Effects of *Spirulina platensis*-derived polysaccharides on the promotion of extracellular matrix production and inhibition of hydrogen peroxide-induced fibroblast injury

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Abstract

Background: The application of algae-derived bioactive ingredients in cosmetics has received increasing attention for the treatment of skin-associated problems. In previous studies, we reported a new strain of *Spirulina platensis* mutant (SPm) with higher polysaccharide content (19.60% vs. 4.72%) after space breeding. In this study, we further explored the mechanism of action of polysaccharides from *S. platensis* (PSP) in skin care.

Methods: The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used to determine the cytotoxicity of the polysaccharides. Enzyme-linked immunosorbent assay (ELISA) was used to measure the hyaluronic acid (HA) and type I collagen (ColI) levels. Biochemical methods were used to determine the levels of oxidative stress-related molecules. Dichlorodihydrofluorescein (DCF) was used to label reactive oxygen species (ROS). The apoptotic rate of Hydrogen peroxide (H_2O_2)-treated fibroblasts was observed using Hoechst 33342 dye.

Results: The MTT assay demonstrated that the polysaccharides had no significant toxicity or proliferation-promoting effects on fibroblasts at less than 160 mg/L. PSP increased the secretion of ColI and HA. Pre-treatment of fibroblasts with PSP

significantly reversed the H_2O_2-induced decrease in superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. PSP increased the concentrations of glutathione (GSH) and decreased the levels of malondialdehyde (MDA) and ROS in fibroblasts after treatment with H_2O_2.

Conclusion: PSP increased the secretion of extracellular matrix (ECM) and protected fibroblasts from oxidative injuries caused by H_2O_2. Our results suggest that PSP has the potential to be used in cosmetics and for the prevention of skin aging and oxidation-related skin problems.

Keywords: *Spirulina platensis*; hydrogen peroxide; human skin fibroblast; extracellular matrix; reactive oxygen species

Introduction

Polysaccharides from *Spirulina platensis* (PSP) are water-soluble biological macromolecules that possess various bioactivities [1]. The molecular structure of PSP is complex and mainly composed of D-glucose, D-galactose, and glucuronic acid linked by glycosidic bonds [2]. Research has shown that PSP exhibits anti-tumor, antioxidant, anti-viral, anti-fatigue, and anti-aging activities along with the regulation of immune function and blood glucose levels [3-11].

Currently, the outdoor cultivation of *S. platensis* is associated with a series of problems, such as yield reduction, difficulty in harvesting, and low content of active substances due to algae degradation, which has become an important factor limiting the applications of *S. platensis*[12]. Space-flight breeding is very common in microorganisms and economic crops, such as rice and corn. In our previous study, a genetically stable mutant of *S. platensis* was screened and obtained by space breeding. It was named H11 and it showed the highest polysaccharide yield. We compared the polysaccharide and polyphenol contents of H11 mutant strains with those of wild strains during outdoor cultivation. Therefore, this laid the foundation for large-scale outdoor cultivation and industrial applications of H11 mutant strains.

Skin aging is accompanied by a decrease in tissue structure, moisture content,

smoothness, elasticity, compliance, and fibroblast damage [13]. Collagen plays an important role in the skin, as it can support the skin and make it elastic and shiny [14]. However, insufficient synthesis and excessive decomposition of collagen in dermal fibroblasts result in the reduction of skin elasticity, which leads to various aging symptoms, such as wrinkles, dehydration, and dull or no luster [15]. Therefore, protecting and filling collagen in the dermis is of great significance for skin care. HA, which has a high moisturizing effect and makes the skin plump and elastic, mainly exists in the dermis in the form of gelatin. However, HA levels in the skin gradually decrease with age. Decreased HA levels result in gradually reduced water retention ability and radiance of the skin. Eventually, these changes led to the emergence of wrinkles [16].

SOD is an anti-peroxide enzyme that is present in the cell under normal conditions; it can scavenge reactive oxygen species (ROS) produced in the cells [17-18]. The free-radical theory of aging indicates that oxidative damage caused by the accumulation of free radicals is the main cause of skin aging, and fibroblasts play an important role in this process [19-20]. Exogenous stimuli, such as H_2O_2, can induce cell damage by disrupting the dynamic balance between ROS levels and antioxidant enzymes. H_2O_2 with a small molecular weight can diffuse into human cells across membranes and is often used as an inducer of cellular oxidative stress and aging [21]. In this study, oxidative damage to normal human skin fibroblasts was induced by H_2O_2 to establish a model of skin aging at the cellular level.

Materials and methods

Extraction of water-soluble polysaccharides from *S. platensis*

H11 *S. platensis* powder (10 g) was dispersed in water. After ultrasonic destruction of the cell structure, the supernatant was collected by centrifugation at 5000 r/min for 10 min after leaching with hot water at 70 °C for 4 h. This process was repeated three times and four volumes of ethanol were added to the combined supernatant. The mixture was incubated at 4 °C for 24 h and centrifuged at 5000 r/min

for 10 min to collect the precipitate. The precipitate was dissolved in water and dried at 60°C. To obtain a polysaccharide extract, the protein was removed using the Savage method, while the pigment was removed with acetone.

Cell culture and MTT assay

Human skin fibroblasts (HSFs) were maintained in DMEM containing 10% FBS and they were incubated at 37°C in a humidified atmosphere with 5% CO_2. To evaluate the safety of the samples, their effects on HSFs were determined using the MTT assay according to the manufacturer's instructions.

H_2O_2-induced oxidative damage in HSFs

The cells were seeded in 96-well plates and cultured in DMEM supplemented with 10% FBS for 24 h. Next, the medium was discarded and varying concentrations of H_2O_2 (50 – 200 μM) diluted in FBS-free DMEM were added for the following 4 h. Cell viability was determined using an MTT assay. Morphological changes were observed using the Hoechst 33342 dye under a fluorescence microscope. The 50% – 70% survival of HSFs indicated that the oxidation-induced damage was established.

Experimental groups

HSFs were seeded in 12-well plates (while 6-well plates for the studies on extracellular matrix) and cultured in DMEM with 10% FBS for 24 h. After removing the medium, cells were treated as follows:

Control group: HSFs were incubated in FBS-free DMEM for 24 h.

Model group in extracellular matrix study: HSFs were incubated with FBS-free DMEM containing PSP (20, 80, 160mg/L) for the next 72 h.

H_2O_2-induced oxidative damage group: HSFs were treated with FBS-free DMEM for 24 h followed by treatment with 100 μM H_2O_2 for 4 h.

PSP protective group: PSP solutions of different concentrations were added to FBS-free DMEM for another 24 h of culture, followed by the addition of 100 μM H_2O_2 for 4 h.

Quantification of hyaluronic acid and type I collagen levels

Hyaluronic acid and type I collagen in the culture medium of HSFs were

quantified using an enzyme-linked immunosorbent assay (ELISA) kit.

Assessment of ROS production

After discarding the medium, HSFs were incubated in FBS-free DMEM containing 10 μM 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) at 37°C for 30 min in the dark. Subsequently, the cells were washed three times with PBS, and ROS levels were measured using a fluorescent microplate reader (E_x/E_m = 504/529 nm).

Determination of MDA, SOD, GSH, and GSH-Px

The supernatant was used to detect MDA content according to the manufacturer's instructions. HSFs were harvested and used to determine the levels of GSH along with the activities of SOD and GSH-Px according to previously described protocols. Protein concentrations were determined using the bicinchoninic acid (BCA) method.

Statistical analysis

All data are expressed as the mean ± S.D. Comparisons between two groups were performed using Student's *t*-test. Statistical significance was set at $p < 0.05$.

Results

PSP could alleviate the oxidative damage induced by H_2O_2

Initially, the cytotoxicity of PSP on HSFs was tested using an MTT assay. As shown in Fig. 1a, incubation of HSFs from 20 to160 mg/L of PSP for 24 h did not significantly affect cell viability. Meanwhile, as shown in Fig. 1b, treatment of HSFs from 12.5 to 400 μM of H_2O_2 for 4 h resulted in a dose-dependent decrease in cell viability, and the survival rate under H_2O_2 stimulation at a concentration of 100 μM was 57 % when compared with the control group.

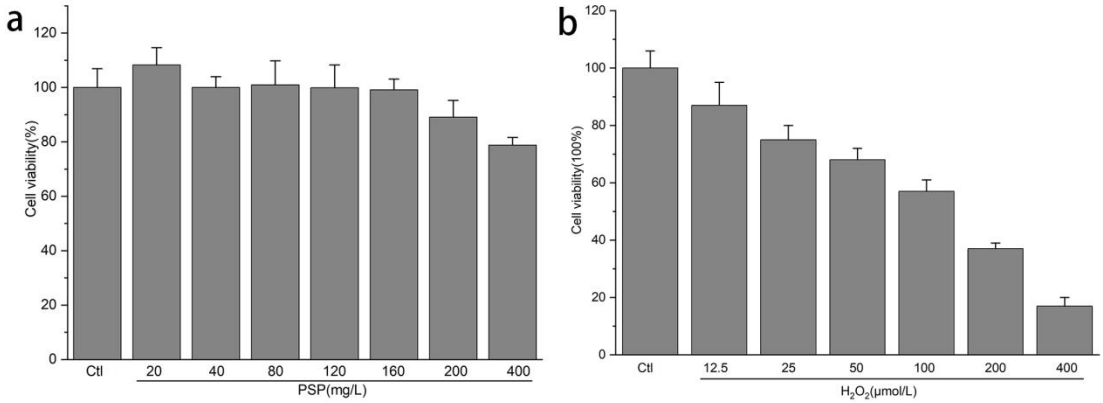

Fig.-1 Effects of PSP and H_2O_2 on cell viability.

Pre-treatment of HSFs from 20 to 160 mg/L PSP for 24 h before exposure to H_2O_2 significantly increased viability in a dose-dependent manner. These results indicate that PSP protects HSFs from oxidative stress-induced injuries. Based on these results, further studies employed an H_2O_2 concentration of 100 μM with PSP concentrations of 20, 80 and 160 mg/L (Fig.- 2).

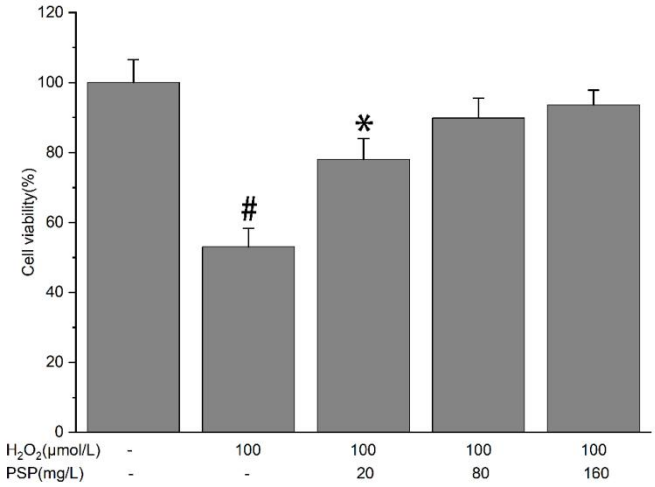

Fig.-2 Inhibition of PSP on H_2O_2-induced viability decrease of HSFs. Results are expressed as the mean±SD (n = 3), #$p < 0.05$ compared to the control group (without the treatment of PSP and H_2O_2); *$p < 0.05$ compared to the 100μmol/L H_2O_2 group.

The H_2O_2-treated HSFs exhibited condensed and fragmented nuclei, and nuclear condensation and the appearance of apoptotic body-like structures became increasingly frequent after 24 h of incubation with H_2O_2. In contrast, in the control group, without treatment with H_2O_2, cells treated with PSP showed no signs of morphological nuclear damage or chromatin condensation. Although PSP markedly

improved the morphological changes in cells treated with H_2O_2, the changes were still not as evident as those in H_2O_2-untreated cells (Fig. – 3).

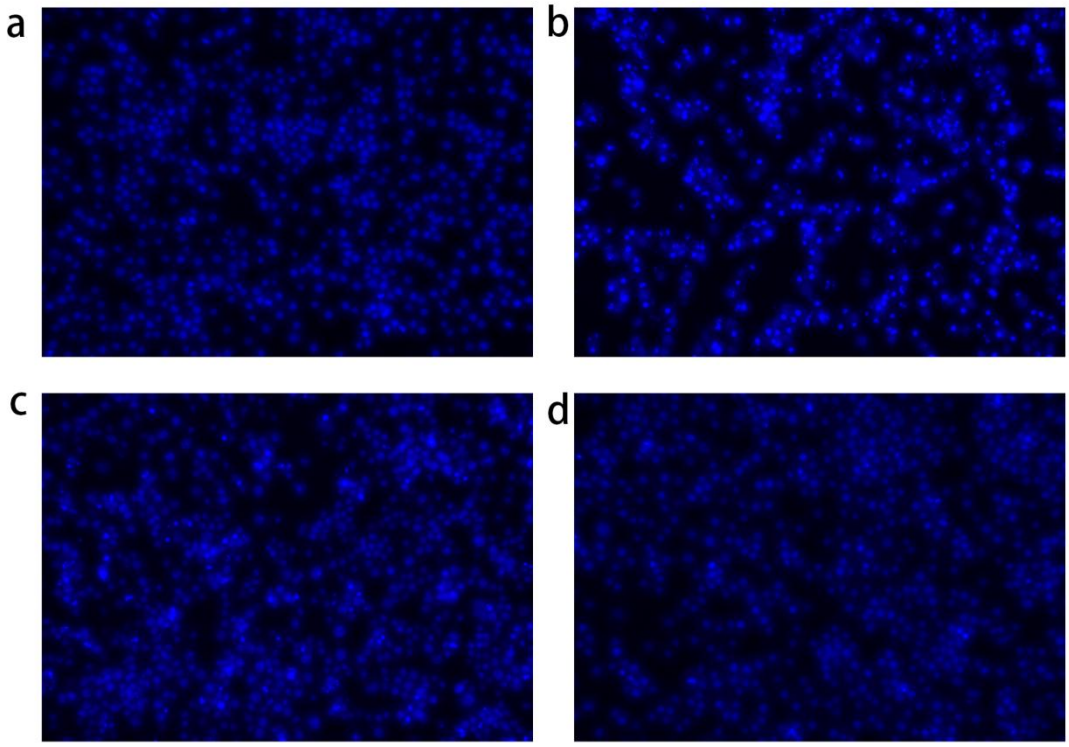

Fig.-3 Effects of PSP on H_2O_2-induced apoptosis of HSFs

(a) HSFs without the incubation of PSP and H_2O_2; (b) HSFs with the incubation of 100μmol/L H_2O_2 for 4 hours; (c) HSFs with the pre-treatment of 80mg/L PSP for 24 hours prior to the incubation of 100μmol/L H_2O_2 for 4 hours; (d) HSFs with the pre-treatment of 160mg/L PSP for 24 hours prior to the incubation of 100μmol/L H_2O_2 for 4 hours.

PSP could increase the production of HA and ColI in HSFs

We studied the effects of PSP on HA production in HSFs by ELISA using cellular supernatant. PSP significantly enhanced HA production, in a dose-dependent manner, in HSFs after 72 h of treatment. In addition, PSP stimulated the production of ColI in HSFs in a concentration-dependent manner. Compared with the control, PSP treatment (160 mg/L) increased HA production by 378.6% and ColI production by 155.8% in HSFs (Fig. – 4a; 4b).

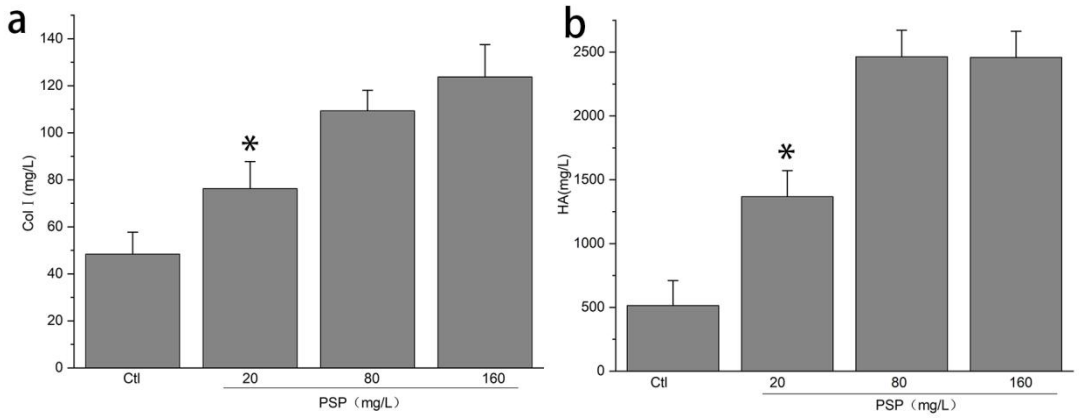

Fig.-4 Effects of PSP on production of hyaluronic acid and type I collagen. Results are expressed as the mean±SD (n = 3), *p < 0.05 compared to the control group.

PSP inhibits H_2O_2-induced ROS and MDA production in HSFs

To determine the effect of PSP on H_2O_2-induced intracellular ROS production, intracellular ROS levels were measured by detecting intracellular DCF levels. The results showed that HSFs had significantly increased intracellular ROS accumulation compared to untreated HSFs after exposure to H_2O_2. DCF intensity in the PSP-treated group was significantly lower than that in the H_2O_2 group (Fig.- 5a; 5b).

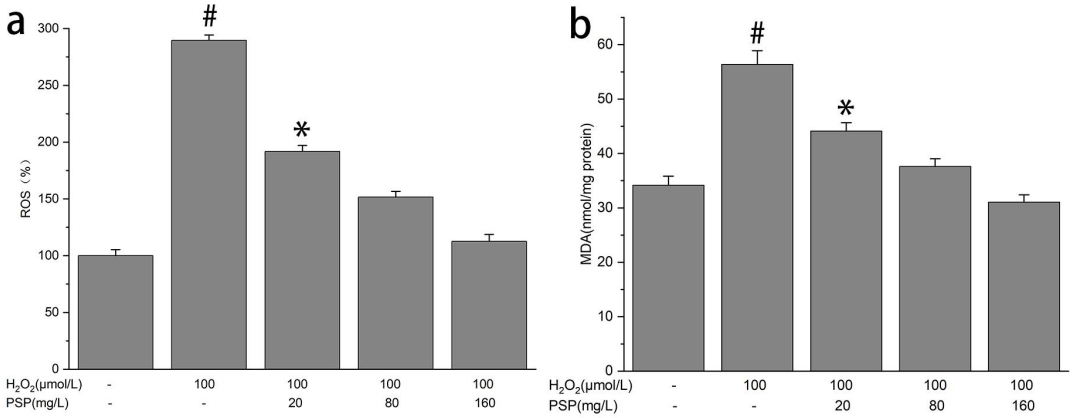

Fig.-5 Effects of PSP pre-treatment on H_2O_2-induced oxidative stress. Results are expressed as the mean±SD (n = 3), #p < 0.05 compared to the control group (without the treatment of PSP and H_2O_2); *p < 0.05 compared to the 100μmol/L H_2O_2 group.

Subsequently, we investigated the levels of MDA, an end-product of lipid peroxidation. Incubation of HSFs with H_2O_2 caused a significant increase in the levels

of MDA. However, pre-treatment with PSP significantly inhibited the changes in MDA induced by H_2O_2.

Effects of PSP pre-treatment on H_2O_2-induced oxidative stress

Compared with the control, H_2O_2-treated HSFs had significantly lower SOD and GSH-Px activities, and GSH levels. However, PSP was effective in restoring SOD and GSH-Px activities and GSH levels in the cells treated with H_2O_2 (Fig. – 6a; 6b; 7).

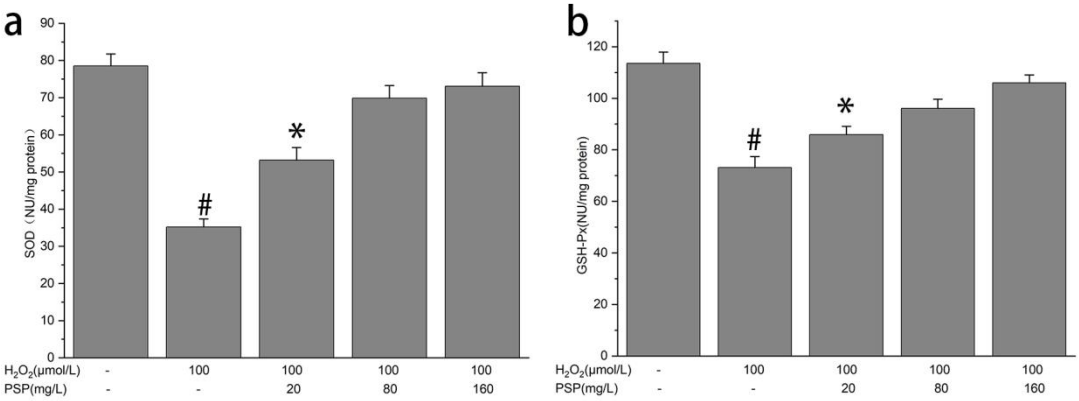

Fig.-6 Protective effects of PSP on H_2O_2-induced oxidative damaged HSFs. Results are expressed as the mean±SD (n = 3), #p < 0.05 compared to the control group (without the treatment of PSP and H_2O_2); *p < 0.05 compared to the 100μmol/L H_2O_2 group.

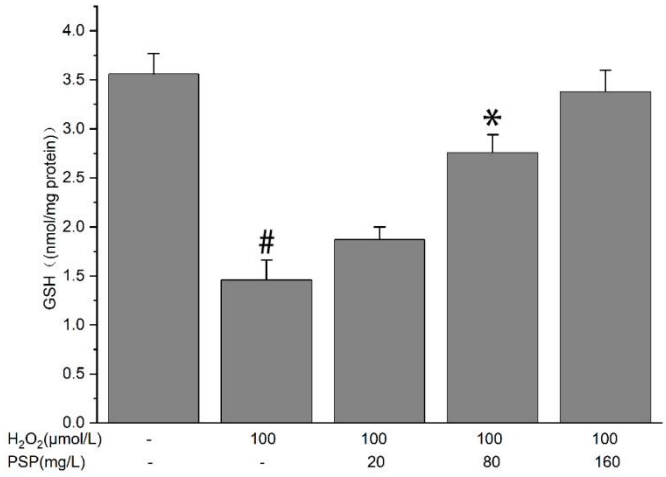

Fig.-7 Effects of PSP on the level of GSH in H_2O_2-treated HSFs. Results are expressed as the mean±SD (n = 3), #p < 0.05 compared to the control group (without the treatment of PSP and H_2O_2); *p < 0.05 compared to the 100μmol/L H_2O_2 group.

Discussion

Oxidative stress refers to the process in which the production of oxidatively reactive substances and the antioxidative system are out of balance. Various harmful stimuli can lead to ROS accumulation and cause tissue damage [22]. For instance, oxidative stress causes lipid peroxidation in biological membranes, denaturation of intracellular proteins and enzymes, and DNA damage. Consequently, cell death via apoptosis and tissue damage ultimately result in diseases.

Normal skin fibroblasts exhibit a strong ability to scavenge ROS. If ROS cannot be cleared in time by the antioxidant system in the skin, they will cause damage to cell membranes, nucleic acids, lipids, proteins, etc. [23], which in turn will cause damage to the fibroblasts. Fibroblasts are the most important cells in the process of skin aging and wrinkle formation. Their biological characteristics include decreased cell proliferation, increased apoptosis, and decreased ability to synthesize antioxidant enzymes and extracellular matrix. Women are more prone to these pathological changes because of their unique physiological characteristics [24]. H_2O_2 is a member of the ROS family, and it is involved in the regulation of a series of physiological and pathological processes, such as cell signal transduction, cell proliferation, aging, apoptosis, and necrosis [25]. In this study, H_2O_2 was used to treat human skin fibroblasts to simulate oxidative damage of HSFs. The results showed that the proliferative activity of human skin fibroblasts in the H_2O_2 group was significantly decreased, the apoptosis rate was increased, the contents of MDA and ROS were increased, and the activities of SOD and GSH-Px along with the levels of GSH were decreased, indicating that incubation of human skin fibroblasts with H_2O_2 could induce oxidative stress in cells. At present, there are no reports on the protective mechanism of PSP on hydrogen peroxide-injured human skin fibroblasts. We used the MTT assay to study the protective effect and mechanism of action of PSP on oxidative stress in human skin fibroblasts. PSP decreased the contents of MDA and ROS in H_2O_2-treated cells but increased the activities of SOD and GSH-Px along with the levels of GSH in HSFs in a dose-dependent manner. Simultaneously, we found

that PSP also increased the production of HA and ColI. The literature describes extensive studies on the benefits of *S. platensis* in the research and development of food additives, functional medicines, health products and other fields and activities.

Conclusion

This study investigated the protective effects of PSP against H_2O_2-induced oxidative stress injury in human skin fibroblast cells. The results demonstrated that PSP improved the cellular antioxidant system and ECM production. Our study demonstrated that PSP has the potential to be used as a component of anti-aging cosmetics.

Acknowledgments

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Conflict of interest statement

None.

Reference

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