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Semen *Ziziphus jujube* Saponins Protects HaCaT Cells against UV Damage and Alleviates the Aging of *Caenorhabditis elegans*

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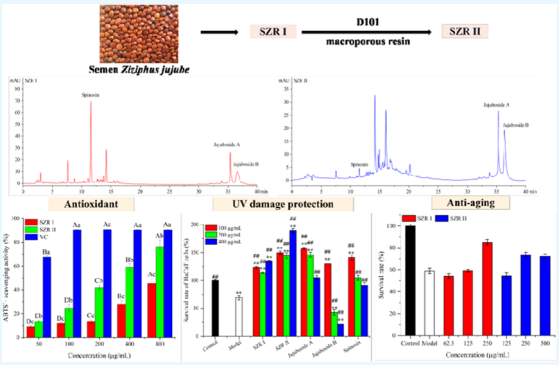
ABSTRACT: The role of Semen *Ziziphus jujube* saponins in sedative and hypnosis has attracted much attention. The study aimed to investigate its possible UV damage protection and anti-aging effects. Total saponins (SZR I) and purified saponins (SZR II) were analyzed and compared by infrared spectroscopy and high-performance liquid chromatography (HPLC). The protective effects of SZR I, SZR II, and their three monomers on HaCaT cells damaged by UV were studied, and their anti-aging activities were observed by *Caenorhabditis elegans* with paraquat-induced oxidative stress. The results showed that SZR I and SZR II differ in chemical composition but both have the same three monomers. The cell survival rate treated with SZR I and SZR II at a concentration of 400 μg/mL increased by 34.45 and 88.98%, respectively, indicating that they could promote the proliferation of UVB-damaged HaCaT cells. Jujuboside A, Jujuboside B, and spinosin from the saponins exhibited similar effects on UVB-damaged HaCaT cells. SZR I and SZR II had little effect on reproductive performance but could delay the senescence caused by heat and oxidative stress of the *C. elegans* model. These results provide useful data that Semen *Z. jujube* saponin is a potential natural product with UV damage protection and anti-aging characteristics.



INTRODUCTION

Skin aging can be classified into two categories: endogenous aging and exogenous aging caused by environmental factors.[1] The obvious characteristics caused by exogenous aging are mainly reflected in skin dryness, fine wrinkles, and laxity.[2,3] Solar ultraviolet (UV) may induce the production of reactive oxygen species (ROS) in the skin, leading to the manifestation of mottled pigmentation, lentigines, and solar dermatitis, which probably become the most important incentive factor of diseases such as solar keratosis, squamous cell carcinoma, and malignant melanoma.[4] ROS can also induce abnormal DNA structure and protein synthesis, leading to cell oxidative damage and accelerating cell aging.[4]

With the continuous development of biological technology, more in-depth research on the mechanism of aging has been carried out from the cellular and molecular biological levels. Human immortalized keratinocytes (HaCaTs) damaged by UVB irradiation were often used to study the effects of drugs on skin photoaging.[5] *Caenorhabditis elegans* also became one of the ideal animal models for screening anti-aging drugs because of its high conservation and similarity with a large number of human pathogenic genes and biological mechanisms.[6] Many drugs with anti-aging activity, such as acacetin, have been shown to prolong the life span of *C. elegans* by upregulating stress response genes SOD-3 and GST-4.[7]

Semen *Ziziphus jujube* is the dry seed of *Ziziphus jujuba*, a traditional Chinese herbal medicine plant,[8] which contains fatty acids, flavonoids, alkaloids, saponins, polysaccharides, and other chemical components and is used to treat anxiety, depression, and other symptoms.[9−12] The sleep latency and exercise recovery time of the mice injected with pentobarbital sodium can be significantly shortened by adding the water extract of Semen *Z. jujube*.[13] Complex mixtures of Semen *Z. jujube* exerted sedative and hypnotic functions by influencing the GABAergic and serotonergic system.[14] Flavonoids and saponins of Semen *Z. jujube* could significantly shorten the walking time and coordinated movement ability of mouse, and polysaccharides did not show any effect in tests.[15] However, the study of Semen *Z. jujube* mainly focuses on its sedative and hypnosis activity, and other activities need to be further excavated.

In this study, the saponins extracted from Semen *Z. jujube* were analyzed and identified. The antioxidant capacity of Semen *Z. jujube* saponins was evaluated, and the effects of saponins and their three monomers on the proliferation of the damaged HaCaT cells induced by UVB irradiation were investigated. Finally, the anti-aging activity of Semen *Z. jujube* was studied by

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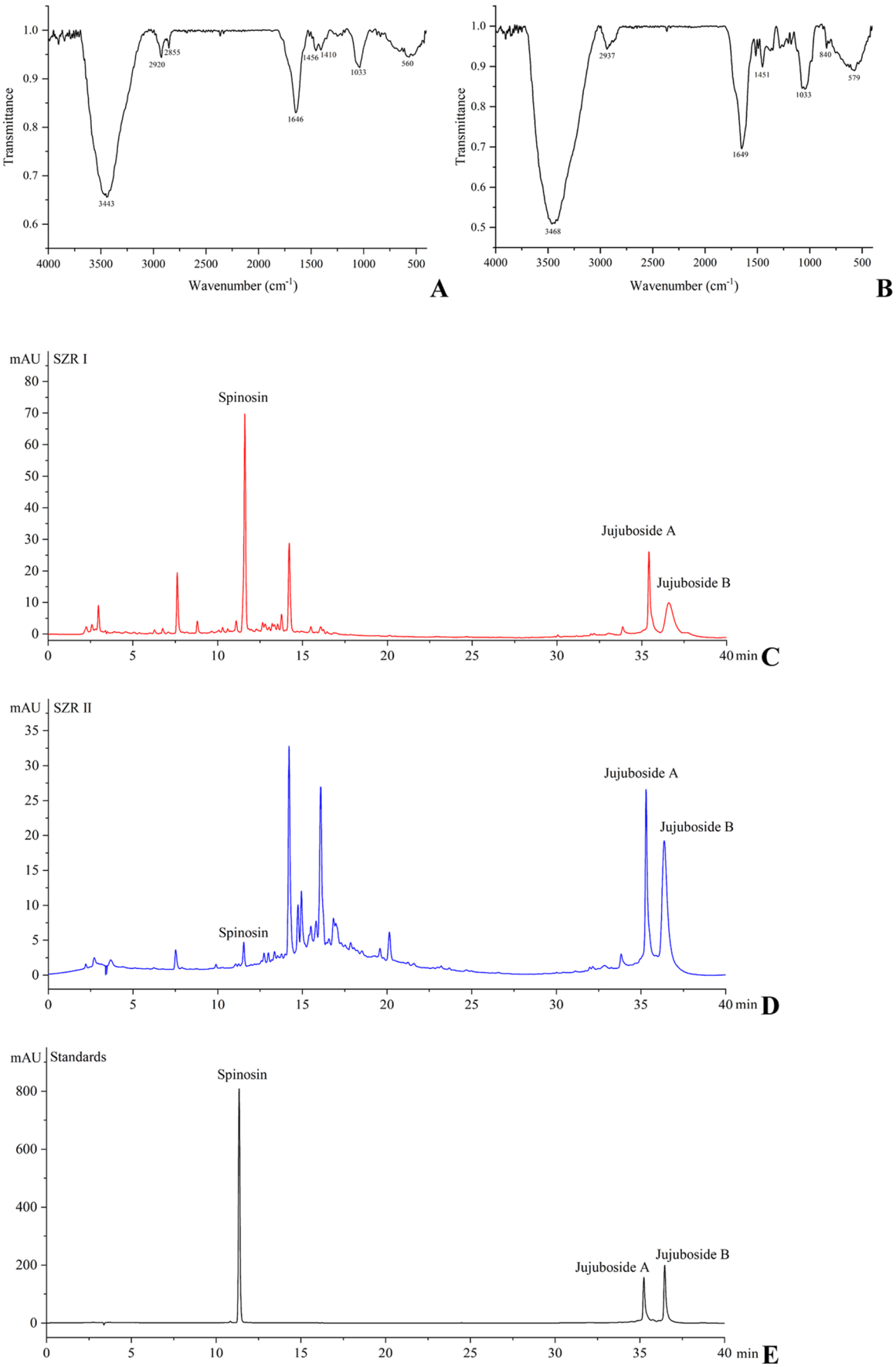

Figure 1. Infrared spectra (A, B) and high-performance liquid chromatography (HPLC) results (C–E) of SZR I, SZR II, and standards.

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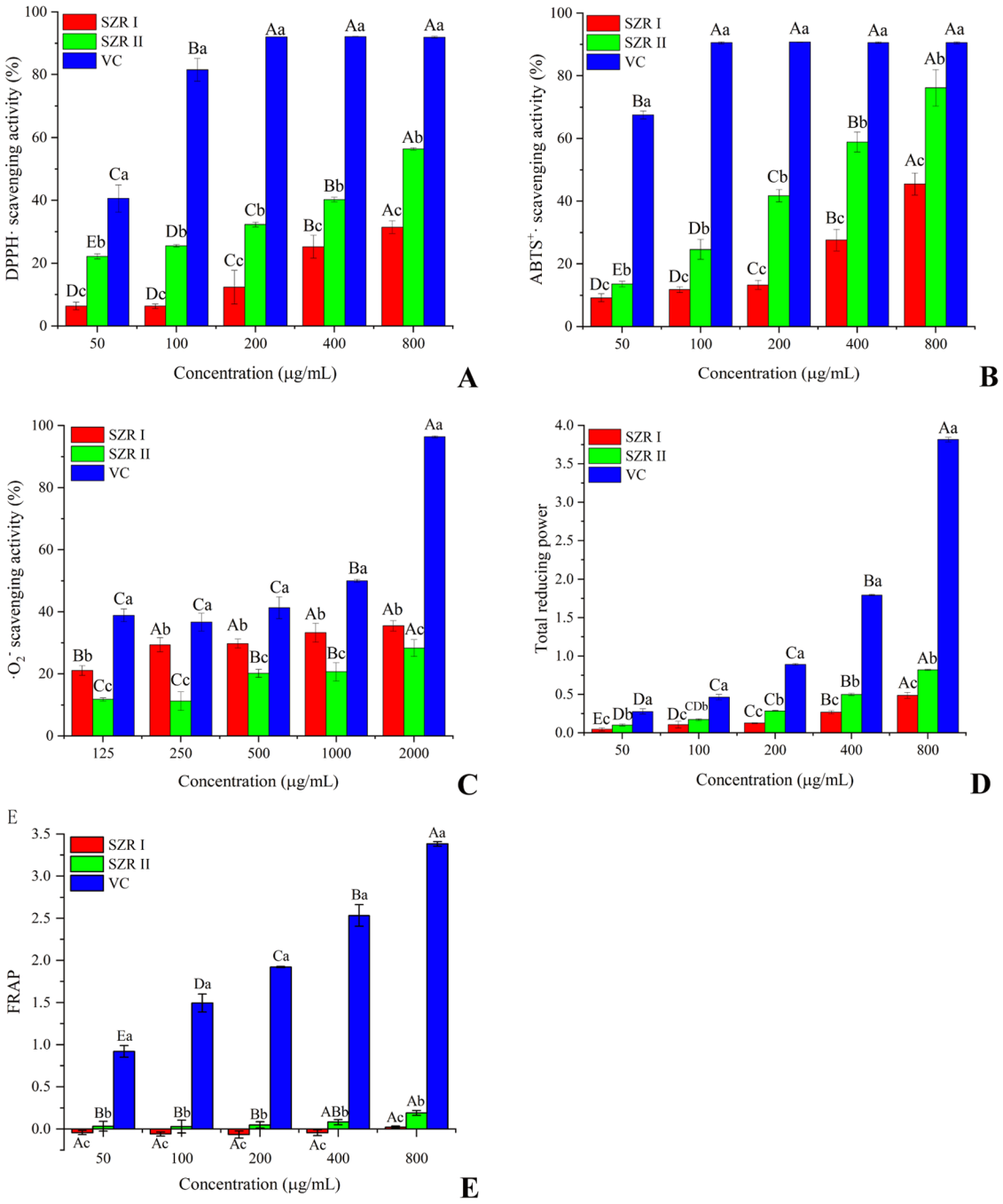

Figure 2. DPPH$^{\bullet}$ scavenging activity (A), ABTS$^{+\bullet}$ scavenging activity (B), $^{\bullet}O_2^-$ scavenging activity (C), total reducing power (D), and FRAP valves (E) of SZR I and SZR II. (Different capital letters indicate that groups treated by the same sample with different concentrations are significantly different from each other, $p < 0.05$. Different lowercase letters indicate significant differences between different samples treated with the same concentration; $p < 0.05$).

using *C. elegans*. The objective of the present research was to investigate the possible UV damage protection and anti-aging activities of Semen *Z. jujube* saponins and provide a more comprehensive theoretical basis for the development and utilization of Semen *Z. jujube*.

RESULTS AND DISCUSSION

Determination of SZR I and SZR II. According to the calculation of the content of saponins in the samples, 90 g of SZR I (n-butanol extract) was obtained from 5 kg of Semen *Z. jujube*, and the saponin content was 52.1%. SZR I was further purified by D101 macroporous resin, and the highest saponin content was 75.2% from 50% ethanol elution fraction, which was named SZR II.

Analysis of Fourier Infrared Spectroscopy. Figure 1A,B shows the infrared spectra of SZR I and SZR II at 4000−400 cm^{-1}, respectively. It could be seen from the figure that there was no significant difference between the maximum absorption peaks of SZR I and SZR II, but there were differences in the location and intensity of absorption peaks. This meant that SZR I and SZR II had the same composition, but the quantity was different. There were OH, C−H, C═C, benzene ring, and C−O

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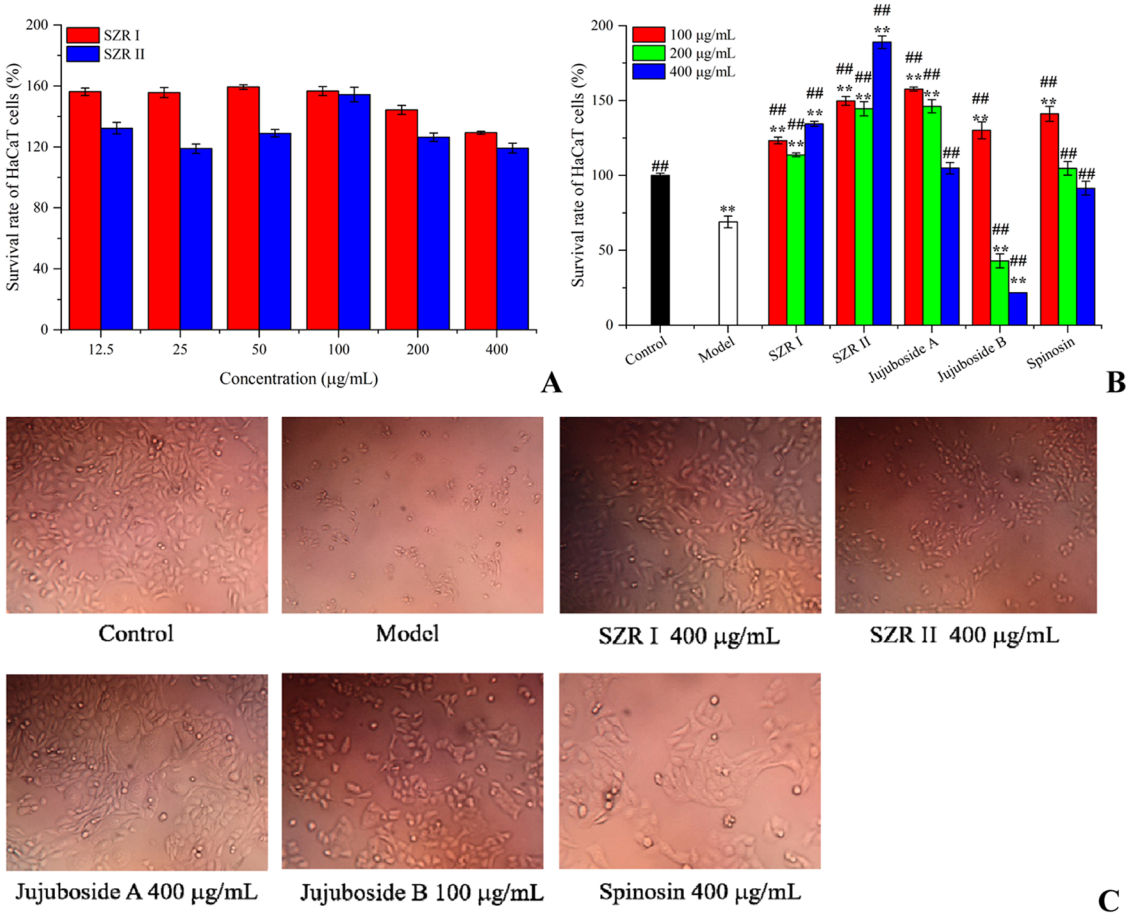

Figure 3. Effects of SZR I and SZR II on the survival rates of normal HaCaT cells (A). Effects of SZR I, SZR II, and its three monomers on survival rate (B) and morphological changes (C) of HaCaT cells after irradiation. (*$p < 0.05$ means compared with blank group, $^{\#}p < 0.05$ means compared with model group).

structures in SZR I and SZR II structures, which may be different in form due to their different locations and absorption intensity. It was worth noting that SZR II had an obvious absorption peak at 840 cm^{-1}, which did not appear in SZR I, indicating that there was a difference in composition between SZR I and SZR II.

Analysis of HPLC. As shown in Figure 1C−E, three major components including Spinosin, jujuboside A, and jujuboside B were identified from SZR I and SZR II by comparing with the retention times and UV spectra of standards. There were differences in the peak response values of the three substances at the same peak time, suggesting that the contents of the three major components of SZR I and SZR II were different. Saponins from Semen *Z. jujube* including jujuboside A and jujuboside B had been identified by previous reports,[27] which showed a new detection method used for the simultaneous determination of the main components in the Semen *Z. jujube*. Spinosin was also detected through previous reports.[28]

Analysis of the DPPH$^{\bullet}$ Scavenging Activity. The DPPH$^{\bullet}$ scavenging rate is a common model to study the free radical scavenging activity of natural extracts.[29] The DPPH$^{\bullet}$ radical scavenging rates of SZR I and SZR II are shown in Figure 2A. The scavenging rate of DPPH$^{\bullet}$ free radicals in each group raised significantly with the increase in concentration, but it was always lower than that of the VC positive control group. At the same concentration, the effect of SZR II on the free radical scavenging rate of DPPH$^{\bullet}$ was significantly higher than that of SZR I. At the concentration of 800 μg/mL, the DPPH$^{\bullet}$ scavenging activities of SZR I, SZR II, and positive control were 31.48 ± 2.04, 56.38 ± 0.42, and 91.96 ± 0.28%, respectively.

Analysis of the ABTS$^{+\bullet}$ Scavenging Activity. ABTS$^{+\bullet}$ is another important index to measure the free radical scavenging activity of natural products.[30] Under the action of oxidants, ABTS can be oxidized to ABTS$^{+\bullet}$, but the presence of antioxidants inhibits the generation of ABTS$^{+\bullet}$. The ABTS$^{+\bullet}$ radical scavenging effects of the two saponins are shown in Figure 2B. The positive control has a good free radical scavenging effect. When the concentration was 100 μg/mL, the free radical scavenging ability reached 90.50 ± 0.35%. Meanwhile, the free radical scavenging rates of the two saponins increased with increasing concentrations. When the concentration was 800 μg/mL, the scavenging rate of ABTS$^{+\bullet}$ free radicals by SZR II reached 76.17 ± 5.78%, while the scavenging rate by SZR I reached 45.44 ± 3.51%. Overall, the ABTS$^{+\bullet}$ free radical scavenging ability of SZR II was stronger than that of SZR I.

Analysis of the $^{\bullet}O_2^-$ Scavenging Activity. Within the experimental concentration range, the scavenging rates of the two saponins to superoxide anion increased significantly in a concentration-dependent manner (Figure 2C). When the concentration was 2000 μg/mL, the superoxide anion scavenging rate of SZR I was 35.47 ± 1.71%, which is only 1/3 of VC. The superoxide anion scavenging capacity of SZR II was lower than that of SZR I, only 28.26 ± 2.72% at the highest concentration.

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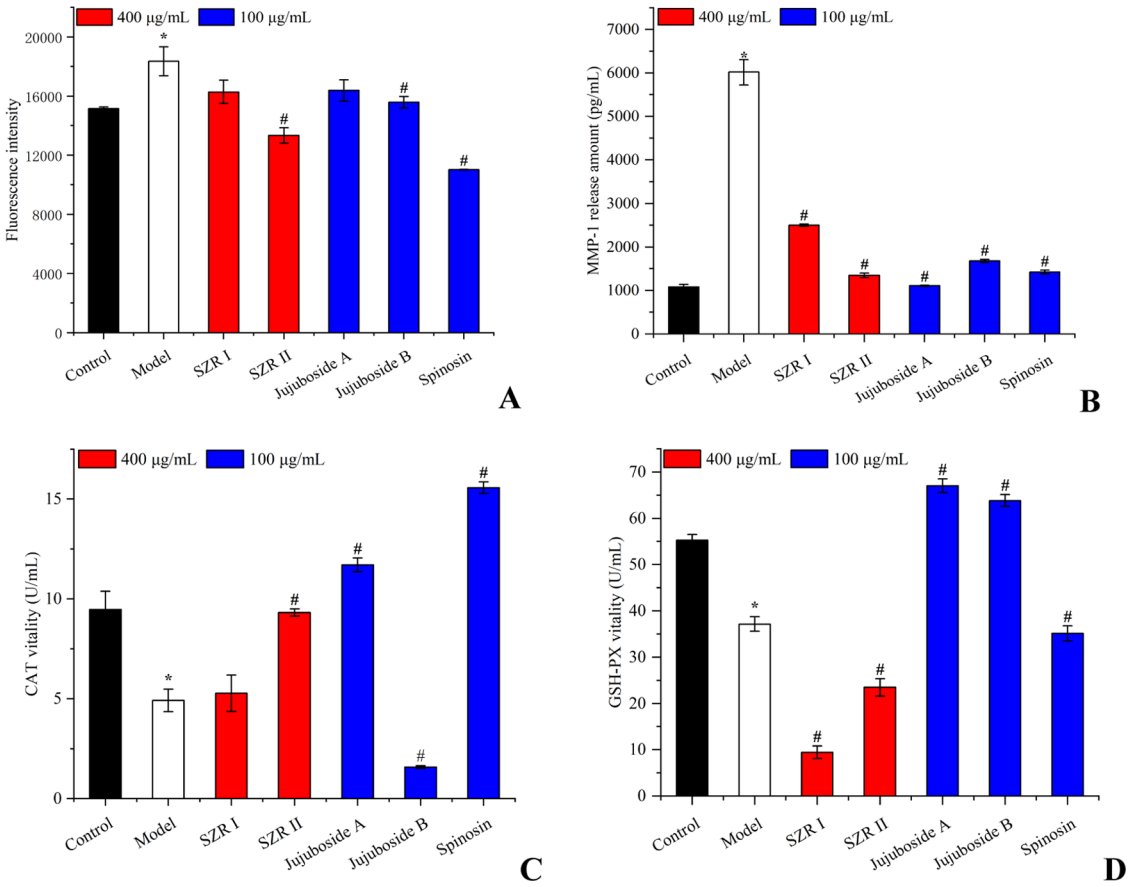

Figure 4. Effects of SZR I, SZR II, and its three monomers on ROS (A), MMP-1 (B), CAT (C), and GSH-PX (D) levels in HaCaT cells after irradiation. ($^{*}p < 0.05$ means compared with blank group, $^{\#}p < 0.05$ means compared with model group).

Analysis of the Total Reducing Power. As shown in Figure 2D, the total reducing power of SZR I and SZR II showed a concentration-dependent increase with a good linear relationship in the range of 50−800 μg/mL. At any concentration, the total reducing power of SZR II was higher than that of SZR I. Overall, although the total reducing power of SZR II was stronger than that of SZR I, it was significantly lower than that of the VC positive control. At 800 μg/mL, the total reducing power value of SZR II was 0.82, which was only 1/4 of the positive control.

Analysis of the FRAP Scavenging Activity. Figure 2E shows the effect of iron ion reducing ability of two saponins. When the concentration was less than 400 μg/mL, the FRAP value of SZR I was negative, indicating that SZR I promoted oxidation and had no reducing power of iron ions. When the concentration was 800 μg/mL, the FRAP value of SZR I and SZR II was 0.019 ± 0.025 and 0.191 ± 0.057, which was much lower than the FRAP value of the lowest concentration of the positive control (0.922), indicating that SZR I and SZR II had a weak iron reduction ability.

Screening of the Safe Concentration of Saponins. The effects of SZR I and SZR II on the survival rate of normal HaCaT cells are shown in Figure 3A. The survival rate was enhanced with the increasing saponin concentration. Both saponins could promote the proliferation of the cells, and the cell survival rate was between 110 and 160%. Overall, SZR I and SZR II had no toxic effects on normal HaCaT cells and had high safety. Since high concentrations of saponins have little effect on cell activity, in order to make the experimental results obvious, 100, 200, and 400 μg/mL were chosen as the experimental concentrations.

Effects on the Survival Rate of HaCaT Cells after Irradiation. The effect of each sample on the viability of HaCaT cells after UVB irradiation is shown in Figure 3B. Compared with the normal group, the cell survival rate of the model group was only 68.9%, which was significantly lower than that of the normal group, indicating that irradiation could induce apoptosis of HaCaT cells. Overall, the anti-photoaging effect of SZR II is better than that of SZR I. Jujuboside A, jujuboside B, and spinosin of 100 μg/mL could promote the proliferation of HaCaT cells, among which jujuboside A had the strongest promoting effect, and the cell survival rate reached 104.78% at 400 μg/mL.

Effect on the Morphological Changes of HaCaT Cells after Irradiation. As shown in Figure 3C, the cells in the model group after UVB irradiation were significantly reduced, and the cell shape changed from a normal polysynaptic shape, irregular triangle shape, to a spindle or round shape. Also, the space between cells became larger, and the cells became smaller compared with the blank group. When the concentration reached 400 μg/mL, the morphology of cells in the SZR I, SZR II, jujuboside A, and spinosin groups was normal, and there was no significant difference in cell size. Compared with the model group, the intercellular space was significantly smaller and the number of cells increased, and jujuboside B at a concentration of 100 μg/mL also showed the same changes, indicating that the sample could maintain cell morphology and reduce cell apoptosis.

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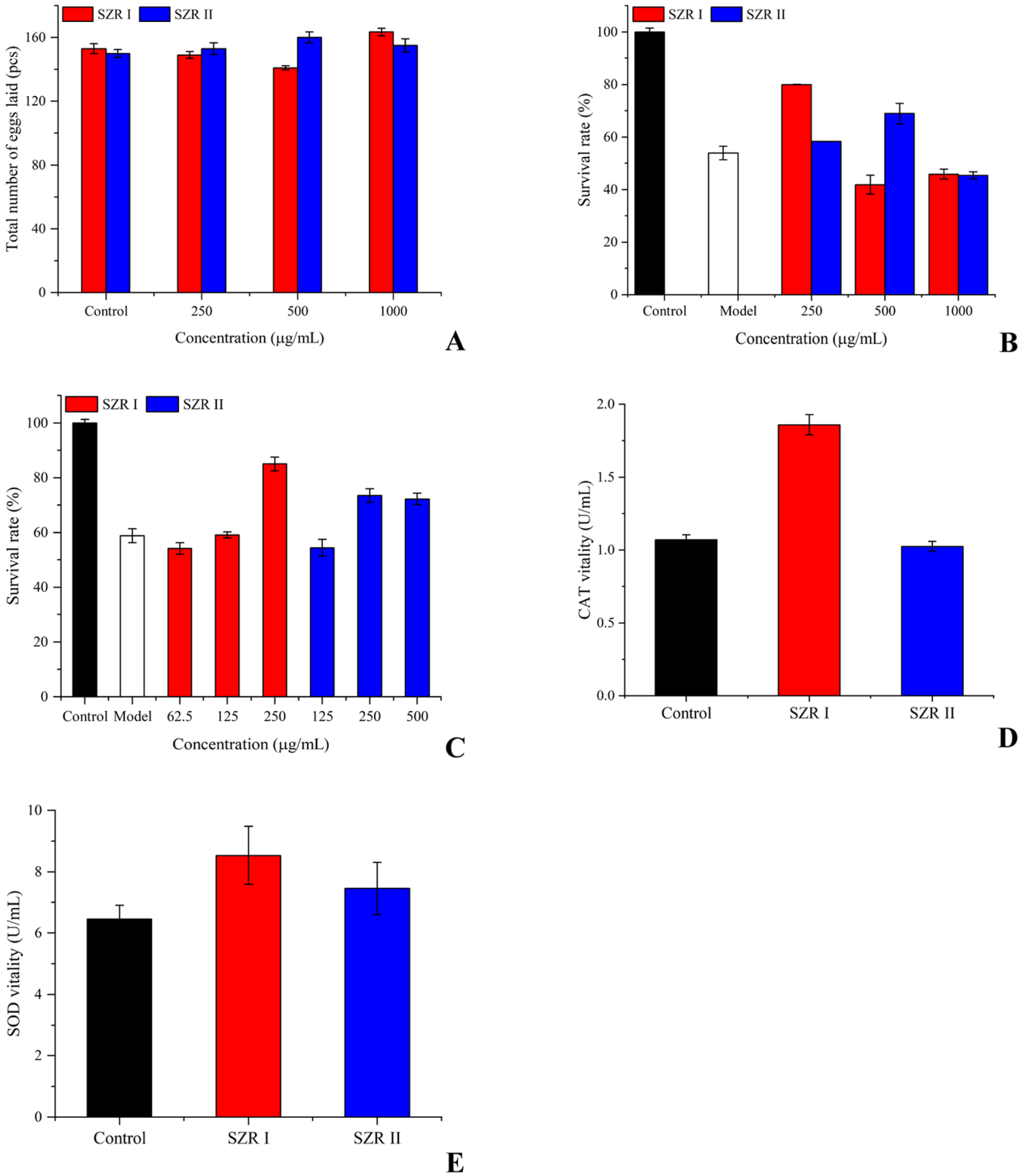

Figure 5. Effects of SZR I and SZR II on the total number of eggs laid (A), resistance to heat stress (B), resistance to oxidative stress (C), vitality CAT (D), and SOD (E).

Effects of the Samples on ROS, MMP-1, CAT, and GSH-PX. UV irradiation can induce the production of ROS, and the increase of the ROS level can increase matrix metalloproteinases (MMPs), resulting in skin damage and skin photoaging.[31] The expression of MMPs may contribute to the exacerbation of skin aging and is often regarded as a major marker of UVB-induced wrinkle formation and skin inflammation.[32] Since jujuboside A, jujuboside B, and spinosin of 100 μg/mL and SZR I and SZR II of 400 μg/mL can significantly improve the cell viability, the above concentrations for each sample were selected to continue the assay of the kit.

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Figure 4A shows the effects of SZR I, SZR II, and its three monomers on intracellular ROS after irradiation. After 100 mJ/cm^2 energy irradiation, the ROS fluorescence intensity of the model group was significantly higher than that of the blank group. Compared with the model group, SZR II, jujuboside B, and spinosin could significantly reduce intracellular ROS, among which spinosin had the best effect. SZR I and jujuboside A could reduce the ROS content in cells, but there was no significant difference compared with the model group.

The effects of saponins and monomer substances on the release of MMP-1 in HaCaT cells after irradiation are shown in Figure 4B. Compared with the blank group, the content of MMP-1 in the model group was significantly increased, and the release amount was about 6500 pg/mL. SZR I and SZR II can significantly reduce the release of intracellular MMP-1, and the effect of SZR II is better than that of SZR I. The three monomers also showed a significant effect on the attenuation of the MMP-1 content.

As shown in Figure 4C, the activity of CAT in cells was significantly reduced after irradiation, suggesting that light damage would affect the activity of CAT enzymes in cells. SZR I slightly increased CAT activity, but SZR II significantly increased CAT activity. Meanwhile, CAT activities improved by jujuboside A and spinosin were even more than that of SZR II, while jujuboside B could not improve the CAT activity.

Figure 4D shows the effects of saponins and monomers on GSH-PX. It could be seen from the figure that compared with the model group, SZR I and SZR II significantly reduced the activity of GSH-PX. However, jujuboside A, jujuboside B, and spinosin significantly improved the activity of GSH-PX enzyme, and the effect of the three compounds was of the order of jujuboside A > jujuboside B > spinosin.

Effects on C. elegans Reproductive Performance. The number of eggs laid is often used to evaluate the reproductive capacity of *C. elegans*. Figure 5A shows the effects of SZR I and SZR II on the number of eggs laid by *C. elegans*. It could be seen from the figure that different concentrations of saponins can slightly increase the number of eggs laid, but there is no significant difference compared with the blank group. It showed that the samples with the concentration of 0.25, 0.5, and 1 mg/mL had little influence on the egg-laying rate of *C. elegans*.

Effects on Heat Stress Injury of C. elegans. As shown in Figure 5B, compared with the blank group, the survival rate (58.7%) of *C. elegans* treated at 35 °C was significantly lower, indicating that too high a growth temperature would reduce the survival rate of *C. elegans* and accelerate senescence. A low concentration of SZR I (0.25 mg/mL) could delay aging caused by heat stress, as the survival rate of *C. elegans* reached 80%, which was significantly higher than that of the model group. A medium concentration (0.5 mg/mL) of SZR II can delay aging. Therefore, 0.25 mg/mL was selected as the maximum experimental concentration of SZR I and 0.5 mg/mL as the highest experimental concentration of SZR II in subsequent experiments.

Effect on Antioxidant Stress Injury of C. elegans. The effects of SZR I and SZR II on oxidative stress damage on *C. elegans* are shown in Figure 5C. The survival rate was basically concentration-dependent. High concentrations of SZR I (250 μg/mL) and SZR II (500 μg/mL) significantly increased the survival rate of *C. elegans*, indicating that SZR I and SZR II can slow down *C. elegans* death caused by oxidative stress.

Effect of Antioxidant Enzymes. Figure 5 shows the effects of SZR I and SZR II on the viability of CAT (D) and SOD (E) in *C. elegans*. SZR I could significantly increase the viability of cellular CAT, but SZR II had no effect. Both SZR I and SZR II can significantly increase SOD activity in *C. elegans* treated with paraquat. The results indicated that SZR I and SZR II had an antioxidant capacity and could delay the aging of *C. elegans* caused by oxidative stress by increasing the scavenging rates of superoxide radicals and hydrogen peroxide.

CONCLUSIONS

SZR I and SZR II had a certain antioxidant activity, and their three monomers were analyzed as the main substances of Semen *Z. jujube*. Two saponins and their three monomers had different degrees of protection on HaCaT cells with UVB irradiation and could reduce the release amount of MMP-1 and ROS levels. SZR II, spinosin, and jujuboside A also enhance the purpose of inhibiting ultraviolet-induced oxidative stress by increasing the CAT vitality. Jujuboside A, jujuboside B, and spinosin can increase the vitality of GSH-PX. SZR I and SZR II could delay the aging of *C. elegans* caused by heat stress and oxidative stress. SZR I was superior to SZR II in enhancing CAT activity and SOD activity in *C. elegan*. Future work will focus on in-depth research on related gene and protein pathways to evaluate anti-photoaging activity from multiple perspectives and continue to evaluate the related indicators such as lipofuscin and life span experiments through the *C. elegans* model to further study the anti-aging mechanisms.

MATERIALS AND METHODS

Experimental Materials and Reagents. SZR I and SZR II were from our laboratory.[16] Ginsenoside Rb1, jujuboside A, jujuboside B, and spinosin standards were purchased from Pufei De Biotech Co. (Chengdu, China). Macroporous resin (D101) was obtained from Dongju Experimental Instrument Co. (Guangzhou, China). Human MMP-1 ELISA was purchased from Neobioscience (Guangzhou, China). Reactive oxygen species (ROS) was purchased from Meilunbio (Dalian, China). Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) were obtained from Nanjing Jiancheng Bioengineering Institute. HaCaT human keratinocyte cell line was purchased from ATCC cell bank. *C. elegans* Bristol N2 and *Escherichia coli* OP50 were purchased from American Caenorhabditis Genetics Center.

Preparation of Saponin Samples. Dried Semen *Z. jujube* powder extracted successively with petroleum ether, 70% ethanol, ethyl acetate and n-butanol, and the n-butanol saponin was named SZR I. SZR I was eluted by different concentrations of ethanol combined with D101 macroporous resin. The component with the highest saponin content was named SZR II.[16]

Determination of Saponins. The determination of saponin content was referred to the reported method.[17] Taking ginsenoside Rb1 as the standard product, the standard curve obtained by the vanillin−glacial acetic acid−perchloric acid method was $Y = 8.11071X + 0.000182$ ($R^2 = 0.99945$). The saponin content in the samples was calculated according to the standard curve.

Two milligrams of SZR I and SZR II samples was prepared separately, mixed well with KBr powder, ground, compressed into tablets, and scanned in the range of 4000−400 cm^{-1}.

HPLC analysis was performed using an Agilent 1260 Series high-performance liquid chromatograph. All chromatography experiments were performed on Unitary C18 (250 mm × 4.6

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mm, 5 μm). The assay conditions were slightly modified from the reported methods.[18]

The mobile phase was comprised of solvent A (water) and solvent B (acetonitrile). The solvent gradient in volume ratios was as follows: 0−12 min, 10−30% (B); 12−25 min, 30−50% (B); 25−32 min, 50−90% (B); 32−40 min, 90−10% (B); the injection volume was 10 μL and the flow rate was 1.0 mL/min. The detection wavelength was set to 270 nm.

Antioxidant Activity. The antioxidant effects of SZR I and SZR II were determined by the DPPH•, $ABTS^{+\bullet}$, and $^{\bullet}O_2^-$ scavenging activity methods based on previous reports, with slight modifications.[19,20] Twenty microliters of SZR I and SZR II samples with different concentrations (50, 100, 200, 400, and 800 μg /mL) and 180 μL of the DPPH• solution (150 μmol/L) were mixed to determine the DPPH• scavenging activity, and the absorbance value was measured at 517 nm after 10 min at 37 °C. $ABTS^{+\bullet}$ and $^{\bullet}O_2^-$ scavenging activity were also measured in similar methods. The percentage of DPPH•, $ABTS^{+\bullet}$, and $^{\bullet}O_2^-$ scavenging was calculated by the following formula

$$\mathrm{SA}\ (\%) = [1 - (A_{\mathrm{sample}} - A_{\mathrm{background}})/A_{\mathrm{blank\ control}}] \times 100\% \quad (1)$$

where SA is the scavenging activity; A_{sample} is the absorbance of the reaction system with the samples; $A_{\mathrm{blank\ control}}$ is the absorbance of the reaction system without the samples; and $A_{\mathrm{background}}$ is the absorbance of the reaction system in which DPPH• was replaced with ethanol.

Total Reducing Power and FRAP Scavenging Activity. The total reducing power and the FRAP scavenging activity assay of the extract were based on previous reports, with slight modifications.[21] The blank control group was added with distilled water, and the positive control group was added with VC. The total reducing power and the percentage of FRAP scavenging activity were calculated using the following formula

$$\mathrm{SA}\ (\%) = A_{\mathrm{sample}} - A_{\mathrm{blank\ control}} \quad (2)$$

where SA is the scavenging activity; A_{sample} is the absorbance of the reaction system with the samples; and A_{blank} is the absorbance of the reaction system without the samples.

UV Damage Protection. HaCaT cells in the normal state were seeded in the plate after digestion and resuspension. All cells used for the study were passaged three to four times.

Five hundred microliters of the samples with different concentrations (25, 50, 100, 200, 400 μg/mL) of saponins were added into 24-well plates containing normal HaCaT cells, and the cell survival rate was determined according to the MTT method.[22]

The samples of SZR I, SZR II, and its three monomers at different concentrations were added 500 μL per well into HaCaT cells, which were irradiated by 100 mJ/cm^2 energy and the cell survival rate was also determined. In addition, the effect of irradiation on the morphology of HaCaT cells was observed by a microscope.

After normal HaCaT cells were irradiated by 100 mJ/cm^2 energy, the cell supernatants in each sample group were collected, and the contents of ROS, MMP-1, CAT, and GSH-PX in the cells were determined. The methods of measuring the above indicators were referred to in the kit instructions.

Cultivation and Synchronization. The synchronization of *C. elegans* was cultured to the L4 stage by the chemical lysis method.[23] *C. elegans*, which entered the spawning stage, was washed from the NGM medium with the M9 buffer. After centrifugation, the M9 buffer and 0.8 mL of lysis buffer were added, followed by rapid shaking for 1 min and 20 s. The above steps were repeated twice and 1 mL of the M9 buffer was added to be incubated at 24 °C for 12 h and centrifuged and the pellet (L1 stage *C. elegans*) was transferred to the precultured NGM medium containing *E.coli* OP50 and cultured for 36 h.

Reproductive Test of *C. elegans*. One *C. elegans*, which was cultured to the L4 stage, was inoculated into the NGM medium containing different concentrations of saponins (0.25, 0.5, 1 mg/mL) for 24 h; then *C. elegans* was picked out to the new NGM medium for cultivation until no longer productive. (5-Fluorouracil was not added to the medium to allow *C. elegans* to ovulate.) All egg-containing culture media were counted after 48 h of culture at 24°, and the sum of all progeny was the number of eggs laid. Each concentration was replicated three times, and the inactivated *E. coli* bacteria solution was used as a blank control.[24]

Heat Stress Test of *C. elegans*. The heat stress experiments referred to the reported method.[25] After 30 L4 stage *C. elegans* were transferred to the NGM medium containing 50 μL of samples of different concentrations at 24 °C for 72 h (in order to prevent the effect of oviposition, the medium was added with 5-fluorouracil at a concentration of 25 mg/L), *C. elegans* were transferred to a new NGM medium containing the samples at 35 °C for 4 h; then, *C. elegans* were placed at 24 °C for 12 h recovery. The microscope was used to count survival. No response to three taps on the head and a stiff body indicated death.

Acute Oxidative Stress Test of *C. elegans*. Paraquat, whose chemical name is 1,1′-dimethyl-4-4′-bipyridine dichloride, could induce the body to produce a large amount of reactive oxygen species (ROS), resulting in lipid peroxidation of cell membranes and causing oxidative damage.[26] In this experiment, paraquat was used as a free radical initiator to study the antioxidant effect of saponins in *C. elegans* under oxidative stress. Thirty L4 stage *C. elegans* were transferred to the NGM medium containing 50 μL of different concentrations of saponins at 24 °C for 108 h (containing 5-fluorouracil at a concentration of 25 mg/L and supplemented new food every day). *C. elegans* were transferred to a 96-well plate containing 50 μL of paraquat (10 mM) with 10 strips per hole at 24 °C for 12 h, and the counting method is the same as above.[25]

Determination of Antioxidant Enzymes. Three thousand L4 stage *C. elegans* were transferred to the NGM medium containing different concentrations of samples and incubated at 24 °C for 72 h (containing 5-fluorouracil at a concentration of 25 mg/L). *C. elegans* were washed with the M9 buffer and incubated with 1 mL of 10 mM paraquat solution for 12 h in a 4 °C incubator. *C. elegans* were pelleted by centrifugation and made up to 1 mL with the M9 buffer and then repeatedly freeze−thawed in liquid nitrogen, and the supernatant was collected by centrifugation for determination. Refer to the relevant kit instructions for accurate operation and determination of CAT and SOD enzyme activities.

Statistical Analysis. All experiments were set up in three parallel. SPSS 22.0 was used for statistical processing. Origin 8.0 was used for plotting. Significance analysis was performed using Duncan's analysis.

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All authors have made substantial contributions to conception and design of the project. All authors have critically revised and approved the final submitted version of the manuscript.

Notes

The authors declare no competing financial interest.

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