

# Anthocyanin from *Lycium ruthenicum* Murr. in the Qaidam Basin Alleviates Ultraviolet-Induced Apoptosis of Human Skin Fibroblasts by Regulating the Death Receptor Pathway

Liwen Wang<sup>1,\*</sup>, Guangmei Wan<sup>1,\*</sup>, Gang Wang<sup>1</sup>, Meihong Zhang<sup>1</sup>, Nanxin Li<sup>1</sup>, Qinning Zhang<sup>2</sup>, Hualing Yan<sup>1</sup>

<sup>1</sup>Department of Dermatology, Qinghai University Affiliated Hospital, Xining, People's Republic of China; <sup>2</sup>Shijingshan Teaching Hospital, Capital Medical University, Beijing, People's Republic of China

\*These authors contributed equally to this work

Correspondence: Hualing Yan, Department of Dermatology, Qinghai University Affiliated Hospital, No. 29, Tongren Road, Chengxi District, Xining, Qinghai Province, People's Republic of China, Email yhlckw@163.com

**Purpose:** The study aimed to investigate the potential protective role of anthocyanin from *Lycium ruthenicum* Murr. in the Qaidam Basin against ultraviolet B (UVB)-induced apoptosis of human skin fibroblasts (HSFs).

**Methods:** HSFs cultured in vitro were randomly divided into a control group, UVB group, and anthocyanin groups (0.1, 0.5, and 1.0 mg/mL). HSFs in the UVB and anthocyanin groups were exposed to 30 mJ/cm<sup>2</sup> UVB to establish a photoaging model. Then, apoptosis rate, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), cysteinyl aspartate specific proteinase-3 (caspase-3), cysteinyl aspartate specific proteinase-7 (caspase-7), and survivin expression were evaluated.

**Results:** UVB irradiation can increase the apoptosis rate of HSFs and expression of TNF- $\alpha$ , caspase-7, and survivin. Anthocyanin pretreatment (0.1, 0.5, and 1.0 mg/mL) decreased UVB-induced apoptosis rate and TNF- $\alpha$  and caspase-7 expression and increased survivin expression. Compared with the control group, the apoptosis rate and expression of TNF- $\alpha$ , caspase-7, and survivin of anthocyanin groups in UVB-irradiated HSFs were high. Among the three doses of anthocyanin (0.1, 0.5, and 1.0 mg/mL) groups, the apoptosis rate and TNF- $\alpha$  expression of anthocyanin at 1.0 mg/mL were the lowest. There was no significant change in caspase-3 expression in each group.

**Conclusion:** Anthocyanin from *Lycium ruthenicum* Murr. in the Qaidam Basin could alleviate UVB-induced apoptosis by regulating the death receptor pathway.

**Keywords:** *Lycium ruthenicum* Murr., anthocyanin, human skin fibroblasts, ultraviolet B, apoptosis

## Introduction

The skin is the body's first line of defense against the external environment.<sup>1</sup> Human skin fibroblasts (HSFs) are the main cell types in the dermis, and they play an important role in the structure and function of the dermis. HSFs are also the main target of skin photoaging.<sup>2,3</sup>

Skin aging can be classified into intrinsic and extrinsic aging. Intrinsic aging is inevitable, while extrinsic skin aging is mainly attributed to high or low temperature, smoking, ultraviolet (UV) irradiation, etc.<sup>4</sup> UVB (280–320 nm) is the most destructive type of UV that can reach the Earth's surface.<sup>5</sup> Long-term ultraviolet radiation causes DNA damage, inflammatory reaction, and apoptosis and induces the expression of apoptosis-related proteins, ultimately leading to skin photoaging and cancer.<sup>5,6</sup> Tumor necrosis factor (TNF)- $\alpha$  and other cytokines synthesized and secreted by HSFs play an important role in UV-induced skin photoaging. TNF- $\alpha$  is a cytokine mediating inflammation, immune response, apoptosis, and other biological effects.<sup>7,8</sup> TNF- $\alpha$  can

initiate a caspase-dependent enzyme cascade through the death receptor pathway to activate apoptosis.<sup>9</sup> Caspase-3 and caspase-7 are effector apoptotic proteases.<sup>10</sup> Activated caspase-3 and caspase-7 lyse proteins essential for the cell structure and vital functions, leading to apoptosis.<sup>11</sup> Survivin, as an inhibitor of apoptosis proteins (IAPs), can bind to caspases or other protein molecules involved in apoptosis and regulate apoptosis by inhibiting the activity of these proteins and promoting their degradation.<sup>12,13</sup> The proportion of apoptotic cells in total cells was determined by detecting the apoptosis rate, and the degree of UVB-induced damage to HSFs and the protective effect of anthocyanin pretreatment on HSFs exposed to UVB radiation were inferred.<sup>14</sup> Therefore, the apoptosis rate, TNF- $\alpha$ , caspase-3, caspase-7, and survivin were used as indicators to observe UVB-induced apoptosis in this study.

Modern pharmacological studies have proved that anthocyanin of *Lycium ruthenicum Murr* has various effects, such as anti-aging, vision-improving,<sup>15</sup> cardiovascular disease-preventing,<sup>16</sup> cognitive ability-improving,<sup>17</sup> and anti-tumor effects.<sup>18</sup> *Lycium barbarum* can alleviate UVB-induced apoptosis of human immortalized keratinocytes,<sup>19</sup> and studies have shown that anthocyanin can reduce the expression of TNF- $\alpha$  and caspase-3 to alleviate cognitive impairment in rats.<sup>20</sup>

According to the experiments of Qinning Zhang et al and others,<sup>21–24</sup> 30 mJ/cm<sup>2</sup> UVB was used to irradiate HSFs in this study. According to the research of Li et al,<sup>21,25–28</sup> three concentrations of anthocyanin, 0.1, 0.5, and 1.0 mg/mL, were used. This study aimed to investigate whether anthocyanin alleviates UVB-induced apoptosis in HSFs. If yes, we also aimed to clarify whether anthocyanin plays an anti-apoptotic role in HSFs exposed to UVB through the death receptor pathway. In the present study, we provide a basis for anthocyanin as a potential anti-photoaging drug.

## Materials and Methods

### Study Design

The study was a purely experimental study. The control group, UVB group, and anthocyanin group (0.1, 0.5, and 1.0 mg/mL) were compared with each other. Three doses of anthocyanin were also compared with each other. The experiments were conducted in the central laboratory of the Affiliated Hospital of Qinghai University, China.

### Chemicals

Anthocyanin was purchased from Xi'an Tongze Biotech Company (Xi'an, China). The UVB lamp was purchased from Sigma (Shanghai, China).

### Subjects

HSFs were obtained from Zhong Qiao Xin Zhou Biotechnology Company (Shanghai, China). According to research on HSFs,<sup>23,24,29,30</sup> HSFs were incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (streptomycin and penicillin) at 37°C in a 5% CO<sub>2</sub> incubator. HSFs were used between passages 3–5 for all experiments. We made the cultured cells into cell suspension with a concentration of 1\*10<sup>5</sup>/mL and inoculated them into 6-well plates, with 2.5\*10<sup>5</sup> cells seeded in each well. Then, the cells used were for the experiments when the cell coverage reached 80–90% confluence.

### Study Protocol

HSFs were grouped into five groups: control group, UVB group, and anthocyanin groups (0.1, 0.5, and 1.0 mg/mL). Anthocyanin powder was mixed with a complete medium to form different concentrations of anthocyanin solution and added to the anthocyanin group, while only the same volume of complete medium was added to the control and UVB groups. After 24 h of incubation, the cells were exposed to 30 mJ/cm<sup>2</sup> UVB. During irradiation, the cells in the control group were covered with tinfoil. After irradiation, a complete medium was added, followed by 24-h culture.

### Detection of TNF- $\alpha$ Expression via Enzyme-Linked Immunosorbent Assay (ELISA)

HSFs were disrupted and lysed using ultrasonic equipment and centrifuged. Subsequently, the supernatant was retained and tested following the instructions provided with the human TNF- $\alpha$  ELISA kit. The experiment was repeated three times for each group.

## Detection of Caspase-3, Caspase-7, and Survivin Protein Expression via Western Blotting

The cells were lysed on ice with radioimmunoprecipitation assay (RIPA) lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) for 30 min, and the supernatant was collected after centrifugation. A bicinchoninic acid (BCA) protein quantitative kit was used to determine the concentration of protein in each group. The supernatant was diluted with protein loading buffer and boiled in a 95°C water bath for 10 min. The total protein (40 µg) was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred onto 0.2-µm polyvinylidene fluoride (PVDF) membrane, and the membrane was blocked with 5% skim milk powder at room temperature for 1 h. Then, the membrane was incubated with antibodies against caspase-3 (1:1000), caspase-7 (1:1000), survivin (1:1000), and β-actin (1:1500) at 4°C overnight. The next day, the membrane was washed and incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:5000) antibody at room temperature for 1 h. After washing again, an enhanced chemiluminescence (ECL) system was employed for protein detection. ImageJ software was used to analyze the grey values of protein bands. The experiment was repeated three times for each group.

## Detection of Apoptosis Rate by Flow Cytometry

The cells were diluted using annexin binding buffer (1×) to achieve a cell density of  $5 \times 10^5$  cells/mL. Next, 400 µL of annexin binding buffer (1×), 5 µL of annexin V solution, and 5 µL of propidium iodide (PI) solution were added to 100 µL of the cell suspension. The cells were incubated away from light and at room temperature for 15 minutes, after which the apoptosis rate for HSFs in each group was determined by flow cytometry. The experiment was repeated three times for each group.

## Statistical Examination

Data represented means ± standard error of the mean (SEM) of at least three separate experiments. SPSS13.0 software was utilized for data processing. One-way analysis of variance (ANOVA) was used for comparison between multiple groups, and an independent sample *t*-test was used for comparison between two groups. Data with  $p < 0.05$  were considered statistically significant.

## Results

### Expression of TNF-α

The expression of TNF-α was detected by ELISA. As shown in Table 1 and Figure 1, the expression of TNF-α in UVB-irradiated HSFs was significantly increased compared with the control group ( $p < 0.01$ ). Moreover, anthocyanin pretreatment significantly attenuated the expression of TNF-α in HSFs ( $p < 0.01$ ). Among different anthocyanin concentrations (0.1, 0.5, and 1.0 mg/mL), the expression of TNF-α in the 1.0 mg/mL anthocyanin group decreased most obviously, but it was still higher than that in the control group ( $p < 0.01$ ).

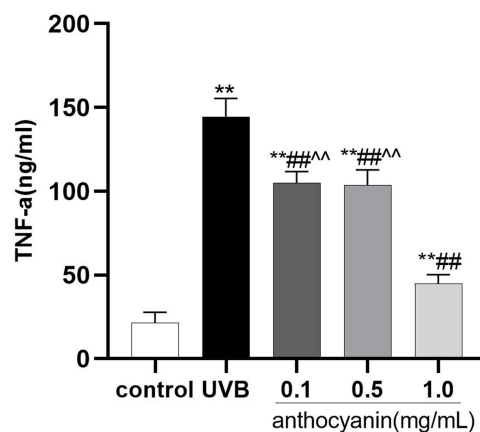
### Protein Expression of Caspase-3, Caspase-7, and Survivin

Western blot was performed to determine the protein expression of caspase-3, caspase-7, and survivin to evaluate the effect of anthocyanin on UVB-induced apoptosis and related proteins in UVB-irradiated HSFs. As shown in Table 1 and Figure 2, there

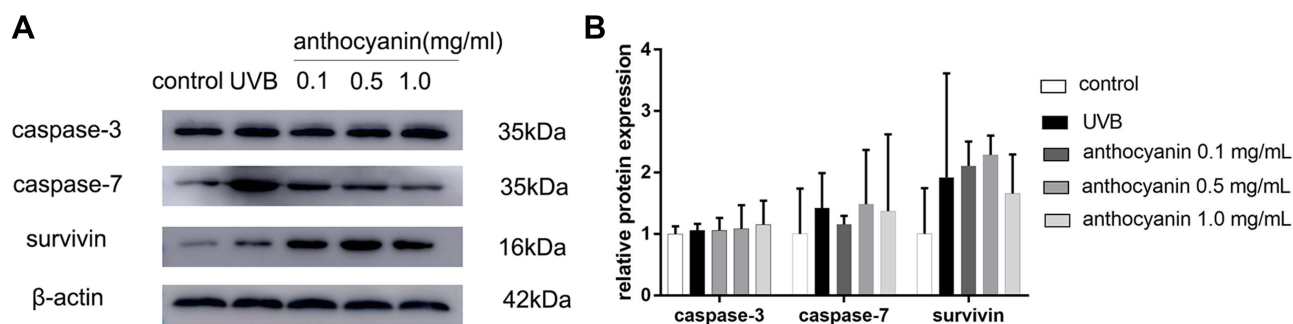
**Table 1** Effect of Anthocyanin on UVB-Induced Human Skin Fibroblasts (Mean ±SEM, n = 3)

Group	Apoptosis Rate (%)	TNF-α (ng/mL)	Expression of Caspase-3	Expression of Caspase-7	Expression of Survivin
Control	2.237±0.041	21.379±6.367	1.000±0.072	1.000±0.425	1.000±0.430
UVB	8.170±0.379**	144.565±10.892**	1.055±0.063	1.422±0.329	1.921±0.979
UVB+0.1 mg/mL anthocyanin	6.803±0.47***^	105.074±6.723***^	1.054±0.119	1.154±0.081	2.108±0.229
UVB+0.5 mg/mL anthocyanin	6.020±0.348***^	103.580±9.210***^	1.085±0.224	1.482±0.510	2.290±0.180
UVB+1.0 mg/mL anthocyanin	3.497±0.312***	44.984±5.273***	1.152±0.224	1.370±0.723	1.659±0.368

**Notes:** Data are shown as the mean ±SEM (n=3). \* $p < 0.05$ , \*\* $p < 0.01$  vs the control group. # $p < 0.05$ , ### $p < 0.01$  vs the UVB group. ^^ $p < 0.01$  vs the 1.0 mg/mL anthocyanin group.



**Figure 1** Effect of anthocyanin on TNF- $\alpha$  in ultraviolet B (UVB)-induced human skin fibroblasts (HSFs). The expression of TNF- $\alpha$  was detected by ELISA. Data are shown as the mean  $\pm$ SEM (n = 3). \*\* $p$  < 0.01 vs the control group. \*\*\* $p$  < 0.01 vs the UVB group. ## $p$  < 0.01 vs the 1.0 mg/mL anthocyanin group.



**Figure 2** Effect of anthocyanin on the expression of caspase-3, caspase-7, and survivin in ultraviolet B (UVB)-induced human skin fibroblasts (HSFs). Western blot was performed to determine the expression of caspase-3, caspase-7, and survivin. (A) Caspase-3, caspase-7, and survivin proteins in HSFs. (B) The expression of caspase-3, caspase-7, and survivin in HSFs. Data are shown as the mean  $\pm$ SEM (n = 3).

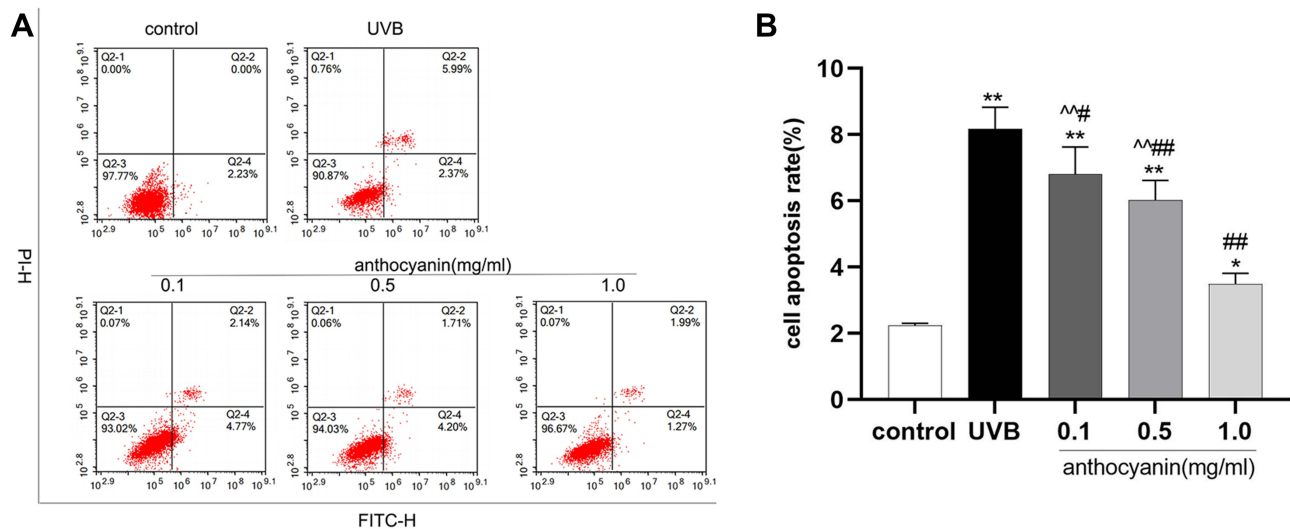
was no significant difference in caspase-3 expression under different treatments ( $p > 0.05$ ). The expressions of caspase-7 and survivin in UVB-irradiated cells showed an upward trend ( $p > 0.05$ ). Caspase-7 expression was decreased in UVB + anthocyanin-treated cells, while the expression of survivin was increased ( $p > 0.05$ ). Among different anthocyanin concentrations (0.1, 0.5, and 1.0 mg/mL), the expression of caspase-7 and survivin was the highest in the 0.5 mg/mL anthocyanin group ( $p > 0.05$ ).

## Apoptosis Rate of HSFs

The apoptosis rate was detected by flow cytometry. As shown in Figure 3 and Table 1, the apoptosis rate in the UVB group was significantly higher than that in the control and anthocyanin groups ( $p < 0.01$ ). There was no significant difference in apoptosis rate between the 0.1 mg/mL and 0.5 mg/mL anthocyanin groups ( $p > 0.05$ ), while the apoptosis rate in the 1.0 mg/mL anthocyanin group was lower than that in the 0.1 mg/mL and 0.5 mg/mL anthocyanin groups ( $p < 0.05$ ). At the same time, we noticed that the apoptosis rate in the anthocyanin (0.1, 0.5, and 1.0 mg/mL) group was higher than that in the control group ( $p < 0.05$ ).

## Discussion

In this study, we investigated the protective effect of anthocyanin on UVB-exposed HSFs. According to N. Xie's research, anthocyanins can reduce arsanilic acid-induced apoptosis.<sup>31</sup> UVB irradiation increases the apoptosis rate of HSFs and TNF- $\alpha$  production, suggesting that UVB irradiation induces apoptosis in HSFs cells;<sup>30</sup> thus, TNF- $\alpha$ -mediated apoptosis could mimic the risk of photoaging in HSFs. TNF- $\alpha$  is an important mediator of UVB-induced apoptosis in HSFs. Nizamutdinova et al found that anthocyanin could reduce inflammation by inhibiting TNF- $\alpha$



**Figure 3** Effect of anthocyanin on apoptosis rate in human skin fibroblasts (HSFs). The apoptosis rate was detected by flow cytometry. **(A)** Apoptosis rate in HSFs. **(B)** Comparison of apoptosis rate in HSFs. Data are shown as the mean  $\pm$  SEM ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$  vs the control group. # $p < 0.05$ , ## $p < 0.01$  vs the UVB group. ^ $p < 0.05$ , ^^ $p < 0.01$  vs the 1.0 mg/mL anthocyanin group.

production in fibroblasts.<sup>32</sup> The apoptosis rate in the anthocyanin (0.1, 0.5, and 1.0 mg/mL) group was lower than that in the UVB group and higher compared to the control group. These results indicated that anthocyanin pretreatment could reduce UVB-induced apoptotic cells but could not achieve complete remission. At the same time, we noticed that the apoptosis rate in the 1.0 mg/mL anthocyanin group was lower than that in the 0.1 and 0.5 mg/mL anthocyanin groups, and there was no significant difference in the apoptosis rate in the 0.1 and 0.5 mg/mL anthocyanin groups. Therefore, 1.0 mg/mL anthocyanin had the best protective effect. In this study, anthocyanin was used as a drug with inhibitory effects on UVB-induced apoptosis. The anti-apoptotic effect of anthocyanin may be related to the regulation of external apoptotic pathways mediated by death receptors.

Apoptosis, also known as programmed cell death, is a programmed autonomous gene-regulated ablation process, which is normal and advantageous in the cellular life cycle and survival.<sup>33</sup> Apoptosis is strictly regulated by intracellular signal transduction factors; hence, apoptosis can respond to physiological and pathological stimuli in a regulated manner.<sup>34</sup> TNF- $\alpha$ , as an inflammation-related cytokine, can not only induce inflammation but also induce apoptosis through the death receptor pathway.<sup>35</sup> When TNF- $\alpha$  is recognized by TNF receptor 1 (TNFR1) on the surface of the cell membrane, TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD) are recruited to transmit death signals and activate the upstream promoter caspase-8.<sup>36,37</sup> Activated caspase-8 directly activates caspase-3, 6, and 7 through a cascade amplification reaction. Activated caspase-3 and 7 can cleave DNA molecules, apoptosis inhibitory effector proteins, extracellular matrix proteins, and cytoskeleton proteins and then promote apoptosis.<sup>34,38</sup> TNF- $\alpha$  is an important factor mediating apoptosis, and caspase-3 and caspase-7 are effector apoptotic proteases. They all play an important role in the external apoptotic pathway mediated by death receptors and can be regarded as relevant factors promoting apoptosis.

We found that the apoptosis rate and TNF- $\alpha$  and caspase-7 expression of UVB-irradiated HSFs increased, indicating that UVB irradiation could activate the caspase protein family through the death receptor pathway and induce apoptosis. Compared with the UVB group, the apoptosis rate and TNF- $\alpha$  and caspase-7 expression in HSFs exposed to anthocyanin were decreased, suggesting that anthocyanin reduced UVB-induced apoptosis.<sup>39</sup> However, compared with the control group, the apoptosis rate and TNF- $\alpha$  and caspase-7 expression in the anthocyanin group were still higher. This suggests that anthocyanin can only alleviate UVB-mediated damage to a certain extent but not completely repair it. Among the three doses of anthocyanin (0.1 mg/mL, 0.5 mg/mL, and 1.0 mg/mL), 1.0 mg/mL anthocyanin led to the lowest apoptosis rate and TNF- $\alpha$  expression and the best photoprotective effect.

Survivin is a bifunctional member of the inhibitor family of apoptosis proteins,<sup>40,41</sup> which can not only inhibit apoptosis but also regulate the cell cycle. Survivin can inhibit apoptosis by inhibiting caspase-3 and caspase-7.<sup>42,43</sup> Overexpression of survivin protects keratinocytes from UVB-induced apoptosis.<sup>44</sup> The expression of survivin in the control group was the lowest among all the study groups, which was related to the low expression of survivin in normal cells.<sup>45</sup> UVB irradiation-induced apoptosis, and the apoptosis rate and survivin protein expression increased in the UVB group. The expression of survivin in HSFs pretreated with anthocyanin was higher than that in the UVB group, suggesting that anthocyanin could promote survivin of HSFs to inhibit UVB-induced apoptosis and reduce UV-induced cell damage. The result was consistent with H. Cai's study, in which they found that anthocyanin increased survivin expression to enhance the viability of mouse islets.<sup>46</sup> Among the three doses of anthocyanin, the expression of survivin with 0.5 mg/mL anthocyanin was the highest, and the apoptosis rate was also low. This suggests that this dose of anthocyanin provided good protection against UVB-induced apoptosis.

In this study, there was no significant difference in the protein expression of caspase-3 in each group. This might be because caspase-8 does not directly activate caspase-3 but activates BH3-only protein, which promotes the subsequent cascade reaction mediated by Bax and Bak, leading to apoptosis.<sup>47</sup> At the same time, it was observed that caspase-3 expression in the 1.0 mg/mL anthocyanin group was the highest among the three anthocyanin groups, but the survivin expression in this group was the lowest among the three doses. Dallaglio et al have shown that silenced survivin could mediate caspase-3 activation.<sup>44</sup> This might be the reason for the highest expression of caspase-3 in the 1.0 mg/mL anthocyanin group. Additionally, although the expression levels of caspase-7 and survivin in each group increased or decreased, the differences were not statistically significant, requiring further exploration.

In conclusion, we reported that anthocyanin of *Lycium ruthenicum* Murr. had photoprotective effects on UVB-irradiated HSFs, such as reducing the apoptosis rate and TNF- $\alpha$  and caspase-7 expression through the death receptor pathway and increasing survivin expression. These results indicated the potential application of anthocyanin from *Lycium ruthenicum* Murr. in the Qaidam Basin as a drug to combat skin photodamage and protect photoaging cells. However, this was an in vitro study, and the protective effect of anthocyanin on photodamaged cells needs to be confirmed by further studies in vivo, with further exploration in the future.

## Abbreviations

UV, ultraviolet; HSF, human skin fibroblast; TNF, tumor necrosis factor; SEM, standard error of the mean; ANOVA, analysis of variance; IAP, inhibitor of apoptosis protein; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethanesulfonyl fluoride; BCA, bicinchoninic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; PI, propidium iodide; TNFR1, TNF receptor 1; TRADD, TNF receptor-associated death domain; FADD, Fas-associated death domain protein.

## Ethics Approval

The cells we used were provided by the biological company, and the study did not require review and approval of the Ethics Committee of Qinghai University Affiliated Hospital based on 2019 Qinghai Province research guidelines.

## Data Sharing Statement

All data used during the current study are available from the corresponding author upon reasonable request.

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## Author Contributions

All authors made significant contributions to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, or interpretation. All authors took part in drafting, revising, or critically

reviewing the article; gave final approval of the version to be published; agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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## Disclosure

All authors declare that they have no conflicts of interest in this work.

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