Protective effect of the *Rubus chingii* Hu. fruit extract on ultraviolet B-induced photoaging via suppression of mitogen-activated protein kinases (MAPKs) *in vitro*

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Received: March 21, 2019; Revised: May 3, 2019; Accepted: June 3, 2019; Published online: July 7, 2019

Abstract: *Rubus chingii* Hu. is an important traditional Chinese medicine with a beneficial effect on the kidney. Traditional Chinese medicine theory believes that supporting the kidney has a key role in anti-aging. To the best of our knowledge, the anti-photoaging activity of *Rubus chingii* Hu. has not been investigated. We investigated the anti-photoaging effects and the underlying mechanism of the ethyl acetate fraction of *Rubus chingii* Hu. fruit ethanol extract (E-EA) in UVB-induced human fibroblasts (HFF-1). Our results show that E-EA (10–40 μg/mL) displayed preventive and therapeutic effects on UVB-induced cytotoxicity in HFF-1 cells. E-EA also presented significant anti-photoaging properties in UVB-induced HFF-1 cells by decreasing the levels of reactive oxygen species (ROS), inhibiting matrix metalloproteinase 1 (MMP-1) production and promoting type-I procollagen production via suppression of the mitogen-activated protein kinase (MAPK) signaling pathway. These results suggested that E-EA might be a promising anti-photoaging agent to protect the skin from UVB damage, with a potential application in cosmetics.

Keywords: *Rubus chingii* Hu.; antioxidant activity; anti-photoaging; reactive oxygen species (ROS); mitogen-activated protein kinase (MAPK) signaling pathway

INTRODUCTION

Skin, the largest organ of body, acts as a physical and immunological barrier against harmful environmental factors such as ultraviolet (UV) radiation, pathogens and toxicants [1]. UV radiation is classified into UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). UVB is the commonest and most harmful external component that threatens the skin and results in skin photoaging [2, 3]. Prolonged exposure to UVB has been reported to cause acute and chronic skin damage such as dryness, reddening, sunburn, roughness, thickening, wrinkling, and skin cancer [4]. Natural plant extracts are widely used as cosmetic ingredients because of their capability to slow skin decline [5], and the search for natural principles is attracting an ever-growing interest in skin care.

UVB stimulation of the skin leads to oxidative stress, which in turn causes overproduction of reactive oxygen species (ROS) and damages the endogenous antioxidant defense system [1,2]. The overproduction of ROS causes lipid peroxidation, DNA damage, and critical structural and functional protein changes, all of which result in cell damage [1,2]. Moreover, the overproduction of ROS activates the mitogen-activated protein kinases (MAPK) signaling pathway, including the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38, and increases the expression of matrix metalloproteins (MMPs), thereby promoting collagen degradation [6,7]. Collagen produced by fibroblasts is the main structural protein in the dermal extracellular matrix (ECM), which is responsible for adherence to connective tissues and the tensile strength of skin [7]. Loss of collagen will
cause wrinkles on the skin [7]. Oxidative stress plays an important role in UVB-induced skin photoaging. Therefore, exogenous supplementation of antioxidants to decrease ROS overproduction was considered as an effective strategy against photoaging [1].

*Rubus chingii* Hu., a member of the Rosaceae family, is a traditional medicinal plant used in China to treat renal asthenia, frequent urination, premature ejaculation and erectile dysfunction [8]. Aging is associated with a decline in health and integrity of the human kidney [9]. According to traditional Chinese medicine theory, reinforcing kidney functions has a key role in anti-aging. A glycoprotein purified from *Rubus chingii* Hu. provides potential anti-aging activities by improving klotho gene expression and improving kidney function [8]. *Rubus chingii* Hu. could protect against photoaging according to its beneficial medicinal effect on the kidneys. In addition, the anti-aging properties of plants are attributed to their antioxidants, which minimize ROS overproduction and protect skin against solar radiation [5]. Improved ROS equilibrium can reduce the risk of kidney damage [10] and cell aging [11]. *Rubus chingii* Hu. has been reported to contain flavonoids with significant antioxidant and protective effects against disease induced by oxidative stress [12-14]. Furthermore, *Rubus chingii* Hu. shows anti-inflammatory activity through the suppression of MAPK activation in macrophages [15]. However, to the best of our knowledge, the anti-photoaging activity of *Rubus chingii* Hu. has not been investigated so far.

In this study, the anti-photoaging properties of *Rubus chingii* Hu. fruit extract were investigated. The optimum solvent fraction was identified by measuring the antioxidant activity of various *Rubus chingii* Hu. fruit fractions. The preventive and therapeutic effects of the fraction on UVB-induced cytotoxicity in human fibroblasts (HFF-1) were evaluated by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) (MTT) method. Furthermore, its effect on ROS, MMP-1 and type-I procollagen expression were investigated and the underlying molecular mechanisms on the MAPK signaling pathway were evaluated in UVB-induced HFF-1 cells. Finally, the main flavonoid components of the optimum solvent fraction were determined by HPLC.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Dried fruit of *Rubus chingii* Hu. was purchased from Leiyunshang Pharmaceutical Co. Ltd (Shanghai, China). Rutin, hyperin, quercetin and kaempferol were purchased from Aladdin Industrial Corporation (Shanghai, China). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), 0.25% (w/v) trypsin and penicillin streptomycin were obtained from Gibco-BRL (Gaithersburg, MD, USA). Enzyme-linked immunosorbent assay (ELISA) kits for MMP-1 and type-I procollagen were purchased from Shanghai Meilian Biotechnology Co. Ltd., China. Antibodies against p-ERK, p-JNK, p-p38, ERK, p38 and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against JNK were obtained from Proteintech (Chicago, IL, USA).

**Preparation of Rubus chingii Hu. extract**

Air-dried fruits of *Rubus chingii* Hu. were collected from Zhejiang, China. Twenty g of the dried fruit were macerated in 200 mL ethanol (80%) for 30 min at room temperature and extracted with 80% ethanol (3×200 mL) for 2 h under reflux conditions. The filtrates were combined, concentrated and lyophilized to obtain the *Rubus chingii* Hu. ethanol extract (E; 3.52 g). Three g of E were suspended in methanol/deionized water (v/v, 1:1, 150 mL) and extracted successively with 150 mL petroleum ether, ethyl acetate and n-butyl alcohol 3 times. The organic phases and water phase were concentrated and lyophilized to obtain the petroleum ether fraction (E-PE; 0.29 g), the ethyl acetate fraction (E-EA; 0.93 g), the n-butanol fraction (E-NB; 0.58 g), and the residual water fraction (E-WR, 0.81 g).

**DPPH radical scavenging assay**

DPPH radical scavenging capacity was evaluated as described [16]. Two mL of the extract (1.25, 2.5, 5, 10, 20, 40, 80, 100 μg/mL) were added to 2.0 mL ethanol solution of the DPPH radical (0.1 mmol/L). The mixture was shaken and left to stand at room temperature and in the dark. After 30 min, the absorbance of the mixture was measured at 517 nm using a UV-vis spectrophotometer (CARY 300, Agilent...
Technologies, USA) against an ethanol blank. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E, was used as positive control. DPPH radical scavenging capacity was calculated by the following formula:

$$I(\%) = \frac{A_b - A_s}{A_b} \times 100$$

where $A_b$ is the absorbance of control, and $A_s$ is the absorbance of sample/positive control. All assays were tested in triplicate, and the results were expressed as $IC_{50}$ (samples at the concentration decrease the absorbance by 50%). The lower the $IC_{50}$ value, the better the free radical scavenging activity.

**ABTS⁺ radical scavenging assay**

ABTS⁺ radical scavenging capacity of samples was determined according to the literature with a little modification [17]. The ABTS⁺ solution was obtained by mixing equal parts ABTS (7.0 mmol/L, aqueous solution) and K₂S₂O₈ (2.45 mmol/L, aqueous solution) for 12 h reaction in dark conditions. Before the test, the ABTS⁺ solution was diluted with deionized water to obtain a diluted ABTS⁺ solution with an absorbance of about 0.7±0.05 (734 nm). One mL of the sample with different concentrations (1.25, 2.5, 5, 10, 20, 40, 80, 100 μg/mL) was mixed with 1.0 mL diluted ABTS⁺ solution for 10 min in the dark. The absorbance (734 nm) of the mixture was measured using the UV-vis spectrophotometer. Trolox was used as the positive control. ABTS⁺ radical scavenging capacity was calculated by the following formula:

$$I(\%) = \frac{A_b - A_s}{A_b} \times 100$$

where $A_b$ is the absorbance of control, and $A_s$ is the absorbance of the sample/positive control. All assays were tested in triplicate, and the results were expressed as $IC_{50}$.

**The total reducing power assay**

The total reducing power was measured by ferric reducing antioxidant power (FRAP) according to the literature with a little modification [18]. 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL K₃Fe(CN)₆ (1%, w/v) were added to 2.5 mL of sample solution (50 μg/mL). The mixture solution was shaken and incubated at 50°C water bath for 20 min, and then cooled by ice water. 2.5 mL trichloroacetic acid (10%, w/v), 2.5 mL deionized water and 0.5 mL FeCl₃ solution (0.1%, w/v) were added to the mixture solution for 10 min. The absorbance (700 nm) was measured against the solvent blank. The total reducing power was calculated using a Trolox standard for the calibration curve of five points from 6.25 to 100 μg/mL ($y = 0.00739 x + 0.024$, $R = 0.99929$). The total reducing power was expressed as Trolox equivalents in g/g of extract. All assays were run in triplicate.

**The total flavonoid content assay**

The total flavonoid content (TFC) was measured colorimetrically [19]. Briefly, 100 μL sample (10 mg/mL) were mixed with 4 mL deionized water, 0.3 mL NaN₃ solution (5%, w/v) and 0.3 mL AlCl₃ solution (10%, w/v). The mixture was mixed and allowed to stand at room temperature for 6 min. To this, 2 mL NaOH solution (1 mol/L) was added and diluted with deionized water to a final volume of 10 mL. The absorbance (510 nm) of the mixture was measured 10 min later against the solvent blank. Quantifications were calculated using a rutin standard for the calibration curve of five points from 10.0 to 80.0 μg/mL ($y=0.01274x-0.03297$, $R=0.99978$). The total flavonoid content was expressed as rutin equivalents in mg/g of extract.

**UV absorption**

The UVB absorption spectra of E-EA and Trolox were determined according to the method described previously [4]. Briefly, the UV absorption spectrum for the samples (200 μg/mL in ethanol) was measured using a UV-vis spectrophotometer over a wavelength range of 200-400 nm. The blank (ethanol) was subtracted from the background reading.

**Cell culture**

HFF-1 cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). The HFF-1 cells were cultured in DMEM with 15% FBS and 100 U/mL penicillin–streptomycin at 37°C, 5% CO₂ in a humidified incubator.
UVB irradiation

The HFF-1 cells were washed with 250 μL PBS twice and covered with 50 μL PBS. Immediately, the HFF-1 cells were irradiated with different doses of UVB (312 nm). The UVB doses were determined with an UV-340 meter (Lutron, Taiwan, China). After irradiation, PBS was removed, and the cells were cultured in FBS-free medium for 24 h. Cells without UVB treatment were used as blank controls.

Drug treatments

HFF-1 cells (5×10^3 cells/well) were seeded in 96-well plates and incubated overnight. Subsequently, HFF-1 cells were treated with E-EA (1.25-400 μg/mL or 10-40 µg/mL) in FBS-free medium for 24 h. Cells treated with UVB without drug treatment served as negative controls.

Cell viability assay

The cell viability of HFF-1 was determined using the MTT assay. After incubation, the cells with or without any treatment were incubated with 200 μL 0.5 mg/mL MTT in FBS-free medium for 4 h. The medium was removed and 150 μL DMSO was added into each well to dissolve the formazan crystals. After shaking for 1 min, the plate was read at 492 nm using a multimode microplate reader (SpectraMax M2, Molecular Devices, Silicon Valley, CA). Cell viability was calculated by the following formula:

\[ I (\%) = \frac{A_s}{A_b} \times 100 \]

where \( A_s \) is the absorbance of the solvent control and \( A_b \) is the absorbance of the sample.

The viability of HFF-1 cells was further determined by the calcein acetoxyethyl ester/propidium iodide (calcein-AM/PI) co-stained assay in which the live cells were stained by calcein-AM to development of green color, and the dead cells were stained by PI to red. Briefly, HFF-1 cells were seeded at 3×10^4 cells/well into 24-well plates and incubated overnight. After the required treatments and 24 h incubation, the cells were labeled with calcein-AM/PI for 30 min and then were observed with a Laser Scanning Confocal Microscope (Nikon, Japan) at an excitation wavelength of 490 nm.

Determination of ROS production

Intracellular ROS production was measured using the fluorescent probe, DCFH-DA. HFF-1 cells were seeded at a density of 1×10^4 cells/well in 96-well plates with 200 µL medium, and incubated overnight. After treatment with E-EA (or Trolox) for 2 h, HFF-1 cells were incubated in 100 μmol/L DCFH-DA with 200 μL FBS-free medium for 30 min and irradiated with 40 µJ/cm² UVB. Intracellular ROS production was measured at 480 nm (excitation) and 525 nm (emission) using a multimode microplate reader. Similarly, HFF-1 cells (5×10^3 cells/well) were seeded in a glass dish and incubated overnight. Cells were treated with E-EA (or Trolox), labeled with DCFH-DA and irradiated with 40 µJ/cm² UVB. After irradiation, a laser scanning confocal microscope (Nikon, Japan) was used to view and photograph the HFF-1 cells at an excitation wavelength 488 nm.

Determination of MMP-1 and type-I procollagen production

MMP-1 and type-I procollagen secreted by HFF-1 cells in culture media were estimated by the enzyme-linked immunosorbent assay (ELISA). Cells with or without E-EA (or Trolox) treatment were irradiated with 60 µJ/cm² UVB. The culture media was collected 12 h later. MMP-1 and type-I procollagen were measured using an ELISA kit as recommended by the manufacturer.

Western blot analysis

The relative amounts of MAPKs in the cell were assessed by Western immunoblotting [20]. HFF-1 cells (5×10^4 cells/well) were seeded in 6-well plates and pretreated with different concentrations of E-EA for 24 h. The cells were exposed to 60 µJ/cm² UVB irradiation. At 30 min after irradiation, total cell lysates were extracted and centrifuged. Sixty µL cell lysate in the supernatant were mixed with 15 µL loading buffer and boiled for 10 min. The mixture was separated by 12% sodium dodecyl sulfate (SDS) polyacrylamide-gel electrophoresis (PAGE). The separated protein samples were transferred to PVDF membranes. After blocking with 5% BSA, the membranes were first incubated with primary antibodies, and then washed in tris-buffered saline (TBS) and 0.1% Tween 20 (TBST), and finally incubated with secondary antibodies. After washing in TBST, the target
proteins on membranes were detected using enhanced chemiluminescence reagents and imaging system. The protein bands were analyzed using ImageJ software.

**HPLC analysis**

HPLC analysis was performed with an Agilent 1260 HPLC system equipped with a Unitary C18 column (4.6 mm×250 mm, 5.0 μm 100 A, ACCHROM, China). The mobile phase was composed of 0.1% trifluoroacetic acid (TFA) aqueous solution and acetonitrile with gradient elution (0-5 min, 20%-30% acetonitrile; 5-20 min, 30%-45%; 20-25 min, 45%-100%). The solvent flow rate was 1.0 mL/min, the injection volume was 10 μL and the detection wavelength was 360 nm.

**Statistical analysis**

Data were expressed as means±SD and evaluated using Student’s T-test and SPSS 23 software packages (IBM Corporation, USA). Significance was defined as p<0.05, p<0.01 and p<0.001.

**RESULTS**

**Antioxidant activity and total flavonoid content**

DPPH and ABTS⁺ radical scavenging assays and the total reducing power assay were used to evaluate the in vitroc activity of Rubus chinensis Hu. extracts (Table 1). DPPH radical scavenging ability, in increasing order, was E-EA>E-NB>E-WR>E. ABTS⁺ radical scavenging ability and the total reducing power were in the order of E-EA>E-NB>E-WR. The IC₅₀ value of E-EA for ABTS⁺ radical scavenging ability was 3.18 μg/mL, which was as low as the positive control Trolox. The TFC of the fractions of Rubus chinensis Hu. was also measured, as shown in Table 1. In these fractions, E-EA contained the highest content of flavonoids, followed by E-NB, E and E-WR.

**UV absorption**

E-EA provided strong UV absorption (Fig. 1). Moreover, the UV absorption of E-EA was stronger than that of Trolox at a wavelength range of 200-400 nm.

**E-EA attenuated UVB-induced cytotoxicity**

The effect of E-EA on UVB-induced toxicity in HFF-1 cells was assessed by comparing to Trolox in both preventive and therapeutic experiments using the MTT assay. Cytotoxicity against HFF-1 cells was measured to determine the non-toxic dose of E-EA in comparison to Trolox. As can be seen in Fig. 2A, cell viability was higher than 85% when the cells were incubated with E-EA at doses ranging from 0 to 50 µg/mL. The preventive and therapeutic effects of E-EA in HFF-1 cells under UVB irradiation were determined. In the preventive approach, HFF-1 cells were pretreated with E-EA (0, 10, 20, 40 µg/mL) before irradiation with UVB. As shown in Fig. 2B, UVB irradiation reduced the viability of HFF-1 cells to 78.81%. However, the

Table 1. TFC, DPPH- and ABTS⁺ scavenging activities and the total reducing power of the fractions.

<table>
<thead>
<tr>
<th>samples</th>
<th>TFC (mg rutin/g)</th>
<th>DPPH-IC₅₀ (μg/mL)</th>
<th>ABTS⁺-IC₅₀ (μg/mL)</th>
<th>FRAP (μg Trolox/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>178.16±0.78c</td>
<td>40.49±0.66d</td>
<td>5.08±0.17d</td>
<td>0.570±0.001d</td>
</tr>
<tr>
<td>E-PE</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-EA</td>
<td>275.23±1.20e</td>
<td>9.47±0.03b</td>
<td>3.18±0.08e</td>
<td>0.960±0.002b</td>
</tr>
<tr>
<td>E-NB</td>
<td>245.66±1.36e</td>
<td>12.72±0.19c</td>
<td>3.89±0.05b</td>
<td>0.741±0.007c</td>
</tr>
<tr>
<td>E-WR</td>
<td>146.76±1.74d</td>
<td>37.12±0.16d</td>
<td>8.72±0.06c</td>
<td>0.335±0.005d</td>
</tr>
<tr>
<td>Trolox</td>
<td>5.43±0.08e</td>
<td>3.16±0.04c</td>
<td>1.03±0.003</td>
<td></td>
</tr>
</tbody>
</table>

TFC was expressed as rutin equivalents in mg/g of extract. DPPH- and ABTS⁺ radical scavenging activities were expressed as IC₅₀ (samples at the concentration decrease the absorbance by 50%). FRAP was expressed as Trolox equivalents in μg/g of extract. Each value is expressed as the mean±SD (n=3). Means with different letters in a column are significantly different at p<0.001. "\" means not determined.

Fig. 1. UV absorption spectrum of E-EA and Trolox at 200 µg/mL.
viability of HFF-1 cells after pretreatment with 10, 20, 40 µg/mL E-EA increased to 87.19%, 94.09% and 97.62%, respectively. In the therapeutic approach, HFF-1 cells were initially irradiated with UVB and then treated with E-EA (0, 10, 20, 40 µg/mL). From Fig. 2C it can be seen that the viability of UVB-induced HFF-1 cells increased from 72.56% to 83.00%, 90.43% and 94.55%, respectively after treatment with 10, 20, 40 µg/mL E-EA. The change in cell viability with or without UVB irradiation and E-EA treatment is also illustrated in Fig. 2D-E by the calcein-AM/PI co-staining assay. The results are in accordance with those of the MTT assay. Many dead cells (red) were observed in the UVB irradiation group. The treatment with E-EA caused a decrease in the amount of dead cells in both the preventive and therapeutic approaches. Furthermore, the percentage of viable cells (green) increased with the increase in E-EA concentration.

**E-EA reduced UVB-induced intercellular ROS generation**

Monitoring the intracellular ROS level in HFF-1 cells was used to investigate the effects of E-EA on UVB-induced photoaging. As shown in Fig. 3A, UVB irradiation stimulated an increase in the intracellular ROS levels in HFF-1 cells from 47.62% to 100%. When HFF-1 cells were pretreated with E-EA, the increase in ROS levels induced by UVB was alleviated. The intracellular ROS labeled with DCFH-DA were also photographed using a laser scanning confocal microscope. Fig. 3B showed that the highest fluorescence intensity was observed in cells induced by UVB (negative control). The fluorescence intensity of HFF-1 cells pretreated with E-EA was reduced.

**E-EA inhibited UVB-induced MMP-1 secretion and increased type-I procollagen expression**

In order to further determine the anti-photoaging activity of E-EA, the effects of increasing concentrations (0, 10, 20, 40 µg/mL) of E-EA on UVB-induced MMP-1 expressions and type-I procollagen degradation were measured. As can be seen in Fig. 4, compared with the blank control, the expression of MMP-1 was increased by 36.50%, and type-I procollagen expression was decreased by 15.63% in HFF-1 cells after UVB irradiation. However, when cells were pretreated with 20 and 40 µg/mL E-EA, MMP-1 expression was inhibited by 24.27% (***p<0.001) and 23.90% (***p<0.001), respectively, and type-I procollagen recovered by 7.81% (*p<0.05) and 12.93% (**p<0.01), respectively, as compared to
Interestingly, the recovery effects of E-EA for UVB-induced MMP-1 and procollagen I production were better than the effect of Trolox at a concentration of 40 µg/mL.

E-EA suppressed UVB-induced MAPK pathway activation

To determine the biological effects of E-EA on MAPK signaling pathways, which likely regulated the UVB-induced expression of MMP-1 and type-I procollagen, we examined the relative changes in phosphorylated ERK, JNK and p38 protein levels examined by Western immunoblotting. As shown in Fig. 5, the phosphorylation levels of MAPKs (ERK, JNK and p38) in UVB-induced HFF-1 cells (negative controls) were increased compared to the controls. However, pretreatment of HFF-1 cells with 10, 20 and 40 µg/mL of E-EA resulted in strong decreases in the ratios of p-ERK/ERK, p-JNK/JNK and p-p38/p38 band intensities.

The HPLC analysis of E-EA

The representative flavonoids rutin, hyperin, quercetin and kaempferol were used as standard substances to characterize the chemical composition of E-EA. Fig.
6 shows that all four flavonoids could be detected by HPLC in E-EA, of which the contents of rutin and hyperin in E-EA were 2.65 mg/g and 69.33 mg/g, respectively. The contents of the other two compounds in E-EA were less than 0.75 mg/g.

**DISCUSSION**

Increases in intracellular free radical levels are closely associated with cell senescence, thus free radical scavenging ability is a potential target for aging prevention [1]. In this work, all fractions, except E-PE, exhibited antioxidant activity in a dose-dependent manner within concentrations of 1.25-100 μg/mL. The E-EA fraction had the best antioxidant capacity in the five fractions of *Rubus chingii* Hu., which was in agreement with previous literature [14]. The anti-aging properties of plants are attributed to contained antioxidant substances, such as polyphenols, flavonoids and anthocyanins [5]. Flavonoids are ubiquitous plant secondary products that exhibit a variety of biological activities and are beneficial to human health [21,22]. In our work, the highest flavonoid content was found in E-EA, suggesting that flavonoids were excellent antioxidants. Several other plant extracts, including *Juniperus communis* L., *Hypericum origanifolium* Willd. are widely documented as effective anti-aging agents because of their high flavonoid and high polyphenol contents [23,24]. The flavonoids contained in plants possess potent antioxidant properties and strong UV absorption, and are widely used in skin protection [1,25]. This suggests that E-EA with its high flavonoid content and strong UV absorption might also provide photoaging resistance for skin care.

It is well known that UVB significantly reduces the viability of human fibroblasts [26]. However, plant extracts can effectively reduce the cytotoxicity of UVB. For example, polyphenol extracts from *Hawthorn Crataegus* L. and hesperidin extract from *Zanthoxylum rhetsa* have been reported to possess a preventive or therapeutic effect on UVB-induced cell death in human dermal fibroblasts [4, 27]. In the present study, UVB irradiation remarkably reduced HFF-1 cell viability in both preventive and therapeutic assays. In the preventive approach, pretreatment with E-EA attenuated the UVB-induced cytotoxicity in HFF-1 cells. In particular, when treated with E-EA at a concentration of 40 μg/mL, cell viability was recovered to the level of the control group. Furthermore, in the therapeutic approach, UVB-induced HFF-1 cell damage was effectively repaired by E-EA. When the concentration of E-EA was 20 and 40 μg/mL, cell viability was higher than obtained with 40 μg/mL Trolox. These results indicated that the E-EA fraction exhibited excellent preventive and therapeutic effects against UVB-induced cell damage in HFF-1 cells.

UVB-induced ROS overproduction is directly related to the occurrence of skin photoaging [2]. The intracellular ROS level of HFF-1 cells irradiated with UVB was significantly increased. The extent of intracellular ROS increase was significantly reduced when HFF-1 cells were pretreated with E-EA at test concentrations (10-40 μg/mL) \( (p<0.001) \). These results showed that E-EA prevented HFF-1 cells from UVB-induced ROS overproduction. Similarly, plant extracts such as clove, sea buckthorn seed and hawthorn have previously been reported to inhibit the production of ROS after UVB induction [27-29]. Aging is associated with cell damage from ROS, and reducing ROS production might be one of the effective strategies to prevent cell aging. Thus, E-EA has the potential to protect the skin against UVB-induced photoaging by reducing ROS overproduction.
ROS overproduction activates the MAPK signaling pathways, which stimulates the transcription of MMP-1 genes in fibroblasts and negatively regulates the transcription of genes encoding type-I procollagen [30]. The results presented herein showed that UVB could significantly upregulate MMP-1 expression and downregulate type-I procollagen in HFF-1 cells via activation of the MAPKs, including ERK, JNK and p38. Nevertheless, treating UVB-induced HFF-1 cells with E-EA significantly decreased the phosphorylation of ERK, JNK and p38, which resulted in the attenuation of MMP-1 production and recovery of type I procollagen expression [31]. These results suggest that E-EA has the ability to protect skin from UVB-induced photoaging by downregulating MMP-1 expression and upregulating type-I procollagen production via inhibition of MAPKs.

Flavonoids act as ROS scavengers because of their abundance in hydroxyl groups [30]. Hyperin was determined to be a major compound in *Rubus chingii* Hu. fruit by HPLC analysis, which is consistent with a previous report [13]. Hyperin has been identified as an antioxidant with ROS scavenging ability that can enhance skin density and elasticity by mediating MMP-1 and type-I procollagen expression [32]. We speculate that the antioxidant and anti-photoaging effects of E-EA are due to the hyperin.

To conclude, our study demonstrated that E-EA, which contains hyperin as a major active compound, was capable of protecting HFF-1 cells from UVB-induced photoaging by scavenging free radicals and reducing ROS levels, decreasing MMP-1 expression and recovering type-I procollagen production via suppression of the activation of MAPKs. The presented results suggest that E-EA can effectively protect the skin from UVB damage and that it has potential applications in cosmetics.

**Acknowledgment:** This work was supported in part by the Fundamental Research Funds for Central Universities, No. 22201817022.

**Author contributions:** LM and YH designed the research study. SJ, SZ and YH performed the research. SJ, SZ and JZ performed the statistical analysis. SJ and YH performed the first drafting of the manuscript. All authors read and approved the manuscript.

**Conflict of interest disclosure:** The authors have no financial interests or potential conflicts of interests to declare.

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