

Pinosylvin-mediated protection against oxidative stress in human retinal pigment epithelial cells

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Purpose: In this work, we investigated the ability of pinosylvin (PS), 3,5-dihydroxy-*trans*-stilbene, to modulate oxidative stress in human RPE cells. PS, a stilbenoid polyphenol, occurs in high concentrations in bark byproducts and therefore represents an attractive bioactive compound for health-promoting applications.

Methods: First, we evaluated the toxicity range of PS by exposing ARPE-19 cells to 0.1–200 μ M concentrations of PS for 24 h followed by the cell viability test. In the next stage, the ARPE-19 cells were preincubated in PS for 24 h followed by hydroquinone (HQ) exposure without PS for another 24 h. The cell viability test was conducted after HQ exposure. To elucidate the potential mechanisms behind PS-mediated protection against oxidative stress, the ARPE-19 cells were treated with 5 μ M PS for 6 h, and mRNA was extracted at four time points (2 h, 6 h, 12 h, 24 h) to determine changes in the expression of nuclear factor-erythroid 2-related factor-2 (*Nrf2*), sequestosome 1 (*p62/SQSTM1*), heme oxygenase-1 (*HO-1*), and glutathione S-transferase pi 1 (*GSTP1*) genes. To clarify the molecular mechanism behind PS-mediated protection further, the ARPE-19 cells were transfected with *p62* and *Nrf2* siRNAs for 24 h, and the roles of *p62*, *Nrf2*, and its target gene *HO-1* in conferring protection against oxidative stress were studied with quantitative real-time PCR (qRT-PCR) and the cell viability test.

Results: PS treatment at concentrations of 5 and 10 μ M significantly enhanced cell survival from oxidative stress. The expression levels of an enzyme with antioxidative, anti-inflammatory, and immunomodulatory properties, HO-1, were increased by PS treatment and correlated strongly with cell survival. PS treatment did not elevate the expression levels of *Nrf2* or its target genes, *p62* or *GSTP1*, even though it had a clear effect on the expression of *HO-1*, another gene controlled by *Nrf2*. RNA interference analysis further confirmed the important role of *Nrf2* and *HO-1* in PS-mediated protection against oxidative stress whereas the role of *p62* seemed to be insignificant at the gene expression and cell viability levels.

Conclusions: Our results suggest that PS treatment conferred protection against oxidative stress through the induction of HO-1 in human RPE cells. Consequently, PS-stilbene compounds, which can be isolated in significant amounts from bark waste, may possess health-promoting properties against aging-related diseases associated with oxidative stress such as age-related macular degeneration (AMD) and Alzheimer's disease. These natural compounds may offer opportunities for high-value use of bark waste in diverse health-related applications.

The high metabolic rates of retinal cells, frequently in the presence of reactive oxygen species (ROS), expose RPE cells to oxidative stress, and this can cause the development of age-related macular degeneration (AMD), a disease that leads to visual impairment and blindness [1]. Antioxidant treatments have exerted protective effects against oxidative stress in the RPE cell model [2,3] suggesting that modifying oxidative environments may represent an approach to promoting the survival of the retina and RPE cells. Activation of antioxidant defense and phase II enzymes is a key system for protecting cells from oxidative damage associated with age-related diseases such as AMD, cardiovascular diseases,

and Alzheimer's disease. Nuclear factor-erythroid 2-related factor-2 (*Nrf2*) is an important transcription factor that plays a key role in the antioxidant response element (ARE)-mediated activation of phase II and antioxidant enzymes such as heme oxygenase-1 (HO-1) and glutathione S-transferase pi 1 (*GSTP1*) [4,5]. It is known [6] that the Kelch-like ECH-associated protein 1 (*Keap1*)-*Nrf2* system plays a central role in cytoprotection against oxidative injury. When cells are in normal conditions without exposure to stress, *Keap1* serves as an adaptor for ubiquitin E3 ligase and promotes proteasomal degradation of *Nrf2*, while *Nrf2* is stabilized when *Keap1* is inactivated upon oxidative stress. Activation of the *Nrf2*-ARE signaling pathway by plant-derived bioactive compounds that can attenuate cellular oxidative stress represents an interesting therapeutic approach against aging-related diseases. Recently, researchers demonstrated [7] that a resveratrol-based diet could significantly ameliorate disorders

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related to cerebral ischemia and reperfusion; resveratrol treatment upregulated protein and mRNA expression of Nrf2 and HO-1. Furthermore, administering the isoflavonoid genistein in the diet of rats exposed to reperfusion neurodegeneration exerted neuroprotective activity and significantly improved spatial learning and memory compared to the vehicle control animals [8]. Genistein treatments attenuated oxidative DNA damage and lipid peroxidation, which was associated with enhanced levels of Nrf2 and HO-1, suggesting that this polyphenol could activate the antioxidant or detoxification of the Nrf2-Keap1 transcription system. Ethyl pyruvate (EP), a simple ester of pyruvic acid, has been reported to possess antioxidative properties and, interestingly, induces translocation of Nrf2 from the cytosol to the nucleus and enhances the expression of HO-1 in a dose-dependent manner [9]. Nrf2 translocation and binding to the ARE located on the HO-1 promoter were observed to begin 30 min after EP treatment.

HO-1 is the inducible isoform of the first and rate-limiting enzyme in heme degradation, and induction of HO-1 has exhibited protective effects against oxidative stress as well as exerting anti-inflammatory and immunomodulatory effects [10]. The beneficial protective activity is related to cell-type specific functions, and an increasing body of evidence indicates that human RPE cells are an attractive target for demonstrating the protective functions of HO-1 [11-14]. The polyphenol-mediated induction of HO-1 appears to be activated via the Nrf2-Keap1 transcription system, at least in some cases. Recently, researchers claimed that the autophagy pathway plays an important role in the oxidative stress defense since this pathway maintains the integrity of the Keap1-Nrf2 system for normal cell function by regulating Keap1 turnover [15]. Sequestosome 1 (p62/SQSTM1) is a multifunctional adaptor protein that has been implicated in selective autophagy and several cell signaling pathways [16,17]. Recent data suggest that p62 plays a key role in an oxidative stress response pathway through direct interaction with the ubiquitin ligase adaptor Keap1 [18], which leads to the activation of the transcription factor Nrf2. Accumulation

of the p62 protein has been identified as a marker for impaired autophagy in various tissues including human RPE cells [16,17]. In certain cases, p62 accumulation disrupts the Keap1-Nrf2 association and stimulates Nrf2 stabilization and nuclear localization, as demonstrated in the pathogenesis of cataract in which defective protein degradation is known to play a key role [19]. Furthermore, Viiri et al. [17] demonstrated that impaired autophagy may contribute to the pathology of AMD.

Considerable data have accumulated that the activation of *Nrf2* target genes (OMIM 600492), particularly *HO-1* (OMIM 141250), are strongly protective against inflammation, oxidative damage, and cell death. Polyphenolic compounds can activate several *Nrf2* target genes including *HO-1* and significantly inhibit the formation of ROS, thus limiting the harmful effects that oxidative stress can exert on target cells. Experimental data indicate that quercetin could protect cells from ultraviolet A (UVA)-induced oxidative damage by elevating intracellular antioxidant activity by enhancing activation of the transcription factor Nrf2 [20]. Furthermore, Johnson et al. [3] showed in RPE cells that citrus flavonoid (eriodictyol) provided long-term protection against oxidative stress by activating Nrf2 and inducing phase II enzymes. Consequently, targeting the Nrf2-ARE signaling pathway may represent a promising avenue for identifying phytochemicals with specific functional properties against aging-related diseases.

Pinosylvin (PS, 3,5-dihydroxy-*trans*-stilbene, Figure 1), a resveratrol analog found in the *Pinus* species, naturally occurs at high concentrations in bark waste. Previously, researchers have demonstrated [21] that PS treatment exerted cancer chemopreventive effects and inhibited oxidative stress and inflammation [22,23]. Thus, limited data suggest PS may be an inexpensive polyphenol potentially with several diverse health-promoting applications. In this work, we used the ARPE-19 cell line to gain new information about the ability of PS to modulate oxidative stress responses and to protect cells from damage.

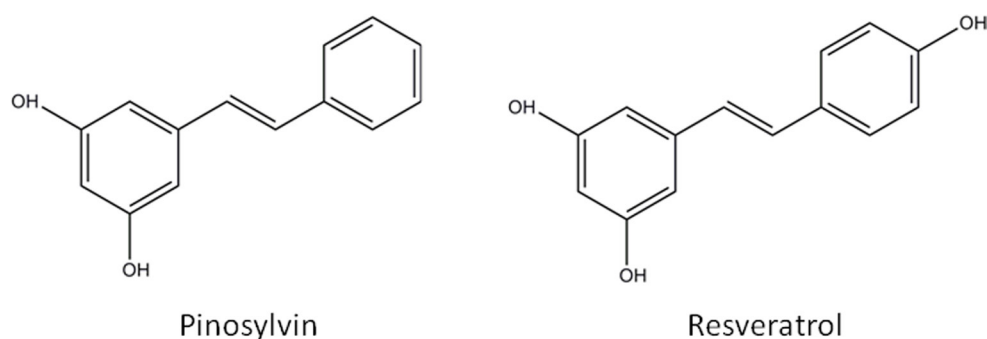


Figure 1. Chemical structures of pinosylvin and resveratrol.

METHODS

Cell culture: The human retinal pigment epithelial (ARPE-19) cell line obtained from American Type Culture Collection (ATCC, Manassas, VA) was used in the study (passages 5–15). Cells were cultured in Dulbecco's modified eagle medium/nutrition mix F-12 (1:1; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 U/ml penicillin (Lonza, Walkersville, MD), 100 µg/ml streptomycin (Lonza), and 2 mM L-glutamine (Lonza). The cells were grown at 37 °C in a humidified 5% CO₂ atmosphere.

Toxicity test and effect of pinosylvin on oxidative stress: ARPE-19 cells were seeded into 96-well plates with approximately 3×10^4 cells/well. The experiments were performed 48 h after seeding using FBS-free medium. To determine the toxicity of PS (Sigma Chemical, St. Louis, MO, dissolved in methanol), the cells were rinsed once with serum-free medium to remove FBS residues and exposed to 0.1–200 µM concentrations of PS diluted in medium. The cells were exposed to PS for 24 h before the cell viability tests.

In the experiments to study the effective dose of PS against oxidative stress, the cells were rinsed once with serum-free medium and incubated in PS (0.1–100 µM) containing medium for 24 h. The influence of PS against oxidative stress at various time points was investigated by incubating cells in 5 µM PS for 2 h, 6 h, 24 h, and 48 h. Before the oxidative stress-provoking agent, hydroquinone (HQ; Sigma-Aldrich), was added, cells were rinsed once using serum-free medium to remove any residual PS. To achieve 30–50% viability, the cells were exposed to 400–600 µM HQ for 24 h, and then survival was assessed with the cell viability test.

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [24], and the absorbance of the lysate was read at 570 nm in a spectrophotometer (PerkinElmer Victor², Turku, Finland). Cells were assessed with microscopy before the viability test to confirm the correlation of the absorbance readings from the MTT assay.

RNA isolation and quantitative real-time PCR: Total RNA isolation (5 µM PS incubation for 2 h, 6 h, 12 h, and 24 h) and cDNA synthesis were performed with the TaqMan Gene Expression Cells-to-CT kit (Ambion, Foster City, CA). Quantitative real-time PCR (qRT-PCR) reactions were conducted using SYBR Green Real-Time PCR Master Mix (Ambion) with 15 µl reaction volume. The qRT-PCR primers were ordered from Oligomer (Helsinki, Finland) using sequences from Kokot et al. [25] for Nrf2, GSTP1, and HO-1, the reference gene sequence for acid riboprotein P0 (RPLP0) from

Malinen et al. [26], and intron-spanning qPCR primers for human p62 mRNA (forward 5'-GCA CAC CAA GCT CGC ATT C-3' and reverse 5'-ACC CGA AGT GTC CGT GTT TC-3') that detect human p62 mRNA isoforms 1–3 (NM_003900.4) were designed using the OLIGO program (Molecular Biology Insights, Cascade, CO) and the BLAST algorithm. Samples were analyzed in triplicate in an ABI Prism 7500 instrument (Applied Biosystems, Foster City, CA) with the following reaction conditions: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s and 72 °C for 20 s, final annealing 72 °C for 5 min, and redensaturation 95 °C for 1 min. Finally, the specificity of the primers was verified by performing a melt curve analysis. Data were obtained using the comparative Ct method and expressed as fold-change normalized against RPLP0.

Nrf2 and p62 RNA interference: ARPE-19 cells were seeded at 2×10^4 cells/well in 96-well plates. The cells were allowed to grow for 24 h and reached approximately 50–80% confluence. The siRNAs (all from Ambion) used were negative control siRNA (cat. no. AM4611; final concentration 50 nM), siRNA against human Nrf2 (cat. no. s9491, final concentration 30 nM), and siRNA against human p62 (cat. no. sl6962, final concentration 30 nM). Cells were transfected in medium without antibiotics and FBS for 24 h using the siPORT Amine (Ambion, cat. no. 4502) transfection agent according to the manufacturer's instructions. After transfection, the cells were washed once with medium (antibiotic and FBS free) and further incubated with a 5 µM concentration of PS in medium without FBS for 6 h. RNA was isolated after PS incubation, and the expression levels of Nrf2 and HO-1 were determined as described in the previous section.

The effect of Nrf2 and p62 RNA interference together with PS and HQ exposure to cell viability was assessed using the MTT assay. Briefly, the cells were plated and transfected as described above. At 24 h post transfection, the cells were treated with a 5 µM concentration of PS and HQ as described. Finally, the cells were assessed with microscopy, and the MTT assay was performed.

Statistical analyses: Data were analyzed using IBM SPSS Statistics for Windows, version 19.0 (IBM, Armonk, NY). All data were subjected to one-way ANOVA (ANOVA) with Bonferroni or Games-Howell corrections and the *t* test. The PCR data were converted to logarithmic values to equalize variances. The MTT assay data are expressed as mean±standard deviation (SD; *n* = 5–6 in each sample group) and the PCR data as mean±standard error of the mean (SEM; *n* = 3 in each sample group). All data were produced and analyzed at least twice to confirm the reliability of the methods and results.

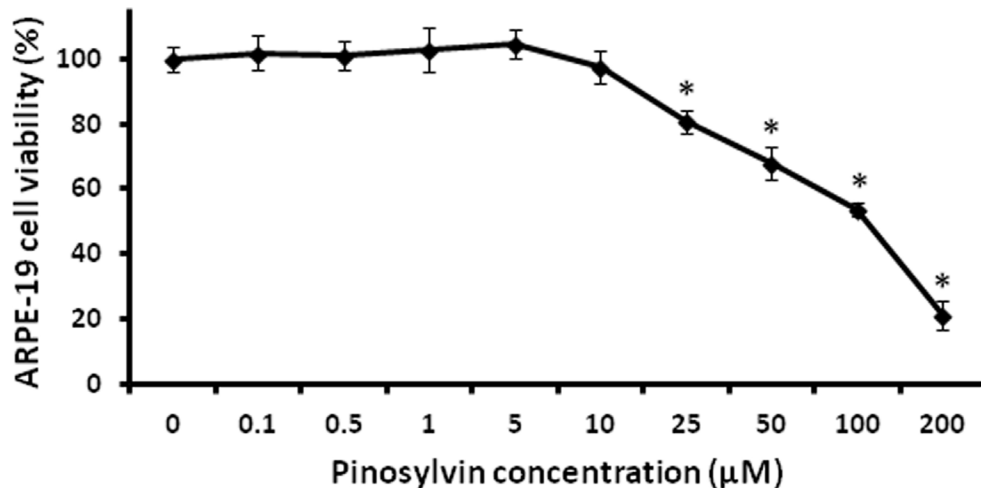


Figure 2. Cytotoxicity of pinosylvin in ARPE-19 cell line. ARPE-19 cells were exposed to pinosylvin (0.1–200 µM) for 24 h before cytotoxicity was determined in the cell viability test. Data as mean value±standard deviation (SD), n = 6. *p<0.05, significantly different from untreated cells (0 µM), ANOVA, and the *t* test.

RESULTS

Toxicity and protective effects of pinosylvin on oxidative stress: To determine the protective role of PS against oxidative stress, human retinal ARPE-19 cells were preincubated in PS before oxidative stress was induced using HQ. First, we evaluated the toxicity range of PS by exposing cells to 0.1–200 µM concentrations of PS for 24 h followed by the cell viability test. At the concentrations between 0.1 and 10 µM, no significant changes in cell viability were detected (Figure 2). The first signs of toxicity appeared at the 25 µM concentration when cell viability was decreased to 80% (p<0.05) and continued decreasing linearly as a result of the increasing PS concentrations. Therefore, 0.1–10 µM PS concentrations were a potential dose range, conferring protection against oxidative stress without disturbing vital cellular processes.

In the next stage, ARPE-19 cells were preincubated in PS for 24 h followed by HQ exposure without PS for another 24 h. The cell viability test was conducted after HQ exposure. HQ at a concentration of 600 µM caused 60–70% cell mortality whereas PS protected cells from HQ-induced oxidative stress at a 5, 10, and 25 µM (p<0.05) concentrations but not at lower or higher concentrations (Figure 3). PS pretreatment doubled the viability of cells at the 5 and 10 µM concentrations (HQ versus PS+HQ treatments). PS concentrations exceeding 10 µM were toxic, and evoked similar or even clearer cell mortality together with HQ exposure as a joint effect of a toxic concentration of PS and oxidative stress (PS 100 µM, p<0.05). The PS dose less than 5 µM did not protect cells from oxidative stress setting an effective dose range of PS as between 5 and 10 µM in the ARPE-19 cell line. Interestingly, our results demonstrate that PS exerted protection against oxidative stress at time points between 6 h and 24 h according to the MTT assay results (Figure 4). Cell

viability did not increase after 2 h of PS treatment, whereas the protective effect of PS was clear after 6 h achieving significantly increased cell viability from 30% to 50% (p<0.05). The impact of PS on cell viability was highest and most statistically significant 24 h after PS treatment (p<0.05). The protective effect of PS diminished after 48 h.

Impact of pinosylvin on *Nrf2*, *p62*, *HO-1*, and *GSTP1* mRNA expression: To elucidate the potential mechanisms behind PS-mediated protection against oxidative stress, we examined the expression of the *Nrf2* transcription factor, which controls the expression of several genes (*HO-1*, *GSTP1*) essential for detoxifying and eliminating reactive oxidants by enhancing cellular antioxidant capacity [4]. The ARPE-19 cells were treated with 5 µM PS, and mRNA was extracted at four time points (2 h, 6 h, 12 h, 24 h) during PS treatment to determine changes in the expression of the *Nrf2*, *p62*, *HO-1*, and *GSTP1* genes (Figure 5). Only a slight peak in *HO-1* expression was detected after 2 h of PS treatment, whereas the expression was significantly increased at time points 6 h, 12 h and 24 h. After 6 h, the *HO-1* mRNA levels started to decrease toward time point at 24 h, when the expression level was significantly decreased compared to time points 6 h and 12 h (p<0.05) but still significantly higher than control (p<0.05). The *Nrf2*, *p62*, and *GSTP1* mRNAs were expressed at relatively constant levels at all time points. Interestingly, the cell viability results (Figure 4) were compatible with the *HO-1* expression data pointing to a strong correlation between cell viability and *HO-1* expression. Thus, PS appears to prevent oxidative damage by inducing *HO-1* expression.

Role of *Nrf2* and *p62* in oxidative stress: To clarify the molecular mechanism behind PS-mediated protection against oxidative stress, ARPE-19 cells were transfected with *p62* and *Nrf2* siRNAs for 24 h and the roles of *Nrf2* and its target

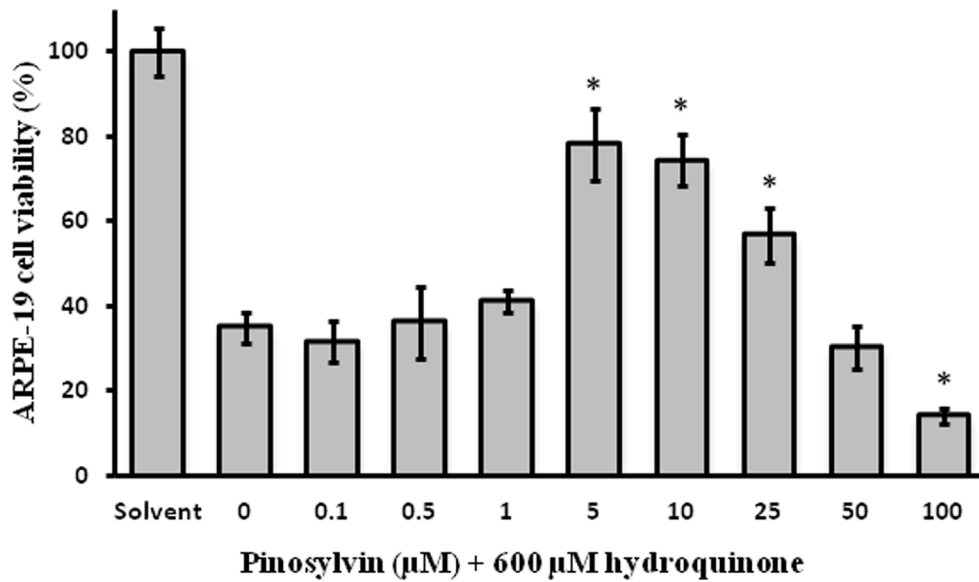


Figure 3. The effect of different pinosylvin doses to combat oxidative stress in the ARPE-19 cell line. ARPE-19 cells were pretreated with pinosylvin at different concentrations for 24 h and exposed to the oxidative stress-causing agent, hydroquinone, for another 24 h before the cell viability test. Data are mean value±standard deviation (SD), n = 5. *p<0.05, significantly different from cells without pinosylvin (0 μM), ANOVA, and the *t* test.

gene *HO-1* were studied in conferring protection against oxidative stress with qRT-PCR and cell viability test (Figure 6 and Figure 7). Nrf2 siRNA suppressed the expression of Nrf2 to approximately 30% (p<0.05) and decreased the basal expression level of the target gene p62 to approximately 50% (p<0.05; Appendix 1). The basal expression level of *HO-1* was not affected by Nrf2 siRNA, although *HO-1* is a target gene for *Nrf2* in conjunction with *p62* [27,28]. p62 siRNA decreased the expression of p62 to approximately 20% (Appendix 2) but did not have any effect on Nrf2 or *HO-1* expression levels. However, PS treatment did not change Nrf2 levels when administered together with Nrf2 or p62 siRNA.

Treatment of cells with PS increased the expression of *HO-1* despite p62 interference whereas Nrf2 siRNA prevented the increase in the level of expression of *HO-1*. Cell viability test data (Figure 7) further confirmed the results of the PCR experiments, that is, there was a correlation between *HO-1* expression and cell viability values. Nrf2 siRNA prevented cell survival from HQ-induced oxidative stress even in the presence of PS treatment whereas p62 siRNA showed no correlation with cell survival.

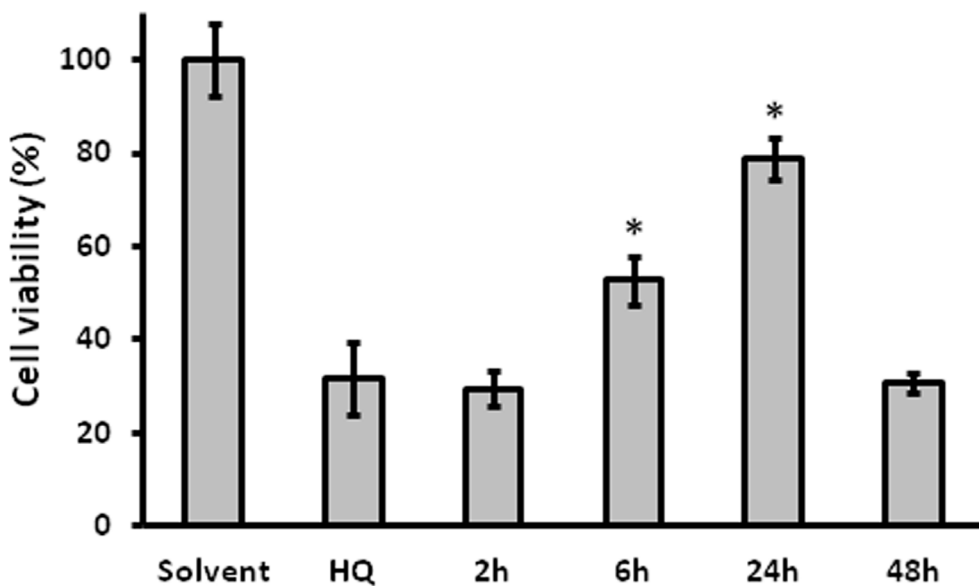


Figure 4. Influence of pretreatment time on the protective effect of pinosylvin against oxidative stress in the ARPE-19 cell line. Cells were pretreated with 5 μM pinosylvin for times ranging from 2 h to 48 h before oxidative stress was evoked by exposure to 500 μM hydroquinone (HQ) for another 24 h before the viability test. Data are mean value±standard deviation (SD), n = 5. *p<0.05, significantly different from cells exposed to HQ, ANOVA, and the *t* test.

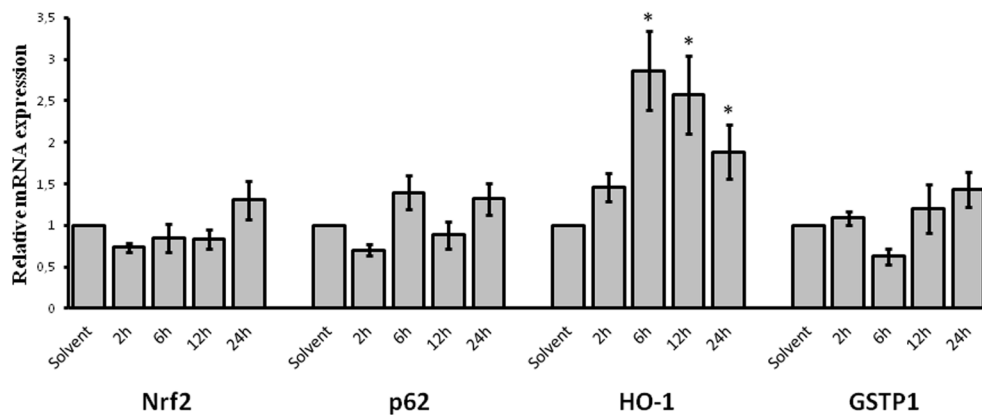


Figure 5. Relative mRNA expression of Nrf2, p62, HO-1, and GSTP1 after 2 h, 6 h, 12 h, and 24 h incubation with 5 μ M pinosylvin. Cells without pinosylvin = 1. Data expressed as the mean relative expression (versus RPLP0) \pm standard error of the mean (SEM), $n = 3$, * $p < 0.05$, significantly different from solvent control (methanol), ANOVA, and the t test.

DISCUSSION

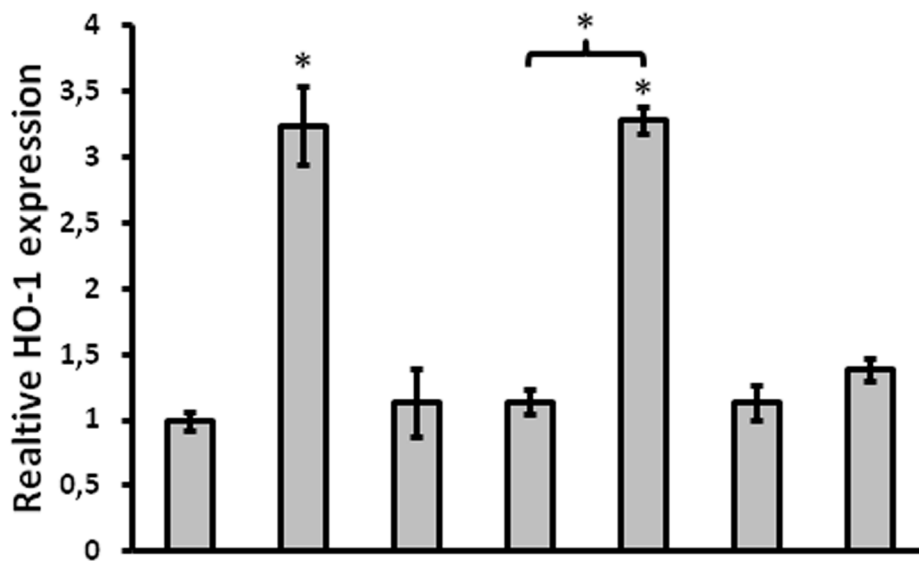
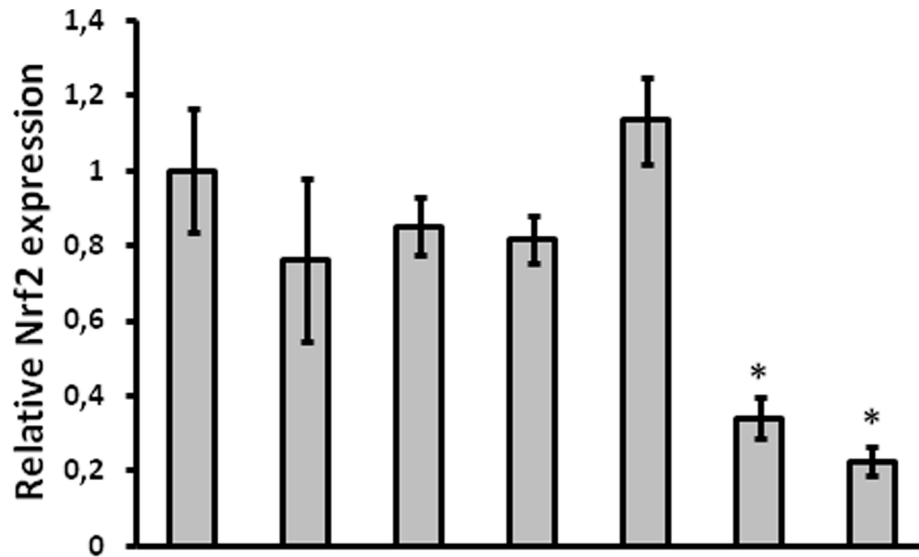
Our data demonstrated that PS can protect RPE cells (ARPE-19) from oxidative stress-induced cell death. At best, PS pretreatment increased cell viability (up to 75–80%) at 5 and 10 μ M concentrations. These results are consistent with the previous observations indicating that quercetin, a well-known flavonoid, can also effectively protect ARPE-19 and retinal ganglion cells (RGC-5) from H_2O_2 -induced cell death [2,12,29,30]. Furthermore, PS provided protection against oxidative stress at time points from 6 h to 24 h resembling the results obtained with the flavonoid, eriodictyol [3], and indicate that plant flavonoid compounds may hold great potential to modulate oxidative environments and thus improve the survival of retina and RPE cells in conditions of aging-related stress.

To elucidate the potential mechanisms behind PS-mediated protection against oxidative stress, we examined the expression of Nrf2, a factor that controls the expression of several genes whose protein products (HO-1, GSTP1) are important in detoxifying and eliminating reactive oxidants, that is, enhancing cellular antioxidant capacity [4]. Our data demonstrate for the first time that PS appears to prevent oxidative damage by inducing HO-1 expression, which suggests a strong correlation between cell viability and HO-1 expression. This finding is important since HO-1 is the key inducible defense molecule exerting cytoprotective, antioxidant, and anti-inflammatory properties. Recently, researchers demonstrated [13] that inhibiting HO-1 could enhance ROS production while the overexpression of HO-1 prevented the toxic effect induced by oxidative stress in rat retinal endothelial cells. Interestingly, plant derived-flavonoids such as resveratrol [31], curcumin [14], naringenin [32], and bilberry anthocyanin [11] have been reported to trigger HO-1 induction, an effect associated with a decrease in ROS levels. Collectively, these data imply that HO-1 is one of the key

defense molecule targets through which PS and other flavonoids protect retinal endothelial cells from oxidative insults.

Interestingly, our data revealed that Nrf2 expression levels after PS treatment remained at a basal level (Figure 5) at all time points analyzed showing decreased rather than increased expression levels. A similar effect was recently reported by Ishikado et al. [33] in which extract of willow bark protected cells against oxidative stress by increasing the HO-1 level without altering the expression level of Nrf2 in human umbilical vein endothelial cells. Furthermore, in this work ARPE-19 cells were transfected with p62 and Nrf2 siRNA to elucidate the role of *Nrf2* and its target gene *HO-1* in conditions of oxidative stress. Interestingly, PS mediated HO-1 expression despite p62 interference whereas Nrf2 siRNA prevented the elevation of HO-1 expression. Apparently, induction of HO-1 expression may be a result of Nrf2 translocation from the cytosol to the nucleus, resulting in increased expression of its target genes. Interestingly, quercetin treatments (1–100 μ M) dose-dependently caused an accumulation of Nrf2 protein suggesting that quercetin-activated ARE-regulated gene expression in response to UV treatment in human keratinocyte cells [20]. Furthermore, eriodictyol mediated the increases in the expression levels of proteins involved in oxidative defense via Nrf2 activation [3]. Thus, polyphenols appear to mediate antioxidative protection via the same mechanisms in many cell model systems.

In addition, we evaluated the role of p62 in PS-mediated protection against oxidative stress by investigating the expression level of p62 mRNA. Accumulation of the p62 protein has been identified as a marker for selective autophagy in many different tissues including human RPE cells [16,17]. Protein degradation is mediated by the autophagic pathway and the ubiquitin-proteasome system during normal unstressed conditions while upon stress, the ubiquitin-proteasome pathway can be overwhelmed, and the autophagic pathway becomes



TR	+	+	+	+	+	+	+
PS	-	+	-	-	+	-	+
Neg. siRNA	-	-	+	-	-	-	-
P62 siRNA	-	-	-	+	+	-	-
Nrf2 siRNA	-	-	-	-	-	+	+

Figure 6. The effect of pinosylvin on the expression of Nrf2 and HO-1 in Nrf2 or p62 knockdown ARPE-19 cells. ARPE-19 cells were transfected with p62 or Nrf2 siRNA for 24 h, and the mRNA expression levels of Nrf2 and its target gene *HO-1* were analyzed with quantitative real-time PCR. Data are expressed as the mean relative expression (versus RPLP0)± standard error of the mean (SEM), n = 3, *p<0.05, significantly different from transfection control (TR) cells, ANOVA, and the *t* test. TR=transfection agent; PS=pinosylvin; Neg. siRNA=negative siRNA.

activated to compensate for increased protein damage. Dysregulation of autophagy may lead to elevated levels of oxidative stress. In certain cases, p62 accumulation disrupts the Keap1-Nrf2 association and stimulates Nrf2 stabilization and translocation into the nucleus, as recently demonstrated in the pathogenesis of cataract where defective protein

degradation was shown to play a key role [19]. In the present work, PS treatment did not affect the expression of p62 while p62 siRNA decreased the expression of p62 to approximately 20% but did not have any effect on Nrf2 or HO-1 mRNA expression levels. One potential explanation may be derived from the observation that in some other cell systems, p62 has

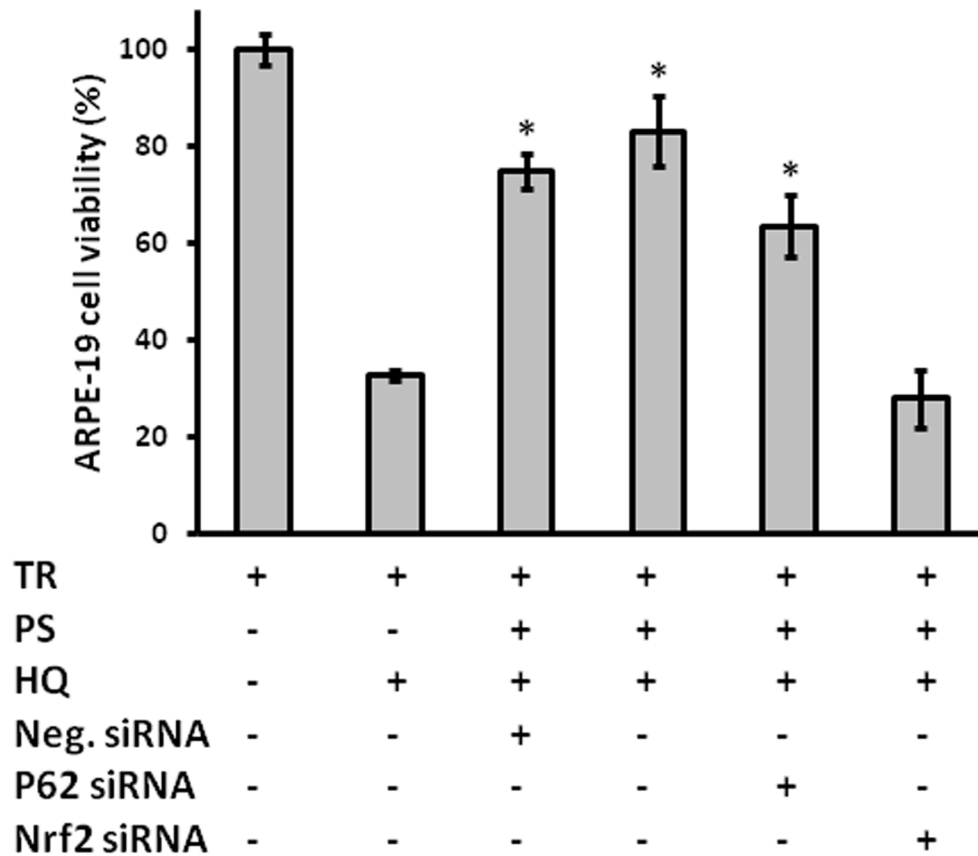


Figure 7. ARPE-19 cells were transfected with p62 and Nrf2 siRNA for 24 h followed by 24 h pretreatment of cells with 5 μ M pinosylvin and finally 24 h oxidative stress evoked with 400 μ M hydroquinone (HQ). Cell viability was measured in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Data are expressed as the mean value \pm standard deviation (SD), n = 5. *p<0.05, significantly different from cells treated with TR and HQ, ANOVA, and the *t* test. TR=transfection agent; PS=pinosylvin; HQ=hydroquinone; Neg. siRNA=negative siRNA.

been shown [15] to be a Nrf2 target, and Nrf2 can induce the accumulation of p62. The inability of PS to modulate Nrf2 expression may consequently attenuate the expression of p62. This is supported by the evidence that there appears to be a positive loop behind the antioxidant response and p62 may also induce the expression of Nrf2 [15]. Disruption of the Keap1-Nrf2 complex and translocation of Nrf2 into the nucleus could be mediated via modification of cysteine residues in Keap1 by PS. This could change the conformation of the repressing complex as is known to occur with some electrophiles and oxidants [34]. Elevated p62 levels have been detected together with the upregulation of heat shock protein 70 (Hsp70), although these proteins exert different functions in attempting to regulate stress responses [16]. In addition, both proteins require a certain stress threshold before they are induced. In this work, an elevated Hsp70 mRNA level was not detected after PS treatment (Appendix 2); that is, PS treatment did not induce stress response. Therefore, we suggest that p62 does not play a major role in preventing oxidative stress, but might be an important protein in triggering cytoprotection at a later stage of oxidative stress and possibly induce adaptive autophagy.

Previously, it has been demonstrated that PS administered at a dose of 10 mg/kg could significantly inhibit the formation of colon cancer, and PS effectively inhibited tumor cell metastasis via modulating metalloproteinases [35] and downregulating cell proliferation signaling pathways [21]. Furthermore, PS also suppressed the production of proinflammatory mediators via inhibiting the nuclear factor-kappaB signaling pathway [36], and administering 30 mg/kg to arthritic rats alleviated the disease and attenuated oxidative stress and inflammation [23]. This present work provides evidence that PS can confer antioxidative protection against oxidative stress via inducing HO-1 in human retinal ARPE-19 cells. Therefore, PS appears to be a promising bioactive compound with therapeutic potential to prevent age-related neurodegenerative diseases associated with aging and oxidative stress such as AMD and Alzheimer’s disease [37]. These natural compounds may open the door for high-value use of bark waste in several health-related applications.

APPENDIX 1. RELATIVE MRNA EXPRESSION OF HSP70 AFTER 2 H, 6 H, 12 H AND 24 H INCUBATION WITH 5 MM PINOSYLVIN.

To access the data, click or select the words “Appendix 1.” Cells without pinosylvin = 1. Data expressed as mean relative expression (vs RPLP0) ± SEM, n=3, *p<0.05, significantly different from solvent control (MeOH), ANOVA and t-test.

APPENDIX 2. THE EFFECT OF PINOSYLVIN ON THE EXPRESSION OF P62 IN NRF2 OR P62 KNOCKDOWN ARPE-19 CELLS.

To access the data, click or select the words “Appendix 2.” ARPE-19 cells were transfected with p62 or Nrf2 siRNA for 24 h and the mRNA expression levels of p62 was analyzed by quantitative real-time PCR. Data expressed as mean relative expression (vs. RPLP0) ± SEM, n=3, *p<0.05, significantly different from transfection control (TR) cells, ANOVA and t-test. TR = transfection agent; PS = pinosylvin; Neg. siRNA = negative siRNA.

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REFERENCES

- Jarrett SG, Boulton ME. Consequences of oxidative stress in age-related macular degeneration. *Mol Aspects Med* 2012; 33:399-417. [PMID: 22510306].
- Hanneken A, Lin FF, Johnson J, Maher P. Flavonoids protect human retinal pigment epithelial cells from oxidative-stress-induced death. *Invest Ophthalmol Vis Sci* 2006; 47:3164-77. [PMID: 16799064].
- Johnson J, Maher P, Hanneken A. The flavonoid, eriodictyol, induces long-term protection in ARPE-19 cells through its effects on Nrf2 activation and phase 2 gene expression. *Invest Ophthalmol Vis Sci* 2009; 50:2398-406. [PMID: 19117929].
- Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem* 2009; 284:13291-5. [PMID: 19182219].
- Zhang M, An C, Gao Y, Leak RK, Chen J, Zhang F. Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. *Prog Neurobiol* 2013; 100:30-47. [PMID: 23025925].
- Taguchi K, Motohashi H, Yamamoto M. Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. *Genes Cells* 2011; 16:123-40. [PMID: 21251164].
- Ren J, Fan C, Chen N, Huang J, Yang Q. Resveratrol pretreatment attenuates cerebral ischemic injury by upregulating expression of transcription factor Nrf2 and HO-1 in rats. *Neurochem Res* 2011; 36:2352-62. [PMID: 21850487].
- Wang R, Tu J, Zhang Q, Zhang X, Zhu Y, Ma W, Cheng C, Brann DW, Yang F. Genistein attenuates ischemic oxidative damage and behavioral deficits via eNOS/Nrf2/HO-1 signaling. *Hippocampus* 2013; 23:634-47. [PMID: 23536494].
- Kim SW, Lee HK, Shin JH, Lee JK. Up-down regulation of HO-1 and iNOS gene expressions by ethyl pyruvate via recruiting p300 to Nrf2 and depriving it from p65. *Free Radic Biol Med* 2013; 65:468-76. [PMID: 23891677].
- Paine A, Eiz-Vesper B, Blasczyk R, Immenschuh S. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. *Biochem Pharmacol* 2010; 80:1895-903. [PMID: 20643109].
- Milbury PE, Graf B, Curran-Celentano JM, Blumberg JB. Bilberry (vaccinium myrtillus) anthocyanins modulate heme oxygenase-1 and glutathione S-transferase-pi expression in ARPE-19 cells. *Invest Ophthalmol Vis Sci* 2007; 48:2343-9. [PMID: 17460300].
- Saviranta NMM, Veeroos L, Granlund LJ, Hassinen VH, Kaarniranta K, Karjalainen RO. Plant flavonol quercetin and isoflavone biochanin A differentially induce protection against oxidative stress and inflammation in ARPE-19 cells. *Food Res Int* 2011; 13:109-13. .
- Castilho A, Aveleira CA, Leal EC, Simoes NF, Fernandes CR, Meirinhos RI, Baptista FI, Ambrosio AF. Heme oxygenase-1 protects retinal endothelial cells against high glucose- and oxidative/nitrosative stress-induced toxicity. *PLoS ONE* 2012; 7:e42428-[PMID: 22879979].
- Woo JM, Shin DY, Lee SJ, Joe Y, Zheng M, Yim JH, Callaway Z, Chung HT. Curcumin protects retinal pigment epithelial cells against oxidative stress via induction of heme oxygenase-1 expression and reduction of reactive oxygen. *Mol Vis* 2012; 18:901-8. [PMID: 22539869].
- Taguchi K, Fujikawa N, Komatsu M, Ishii T, Unno M, Akaike T, Motohashi H, Yamamoto M. Keap1 degradation by autophagy for the maintenance of redox homeostasis. *Proc Natl Acad Sci USA* 2012; 109:13561-6. [PMID: 22872865].
- Viiri J, Hyttinen JM, Ryhanen T, Rilla K, Paimela T, Kuusisto E, Siitonen A, Urtti A, Salminen A, Kaarniranta K. P62/sequestosome 1 as a regulator of proteasome inhibitor-induced autophagy in human retinal pigment epithelial cells. *Mol Vis* 2010; 16:1399-414. [PMID: 20680098].
- Viiri J, Amadio M, Marchesi N, Hyttinen JM, Kivinen N, Sironen R, Rilla K, Akhtar S, Provenzani A, D'Agostino VG, Govoni S, Pascale A, Agostini H, Petrovski G, Salminen A, Kaarniranta K. Autophagy activation clears ELAVL1/HuR-mediated accumulation of SQSTM1/p62 during proteasomal inhibition in human retinal pigment epithelial cells. *PLoS ONE* 2013; 8:e69563-[PMID: 23922739].

18. Nezis IP, Stenmark H. p62 at the interface of autophagy, oxidative stress signaling, and cancer. *Antioxid Redox Signal* 2012; 17:786-93. [PMID: 22074114].
19. Wignes JA, Goldman JW, Wehl CC, Bartley MG, Andley UP. p62 expression and autophagy in alphaB-crystallin R120G mutant knock-in mouse model of hereditary cataract. *Exp Eye Res* 2013; [PMID: 23872361].
20. Kimura S, Warabi E, Yanagawa T, Ma D, Itoh K, Ishii Y, Kawachi Y, Ishii T. Essential role of Nrf2 in keratinocyte protection from UVA by quercetin. *Biochem Biophys Res Commun* 2009; 387:109-14. [PMID: 19576171].
21. Park EJ, Chung HJ, Park HJ, Kim GD, Ahn YH, Lee SK. Suppression of Src/ERK and GSK-3/beta-catenin signaling by pinosylvin inhibits the growth of human colorectal cancer cells. *Food Chem Toxicol* 2013; 55:424-33. [PMID: 2333577].
22. Jeong E, Lee HR, Pyee J, Park H. Pinostylin induces cell survival, migration and anti-adhesiveness of endothelial cells via nitric oxide production. *Phytother Res* 2013; 27:610-7. [PMID: 22736379].
23. Macickova T, Drabikova K, Nosal R, Bauerova K, Mihalova D, Harmatha J, Pecivova J. In vivo effect of pinosylvin and pterostilbene in the animal model of adjuvant arthritis. *Neuroendocrinol Lett* 2010; 31:Suppl 291-5. [PMID: 21187826].
24. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 1989; 119:203-10. [PMID: 2470825].
25. Kokot A, Metze D, Mouchet N, Galibert MD, Schiller M, Luger TA, Bohm M. Alpha-melanocyte-stimulating hormone counteracts the suppressive effect of UVB on Nrf2 and nrf-dependent gene expression in human skin. *Endocrinology* 2009; 150:3197-206. [PMID: 19282378].
26. Malinen M, Saramaki A, Ropponen A, Degenhardt T, Vaisanen S, Carlberg C. Distinct HDACs regulate the transcriptional response of human cyclin-dependent kinase inhibitor genes to trichostatin A and 1alpha,25-dihydroxyvitamin D3. *Nucleic Acids Res* 2008; 36:121-32. [PMID: 17999998].
27. Jain A, Lamark T, Sjøttem E, Larsen KB, Awuh JA, Overvatn A, McMahon M, Hayes JD, Johansen T. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J Biol Chem* 2010; 285:22576-91. [PMID: 20452972].
28. Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S, Yamamoto M. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem* 2000; 275:16023-9. [PMID: 10821856].
29. Maher P, Hanneken A. Flavonoids protect retinal ganglion cells from oxidative stress-induced death. *Invest Ophthalmol Vis Sci* 2005; 46:4796-803. [PMID: 16303981].
30. Kook D, Wolf AH, Yu AL, Neubauer AS, Priglinger SG, Kampik A, Welge-Lüssen UC. The protective effect of quercetin against oxidative stress in the human RPE in vitro. *Invest Ophthalmol Vis Sci* 2008; 49:1712-20. [PMID: 18385095].
31. Zheng Y, Liu Y, Ge J, Wang X, Liu L, Bu Z, Liu P. Resveratrol protects human lens epithelial cells against H2O2-induced oxidative stress by increasing catalase, SOD-1, and HO-1 expression. *Mol Vis* 2010; 16:1467-74. [PMID: 20806083].
32. Chen S, Ding Y, Tao W, Zhang W, Liang T, Liu C. Naringenin inhibits TNF-alpha induced VSMC proliferation and migration via induction of HO-1. *Food Chem Toxicol* 2012; 50:3025-31. [PMID: 22709785].
33. Ishikado A, Sono Y, Matsumoto M, Robida-Stubbs S, Okuno A, Goto M, King GL, Keith Blackwell T, Makino T. Willow bark extract increases antioxidant enzymes and reduces oxidative stress through activation of Nrf2 in vascular endothelial cells and *Caenorhabditis elegans*. *Free Radic Biol Med* 2013; [PMID: 23277146].
34. Kansanen E, Kuosmanen SM, Leinonen H, Levonen A. The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. *Redox Biology* 2013; 1:45-9. [PMID: 24024136].
35. Park EJ, Park HJ, Chung HJ, Shin Y, Min HY, Hong JY, Kang YJ, Ahn YH, Pyee JH, Lee SK. Antimetastatic activity of pinosylvin, a natural stilbenoid, is associated with the suppression of matrix metalloproteinases. *J Nutr Biochem* 2012; 23:946-52. [PMID: 21937212].
36. Lee J, Jung E, Lim J, Lee J, Hur S, Kim SS, Lim S, Hyun CG, Kim YS, Park D. Involvement of nuclear factor-kappaB in the inhibition of pro-inflammatory mediators by pinosylvin. *Planta Med* 2006; 72:801-6. [PMID: 16783695].
37. Kaarniranta K, Salminen A, Haapasalo A, Soininen H, Hiltunen M. Age-related macular degeneration (AMD): Alzheimer's disease in the eye. *J Alzheimers Dis* 2011; 24:615-31. [PMID: 21297256].

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