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Protective Effects of Forsythiaside A, Forsythiaside B, and Phillyrin against UVA-Induced Cell Damage

Fu-Jun Jin[1], Hong-Wei Zhao[2], Xiao Yuan[1], Zhi-Yun Wu[2], Qiao-Li Wang[1], Chung-Wah Ma[2], Zhe Ren[1,*], Yi-Fei Wang[1,*]

[1]Guangzhou Jinan Biomedicine Research and Development Center, National Engineering Research Center of Genetic Medicine, Jinan University, Guangzhou, PR China
[2]Infinitus (China) Company Co., Ltd, Guangzhou, PR China
*Corresponding author: rz62@163.com; twang-yf@163.com

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Abstract Chronic exposure to ultraviolet (UV) radiation affects diverse constituents of skin and induces photoaging. Current therapeutic approaches cannot fully reverse the pathophysiology of photoaging, thus necessitating the discovery of new strategies to protect skin from UV radiation. Natural products have long been reported to possess photoprotective effects. Herein, we investigated the potential anti-UVA effects of forsythiaside A, forsythiaside B, and phillyrin extracted from *Forsythia suspensa (Thunb.) Vahl*. In vitro, all three compounds significantly prevented cytotoxicity, DNA damage, apoptosis, and cell-cycle arrest caused by a low dose of UVA. All three compounds also inhibited the expression of inflammatory cytokines. Collectively, our results suggest that these three compounds could be preventives against UVA-induced skin damage.

Keywords: *Forsythiaside A, Forsythiaside B, Phillyrin, Anti-UVA, Anti-inflammatory*

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1. Introduction

Aging is one of the most elementary biological constants, ultimately culminating in the inability to adapt to metabolic stress and a decline of biological function [1]. The skin is the largest organ of the body and is a physiological barrier against damaging environmental effects. Skin aging is characterized as either intrinsic or extrinsic aging senescence, with the latter mainly due to environmental factors like UV radiation, smoking, wind, sun, harmful chemicals [2,3].

Photoaging is induced by chronic skin exposure to solar UV radiation and is a critical initiator of many skin diseases [4]. Ultraviolet rays damage the skin by causing oxidative stress, inflammation, DNA damage, production of proteolytic enzymes, immunosuppression, and stimulation of growth factors that promote cancer cell growth [5,6,7]. The clinical symptoms of skin photoaging include mottled dyspigmentation, wrinkling, sagging, fragility, easy bruising, loss of elasticity, accumulation of precancerous lesions, and epithelial neoplasms [5]. Solar rays can be divided into long wave (UVA; 320–400 nm), mid wave (UVB; 290–320 nm) and short wave (UVC; 200–290 nm) [8]. UVC radiation is strongly absorbed by the ozone layer, barely reaching the earth's surface. UVB radiation constitutes only 5% of total solar UV radiation but is primarily responsible for skin cancers and other skin diseases [9]. The remaining 90–95% of the solar UV spectrum is UVA, which can penetrate deep into our dermis and epidermis. UVA is responsible for chronic damage associated with photoaging as well as malignant cancers [10]. As the ozone layer is progressively destroyed, more UV radiation reaches the earth, intensifying photoaging. New UV-protective agents are therefore urgently needed to provide increased skin protection.

Forsythia suspensa (Thunb.) Vahl, is a widely distributed climbing plant and an important traditional Chinese medicine [11]. It has significant antiviral, anti-bacterial, antioxidant, and anti-inflammatory activities [12,13]. Lignans, phenylethanoid glycosides, flavonoids, volatile oils, and terpenes have all been extracted from the plant, among which forsythiaside A, forsythiaside B, and phillyrin are thought to be the main bioactive constituents. Forsythiasides A and B have potent neuroprotective and cardioprotective effects [14,15,16]. To our knowledge, however, UV-protective activity has not been reported for these compounds. We therefore sought to evaluate whether these compounds might protect against UVA-induced skin damage.

2. Materials and Methods

2.1. Chemicals

TRIzol® Reagent, low-melting-point agar, and regular agar were obtained from Invitrogen (Invitrogen, CA, USA). 2-(2, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), lipopolysaccharide (LPS), propidium iodide (PI), and annexin V-FITC were purchased from Sigma-Aldrich (Sigma-Aldrich, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), and penicillin-streptomycin were bought from Gibco (Gibco, CA, USA). All other reagents used were of pharmaceutical grade.

2.2. Cell Culture

NIH/3T3 fibroblast cells and RAW 264.7 macrophages were obtained from Cell Bank of the Chinese Academy of Sciences, Shanghai, PR China and were cultured in DMEM supplemented with 10% heat-inactivated FBS and 100 U/mL penicillin/streptomycin in a humidified incubator with a 5% CO_2 atmosphere at 37°C.

2.3. Preparation of Phillyrin, Forsythiaside A, and Forsythiaside B

250 g of *F. suspense* was ground into 30–60 mesh powder, extracted with 2000 g 70% ethanol, and extracted again with 1500 g 70% ethanol. The combined extracts were filtered and evaporated, and the residue was suspended in a 2-fold weight ratio of water and loaded onto a 0.4 L macropore resin PIPO-02 column (C1). C1 was washed with purified water until the eluate became light in color, and a second PIPO-02 column (C2) was connected. The columns were washed with 20–30 column volumes of methanol/ethyl acetate (4:1): 0.2% formic acid (5:98), after which a third PIPO-02 column (C3) was connected and washed with methanol/ethyl acetate (4:1): 0.2% formic acid (10:90) until forsythiaside A was detected in the eluate. C1 was then disconnected, and a 70 mL C-18 column was connected to C3 and eluted with methanol/ethyl acetate (4:1): 0.2% formic acid (10:90) until forsythiaside A was detected in the eluate. The C18 column was then disconnected from C3 and washed with methanol. After evaporating the fractions, the purity of forsythiaside A was 98%. Next, a second C18 column was connected to column 3 and washed with methanol/ethyl acetate (4:1): 0.2% formic acid (10:90) until forsythiaside B was detected in the eluate. The C-18 column was then disconnected from C3 and washed with methanol. The collected methanol fractions were evaporated and refrigerated to yield forsythiaside B at 98% purity. Finally, column C1 was connected to another PIPO-02 column (C4) and eluted with methanol/ethyl acetate (4:1): 0.2% formic acid (15:85) and then eluted with methanol/ethyl acetate (4:1): 0.2% formic acid (20:80). The combined eluate fractions were concentration and crystallized, and the raw crystals were recrystallized in 70% ethanol to yield phillyrin at 98% purity. The identity and purity of each compound were confirmed by HPLC and by comparison to known standards supplied from PI & PI Biotech Inc.

2.4. Cytotoxicity Assays

The cytotoxicity of forsythiaside A, forsythiaside B, and phillyrin toward NIH/3T3 fibroblast cells and RAW 264.7 macrophages was determined using MTT assays. Cells were plated in 96-well plates in 100 µL growth media (DMEM with 10% FBS), cultured overnight, and exposed to a range of compound concentrations for 48 h. 10 µl of a 5 mg/mL MTT stock solution was added to each well, and the plate was incubated for 4 h in the dark. The MTT solution was discarded and 100 µL dimethyl sulfoxide (DMSO) was added to each well. Plates were gently shaken for 15 min at room temperature, after which the OD at 570 and 630 nm were measured with an enzyme immunoassay reader (EIA) (Bio-Rad USA). The normalized cell viability for each compound concentration was determined by comparison to a compound-free control.

2.5. UVA Irradiation

NIH/3T3 fibroblast cells were grown in 96-well plates to 60–70% confluence, after which the medium was discarded, and the cells were covered with a thin layer of phosphate-buffered saline (PBS). Plates were then treated with 1.4 J/cm^2 UVA for 15, 30, 45, or 70 min generated from a model HB 406/A Philips Original Home Solarium (Philips, Groningen, Holland), and the dose was confirmed with a UV Power Pack Radiometer (EIT Inc., Sterling, VA). PBS was then removed and replaced with growth medium, and the plates were cultured for another 24 h. As a control, several plates were mock treated without radiation. 48 h after UVA irradiation the viability of cells in each group was measured by MTT. The 50% cytotoxic irradiation dose (CC_{50}) was calculated and used in subsequent UVA irradiation experiments.

2.6. Evaluation of UVA Protective Activity of each Compound

NIH/3T3 fibroblast cells were grown in 96-well plates to 60–70% confluence and pretreated for 24 h with the indicated concentrations of each compound in growth medium. The medium was then replaced by sterile PBS, and the plates were treated with a CC50 UVA dose. PBS was then removed and replaced with growth medium without compound. As a comparator, a control plate received UVA exposure without compound pretreatment. 24 h after UVA irradiation the cell viability of each group was measured by MTT assay and calculated in comparison to the control.

2.7. Comet Assay

NIH/3T3 fibroblast cells were grown in 12-well plates to 60–70% confluence. The medium was discarded and replaced with 1 mL of growth medium per well that contained the standardized test dose of each compound: 100 µg/mL forsythiaside A, 6.25 µg/mL forsythiaside B, or 200 µg/mL phillyrin. The plates were cultured for another 24 h and treated with the CC50 UVA dose for 24 h. The cells were digested, collected by centrifugation, and suspended in PBS at a concentration of 1×10^6 cells/mL. Cell suspensions were mixed with 0.8% low-melting-point agar and placed onto a slide precoated with 0.7% regular agar. After the agar solidified, slides were soaked in a prechilled, fresh lysing solution (pH 10) for 1.5–2 h at 4°C. After rinsing with fresh 0.4 M Tris buffer (pH 7.5), slides were soaked for 15 minutes in a reservoir filled with electrophoresis buffer (pH > 13) and subjected

to electrophoresis for another 15 min (25 V, 300 mA). Slides were neutralized three times by a 5 min soak with Tris-HCl (pH 7.5). Slides were then stained with PI and photographed under a fluorescent microscope (OLYMPUS IX71). The percentages of tail DNA, tail length, and Oliver tailMor, which indicates damaged DNA, were analyzed using the Casp software program (version 1.2.2).

2.8. Apoptosis and Cell cycle Analysis

NIH/3T3 fibroblasts were grown in a 6-well cell culture plate to 60–70% confluency and treated with growth medium containing the standardized test dose of each compound for 24 h. The plates were then exposed to 1.4 J/cm^2 UVA for 45 min. After another 24 h, the cells were harvested in cold PBS, fixed in 70% ethanol, and stored overnight at 4°C. The cells were washed once with PBS and resuspended in 1 mL of 50 mg/mL PI staining reagent containing 100 µg/mL RNase, then incubated in the dark for 30 min. The percentage of cells in each cell cycle phase was measured by fluorescence activated cell sorting (FACS). For apoptosis analysis, the cells were harvested and washed twice with cold PBS, after which they were resuspended in 100 µL incubation buffer with annexin V-FITC and PI. After incubation in the dark for 15 min, apoptosis was analyzed by FACS.

2.9. LPS-stimulated Inflammation Assay

RAW 264.7 macrophages were cultured in 6-well plates to 60–70% confluence. The medium was discarded and cells were washed twice with PBS. Serum-free DMEM medium was added to each well. After 24 h, the medium was replaced with growth medium that contained the standardized test dose of each compound, and the plates were incubated for 4 h. The medium was then replaced with LPS induction medium (100 ng/mL) and cultured for another 24 h, after which the cells were collected for total RNA isolation.

2.10. RNA Isolation, Reverse Transcription, and Quantitative Real-time PCR (qPCR)

Total RNA was extracted using TRIzol® Reagent, and RNA concentrations were measured using a spectrophotometer (Thermo, USA) monitoring at 260 and 280 nm. 500 ng of extracted RNA was reverse transcribed using a PrimeScript RT reagent kit (Takara, Japan), and qRT-PCR assays were conducted using SsoFast™ EvaGreen® Supermix (Bio-Rad, USA) according to the manufacturer's instructions. Primer pairs used were specific for IL-1β (F: 5'-GAG CCT GTG TTT CCT CCT TG-3' and R: 5'-CAA GTG CAA GGC TAT GAC CA-3'), IL-6 (F: 5'-CTG ACA ATA TGA ATG TTG GG-3' and R: 5'-TCC AAG AAA CCA TCT GGC TAG G-3'), TNF-α (F: 5'-GGG AGC AAA GGT TCA GTG AT-3' and R: 5'-CCT GGC CTC TCT ACC TTG TT-3'), iNOS (F: 5'-AAG CAG CTG GCC AAT GAG-3' and R: 5'-CCC CAT AGG AAA AGA CTG CA-3'), MCP-1 (F: 5'-GTC TCT GCA ACG CTT CTG TGC C-3' and R: 5'-AGT CGT GTG TTC TTG GGT TGT GG-3'), GAPDH (F: 5'-GTC ATT GAG AGC AAT GCC AG-3' and R: 5'-GTG TTC CTA CCC CCA ATG TG-3'). The relative expression of each genes was normalized to the housekeeping gene GAPDH.

2.11. Statistical Analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA), and results given are the mean ± SEM. The statistical significance of specific comparisons was determined by the Student's t test; *p* values of less than 0.05 were considered significant.

3. Results

3.1. Cytotoxicity and UVA-protective Activity of *F. suspensa* Bioactives

Forsythiaside A, forsythiaside B, and phillyrin were isolated from *F. suspensa*, and their chemical structures were unequivocally identified (Figure 1). The cytotoxicity of each was determined in NIH/3T3 cells by MTT assay. The maximal concentration at which no cytotoxicity was observed were > 200 µg/mL forsythiaside A, 50 µg/mL forsythiaside B, and > 200 µg/mL phillyrin (Figure 2A).

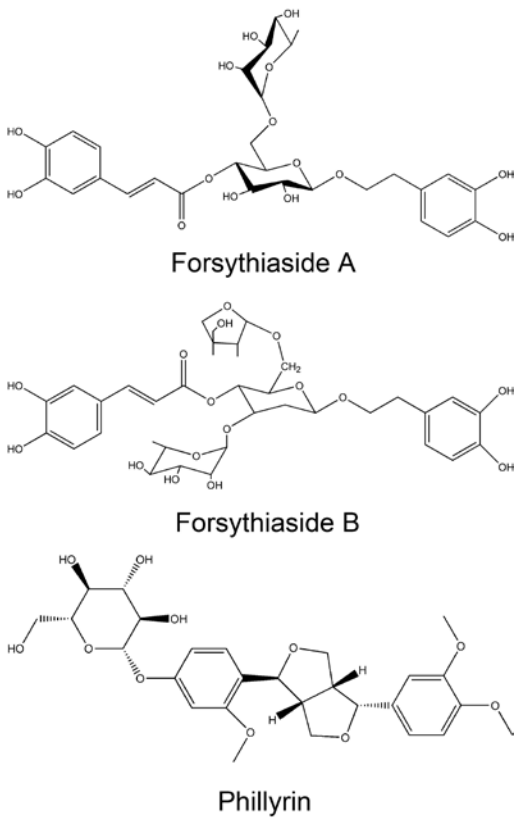

Figure 1. Chemical structures of Forsythiaside A, forsythiaside B, and phillyrin extracted from *F. suspensa*

To specially measure the UVA-protective activity of each compound on the dermis damage caused by UVA irradiation. The mouse fibroblast NIH/3T3 cells were chosen as a model of the dermis and be used in the sequent experiment. Firstly, we measured the cytotoxicity of irradiation with 1.4 J/cm^2 UVA for 15, 30, 45, and 70 min, followed by MTT assay for viability. The 45 min dose showed half maximal inhibition (Data not shown) and was used for UVA-protection assays. Next, the UVA-protective activity of each compound was measured after

pretreatment with compounds and UVA exposure. All three compounds exhibited excellent UVA-protective activity (Figure 2B). Compared to a 50% viability reduction in the absence of compound, viability was only decreased by 25%, 29%, and 32% when cells were pretreated with 100 µg/mL forsythiaside A, 6.25 µg/mL forsythiaside B, or 200 µg/mL phillyrin, respectively. These concentrations were selected as a standardized test concentration for further characterizations.

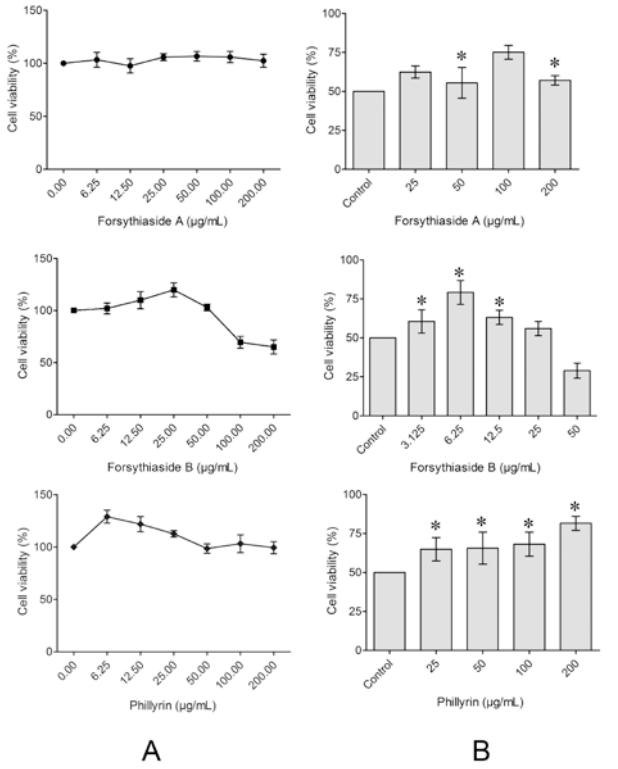

Figure 2. The UVA-protective activity of *F. suspensa* bioactives. (A) The cytotoxic activity of the compounds on NIH/3T3 cells. (B) The protective activity of a 24 h pretreatment with each compound against 48 h UVA exposure. Data are the mean values of at least three independent experiments ± SEM. *$p < 0.05$: significant difference compared to the control group

3.2. *F. suspensa* Bioactives Protect NIH/3T3 Cells from UVA Induced DNA Damage

UVA irradiation can cause DNA damage by generating reactive oxygen species that form DNA adducts, cause double-strand breaks (DSBs), and make interstrand DNA crosslinks [17]. UVA irradiation caused severe DNA damage in NIH/3T3 cells in the absence of a protective compound, as evidenced by the appearance of comet tails of broken DNA after single-cell gel electrophoresis (Figure 3). In contrast, pretreatment with the standard test concentration of each compound (100 µg/mL forsythiaside A, 6.25 µg/mL forsythiaside B, or 200 µg/mL phillyrin) for 24 h greatly decreased UVA-induced DNA damage. In fact, the percentage of DNA with tails decreased by ~94%, and the tail length reduced 89%. The oliver tailMor also dropped to 89% after pretreatment with forsythiaside A. Quantitatively, forsythiaside B inhibited the formation of DNA with tails, tail length, and the oliver tailMor by 88%, 87%, and 95%, respectively. Similarly, phillyrin inhibited the three measurable by 74%, 73%, and 89%.

3.3. *F. suspensa* bioactives Inhibit UVA-Induced Apoptosis and G1 Cell Cycle Arrest

We next evaluated whether the *F. suspensa* bioactives could protect against UVA-induced apoptosis and cell cycle arrest using annexin-V and PI staining after exposure to UVA for 24 h in the presence of absence of the compounds at their standard test concentrations. As shown in Figure 4, the proportion of apoptosed cells increased 3.86% after low dose UVA irradiation in the absence of compound treatment, whereas no significant increase in apoptosis was observed after compound pretreatment. By flow cytometry, UVA irradiation increased the proportion of cells in G1 phase by ~38% but only by 20%, 6.3%, and 9.4% after pretreatment with each bioactive. Collectively, these results demonstrated a protective effect against apoptosis and G1 cell-cycle arrest.

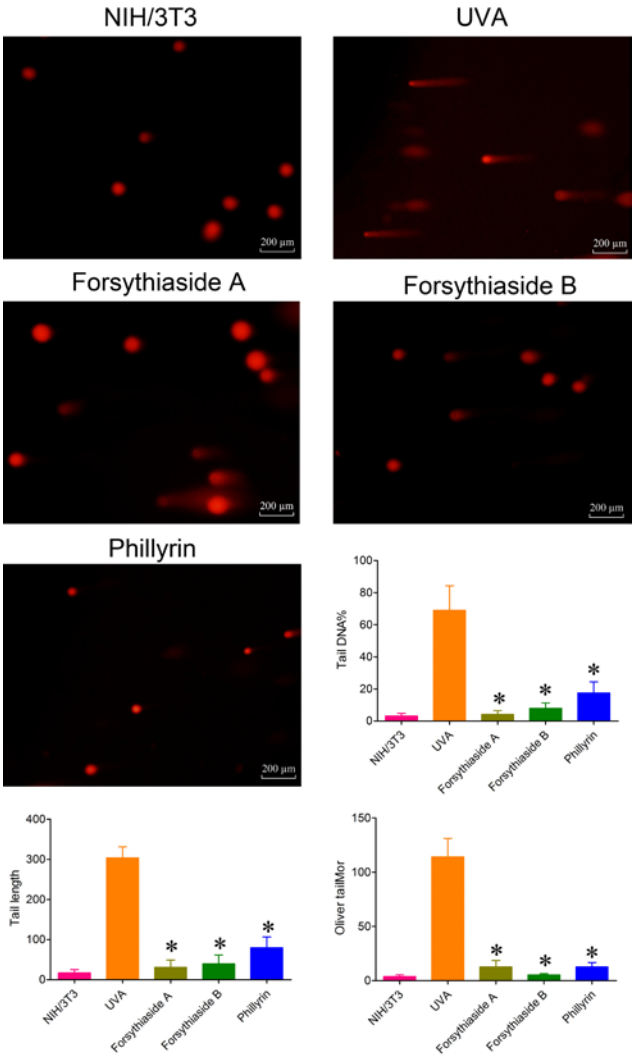

Figure 3. The protective activity of *F. suspensa* bioactives against DNA damage. NIH/3T3 cells were pretreated for 24 h with 100 µg/mL forsythiaside A, 6.25 µg/mL forsythiaside B, or 200 µg/mL phillyrin followed by exposure to UVA for 24 h, followed by single-cell electrophoretic assay of cells from each group. The percentages of tail DNA, tail length and oliver tailMor were analyzed using the Casp software program. Data shown are the mean values of at least three independent experiments ± SEM. *$p < 0.05$: significant difference compared to the control group

3.4. Anti-inflammatory Effect of *F. suspensa* Bioactives

One of the principal mechanisms by which UVA irradiation causes cell damage is by inducing inflammation. To determine the anti-inflammatory activity of our compounds, we challenged RAW 264.7 cells with LPS in the presence or absence of compound treatment, and the expression levels of IL-1, IL-6, TNF-α, MCP-1, and iNOS cytokines were monitored by qRT-PCR. Importantly, we confirmed the lack of cytotoxicity for each compound against RAW 264.8 cells, maximal nontoxic concentrations of forsythiaside A, forsythiaside B, and phillyrin that were > 200 μg/mL, 50 μg/mL and 200 μg/mL, respectively (Figure 5A). We then found that 100 μg/mL forsythiaside A significantly inhibited MCP-1 and iNOS expression by 60% and 23% (Figure 5B). Likewise, 6.25 μg/mL forsythiaside B inhibited MCP-1 expression by 56%, and finally, pretreatment with 200 μg/mL phillyrin reduced the expression of IL-1, IL-6, MCP-1, and TNF-α by 41%, 77%, 70%, and 53%, respectively. Thus, all of the *F. suspensa* bioactives exhibited significant anti-inflammatory activity.

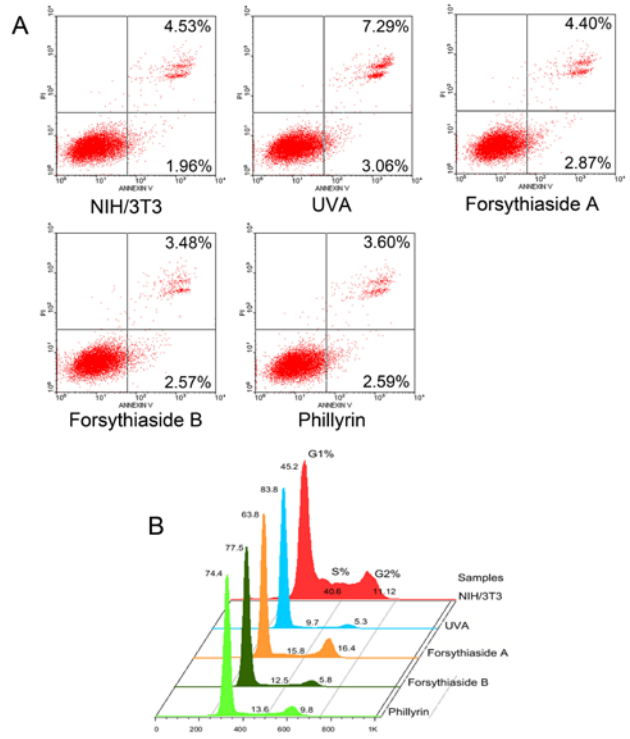

Figure 4. The protective effect of *F. suspensa* bioactives against UVA-induced apoptosis and cell-cycle arrest. (A) After exposure to UVA for 24h, untreated cells or cells pretreated as described in Figure 3 were irradiated, followed by staining with annexin-V/PI and quantification of apoptosis by FACS. (B) To assess cell-cycle arrest, the cells were stained with PI staining reagent, and the percentages of cells in each phase of the cell cycle were measured by FACS

4. Discussion

Photoaging is caused by chronic exposure to solar radiation and is a complex biological process that affects diverse skin constituents [18]. UV irradiation can trigger DNA damage, depletion of the antioxidant defense system, release of pro-inflammatory mediators, cell-cycle arrest, apoptosis, and immunosuppression [19]. The use of chemopreventive agents, especially natural products like plant polyphenols, has shown some effectiveness for preventing the damaging effects of UV irradiation [10].

The majority of UV damage to skin cells is thought to occur via an oxygen-dependent mechanism involving multiple cellular targets, including proteins, membranes, and DNA [20]. The genotoxicity of UVA most likely occurs via indirect mechanisms that involve reactive oxygen species like superoxide anion ($O2^-$), hydroxyl (OH^-), and hydrogen peroxide (H_2O_2) [21,22]. The damage caused includes pyrimidine dimers and double- or single-strand breaks, which can be removed by nucleotide excision repair (NER) or recombinational repair [23,24]. In many cases, cells will arrest at the G1 phase to enable DNA repair, thereby protecting cells against the propagation of mutations [25], and cell cycle regulators like p53, p21 play an important role in this response [26,27].

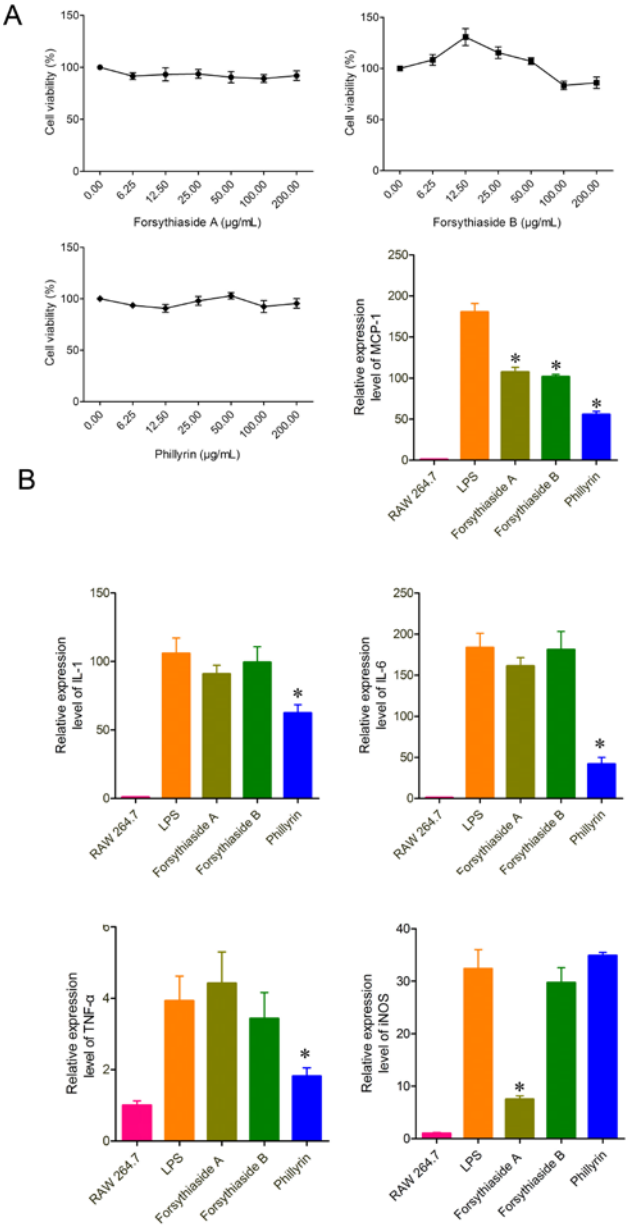

Figure 5. The anti-inflammatory activity of *F. suspensa* bioactives. (A) The cytotoxicity of the tested compounds toward RAW 264.7 cells. (B) Relative mRNA expression levels of IL-1, IL-6, TNF-α, MCP-1, and iNOS after compound pretreatment and LPS stimulation. Compound concentrations were the same as in Figs. 3 and 4. The data presented are the average values from at least three independent experiments ± SEM. *$p < 0.05$ indicates a significant difference compared to the control group

DNA damage caused by ROS and lipid peroxidation can also induce apoptosis, with ROS being the driver of UVA-induced apoptosis. This occurs by ROS repressing the expression of anti-apoptosis genes like bcl-2 and promoting the expression of apoptosis-related genes like p53 and c-myc [28]. Herein, we showed that a low dose of UVA irradiation was cytotoxic toward NIH/3T3 cells, caused significant DNA damage, and induced apoptosis and cell-cycle arrest.

We also evaluated the ability of three *F. suspensa* bioactive components, phillyrin, forsythiaside A, and forsythiaside B, to protect against these UVA-induced effects. For each determination, cells were pretreated with a non-toxic concentration of each compound and then exposed to a damaging dose of UVA. We found that all three bioactives were protective against UVA cytotoxicity. This protective effect is consistent with literature reports that these three compounds have antioxidative activity [14,15,16].

Inflammation is also thought to be an important trigger of the pathogenesis of multiple age-related diseases including atherosclerosis, autoimmune disorders, and neurodegenerative diseases [29]. Some studies have even reported that inflammatory response to UVA exposure causes protein denaturation, DNA damage, and oxidative stress [30]. Macrophages are an important trigger of this response by secreting inflammatory mediators when activated [31]. We therefore evaluated whether the *F. suspensa* bioactives have anti-inflammatory activity in vitro with LPS-challenged RAW 264.7 cells. We found that phillyrin greatly inhibited the LPS-stimulated expression of IL-1, IL-6, TNF-α, and MCP-1. Similarly, forsythiaside A significantly inhibited the LPS-stimulated expression of MCP-1 and iNOS, and forsythiaside B significantly inhibited the expression of MCP-1. These results are consistent with a previous reports in which these three compounds inhibited inflammation by suppressing activation of the NF-κB and MAPK pathways [14,15,16,32]. A series of studies over the past decade have also shown that chronic oxidative stress can cause activation of a variety of transcription factors such as NF-κB and p53, which may explain the anti-inflammatory activity of the compounds [33].

5. Conclusions

In conclusion, as our environment continues to change and increase our exposure to UVA irradiation, photoaging is becoming a more serious problem. Consequently, it is becoming more important to develop new preventive and therapeutic strategies against UVA skin damage. Herein, we showed that forsythiaside A, forsythiaside B, and phillyrin significantly attenuate cell damage caused by UVA and that this protective effect is characterized by protection against DNA damage, apoptosis, and G1 phase cell-cycle arrest. Moreover, the compounds significantly repress the expression of multiple inflammatory cytokines. With these promising activities and low inherent cytotoxicity, these compound may be promising and safe chemical additives for sunscreens.

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Conflict of Interest

The authors have no competing interests

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