The medicinal herb *Lycium barbarum* fruit has been widely used for improving and maintaining the health of the eyes in the Far East for many centuries. This study is aimed at investigating whether protective effects generated from the aqueous (LBA) and ethanol (LBE) extracts of the *L. barbarum* fruit existed against oxidative stress-induced apoptosis in human retinal pigment epithelial cells. *L. barbarum* extracts LBA and LBE exerted the activity of ROS scavenging and rescued UVB irradiation-induced growth inhibition in retinal pigment epithelial ARPE-19 cells. Compared to LBA, the ethanol extract LBE exerted a superior protective activity on UVB-induced growth arrest in ARPE-19 cells. Both *L. barbarum* extracts significantly reduced cell cycle G2-arrest population in ARPE-19 cells. Furthermore, the cytometer-based Annexin V/propidium iodide staining assay further showed that both *L. barbarum* extracts protected ARPE-19 cells from UVB-induced apoptosis. *L. barbarum* extracts also reduced the activation of γH2AX, a sensor of DNA damage in ARPE-19 cells. By using Ingenuity Pathway Analysis (IPA), the bioinformatics revealed that the protective effects of both LBA and LBE extracts might be involved in three signaling pathways, especially the Toll-like receptor (TLR) pathway associated with cellular proliferation. Our study suggests that both ethanol and aqueous extracts of *L. barbarum* exhibit antioxidant activity and rescue UVB-induced apoptosis of ARPE-19 cells. Collectively, the ethanol extract exerts a superior effect on rescuing UVB-induced growth arrest of ARPE-19 compared to the aqueous extract, which might be associated with the activation of TLR signaling. Our present work will benefit the preventive strategy of herbal medicine-based vision protection for treating eye diseases such as age-related macular degeneration in the future.
1. Introduction

Age-related macular degeneration (AMD), a progressive macular retinal disease with degenerative changes, can be divided into atrophic and exudative, characterized by the progressive atrophy of retinal pigment epithelial (RPE) cells and the formation of choroidal neovascularization (CNV) [1]. RPE cells are located between the layers of photoreceptor cells and provide nutrition to the latter. If oxidative damage occurs in RPE cells, the breakdown of photoreceptor cells would quickly follow and visual acuity might become damaged [2].

The fruit of Lycium barbarum (LB) wolfberry is a traditional Chinese herbal medicine that has multiple functions in pharmacology [3] like antioxidation [4–6], antiaging [7, 8], neuroprotection [9–12], cytoprotection [13, 14], and immunomodulating [5, 15]. A previous study showed that LBP (Lycium barbarum polysaccharides) extracted from the fruit of L. barbarum might be responsible for the above biological activities [16]. LBP was also shown to exert a protective effect against oxidative damage in cells [17–20]. Based on the antioxidant activity of L. barbarum, many studies have demonstrated that LBP has a protective effect against oxidative injury in cells [17–20], and many studies have focused on the bioactivities of this extract of L. barbarum. However, the effects of the ethanol fraction of LB extracts have been little addressed by previous studies. In the study, we prepared both aqueous (LBA) and ethanol (LBE) extracts of LB and investigated the protective effects of LBA or LBE on human retinal pigment epithelial (ARPE-19) cells from UVB damage, notably proliferation inhibition and apoptosis. We also discussed the possible mechanism underlying L. barbarum extract-mediated protective effect on retinal pigment epithelial cells.

2. Materials and Methods

2.1. Plant Material and Extraction. A total of 500 g of dried fruits of L. barbarum were placed in boiling 3 L water (100°C) for 4 h according to a traditional method described as in the previous study [21]. After filtration, using Whatman no. 3 filter paper, the aqueous extract of L. barbarum was lyophilized. For the ethanol extracts, 500 g of dried fruits was placed in 3 L of ethanol for 3 h at 70°C. The solution was filtered with Whatman no. 3 filter paper and then evaporated at 35°C with reduced pressure.

2.2. Cell Culture. Aring retinal pigment epithelia cell line-19 (ARPE-19), a monolayer of polarized epithelial cells located between the sensory retina and choriocapillaris, is differentiated and mitotically inactive under normal physiological conditions. The ARPE-19 (No. 60,383), obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan), was grown in DMEM medium (Dulbecco's Modified Eagle's Medium, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in an incubator with 5% CO₂ at 37°C. Cells were pretreated with L. barbarum extracts (from 0 to 200 μg/mL) for 2 h; then, cells were exposed to 50 μl/cm² of UVB cultured for 24 h. The experiment of different exposing dose of UVB was performed in triplicate and repeated three times to ensure reproducibility.

2.3. Assessment of Cell Viability. The cell viability was assessed using a colorimetric tetrazolium 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay. Briefly, 1 × 10⁵ cells were seeded into a 96-well plate, and the cells were treated with different exposing doses of UVB (from 0 to 60 ml/cm²) for indicated periods (24 and 48 hr, respectively). The final concentration of MTT in each well was 0.5 mg/mL, and the cells were further incubated. Afterward, the media containing MTT were removed, and the crystal formazan was dissolved entirely with Dimethyl sulfoxide (DMSO). The absorption length of light was measured at 570 nm using a microplate reader (Thermo, Massachusetts, USA), and the relative cell viability was presented as the percentage of absorbance values in treated cells to that of control cells.

2.4. Assessment of Cell Cycle Distribution. The assay of flow cytometer-based propidium iodide (PI) staining was used for detecting cell cycle distribution as described previously [22]. Briefly, ARPE-19 cells were pretreated with LBA and LBE for 2 h; then, the cells were irradiated with 50 ml/cm² UVB for 24 hr. Afterward, trypsin-suspended cells were washed and ethanol-fixed. After centrifugation, the cells were stained with 10 μg/mL PI (Sigma, St. Louis, MO) and 10 μg/mL RNase A at room temperature in the dark. The PI-stained cells were further analyzed by FACS, a flow cytometer (Becton-Dickinson, M ansfeld, MA), and WinMDI/PC-freeware V2.9 (Joseph Trotter, La Jolla, CA, USA).

2.5. Assessment of Mitochondrial Membrane Potential (ΔΨm). The changes in the mitochondrial membrane potential (ΔΨm) were assessed using a mitochondrial permeable lipophilic cationic dye 5,5,6,6-Tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). In healthy cells, JC-1 accumulates in the mitochondria and emits red fluorescence (560 nm); however, in mitochondrial cells with changed membrane potential, the JC-1 accumulates in the cytoplasm and emits fluorescence (530 nm). ARPE-19 cells were pretreated with LBA and LBE and incubated with 10 mg/mL JC-1 at 37°C in the dark. Cells were washed twice with serum-free medium and detected using a fluorescence microscope (Olympus IX71 CTS).

2.6. FITC-Annexin V/PI Apoptosis Assay. The assessment of apoptotic cells was performed according to previous work [22]. Briefly, cells were pretreated with LB extracts 2 hr, respectively, prior to UVB irradiation for 24 hr. The changes of early and late apoptosis were determined using an Annexin V-Fluorescein isothiocyanate (FITC)/PI Apoptosis Detection kit (BioVision, CA 94043, USA). Briefly, cells were resuspended in binding buffer then incubated in the dark with FITC-labeled Annexin V and propidium iodide for 15 min at room temperature; finally, the samples were diluted with phosphate-buffered saline (PBS). Flow cytometry was carried out on a FACS instrument (FACS Calibur; Becton Dickinson, Mountain View, CA, USA), and data were
processed with WinMDI/PC-software V2.9 (written by Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). Cells labeled with annexin conjugated with the FITC fluorescence were recognized as apoptotic populations.

2.7. Determination of Intracellular ROS. We measured the changes in intracellular ROS according to the previous work with minor modifications [23]. \(5 \times 10^5\) cells were seeded in a six-well plate in triplicate. The cells were pretreated with or without LBA and LBE 2 hr prior to the UVB irradiation. After 12 hr, the cells were washed twice with PBS, collected, and then further incubated with \(10 \mu M\) dichloro-dihydro-fluorescein diacetate probe (DCFH-DA, Molecular Probes Inc., Eugene, OR, USA) at 37°C for 30 min. The fluorescence intensity of DCFH was quantified by flow cytometry (the length of excitation is 485 nm, and the length of emission is 525 nm). The results were presented as the percentage of the control cells (100%).

2.8. Assessment of DNA Damage. Briefly, cells were pretreated with LB extracts 2 hr, respectively, prior to UVB irradiation. Afterward, cells were harvested and fixed with 70% ethanol at −20°C overnight; then, the cells were washed twice with BSA-T-PBS solution (1% BSA and 0.2% Triton X-100 in PBS). The cells were then incubated with 0.2 μg/mL antibodies against phosphorylated-Ser\(^{159}\) H2AX (γH2AX), and the secondary antibody was subsequently conjugated with Alexa Fluor 488 (Jackson Laboratory, Bar Harbor, Maine, USA). The intensity of fluorescence was measured by flow cytometry (FACSCalibur, Becton-Dickinson). The results were quantified using the software Cell Quest (Becton-Dickinson).

2.9. Microarray Analysis. RNA molecules were isolated using MirVana Total RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the instructions of the manufacturer. The microarray and the data analysis were performed by Welgene Biotech (Taipei, Taiwan) using the SurePrint G3 GE 8 × 60 K Microarray, 8 × 60 K, AMADID 028005 (Agilent Technologies, USA [24]). The arrays were scanned with G2505C Microarray Scanner (Agilent). The information of probes on the arrays was extracted from the image data using Feature extraction 10.5.1.1 (Agilent) for quantifying signal and the intensity of background.

2.10. Ingenuity Pathway Analysis. The molecular functions of the unique gene analysis of the UVB-induced genes were analyzed using the software Ingenuity Pathway Analysis® (IPA, Ingenuity Systems, Redwood City, California, USA). The changed genes which met the criteria and were correlated with the biological functions of the Ingenuity Pathways Knowledge Base (Ingenuity Systems) were included.

2.11. Statistical Analysis. All experiments were conducted in triplicate, and the data were represented as mean ± SD. The data were subjected to an analysis of variance (ANOVA) and Duncan’s multiple range tests. \(p < 0.05\) was considered significant.
3.5. L. barbarum Extracts Attenuate UVB-Induced Loss of Mitochondrial Membrane Potential.

Mitochondria-mediated apoptosis is mainly responsible for apoptosis; we further determined whether LB extract protects UVB-induced apoptosis through the regulation of mitochondrial signaling. The JC-1 fluorescence dye was used for detecting the loss of mitochondrial membrane potential (MMP), a marker of mitochondrial-mediated apoptosis [30]; therefore, we assessed the MMP membrane potential ($\Delta \Psi m$) in UVB-irradiated cells using JC-1 staining. As shown in Figure 5, the results show that L. barbarum extract pretreatment had strong intensity of red fluorescence (J-aggregation) and weak intensity of green fluorescence (JC-1 monomer) compared to UVB irradiation alone, indicating that both L. barbarum extracts LBA and LBE prevent the loss of MMP in UVB-irradiated cells.

3.6. LBA and LBE Protected UVB-Induced DNA Damage of ARPE-19 Cells. H2AX, a phosphorylated histone variant H2AX at site Ser139, is a marker of DNA damage [31]. The assessment of γH2AX is widely used for detecting DNA damage. As shown in Figure 6, the protective ability of both LBA and LBE pretreated with various concentrations at 25 and 50 μg/mL shows 24.6 ± 2.1% of UVB-irradiated, 8.7 ± 0.4% and 6.6 ± 0.9% of LBA, and 7.8 ± 0.6% and 6.6 ± 0.4% of LBE significantly attenuated the activation of γH2AX in a dose-responsive manner, suggesting a protective role of both LBA and LBE in UVB-induced apoptosis of human ARPE-19 cells.

4. Discussion

Chronic photooxidative stress from the environment could cause various damages including the integrity of the membrane, increased ROS, DNA damage, and cell death of RPE. Dysfunction and degeneration of RPE cells are crucially involved in the pathogenesis of AMD or other RPE degeneration-associated diseases [32, 33]. The apoptotic death of RPE cells, followed by photoreceptor cell death, is thought to mainly contribute to the pathogenesis of the dry form of AMD [33]. The progression of AMD pathogenesis is highly correlated with various oxidative stresses and could be prevented or delayed by supplying vitamin C or other antioxidants [34, 35]. L. barbarum (wolfberry) has been used as a traditional antiaging herb in Chinese pharmacopeia over a long history [36]. LBA, the primary active ingredient of lycium barbarum, isolated from the aqueous extracts of L. barbarum, has multibioactivity properties on modulating cellular physiology, and LBA could exert protective effects on hepatocytes and neurons of the eye [37–40]. However, little is known regarding ethanol extracts of LB.

To confirm whether LB extracts LBA and LBE could protect ARPE-19 cells against UVB-induced cell damage, we first examine the viability of ARPE-19 cells after the irradiation of UVB. Our results showed that the pretreatments of both LBA and LBE significantly mitigated the proliferation of ARPE-19 cells. Under normal conditions, reactive oxygen species (ROS) could act as a second messenger in cell signaling and regulates various biological functions [25–27]; however, a high level of endogenous ROS is highly correlated with the pathophysiology of retinal degeneration diseases including...
Here, we demonstrated that both aqueous and ethanol extracts of *L. barbarum* could enhance antioxidant activities and attenuate the levels of endogenous ROS in ARPE-19 cells. Furthermore, both aqueous and ethanol extracts of *L. barbarum* have potent antioxidant activities and rescue cells from UVB-induced apoptosis. Moreover, ethanol extracts of *L. barbarum* display stronger antioxidant effects than the aqueous extract of *L. barbarum*. For bioactive constituents, *L. barbarum* is rich in antioxidants, including hydrophilic polysaccharides [41] and the hydrophobic flavonoids carotenoid and riboflavin [42]. Despite polysaccharide fractions, LBP has been shown to have antioxidant activity, and a previous study revealed that crude polysaccharide extracts exhibited stronger antioxidant activity than purified polysaccharide fractions (LBP) because antioxidants such as carotenoids, riboflavin, ascorbic acid, thiamine, and nicotinic acid are more abundant in crude extracts. In our study, the results showed that both LBA and LBE reduced the level of...
ROS in human ARPE-19 cells after UVB irradiation. Moreover, the JC-1 staining for detecting the loss of mitochondrial membrane potential (MMP) as a marker of mitochondrial-mediated apoptosis was conducted, and the results showed that the pretreatment of both LB extracts prevented the loss of MMP following UVB irradiation.

Therefore, we further determined whether LBA and LBE occluded cell apoptosis under UVB-irradiated ARPE-19 cells using a cytometer-based Annexin V/PI double staining assay. Consistently, our results showed that UVB-induced apoptosis of ARPE-19 cell at 24 hr; however, the percentages of UVB-induced apoptotic cells were decreased with both LB extract pretreatments. Both extracts exerted protective effects on UVB-induced apoptosis in an ARPE-19 cell line.

It is well known that irradiation of UVB or visible light causes photochemical lesions or other damages through the induction of DNA damage [43]; therefore, examination of whether LB extracts could protect ARPE-19 cells from DNA damage using the assessment of γH2AX, a marker of DNA damage [31], is desirable.
Our results showed that the pretreatments of both LBE and LBA significantly attenuated the activation of γH2AX in a dose-responsive manner, suggesting a protective role of LB extracts in UVB-induced DNA damage of human ARPE-19 cells. Because of the great benefits of L. barbarum, many studies have been conducted extensively on the preventive effects of LB extracts against eye diseases [44–46]. For example, the work of Li et al. suggested that LBP is neuroprotective and could delay secondary degeneration of retinal ganglion cells (RGCs), which might be associated with the downregulation of oxidative stress and the MAPK JNK/c-Jun pathway in retinal tissue [46]. Furthermore, the work of Liu et al. demonstrated that the treatment of LBP not only improves morphology and function of retinal tissue in rd1 mice, an in vivo photoreceptor degenerating model of retinitis pigmentosa, but also delays the functional degeneration of RGCs [45]. However, little is known regarding the bioactivity and mechanism of L. barbarum against DNA damage; consequently, our study is the first to reveal L. barbarum extracts as protective agents for UVB-induced DNA damage.

Previously, Lin et al.’s work analyzed the interrelationship between Lycium barbarum and gene expression in mouse spleen using oligo-microarray, and their result showed that three genes, Bcl-2, NFkB, and TNF, were upregulated whereas two proapoptotic genes (apoptotic protease-activating factor-1 (apaf-1) and caspase-3) were downregulated by the treatment of Lycium barbarum [47]. Given the superior cytoprotection on UVB irradiation-induced growth arrest by ethanol extract LBE, we further investigated the mechanism using human global gene expression profiles of RNA isolated from ARPE-19 cells pretreated with L. barbarum extracts LBE or LBA prior to the irradiation of UVB analyzed by a cDNA microarray at 24 hr. We identified that a total of 403 genes in LBE-treated cells and a total of 558 genes in LBA-treated cells were changed, and 308 genes might be involved in the signaling pathways associated with the protective effect of L. barbarum extracts using analysis by IPA bioinformatics software (Figure 7(a)). The analysis of IPA further suggested that three signaling pathways of the top-level functional annotation categories of genes including PPAR, Integrin survival signaling, and Toll-like receptor (TLR) signaling pathways were affected by the pretreatment of both L. barbarum extracts LBE and LBA (Figures 7(b) and 7(c)).

Peroxisome proliferator-activated receptors (PPARs) are important nuclear transcription factors for oxidative defense system in eukaryotic cells. The PPAR family has at least three members including PPARa, PPARβ/δ, and PPARγ [48]. For example, PPARγ has been shown to have an antioxidant function through transcriptional regulating a number of antioxidation-associated gene catalase (CAT), glutathione peroxidase (GPX-3), heme oxygenase-1 (HO-1), and manganese superoxide dismutase (MnSOD) [49].

Integrins are transmembrane proteins and the heterodimerization of integrin receptors have been shown to regulate cell survival, differentiation, and migration of metazoa through communicating signals via the plasma membrane [50]. Neuronal survival is exhibited by olfactory ensheathing cell, a type of glia that supports axon outgrowth olfactory system through integrin/milk fat globule-EGF factor 8 (MFG-E8) a secreted glycoprotein) signaling pathway [51]. Moreover, a recent study demonstrated that the sensory axons of the spinal cord can regenerate by expressing tenascin-binding α9-integrin together with kindlin-1, the integrin activator [52].

Toll-like receptors (TLRs) are single and noncatalytic receptors with membrane-spanning domains and the TLRs can recognize microbial pathogen and initiate signal transduction pathways of innate immunity [53]. Beyond the role of TLRs in activating innate immunity, TLR seems to play a critical role in neuron survival [54–57]. Patel and Hackam’s work suggested that the oxidative stress-activated TLR3 triggers neuron protection rather than pathogenic signaling in the retina tissue of mice [58]. Bsibsi et al.’s work showed that inflammation-induced TLR-3 activation triggers a neuroprotective response rather than a proinflammatory response in human astrocytes [59]. Furthermore, Jeong et al.’s work showed that polyinosinic poly-cytidylic acid- (poly(I:C)-) induced activation of TLR3 might favor the survival of microglia after cerebral ischemia, suggesting the neuroprotective role of TLR3 [60]. Controversially, the activation of TLR might also create a degenerative effect in neuron cells; for example, Chintala et al.’s work showed that poly(I:C)-induced TLR3 activation upregulated the protein level of MAPK JNK3 and promoted the degeneration of RGCs in mouse eye [61]. Similarly, Gao et al.’s work showed that poly(I:C)-induced TLR3 evoked an inflammatory response and cell death both in murine photoreceptor 661W cells and an in vivo model [62]. Therefore, the role of TLR in pro-survival or pro-cell death of neuron cells may depend on the types [55] of stimuli, the level [60] of stimuli, and the downstream of TLR signaling [58].

In the results of microarray analysis combining the IPA bioinformatics approach, the activation level of the TLR pathway is higher in LBE pretreatment than that in LBA pretreatment (Figures 7(b) and 7(c)). Given the pivotal role of the TLR pathway in cell proliferation and cell survival [63], it is worth further investigating the three signaling pathways, especially the TLR pathway in LB extract-mediated cytoprotection in human retinal pigment epithelial cells.

4.1. LBA and LBE Rescue UVB-Induced G2/M-Arrest after Irradiated ARPE-19. In the study, 50 μl/cm² of UVB irradiation significantly caused the accumulation of cell cycle G2/M population in human ARPE-19 cells. Interestingly, Chou et al.’s work showed that 10 to 30 μl/cm² UVB caused S-arrest in ARPE-19 cells [64]; we suggest that different doses of UVB irradiation might result in the discrepancy in the arrest of cell cycle S and G2/M phases. Furthermore, the pretreatment of both LBA and LBE rescues UVB irradiation-induced G2/M-arrest in ARPE-19 (Figure S1).

5. Conclusions

We demonstrated that both aqueous and ethanol extracts of L. barbarum exhibit antioxidant activities and prevent UVB irradiation-induced DNA damage and apoptosis of ARPE cells. Interestingly, ethanol extracts of L. barbarum exert...
Figure 7: Activation of genes involved in *L. barbarum* extract-mediated protective effect on UVB irradiation. The human global gene expression profiles of RNA were isolated from ARPE-19 cells pretreated with *L. barbarum* extracts LBE or LBA prior to the irradiation of UVB then analyzed by a cDNA microarray at 24 hr. (a) A total of 403 genes in LBE and a total of 558 genes in LBA were changed, and 308 genes might be involved in the signaling pathways associated with the protective effect of *L. barbarum* extracts using the analysis of IPA bioinformatics software. (b). Top-level functional annotation categories of genes including peroxisome proliferator-activated receptor (PPAR), the nuclear receptor as a transcription factor, Integrin, and Toll-like receptor (TLR) signaling pathways were affected by the pretreatment of both *L. barbarum* extracts LBE and LBA. Remarkably, the activation level of the TLR pathway is higher in the LBE pretreatment than in the LBA pretreatment. (c) Genes might be involved in the activation of the TLR pathway. A pink color indicates the upregulation of the genes. A green color indicates the downregulation of the genes.
stronger antioxidant than aqueous extract, suggesting that polyphenolic components might enhance the antioxidant activities of the ethanol extract of *L. barbarum*. Based on the results of the present study, we conclude that both aqueous and ethanol extracts of *L. barbarum* protect ARPE-19 cells from UVB exposure-induced growth and apoptosis through reducing endogenous ROS and DNA damage (Figure 8). This study is the first to depict the cytoprotective activity of *L. barbarum* extracts on cytoprotection in UVB-induced DNA damage in ARPE-19 retinal cells that might be correlated with three signaling pathways including PPAR, Integrin, and TLR. Further investigation on the ethanol extracts of *L. barbarum* will benefit the prevention of retinal degenerative-associated diseases such as AMD in the future.

**Data Availability**

The data used to support the findings of this study are available from the corresponding author upon request.

**Conflicts of Interest**

The authors declare no conflict of interests.

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**Supplementary Materials**

Figure S1: the effect of *L. barbarum* extract pretreatments on UVB-induced G2-arrest in ARPE-19 cells. ARPE-19 cells were pretreated with 50 μg/mL *L. barbarum* extracts LBA or LBE, respectively, for 2 hr prior to the irradiation of UVB described in the section of Materials and Methods.

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