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Pinus densiflora bark extract prevents selenite-induced cataract formation in the lens of Sprague Dawley rat pups

Jun Kim, Se-Young Choung

Department of Life and Nanopharmaceutical Sciences, Graduate School, Kyung Hee University, Kyungheedae-ro, Dongdaemun-gu, Seoul, Korea

Purpose: Rat pups treated with sodium selenite are typically used as an in vivo model to mimic age-related nuclear cataract. Reactive oxygen species (ROS) production, lipid peroxidation, reduction of antioxidant enzymes, crystalline proteolysis, and apoptosis are considered factors that contribute to pathogenesis of age-related nuclear cataract. In the present study, we investigated whether *Pinus densiflora* bark extract has potential to prevent cataract formation and elucidated the underlying mechanism.

Methods: Sprague Dawley rats were divided into six groups (n=10). Group 1 rat pups (the control) were treated with only normal saline. The rat pups in groups 2 to 6 were given a subcutaneous injection with sodium selenite (18 µmol/kg bodyweight) on postnatal (P) day 10. Group 3 rat pups (the positive control) were given gastric intubation with curcumin (80 mg/kg bodyweight) on P9, P10, and P11. The rat pups in groups 4 to 6 were given gastric intubation with *P. densiflora* bark extract 40 mg/kg, 80 mg/kg, and 120 mg/kg, respectively, on P9, P10, and P11.

Results: This study showed that *P. densiflora* bark extract dose-dependently prevented cataract formation. Water-soluble protein, glutathione, superoxide dismutase, glutathione peroxidase, and catalase activity levels were found to be high, and conversely, water-insoluble protein, malondialdehyde, and Ca^{2+}-ATPase were found to be low in the groups treated with *P. densiflora* bark extract compared to group 2. Real-time PCR analysis showed αA-crystalline, lens-specific m-calpain (*Lp84*), lens-specific intermediates (filensin and phakinin), and antiapoptotic factor (*Bcl-2*) were downregulated, and the apoptotic factors (caspase-3 and Bax) and plasma membrane Ca^{2+}-ATPase (*PMCA-1*) were upregulated in group 2 compared to group 1. P. densiflora bark extract regulated the imbalance of these genes. The increased cleavage form of caspase-3 was lowered in the groups treated with *P. densiflora* bark extract. In conclusion, *P. densiflora* bark extract prevented selenite-induced cataract formation via regulating antioxidant enzymes, inhibiting *m*-calpain-induced proteolysis, and apoptosis, and thus, maintained the transparency of the lens.

Conclusions: These results suggested that *P. densiflora* bark extract could be a new agent for preventing age-related nuclear cataract.

The eye is susceptible to oxidative damage due to intense light exposure, robust metabolic activity, high oxygen levels, and tension [1]. With aging, accumulation of oxidized lens components and decline of antioxidant systems contribute to the eye becoming prooxidant [2]. A prooxidant state has been considered the major factor of severe ocular diseases, such as cataract, macular degeneration, diabetic retinopathy, and retinitis pigmentosa [3]. Among these diseases, cataract is the primary cause of visual loss and the major cause of visual impairment in the elderly [4]. The treatment of cataract involves surgical removal of the old lens and implantation of a new artificial intraocular lens. Even though the surgery is known for being cost-effective while also increasing quality of life and productivity in patients, it is still suboptimal in developing countries, and a large backlog of cataract cases continues to grow [5]. The postoperative vision outcomes can also have high complication rates, including posterior capsule opacification, detached retina, cystoid macular edema, and double vision [6]. In the case of the elderly, underlying diseases, such as renal dysfunction and diabetes, should be considered before or after cataract surgery [7,8]. To decrease the burden of surgery, preventive interventions of lens opacification are continually sought.

Age-related cataract is characterized by reactive oxygen species (ROS) production, reduction of endogenous antioxidants, lipid peroxidation, biochemical changes in crystalline, abnormal cross-linked proteins, activation of m-calpain, apoptosis of lens epithelial cells, etc [3,9]. In the aging process, intracellular ROS production damages not only membrane transport proteins, lipids, and other intermediates but also mitochondrial DNA. This damage leads to mitochondrial dysfunction, cell apoptosis, consequent ROS production, and decline of cellular antioxidant capacity [10,11]. The activity of Ca^{2+}-ATPase, a transport protein in the cell membrane, is decreased by oxidative stress, followed by

Correspondence to: Se-Young Choung, Department of Life and Nanopharmaceutical Sciences, Graduate School, Kyung Hee University, 26, Kyungheedae-ro, Dongdaemun-gu, Seoul, 02447, Korea; Phone:+82-2-961-9198; FAX: +82-2-961-0372; email: sychoung@khu.ac.kr

calcium accumulation and the activation of m-calpain. The increased calpain activity causes proteolysis, coprecipitation, and insolubilization of crystallines, which then leads to cataract light scattering [12]. An overdose of sodium selenite induces oxidative damage causing cataract formation in rat pups. The selenite rat model partially mimics age-related nuclear cataract and has several similarities to human cataract, e.g., decline of Ca^{2+}-ATPase activity, antioxidant enzymes and water-soluble proteins, calpain activation, proteolysis of crystalline, and disruption of the cytoskeleton [13,14]. Although the selenite cataract model is not the same as human cataract, this model is still a useful in vivo model for initial screening of potential anticataract agents.

Korean red pine (*Pinus densiflora*) is widely distributed in Asian countries, such as Korea, Japan, and China. Various parts of *P. densiflora*, including the cortices, cones, bark, pollen, and needles, have been used as traditional medicine in many diseases. Among them, the bark of *P. densiflora* has been used to relieve cough, chest pain, abscess, small pox, melena, and dysentery. According to Boncho Gangmok, a Korean key reference book for the study of Eastern medicine, *P. densiflora* is effective in hemostasis and anti-inflammation. Recently, many studies have found new effects of *P. densiflora* bark in several diseases. Similar to the French maritime pine bark extract (commercially called Pycnogenol), the Korean red pine bark extract contains similar amounts of procyanidins and polyphenols (catechin and taxifolin) according to high-performance liquid chromatography (HPLC) analyses [15-17]. Procyanidins and polyphenols are well known antioxidants [18,19]. Many studies indicated that the French maritime pine bark extract has protective effects against oxidative stress through scavenging of free radicals and increasing synthesis of antioxidative enzymes [20]. However, few studies have investigated antioxidant and preventive effects of *P. densiflora* and French maritime pine bark extract in ocular diseases. Both pine bark extracts have been little studied for preventive effects on age-related cataract. To verify the efficacy of *P. densiflora* bark extract on cataract formation, we used curcumin as the positive control. Previous studies have indicated that curcumin has antioxidant effects and other pharmacological properties. Curcumin also has therapeutic and preventive effects in ocular diseases, such as age-related cataract, age-related macular degeneration, diabetic retinopathy, and glaucoma [21,22]. Using the selenite-induced cataract model, we investigated the effects of *P. densiflora* bark extract on lens opacification, the antioxidant system, lipid peroxidation, and regulation of mRNA expression in the lens.

METHODS

Materials and chemicals: Pinus densiflora bark was collected from 30-year-old *Pinus densiflora Sieb.* and *Zucc.* in Samcheok (Gangwon-do, Republic of Korea), sliced, and crushed into a fine powder. Then, extraction was performed using 60% EtOH in 50 °C for 3 h. The extraction step was repeated at least twice. After the filtrate was evaporated under low pressure, the extract was spray-dried. To remove the nonpolar materials, 3 h filtration with alcohol was performed at 30 °C. The filtrate was dried in 50 °C for 3 h to obtain the *Pinus Densiflora* bark powder. The content of taxifolin, procyanidin B1 and (+)-catechin were determined by HPLC, as described previously [17]. Curcumin (≥90%) was obtained from Carl Roth GmbH + Co. (Karlsruhe, Germany). Sodium selenite (Na_2SeO3, ≥98%) was obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents used in the experiment were of analytical grade.

Animals: Sprague-Dawley (SD) rat pups, aged 6 days, were obtained from DBL (Cheongju, Republic of Korea). Sixty rat pups weighing 16–19 g were housed along with their mothers in normal cages and maintained at 25±1 °C with a 12 h:12 h light-dark cycle. The mothers were fed a standard laboratory animal diet and water ad libitum. The experiments involving handling the animals were conducted in strict accordance with ARVO guidelines, and the protocol was approved by the Institutional Animal Care and Use Committee guideline of Kyung Hee university (KHUASP(SE)-16–071).

Design: The rat pups were randomly divided into six groups (10 rat pups in each group) as follows.

Group 1: control animals treated with only normal saline.

Group 2: animals treated with sodium selenite (18 µmol/kg bodyweight).

Group 3: animals treated with sodium selenite and curcumin (80 mg dry extract/kg bodyweight).

Group 4: animals treated with sodium selenite and *P. densiflora* bark extract (40 mg dry extract/kg bodyweight).

Group 5: animals treated with sodium selenite and *P. densiflora* bark extract (80 mg dry extract/kg bodyweight).

Group 6: animals treated with sodium selenite and *P. densiflora* bark extract (120 mg dry extract/kg bodyweight).

Group 2 to 6 animals were given subcutaneous (SC) injection of 18 µmol/kg sodium selenite on P10. Group 1 animals were given an equivalent amount of normal saline, SC. Groups 3 to 6 were given gastric intubation of each sample from P9 to P11 while groups 1 and 2 were given normal saline. On P16, cataract formation was evaluated with an ophthalmoscope, and lens opacification was observed with

and without a white-light lamp. The rat pups were sacrificed by cervical dislocation, and the eye lenses were obtained with the posterior approach on P30. Dosage was determined according to Manikandan et al. [23]. Curcumin prevented cataractogenesis at a concentration of more than 75 mg/kg bodyweight [23]. The 80 mg/kg bodyweight was selected as the dosage for the curcumin and *P. densiflora* bark extract.

Evaluation of cataract staging: : Cataract staging was evaluated on P16 and determined based on a scale of 0 to 6 according to Hiraoka et al. [24], as follows:

stage 0: normal transplant lens

stage 1: initial sign of nuclear opacity, little light scattering is observed with a white-light lamp

stage 2: little light scattering with the naked eye, slight nuclear opacity, including swollen fibers or posterior subcapsular scattering with a white-light lamp

stage 3: diffuse nuclear opacity with the naked eye

stage 4: partial nuclear opacity with the naked eye

stage 5: dense opacity that does not involve the lens cortex

stage 6: mature dense opacity.

Lens homogenate preparation and protein estimation: : One of both eyes was used to measure proteins, glutathione (GSH) and malondialdehyde (MDA) levels, and antioxidant enzymes. The resected lens tissues were rinsed with ice-cold PBS (1X; 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4), blotted, weighted. The lens tissues were homogenized (10% w/v) in ice-cold PBS (pH 7.4) and centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant contained water-soluble proteins and enzymes. The pellet was dissolved in sodium hydroxide and rehomogenized. Then, the pellet was centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant contained water-insoluble proteins. Water soluble and insoluble proteins were measured with the Pierce BCA protein assay kit from Thermo Fisher Scientific Co. (catalog#23225, Fair Lawn, NJ). The results were expressed as nanograms of protein per milligrams of lens tissue a using standard concentration of BSA (BSA; 25–2,000 mM).

Estimation of Ca^{2+}-ATPase activity: Ca^{2+}-ATPase activity was determined in the supernatant that contained water-soluble proteins. Ca^{2+}-ATPase activity was estimated with the Ca^{2+}-ATPase ELISA kit from MyBioSource (catalog#: MBS728130, San Diego, CA) according to manufacturer's protocol. The Ca^{2+}-ATPase activity was expressed as micromoles of Pi liberate per milligrams of lens protein per hour using a standard concentration of Ca^{2+}-ATPase (0–10 ng/ml).

GSH and MDA levels in lens: : Glutathione and malondialdehyde levels were determined in water-soluble proteins. Total glutathione levels were estimated with the GSH ELISA kit from Elabscience (catalog#: E-EL-0026, Bethesda, MD) according to the manufacturer's protocol. The GSH concentration was expressed as nanomoles of GSH per milligrams of tissue using standard concentrations of GSH (0.005–0.325 mM). Malondialdehyde levels were estimated with the MDA ELISA kit from Elabscience according to the manufacturer's protocol (catalog #: E-EL-0060). The MDA concentration was expressed as nanomoles of MDA per grams of tissue using standard concentrations of MDA (0.434–0.1388 µM). GSH and MDA standard curves were drawn using Curve expert professional software (v.2.3.0, Starkville, MS).

Antioxidant enzyme activity in the lens: : Enzyme activities were measured in the supernatant which contained water-soluble proteins. Superoxide Dismutase (SOD) activity was estimated with the SOD assay kit from Cayman Chemical Co (catalog #: 706,002, Ann Arbor, MI). The SOD activity was expressed as units per milligrams of tissue using standard concentrations of SOD (0–0.05 U/ml). One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Glutathione peroxidase (GPx) activity was estimated with the GSH-PX assay kit from Elabscience (catalog #: BC0096). The GPx activity was expressed as the enzyme unit per minute per gram of tissue, where one unit of GPx is defined as 1 g tissue makes the concentration of glutathione in the reaction system decrease 1 µmol of H_2O_2 per minute at pH 4.5 at 25 °C. Catalase (CAT) activity was estimated with the CAT Colorimetric assay kit from BioVision (catalog #: K773–100, Exton, PA). The CAT activity was expressed as milliunits per gram of tissue, where one unit of catalase is the amount of catalase that decomposes 1 µmol of H_2O_2 per minute at pH 4.5 at 25 °C. All assay kits were performed according to the manufacturer's protocol.

RNA extraction and quantitative real-time PCR: : Total RNA was harvested from the eye lenses using easy-BLUE reagent form iNtRON biotechnology (Seongnam, Republic of Korea) according to the manufacturer's instruction. cDNA was synthesized from RNA using a first-strand cDNA synthesis kit from TaKaRa (Tokyo, Japan). After cDNA was synthesized, quantitative real-time PCR was performed with SYBR Premix Ex Taq from TakaRa using an ABI Step One PlusTM Real-Time PCR system (Applied Biosystems, Foster city, CA). The mixtures were incubated for an initial duration at 95 °C for 10 min, followed by 60 cycles of 95 °C for 5 s, and Tm temperature for 30 s and 72 °C for 30 s. All gene expression levels were normalized to glyceraldehyde 3-phosphated

dehydrogenase (GAPDH). The data were calculated using the 2-Δ(ΔCt) method [25]. The primer sequences are shown in Table 1.

Immunoblot analysis: The resected lenses were homogenized in lysis buffer (20 mM Tris-HCL (pH 7.4), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 M EDTA (pH 8.0), 0.32 mM sucrose, protease inhibitor cocktail, 1 mM NaF, 1 mM Na_3VO_4). Total tissue lysates were dispersed ultrasonically for 30 min and centrifuged 16,000 × *g* at 4 °C for 15 min. The proteins were separated with 12.5% PAGE (PAGE) and then transferred to a nitrocellulose membrane. The primary antibodies of caspase-3 (Cell Signaling Technology, Danvers, MA) were used at a dilution of 1:1,000 and incubated at 4 °C overnight. After incubation, the membranes were incubated with horseradish peroxidase (HRP)–labeled anti-rabbit second antibodies for 2 h at 1:5,000 in blocking solution. Expression levels were quantified by band density, followed by normalization to the beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA) control. The bound antibody was visualized using a LAS3000 Luminescent image analyzer (Fuji Film, Tokyo, Japan). Expression levels of band intensity were quantified by image Lab statistical software (Bio-Rad, Richmond, CA).

Statistical analyses: The results were expressed as the mean ± standard deviation (SD). The results were statistically analyzed with a Student *t* test, Mann–Whitney U test, and one-way ANOVA (ANOVA) using Tukey's honestly significant difference test. Statistically significant differences were assessed with SPSS (Chicago, IL). A p value of less than 0.05 was considered statistically significant.

RESULTS

Effect of *P. densiflora* bark extract on cataract formation and classification of cataractous lenses

Before identifying the underlying mechanism of age-related nuclear cataract, we first confirmed the protective effect of *P. densiflora* bark extract on selenite-induced cataract formation. All the rat eyes were evaluated and classified based on a scale 0 to 6 on P16 (Table 2). All ten rat pups in group 2 (selenite only) exhibited mature opacification in the lenses (mean 4.7, median 5, $p<0.001$ compared to group 1). Administration of *P. densiflora* bark extract prevented the development of opacification in the lens dose-dependently compared to group 2 (groups 4 to 6, mean 3.9, 2.8, 2.2, median 4, 3, 2, $p<0.05$, $p<0.05$, $p<0.001$, respectively). The lens opacification observed in the curcumin-treated group (group 3), which served as the positive control, was between that observed in groups 4 and 5 according to the mean (3.6) and the median (3). Figure 1 shows a representative image of each group. The degree of opacification in the lens was determined using a white-light lamp in groups 5 and 6 (Figure 1B). With the light lamp, dark rings were observed near the equator of the lens, which cannot be found with the naked eye. In comparison with the healthy eye lens, the dark parts in the lens may be light scattering and increased as the stage progressed. These data suggest that *P. densiflora* bark extract has a preventive effect on selenite-induced cataract formation in Sprague Dawley rats.

Effect of P. densiflora bark extract on lens crystalline and intermediate filaments: The transparency of the lens is determined by crystalline proteins and lens-specific intermediate filaments. The α-crystalline, a predominant protein in the lens, makes up almost 50% of the water-soluble fraction of the lens. Having higher solubility, stability, and chaperone function, α-crystalline serves to prevent protein misfolding and aggregation in the lens. Oxidative stress by selenium can impose abnormal changes on the conformation and chaperone functionality of α-crystalline [23,26]. Filensin and phakinin, two beaded filaments of the lens intermediate, play an essential role in the maintenance of transparency by forming a complex with α-crystalline. In a previous report, these two filament transcripts are degraded in selenite-induced cataract [27]. In this study, an analysis of lens αA-crystalline and filaments mRNA revealed a substantial loss of transcripts in the selenite-induced groups (Figure 2). Restoration of the mRNA levels in group 5 (80 mg/kg *P. densiflora* bark extract) may indicate that *P. densiflora* bark extract protected selenite-induced abnormal changes in the lens crystalline and intermediate filaments.

Effect of P. densiflora bark extract on glutathione, malondialdehyde, and antioxidant enzymes: ROS-induced oxidative stress is one of the major causes in the pathogenesis of cataract. To identify antioxidant effects of *P. densiflora* bark extract on selenite-induced cataract, we analyzed GSH, MDA, and antioxidant enzymes activities (GSH, SOD. GPx, and CAT). The GSH level and antioxidant activities were reduced and the MDA level was increased significantly in group 2 (selenite only) compared to the normal control. Administration of *P. densiflora* bark extract (40, 80, and 120 mg/kg) dose-dependently provided protection from decreases in the GSH level (37%, 72%, and 91%, respectively), SOD (74%, 80%, and 88%, respectively), GPx (47%, 70%, and 90%, respectively), and CAT (27%, 51%, and 88%, respectively). *P. densiflora* bark also suppressed lipid peroxidation (MDA; 27%, 73%, and 89%, respectively). Results are shown in Table 3. We observed that *P. densiflora* bark extracts boosted antioxidant enzymes and inhibited lipid peroxidation.

Table 1. Details of primers used for quantitative real-time PCR.

No.	Gene	NCBI ID	Sequence (Forward/Reverse; 5'-3')	TM (°C)/ Cycle
1	αA-crystalline	NM_012534.2	TGGCAAACACACGAGAGGCA	GCCAGCATCCAAGCCAGACT	60/60
2	Filensin	NM_031555.1	CCCTGGAACAAGCTATTAAGCATG	TTCCGGAGGTTTTCGATCTG	58/60
3	Phakinin	NM_001277434.1	CTCCAGGCTGAGACAGAAATCTTTA	TCATGCCAGTGCTTGGCAT	58/60
4	PMCA-1	NM_053311.1	CGCCATCTTCTGCACAATT	CAGCCATTGTTCTATTGAAAGTTC	55/60
5	Lp82	NM_017117.1	GTGTTTCCGGCGGGCACCCA	CGCCGCCAATAAATAAAGCGCG	63.5/60
6	Caspase-3	NM_012922.2	GGTTCATCCAGTCACTTTGC	CCAGGGAGAAGGACTCAAAT	57/60
7	Bax	NM_017059.2	CGATGAACTGGACAACAACA	AGCAAAGTAGAAAAGGGCAA	54/60
8	Bcl-2	NM_016993.1	GGGATGCCTTTGTGGAACTA	CTCACTTGTGGCCCAGGTAT	58/60
8	GAPDH	NM_017008.4	GCCAGCCTCGTCTCATAGACA	AGAGAAGGCAGCCCTGGTAAC	61/60

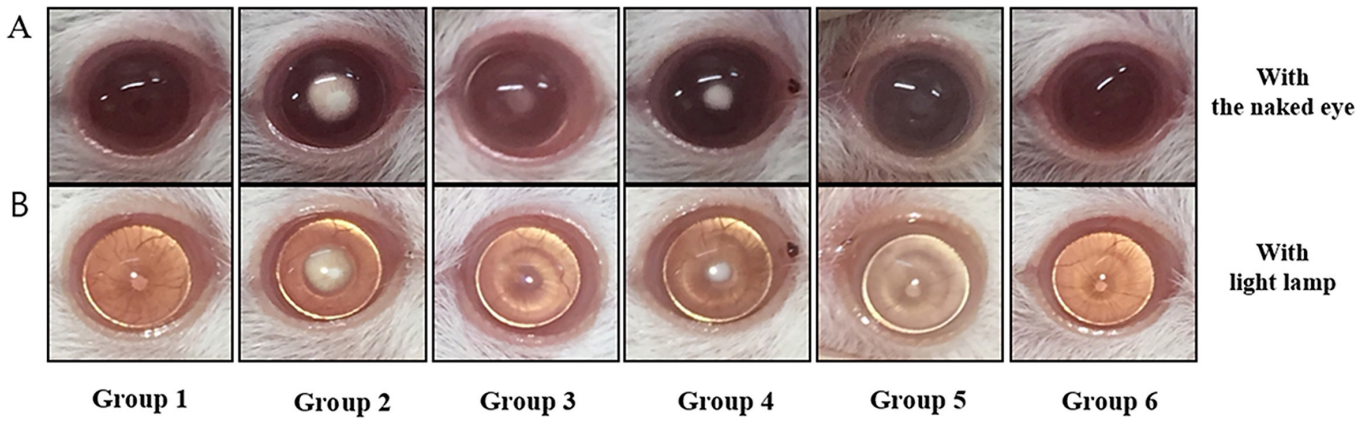

Figure 1. Effect of Pinus densiflora bark extract on morphological changes in lenses of 16-day-old rats. Anterior views were taken (**A**) without a white-light lamp and (**B**) with a white-light lamp. Lenses of group 1 were totally clear (stage 0), those of group 2 developed stage 5 cataract, and those of groups 3 and 4 developed stage 4 cataract. Lenses of groups 5 and 6 were protected by *P. densiflora* bark extract (stages 3 and 2 each). Group 1: normal control, Group 2: selenite-treated, Group 3: selenite/curcumin-treated (80 mg/kg), Groups 4 to 6: selenite/*P. densiflora* bark extract-treated (40, 80, and 120 mg/kg, respectively) animals.

P. densiflora bark extracts inhibits calpain-induced proteolysis and lens protein insolubilization: Calcium homeostasis is well maintained by a balance between inward and outward movements of calcium ions. Calcium levels become high in most cataracts due to the decline in Ca^{2+}-ATPase activity. The elevated calcium level leads to the activation of lenticular calpain Lp82, which, in turn, proteolyzes lens soluble proteins, including several types of crystallines. The conversion of water-soluble to insoluble proteins is caused by aggregation and coprecipitation of crystallines. Low water-soluble and high water-insoluble protein levels are factors that can indicate cataract formation [28]. In this study, water-insoluble protein levels were increased, and water-soluble protein levels were decreased in group 2 (Figure 3A,B). *P. densiflora* bark extract dose-dependently prevented both protein levels from changing significantly. In group 2, Ca^{2+} ATPase activity and mRNA expression of PMCA-1 were found to be higher compared to the normal control group (Figure 3C,D). Several studies revealed that the Ca^{2+} ATPase activity and mRNA transcript level of PMCA-1 increase during cataractogenesis due to a compensatory mechanism, which possibly occurred because of increased calcium levels in lenticular cells [29,30]. The increased levels of Ca^{2+} ATPase activity and gene expression recovered significantly upon treatment with *P. densiflora* bark extract in rat pups. The gene expression of lenticular calpain *Lp82* was decreased in selenite-induced cataractous lenses. We observed that *P. densiflora* bark extract treatment significantly increased *Lp82* expression compared to group 2 (Figure 3D). These results suggest that *P. densiflora* bark extract protects against selenite-induced lens protein proteolysis, aggregation, and insolubilization.

Table 2. Classification of morphological cataractous lenses.

Groups (n=10)	Number of lenses with different degree of opacification							Median (range)	Mean
	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6		
Group 1	10	-	-	-	-	-	-	0(0)	0
Group 2	-	-	-	-	3	7	-	5(1)	4.7###
Group 3	-	-	1	3	5	1	-	4(3)	3.6**
Group 4	-	-	1	2	4	3	-	4(3)	3.9*
Group 5	-	1	3	3	3	-	-	3(3)	2.8*
Group 6	-	3	4	2	1	-	-	2(3)	2.2***¥¥

Degree of lens opacification was observed and determined based on a scale 0 to 6 according to [24] on PN day 16. Group 1: normal control, Group 2: selenite-treated, Group 3: Selenite/Curcumin-treated (80 mg/kg), Group 4 to 6: Selenite/P.D bark extract-treated (40, 80, 120 mg/kg, respectively) animals. The Mann–Whitney U-test was performed for pair wise comparisons. ###p<0.001 significant different from group 1. *p<0.05, **p<0.01, ***p<0.001 significant different from group 2. ¥¥p<0.01, significant different from group3.

Table 3. Effect of P.D bark on the MDA, GSH, SOD, GPx and CAT levels of the lenses in rats.

Group	Parameters in lens				
	GSH (nmol/mg Tissue)	MDA (nmol/g Tissue)	SOD (U/mg Tissue)	GPx (U/min/g Tissue)	CAT (mU/g Tissue)
Group 1	0.44±0.02	42.4±5.1	0.262±0.015	14.28±1.11	0.72±0.22
Group 2	0.26±0.06###	103.2±21.8###	0.184±0.011###	8.43±1.88###	0.34±0.14##
Group 3	0.36±0.04#	78.2±24.5###	0.241±0.012#***	12.48±3.94*	0.50±0.20
Group 4	0.33±0.08#	86.7±25.4###	0.242±0.013#***	11.19±2.45	0.41±0.14##
Group 5	0.39±0.05**	58.9±13.3***	0.246±0.009***	12.50±2.00*	0.50±0.16
Group 6	0.41±0.06***¥	48.8±8.7***¥¥	0.255±0.010***	13.71±0.81***	0.63±0.14*

Levels of GSH and MDA and activities of SOD, GPx and CAT in the cataractous lenses of experimental groups. Each value represents mean ± SD of eight animals and three independent experiments. #p<0.05, ##p<0.01, ###p<0.001 significant different from group 1. *p<0.05, **p<0.01, ***p<0.001 significant different from group 2. ¥p<0.05, ¥¥p<0.01 significant different from group 3. Group 1: normal control, Group 2: selenite-treated, Group 3: Selenite/Curcumin-treated (80 mg/kg), Group 4 to 6: Selenite/P.D bark extract-treated (40, 80, 120 mg/kg, respectively) animals.

Inhibition of apoptosis in selenite-induced cataractous lenses by P. densiflora bark extract: We hypothesized that selenite increases ROS and lipid peroxidation, causes continual intracellular damages, and results in lens cell apoptosis. The loss of lens epithelial cells leads to imbalance in normal lens homeostasis. In this study, apoptosis-related genes (*caspase-3* and *Bax*) were significantly upregulated, while an antiapoptotic gene (*Bcl-2*) was significantly downregulated in the selenite-induced cataract model (Figure 4A). These imbalances in the expression of these genes were counteracted by treatment with *P. densiflora* bark extract. Then, we performed western blot analysis to clarify the regulatory effect of *P. densiflora* bark extract on selenite-induced apoptosis. Caspase-3 activation causes structural and regulatory component proteolysis in cells, resulting in apoptosis [31]. The cleaved form of caspase-3 is commonly used as an apoptotic factor in many studies. Selenite cataract (group 2) exhibited increased levels of cleaved caspase-3 compared to normal control. In contrast, the group treated with *P. densiflora* bark extract exhibited significantly lower levels of both proteins than group 2 (Figure 4B). Together, these data suggest that *P. densiflora* bark extract inhibits apoptosis in selenite-induced cataractous lenses.

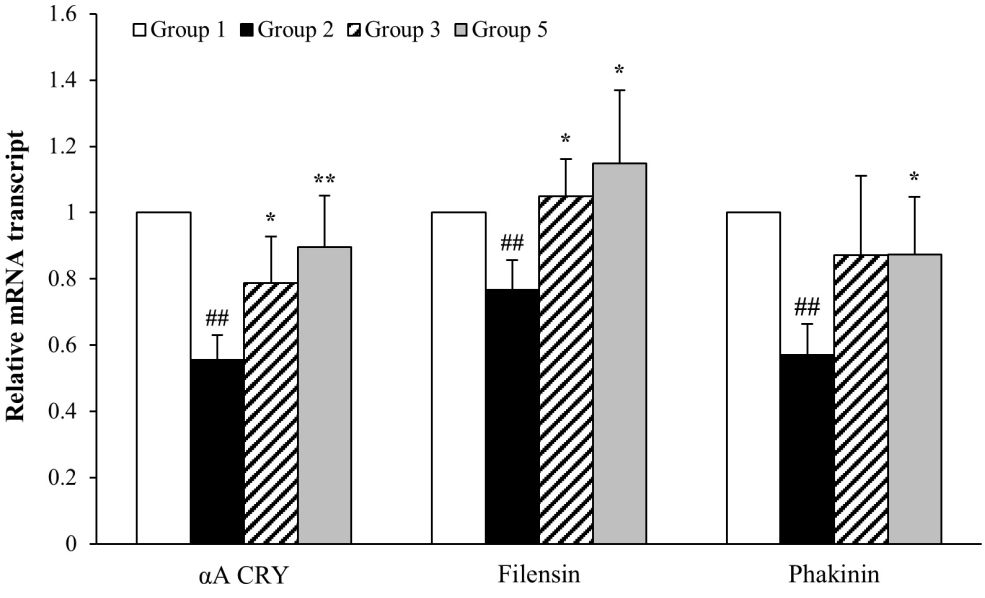

Figure 2. mRNA expression of αA-crystalline, filensin, and phakinin. mRNA levels were analyzed with comparative real-time PCR and normalized to GAPDH. Each value represents mean ± standard deviation (SD) of three independent experiments (n=7). #p<0.05, ##p<0.01 statistically significant different from group 1. *p<0.05 statistically significant different from group 2. αA-CRY: αA-crystalline. Group 1: normal control, Group 2: selenite-treated, Group 3: selenite/curcumin-treated (80 mg/kg), Groups 4 to 6: selenite/*Pinus densiflora* bark extract-treated (40, 80, and 120 mg/kg, respectively) animals.

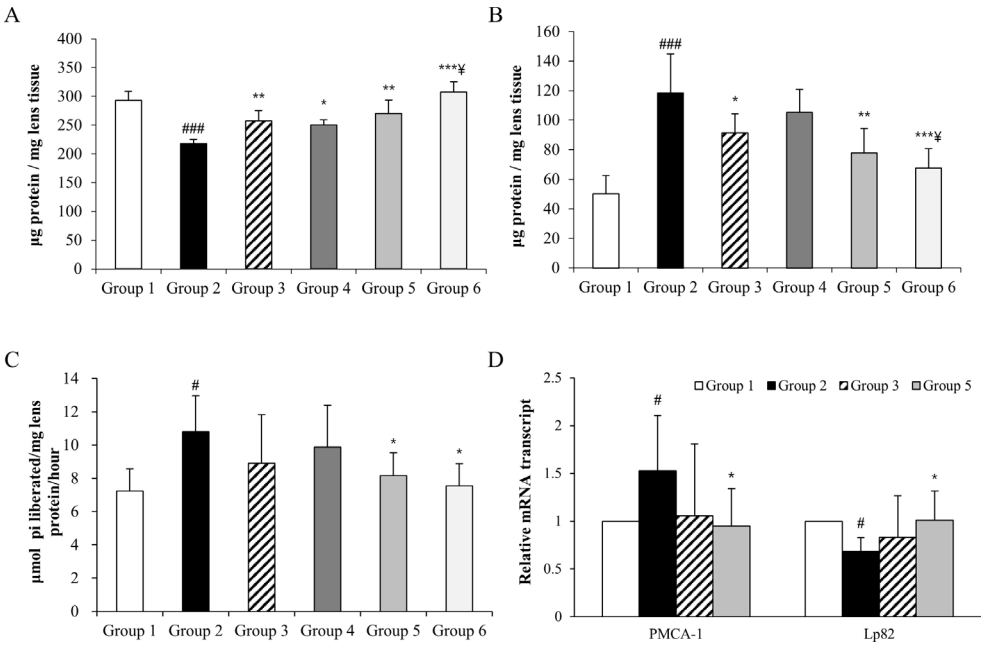

Figure 3. Inhibitory effect of *P. densiflora* bark extract on calpain-induced proteolysis and lens protein insolubilization. **A**: Water-soluble and (**B**) -insoluble protein levels and (**C**) activity of Ca^{2+}-ATPase were measured in the cataractous lenses of the experimental groups. The data were presented as the mean ± standard deviation (SD) of three independent experiments (n=7). **D**: mRNA expression of PMCA-1 and Lp82 was analyzed with comparative real-time PCR (normalized to GAPDH). Each value represents mean ± SD of three independent experiments (n=4). #p<0.05, ###p<0.001 statistically significant different from group 1. *p<0.05, **p<0.01, ***p<0.001 statistically significant different from group 2. ¥p<0.05 statistically significant different from group 3. Group 1: normal control, Group 2: negative control, Group 3: curcumin treated (80 mg/kg), Groups 4 to 6: *P. densiflora* bark extract treated (40, 80, and 120 mg/kg, respectively) animals.

DISCUSSION

Pycnogenol, a standardized extract of French maritime pine bark, is composed of flavonoids, procyanidins, and phenolic acids. The mixture of the components has shown greater pharmacological effects than its purified components, such as cardiovascular benefits, antihypertensive effects, alleviation of allergies and asthma, reduction of thromboxane, and antidiabetic effects [18,20,32]. However, a recent study showed that the Korean red pine (*P. densiflora*) bark extract is also composed of flavonoids and phenolic acids, and has considerable antioxidant effects [33]. Several studies discovered antiadipogenic and anti-inflammatory effects of *P. densiflora* bark extract, but further studies are required to validate the effectiveness [34,35]. French maritime and

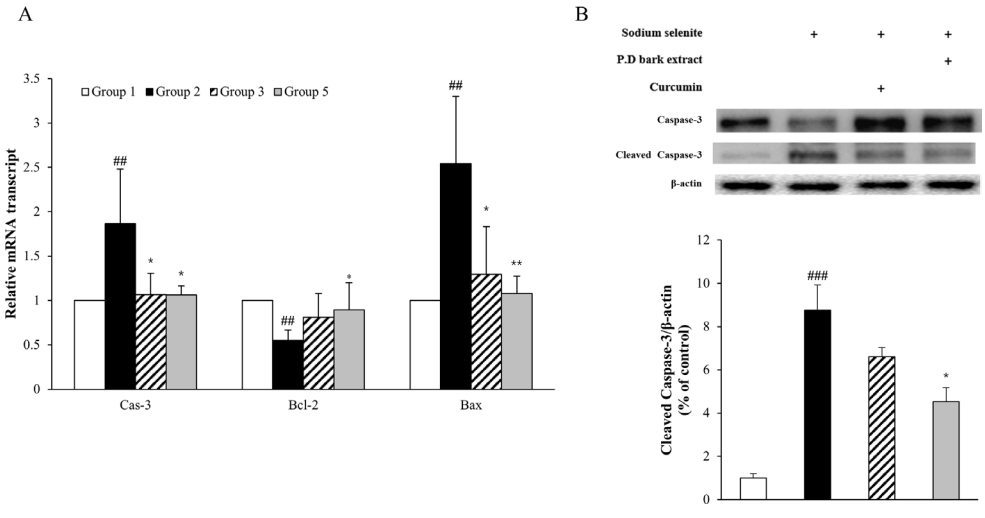

Figure 4. *P. densiflora* bark extract inhibited selenite-induced apoptosis in rat pup lenses. **A**: The levels of caspase-3, Bcl-2, and Bax mRNA were normalized to the GAPDH mRNA level. **B**: western blotting analysis indicated protein expression of caspase-3 (35 kDa) and the cleaved form of caspase-3 (17 kDa). The protein levels were quantified with band density. The results are presented as the mean ± standard deviation (SD) of three independent experiments (n=4). #p<0.05, ##p<0.01, ###p<0.001 statistically significant different from group 1. *p<0.05, **p<0.01, ***p<0.001 statistically significant different from group 2. Cas-3: caspase-3. Group 1: normal control, Group 2: selenite-treated, Group 3: selenite/curcumin-treated (80 mg/kg), Groups 4 to 6: selenite/*P. densiflora* bark extract-treated (40, 80, and 120 mg/kg, respectively) animals.

Korean red pine bark extract have been studied in many diseases but rarely in ocular diseases. The role of pine bark extract in age-related cataract is still unclear. That being said, other antioxidant substances can be used to prevent oxidative stress–related ocular diseases, such as diabetic retinopathy, age-related macular degeneration, glaucoma, and cataract [10,36]. In the present study, we investigated and evaluated the anticataract effects of Korean red pine bark extract within the selenite cataract model.

The sodium selenite-induced cataract model is a convenient and rapid model for studying mechanisms of age-related nuclear cataract. Oxidative stress and apoptosis have been considered the major underlying pathways in the initiation and progression of cataracts. Overdose of sodium selenite induces ROS generation, damage of membrane Ca^{2+}-ATPase, calpain-mediated proteolysis, insolubilization of crystallines, degradation of cytoskeletal elements, and decline of antioxidant enzymes [29]. Intracellular ROS overproduction can change the redox set point of lens epithelial cells quickly, causing the cells to become an oxidizing environment. Due to this environment, ROS easily oxidize DNA, membrane pump systems, lipids, and proteins, followed by loss of lens epithelial cells by apoptotic mechanisms via caspase-3 activation and regulation of Bcl-2 and Bax [37,38]. Caspase-3 has been investigated to play a significant role in regulating apoptosis. Activated caspase-3 leads to the cleavage of several cytoplasmic and nuclear substrates [39]. The relative equipoise of Bcl-2 and Bax is an important determinant of apoptosis in mammalian cells [26]. In the present study, the mRNA expression levels of caspase-3 and Bax were found to be high in the selenite-treated group but remained at near-normal levels in the P. *densiflora*–treated group. The mRNA expression level of Bcl-2, an antiapoptotic factor, was also maintained at near-normal levels in the group treated with *P. densiflora* bark extract.

The effect of *P. densiflora* bark extract on cataract formation was evaluated through the observation of the lenses. Cataract staging was determined with the naked eye and with a white lamp. Stages 3 to 6 can be easily characterized with the naked eye according to [24]. However, stages 2 and 3 needed light to determine the degree of opacification. Using a white lamp, we can distinguish the differences between the two stages. Morphological examinations and cataract staging showed that lens opacification was decreased dose-dependently by treatment with *P. densiflora* bark extract. Further chemical estimations were also performed to investigate intracellular mechanisms.

ROS are generated in the lens continuously by exposure to solar ultraviolet light and cellular metabolism [40]. To protect from oxidative stress, the eye lens has high concentrations of antioxidant components, such as ascorbic acid, GSH, and several other antioxidant enzymes [41]. In this study, GSH, MDA, SOD, GPx, and CAT were measured to investigate the antioxidant effect of *P. densiflora* bark extract. MDA results from lipid peroxidation and is usually considered a biomarker for evaluating the level of oxidative stress [42]. GSH levels and enzyme activities of SOD, GPx, and CAT were found to be low in the selenite-induced group, while the MDA level was higher than that of the normal control group. The levels of MDA and GSH and the activities of SOD and GPx showed similar levels to those found by Ferlemi et al. [43]. Antioxidant enzyme activities were maintained at near-normal levels, and MDA levels were also dose-dependently decreased in the *P. densiflora* bark extract groups. We can now assume that *P. densiflor*a bark extract enhanced SOD, GPx, and CAT activities and inhibited lipid peroxidation and GSH depletion by scavenging ROS directly.

Ca^{2+}-ATPase activity was significantly decreased after subcutaneous injection of sodium selenite; however, it increased at near-normal levels after 72 h of the injection. Elevation of Ca^{2+}-ATPase activity and mRNA transcript levels were also observed in the Upjohn Pharmaceuticals Limited rat which is the hereditary cataract model [44]. Administration of *P. densiflora* bark extract normalized Ca^{2+}-ATPase activity and gene expression of PMCA-1 by protecting proteins from sulfhydryl oxidation, and thus prevented calpain-mediated proteolysis. Consequently, the insoluble protein levels of the groups treated with *P. densiflora* bark extract were decreased in a dose-dependent manner. Lp82 calpain, found only in the rodent rat lens, provides information for understanding the role of calpain-3 in the human lens. In the selenite cataract model, Lp82 calpain is activated by an influx of Ca^{2+} ions, thus resulting in proteolysis of crystalline. After the progression of cataracts, autolysis of Lp82 calpain occurs, and the gene expression level is lowered compared to that of the normal control [30]. Administration of P. *densiflora* bark extract normalizes Lp82 gene expression and can indicate that calpain of the group treated with *P. densiflora* bark extract may be less activated than that of the selenite-treated group.

The results for the lens morphology, protein contents of the lens, GSH, MDA, antioxidant enzyme activities, and gene expression in the selenite-treated groups are in accordance with several studies of the selenite cataract in vivo [27,28,43]. The mechanism of pathogenesis in age-related cataract includes oxidative stress–induced proteolysis of crystalline and apoptosis pathway. There are two approaches to preventing cataract formation: (1) the prevention of ROS production and (2) the inhibition of calpain-induced

proteolysis. The results for GSH, MDA, and antioxidant enzyme activities showed that *P. densiflora* bark extract boosts antioxidant potential and blocks the burst of ROS production. Early blocking of ROS production could inhibit the onset of cataract formation and maintain normal status in antioxidant enzyme activities. *P. densiflora* bark extract also inhibited calpain-induced proteolysis through regulation of Ca^{2+}-ATPase activity, PMCA-1, and *Lp82* gene expression. The decreased tendency of proteolysis was also observed in the water-soluble and -insoluble protein contents. Therefore, *P. densiflora* bark extract inhibited selenite-induced apoptosis in rat lenses in two ways.

In conclusion, the results of this study appear to suggest the effectiveness of *P. densiflora* bark extract in preventing cataract formation by modulating antioxidant enzymes, Ca^{2+}-ATPase activity, gene expression of αA-crystalline, intermediate filament (filensin and phakinin), plasma membranes Ca^{2+}-ATPase, and lens-specific calpain and apoptosis-related genes. *P. densiflora* bark extract possibly acts as a scavenger of free radicals, protects against oxidative stress, and inhibits calpain-induced proteolysis. The results suggest that *P. densiflora* bark extract could be an anticataract agent; however, further investigation is needed for application in human cataract.

REFERENCES

1. Shoham A, Hadziahmetovic M, Dunaief JL, Mydlarski MB, Schipper HM. Oxidative stress in diseases of the human cornea. Free Radic Biol Med 2008; 45:1047-55. [PMID: 18718524].
2. Berthoud VM, Beyer EC. Oxidative stress, lens gap junctions, and cataracts. Antioxid Redox Signal 2009; 11:339-53. [PMID: 18831679].
3. Kruk J, Kubasik-Kladna K, Aboul-Enein HY. The Role Oxidative Stress in the Pathogenesis of Eye Diseases: Current Status and a Dual Role of Physical Activity. Mini Rev Med Chem 2015; 16:241-57. [PMID: 26586128].
4. Pascolini D, Mariotti SP. Global estimates of visual impairment: 2010. Br J Ophthalmol 2012; 96:614-8. [PMID: 22133988].
5. Chang MA, Congdon NG, Baker SK, Bloem MW, Savage H, Sommer A. The surgical management of cataract: barriers, best practices and outcomes. Int Ophthalmol 2008; 28:247-60. [PMID: 17712529].
6. Brian G, Taylor H. Cataract blindness–challenges for the 21st century. Bull World Health Organ 2001; 79:249-56. [PMID: 11285671].
7. Kim SJ, Equi R, Bressler NM. Analysis of macular edema after cataract surgery in patients with diabetes using optical coherence tomography. Ophthalmology 2007; 114:881-9. [PMID: 17275910].
8. Luo LH, Xiong SH, Wang YL. Results of cataract surgery in renal transplantation and hemodialysis patients. Int J Ophthalmol 2015; 8:971-4. [PMID: 26558211].
9. Petrash JM. Aging and age-related diseases of the ocular lens and vitreous body. Invest Ophthalmol Vis Sci 2013; 54:ORSF54-9. [PMID: 24335070].
10. Brennan LA, Kantorow M. Mitochondrial function and redox control in the aging eye: role of MsrA and other repair systems in cataract and macular degenerations. Exp Eye Res 2009; 88:195-203. [PMID: 18588875].
11. Wei YH, Lee HC. Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. Exp Biol Med 2002; 227:671-82. [PMID: 12324649].
12. Liao JH, Chen CS, Hu CC, Chen WT, Wang SP, Lin IL, Huang YH, Tsai MH, Wu TH, Huang FY, Wu SH. Ditopic complexation of selenite anions or calcium cations by pirenoxine: an implication for anti-cataractogenesis. Inorg Chem 2011; 50:365-77. [PMID: 21138325].
13. Nakazawa Y, Oka M, Bando M, Takehana M. Hesperetin prevents selenite-induced cataract in rats. Mol Vis 2015; 21:804-10. [PMID: 26283862].
14. Shearer TR, Ma H, Fukiage C, Azuma M. Selenite nuclear cataract: review of the model. Mol Vis 1997; 3:8-[PMID: 9238097].
15. Choi J-H, Choi M-K, Han O-T, Han S-J, Chung S-J, Shim C-K, Kim D-D. Evaluation of Skin Absorption of Catechin from Topical Formulations Containing Korean Pine Bark Extract (Pinexol®). J Pharm Investig 2007; 37:359-64. .
16. Saliou C, Rimbach G, Moini H, McLaughlin L, Hosseini S, Lee J, Watson RR, Packer L. Solar ultraviolet-induced erythema in human skin and nuclear factor-kappa-B-dependent gene expression in keratinocytes are modulated by a French maritime pine bark extract. Free Radic Biol Med 2001; 30:154-60. [PMID: 11163532].
17. Hwang YJ. Yj, Tam LT, Youn SH, Ahn HS, Kwon SH, Min BK, Yun SH, An YE, Lee MW. Quantitative analysis of taxifolin, (+)-catechin and procyanidin B1 from the preparation of Pinus densiflora (PineXol). Korean J Pharmacogn 2016; 47:246-50. .
18. Packer L, Rimbach G, Virgili F. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, pycnogenol. Free Radic Biol Med 1999; 27:704-24. [PMID: 10490291].
19. Eghbaliferiz S, Iranshahi M. Prooxidant Activity of Polyphenols, Flavonoids, Anthocyanins and Carotenoids: Updated Review of Mechanisms and Catalyzing Metals. Phytother Res 2016; 30:1379-91. [PMID: 27241122].
20. Rohdewald P. A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. Int J Clin Pharmacol Ther 2002; 40:158-68. [PMID: 11996210].
21. Pescosolido N, Giannotti R, Plateroti AM, Pascarella A, Nebbioso M. Curcumin: therapeutical potential in ophthalmology. Planta Med 2014; 80:249-54. [PMID: 24323538].

22. Raman T, Ramar M, Arumugam M, Nabavi SM, Varsha MK. Cytoprotective mechanism of action of curcumin against cataract. Pharmacol Rep 2016; 68:561-9. [PMID: 26894964].

23. Manikandan R, Thiagarajan R, Beulaja M, Arumugam M. Effect of curcumin on selenite-induced cataractogenesis in Wistar rat pups. Curr Eye Res 2010; 35:122-9. [PMID: 20136422].

24. Hiraoka T, Clark JI. Inhibition of lens opacification during the early stages of cataract formation. Invest Ophthalmol Vis Sci 1995; 36:2550-5. [PMID: 7591646].

25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Method. Methods 2001; 25:402-8. [PMID: 11846609].

26. Kumari RP, Ramkumar S, Thankappan B, Natarajaseenivasan K, Balaji S, Anbarasu K. Transcriptional regulation of crystallin, redox, and apoptotic genes by C-Phycocyanin in the selenite-induced cataractogenic rat model. Mol Vis 2015; 21:26-39. [PMID: 25593511].

27. Sreelakshmi V, Abraham A. Cassia tora leaves modulates selenite cataract by enhancing antioxidant status and preventing cytoskeletal protein loss in lenses of Sprague Dawley rat pups. J Ethnopharmacol 2016; 178:137-43. [PMID: 26692278].

28. Bhadada SV, Bhadada VJ, Goyal RK. Preventive Effect of Tephrosia purpurea on Selenite-Induced Experimental Cataract. Curr Eye Res 2016; 41:222-31. [PMID: 25848963].

29. El Okda EA, Mohamed MM, Shaheed EB, Abdel-Moemin AR. Switching to instant black coffee modulates sodium selenite-induced cataract in rats. Ger Med Sci 2016; 14:Doc05- [PMID: 27158251].

30. Sundararajan M, Thomas PA, Teresa PA, Anbukkarasi M, Geraldine P. Regulatory effect of chrysin on expression of lenticular calcium transporters, calpains, and apoptotic-cascade components in selenite-induced cataract. Mol Vis 2016; 22:401-23. [PMID: 27168717].

31. Pan S, Berk BC. Glutathiolation regulates tumor necrosis factor-alpha-induced caspase-3 cleavage and apoptosis: key role for glutaredoxin in the death pathway. Circ Res 2007; 100:213-9. [PMID: 17185628].

32. Maimoona A, Naeem I, Saddiqe Z, Jameel K. A review on biological, nutraceutical and clinical aspects of French maritime pine bark extract. J Ethnopharmacol 2011; 133:261-77. [PMID: 21044675].

33. Lee YJ, Han OT, Choi H-S, Lee BY, Chung H-J, Lee O-H. Antioxidant and Anti-adipogenic Effects of PineXol®. Korean J Food Sci Technol 2013; 45:97-103. .

34. Ahn H, Go GW. Pinus Densiflora Bark Extract (PineXol) Decreases Adiposity in Mice by Down-Regulation of Hepatic De Novo Lipogenesis and Adipogenesis in White Adipose Tissue. J Microbiol Biotechnol 2017; 27:660-7. [PMID: 28081360].

35. Kim KD, Kim HJ, Park K-R, Kim S-M, Na Y-C, Shim BS, Kim S-H, Choi S-H, Ahn KS, Ryu S-H. Pinexol inhibits in vitro inflammatory biomarkers by blocking NF-κB signaling pathway and protects mice from lethal endotoxemia. Orient Pharm Exp Med 2011; 11:61-70. .

36. Schonlau F, Rohdewald P. Pycnogenol for diabetic retinopathy. A review. Int Ophthalmol 2001; 24:161-71. [PMID: 12498513].

37. Asha R, Gayathri Devi V, Abraham A. Lupeol, a pentacyclic triterpenoid isolated from Vernonia cinerea attenuate selenite induced cataract formation in Sprague Dawley rat pups. Chem Biol Interact 2016; 245:20-9. [PMID: 26697995].

38. Ou Y, Yuan Z, Li K, Yang X. Phycocyanin may suppress D-galactose-induced human lens epithelial cell apoptosis through mitochondrial and unfolded protein response pathways. Toxicol Lett 2012; 215:25–30. <jrn>39. Brauns SC, Dealtry G, Milne P, Naude R, Van de Venter M. Caspase-3 activation and induction of PARP cleavage by cyclic dipeptide cyclo(Phe-Pro) in HT-29 cells. Anticancer Res 2005; 25:6B4197-202. [PMID: 16309216].

39. Brauns SC, Dealtry G, Milne P, Naude R, Van de Venter M. Caspase-3 activation and induction of PARP cleavage by cyclic dipeptide cyclo(Phe-Pro) in HT-29 cells. Anticancer Res 2005; 25:6B4197-202. [PMID: 16309216].

40. Lux-Neuwirth O, Millar TJ. Lipid soluble antioxidants preserve rabbit corneal cell function. Curr Eye Res 1990; 9:103-9. [PMID: 2335108].

41. Anbaraki A, Khoshaman K, Ghasemi Y, Yousefi R. Preventive role of lens antioxidant defense mechanism against riboflavin-mediated sunlight damaging of lens crystallins. Int J Biol Macromol 2016; 91:895-904. [PMID: 27316765].

42. Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutrition, metabolism, and cardiovascular diseases. Nutr Metab Cardiovasc Dis 2005; 15:316-28. [PMID: 16054557].

43. Ferlemi AV, Makri OE, Mermigki PG, Lamari FN, Georgakopoulos CD. Quercetin glycosides and chlorogenic acid in highbush blueberry leaf decoction prevent cataractogenesis in vivo and in vitro: Investigation of the effect on calpains, antioxidant and metal chelating properties. Exp Eye Res 2016; 145:258-68. [PMID: 26808488].

44. Nabekura T, Tomohiro M, Ito Y, Kitagawa S. Changes in plasma membrane Ca2+ -ATPase expression and ATP content in lenses of hereditary cataract UPL rats. Toxicology 2004; 197:177-83. [PMID: 15003327].

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