stimulated BV2 Microglia, Evid. Based Complementary Altern. Med., 2019; 2019: Article ID 7585896.
19. Chono I, Miyazaki D, Miyake H, Komatsu N, Ehara F, Nagase D, Kawamoto Y, Shimizu Y, Ideta R, Inoue Y. High interleukin-8 level in aqueous humor is associated with poor prognosis in eyes with open angle glaucoma and neovascular glaucoma. Sci Rep 2018; 8(1): 14533.
20. Song H, Fu Y, Wan D, Xia W, Lyu F, Liu L, Shen L. Mytoxin B and Myrothecine A Induce Apoptosis in Human Hepatocarcinoma Cell Line SMMC-7721 via PI3K/Akt Signaling Pathway. Molecules. 2019; 24(12): 2291.
21. Parrish AB, Freel CD, Kornbluth S. Cellular mechanisms controlling caspase activation and function. Cold Spring Harb Perspect Biol. 2013; 5(6): a008672.
22. Hata AN, Engelman JA, Faber AC. The BCL2 Family: Key Mediators of the Apoptotic Response to Targeted Anticancer Therapeutics. Cancer Discov. 2015; 5(5): 475-487.