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Comparison of in Vitro Proliferation and Antioxidant Activity Between Ginseng-Derived Exosome-Like Nanoparticles and Ginseng Original Liquid

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Abstract

To compare the proliferation and antioxidant activity of Ginseng-derived exosome-like nanoparticles (GELNs) and Ginseng extracts (GEXs) in vitro. GEXs were prepared from natural plant ginseng, and GELNs were extracted by ultracentrifugation combined with sucrose density gradient centrifugation. The CCK-8 method was used to compare the effects of GELNs and GEXs on the proliferation of skin cells. The scratch test was used to compare the effects of GELNs and GEXs on the migration ability of skin cells, and then to verify the ability of GELNs and GEXs to promote proliferation, the antioxidant activity of GELNs and GEXs was compared by OH + DPPH + ABTS + FRAP method. The results showed that although GELNs and GEXs could promote the proliferation of human keratinocytes cells (HaCaT) at a certain concentration, GELNs not only showed better ability to promote the proliferation of skin cells, but also showed good biosafety. In addition, under the same protein concentration, GELNs showed stronger ability to scavenge OH, DPPH, ABTS free radicals and reduce iron ions than GEXs, that is, GELNs had relatively stronger antioxidant activity. The GELNs obtained by purification technology potentially improved the bioavailability and biosafety of ginseng, in order to provide a theoretical basis for its in-depth study as a natural antioxidant.

Keywords: Ginseng, exosome-like nanoparticles, HaCaT cells, cell proliferation, antioxidant activity

1. Introduction

1.1 Antioxidation

Antioxidation, also known as antioxidant free radicals, refers to the process in which the body resists the attack of external free radicals through the antioxidants produced by itself, thereby maintaining the dynamic balance of the antioxidant system(Bailey et al., 2020). It is a self-protection mechanism in organisms. In recent years, due to environmental pollution, light pollution and mental stress and other factors, the free radicals in the human body are rampant. Scientific research shows that the production of excessive free radicals will increase the prevalence of diseases such as cancer, aging and diabetes. The demand for antioxidants has become increasingly prominent(Oschman, 2023). Natural antioxidants derived from natural plants have gradually replaced synthetic antioxidants because of their safety, high efficiency and wide distribution. Screening and developing antioxidant components from natural plants has become the development direction of modern pharmaceutical industry.

1.2 Exosome

Exosome is a small cell vesicle with a phospholipid bilayer structure with a diameter of 30-100 nm. It has good biocompatibility, biological stability and bioavailability, and plays a role in intercellular communication, transfer of biomolecules and pathological processes of the body(Kalluri & LeBleu, 2020). At present, great progress has been made in the research of animal exosomes. However, its source makes it have the problem of allogeneic immunogenicity, which limits its clinical application to a certain extent. In recent years, scientists have expanded the source of exosomes to the field of plants, and have successfully confirmed the biological activities of

blueberry(De Robertis et al., 2020), lemon(Yang et al., 2020), ginger(Zeng et al., 2024) and other plants. At the same time, it has been found that compared with animal-derived exosomes, plant-derived exosome-like nanoparticles (PELNs) not only have lower immunogenicity, but also have shorter extraction cycle, higher yield and easier access68(Han et al., 2022; Suharta et al., 2021), showing great application potential in the field of disease treatment.

1.3 Active Ingredients of Ginseng

Ginseng (Panax ginseng C.A.Mey.), a perennial herb of Panax in Araliaceae, is a precious Chinese herbal medicine with a long history. It has always been a research hotspot in China's pharmaceutical industry. It has the effects of anti-cancer, anti-oxidation, anti-depression, hypoglycemic and anti-aging(L. Wang et al., 2020). At present, a variety of active ingredients from ginseng have been confirmed to have certain antioxidant activity, including ginsenoside Rg1(Gao et al., 2020), Rb1(Fan et al., 2020), Re(Lee et al., 2020), ginseng polysaccharide(Kim et al., 2020; K. Wang et al., 2020), etc. However, the application of these components has its unique limitations, such as cumbersome extraction process, low yield, poor water solubility, low bioavailability, and insignificant therapeutic effect(Hu et al., 2023; M. Wang et al., 2020).

In summary, this study used ultracentrifugation combined with sucrose density gradient centrifugation to extract and purify Ginseng-derived exosome-like nanoparticles (GELNs) from Ginseng extracts (GEXs), and investigated the in vitro proliferation and antioxidant activity of GELNs and GEXs, in order to provide a reference for the development of new natural nano-level antioxidants.

2. Method

2.1 Material

The roots of Panax ginseng were collected from Changchun City, Jilin Province, China. HaCaT cells, derived from the Concordia cell bank.

DMEM high glucose medium, penicillin streptomycin, fetal bovine serum, 0.25 % trypsin, from Gibco (USA). PBS buffer, 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical scavenging assay kit, from Solarbio (China). CCK8 Cell Proliferation Toxicity Assay Kit from Tongren Institute of Chemistry. Total antioxidant capacity detection kit from Shanghai Yuanye (China). BCA protein concentration detection kit and total antioxidant capacity detection kit (FRAP method) were derived from Beyotime (China).

2.2 Extraction and Purification of GELNs

Fresh ginseng was rinsed clean and dried for later use. An appropriate amount of PBS was added and placed in a homogenizer to prepare GEXs. After filtration, differential centrifugation was used to centrifuge at 300×g, 2000×g, and 10 000×g for 10 min, 20 min, and 30 min to remove cell debris, fibers, and large particles. The supernatant was added to the SW32 Ti rotor and centrifuged at 100 000 × g for 2 h. The precipitate was resuspended with 20 mmol/L Tris-HCl and fully vortexed. The samples were further purified by sucrose density gradient method. The samples were placed in a centrifuge tube, and different concentrations of pure water sucrose solution (15 %, 30 %, 45 %, 60 %) were added. After centrifugation at 100 000 × g for 2 h, 30 % and 45 % intermediate sample bands were gently sucked. After dilution with 20 mmol/L Tris-HCl, sucrose was removed by centrifugation at 100000 × g for 2 h, and the purified GELNs were obtained by resuspending precipitation(Cho et al., 2021).

2.3 Study on the Proliferation Activity of GELNs and GEXs in vitro

2.3.1 Cell Culture

HaCaT cells were cultured in DMEM complete medium containing 10 % fetal bovine serum (FBS) and 1 % double antibody (penicillin-streptomycin solution) at 37 ℃ in a 5 % CO_2 cell incubator. When the cell density grows to 80 %-90 %, discard the old liquid, PBS washed twice. Appropriate amount of 0.25 % trypsin was added for digestion. After the cells began to become round, complete medium was added to terminate the digestion, and the cells were blown evenly. The cells were transferred to a centrifuge tube, centrifuged at 1200 × g for 5 min, the supernatant was discarded, and the complete medium was added to make a cell suspension. The cells were passaged at a ratio of 1:2 or 1:3 until the cells grew to the logarithmic growth phase for subsequent research.

2.3.2 Cell Viability Assay

HaCaT cells in logarithmic growth state were digested with 0.25 % trypsin and made into cell suspension. The cell suspension was inoculated in 96-well plates and cultured in a cell incubator at 37 ℃ and 5 % CO_2 for 24 h, and the old liquid was discarded. GELNs and GEXs solutions with mass concentrations of 10,20,30,40,50,60,70,80,90,100,200μg/mL diluted with serum-free medium were added. Six duplicate wells were set for each concentration, and the control wells were not treated. They were re-incubated in an incubator for 24 h

and the old solution was discarded. 100 μL serum-free medium and 10μL CCK-8 solution were added to each well, and a blank hole containing only medium was set up, and the incubation was continued in the incubator for 2 h. The absorbance value (A) at 450 nm was measured by microplate reader.

2.3.3 Cell Wound Scratch Assay

HaCaT cells in logarithmic growth phase were digested with 0.25 % trypsin and made into cell suspension, which was inoculated in 6-well plates and cultured in a cell incubator at 37 ° C and 5 % CO_2 for 24 h. The blank control group and the experimental group were set up in the experiment. The cells were scratched with a 10 μL gun head, washed with PBS three times, and the scratch pictures were recorded by fluorescence microscope at 0 h. The blank control group was added with fresh serum-free medium. The experimental group was added with 12.5,25,50,100 μg / mL GELNs and GEXs solutions diluted with serum-free medium. Each concentration was set up with three holes. After re-incubation in the medium for 24 h, the 24 h scratch pictures were recorded at the same position using a fluorescence microscope. The scratch area was analyzed by ImageJ software, and the scratch healing rate was calculated.

2.4 Study on Antioxidant Activity of GELNs and GEXs

Dilution of GELNs and GEXs samples: The samples were dissolved in PBS, and the protein concentration was determined by BCA kit. The protein concentration was quantified by protein concentration, and the concentration from low to high was 0.125, 0.25, 0.5, 1 and 2mg/mL, respectively. Dilution of GELNs and GEXs samples: The samples were dissolved in PBS, and the protein concentration was determined by BCA kit. The protein concentration was quantified by protein concentration, and the concentration from low to high was 0.125,0.25,0.5,1 and 2 mg / mL, respectively. The antioxidant activity of the samples was determined by hydroxyl radical, DPPH, ABTS scavenging ability test kit and total antioxidant capacity test kit (FRAP method).

2.5 Statistical Analysis

GraphPad Prism8 was used for statistical analysis and plotting. One-way analysis of variance and Tukey test, two-way analysis of variance and Bonferroni test were used to compare the differences between groups. The data were expressed as mean±standard deviation (x±s), and P<0.05 was considered statistically significant.

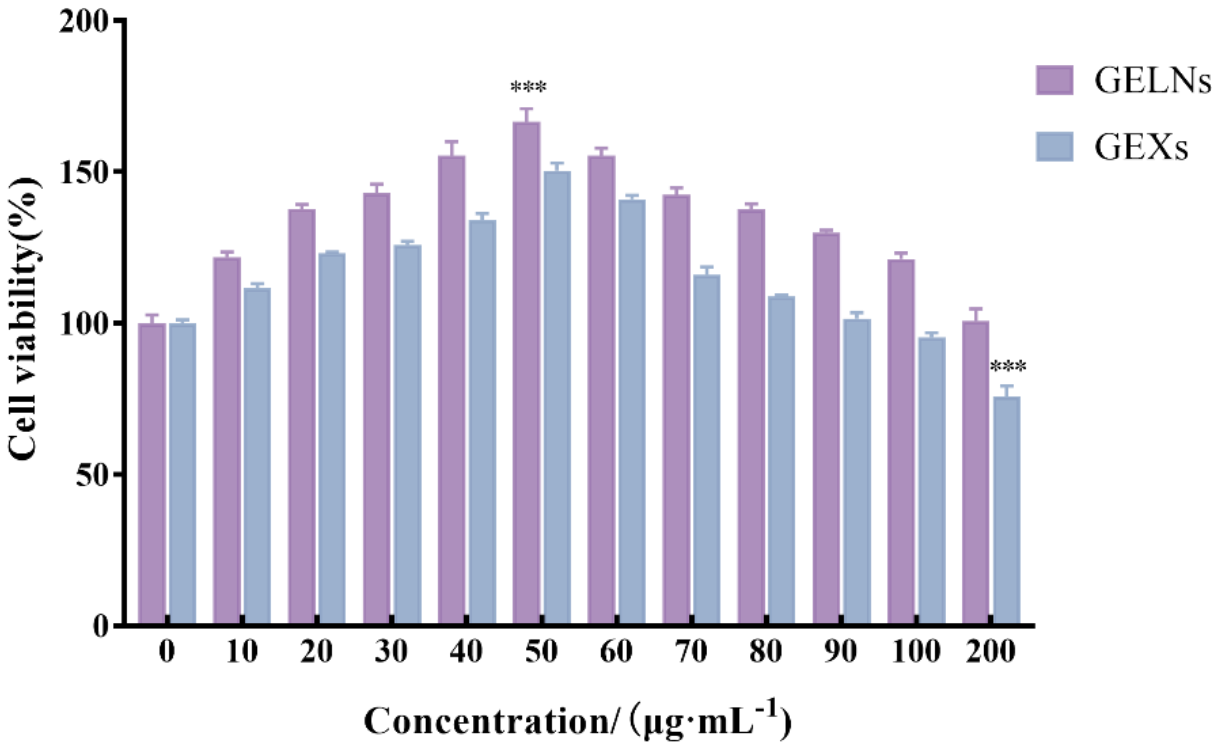

Figure 1. Effects of different concentrations of GELNs and GEXs on survival rate of HaCaT cells. Values represent the mean±S.D. (n = 4 per group). ***P＜0.001 versus non-administered group.

3. Results

3.1 The Effects of GELNs and GEXs on Cell Viability

In order to determine whether GELNs and GEXs will affect the viability of HaCaT cells or cause HaCaT cytotoxicity, CCK-8 assay was used to evaluate the cytotoxic activity. The results are shown in Fig.1.Compared with the blank control group, when 10-200μg/mL GELNs and GEXs acted on HaCaT cells, GELNs did not show obvious toxicity, and even promoted cell proliferation to a certain extent.50 μg / mL GELNs made the cell survival rate as high as 166.66±4.24 %, showing significant proliferation promotion ability (P<0.001). Although GEXs also promoted cell proliferation in the concentration range of 10-100μg/mL, the effect was relatively weak compared with GELNs at the same concentration. When the concentration reached 200μg/mL, the cell survival rate decreased to 75.83±3.48 %, resulting in cytotoxicity. The above results showed that compared with GEXs, GELNs not only had no obvious toxic damage to HaCaT cells, but also showed excellent proliferation effect. Therefore, at the non-toxic mass concentration of GEXs, the concentration gradients of 12.5, 25, 50, 100μg/mL were selected to continue the cell scratch test.

3.2 Evaluation of the Effect of GELNs and GEXs on Cell Scratch Healing

The effects of different concentrations of GELNs and GEXs on the scratch healing of HaCaT cells were further studied by cell scratch test. The results showed that the scratches of the blank control group, GELNs group and GEXs group showed a healing trend within 24 hours (Fig.2a-b). Compared with the blank control group, the healing rate of GELNs group and GEXs group showed an upward trend, and reached the maximum healing rate at 50μg/mL, which were 55.44±1.72% and 48.94±1.65%, respectively (Fig.2c). At the same concentration, the healing rate of GELNs group was significantly higher than that of GEXs group (P < 0.05). The results showed that GELNs showed more significant ability to promote cell scratch healing than GEXs. In summary, GELNs have good biosafety and excellent proliferation-promoting ability.

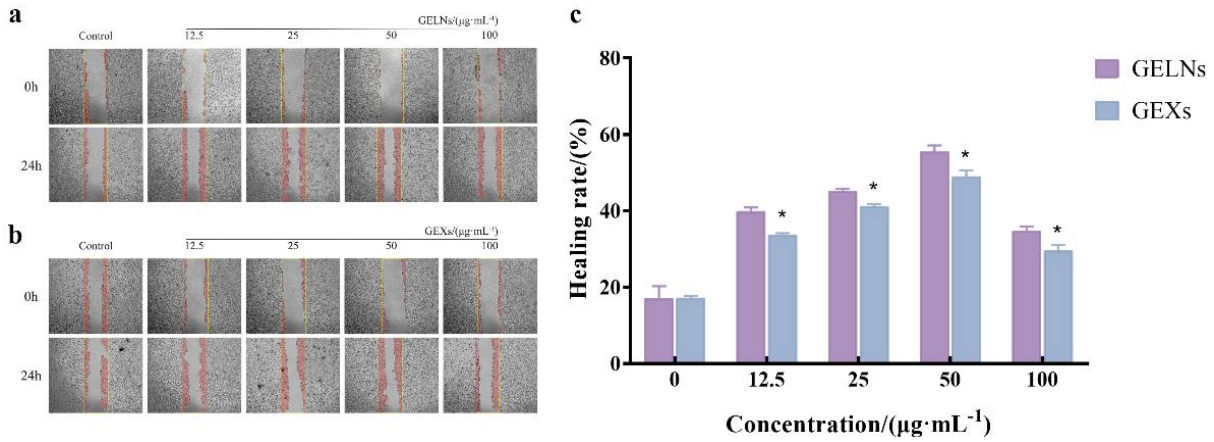

Figure 2. Effects of different concentrations of GELNs and GEXs on survival rate of HaCaT cells (a)GELNs scratch healing map. (b)GEXs scratch healing map. (c) Scratch healing rate comparison chart. Values represent the mean±S.D. (n = 3 per group). *P＜0.05 versus GELNs group.

3.3 Evaluation of Antioxidant Capacity of GELNs and GEXs

3.3.1 Hydroxyl Radical Scavenging Ability

Hydroxyl radical is a highly toxic free radical produced in human metabolism. It can oxidize proteins, nucleic acids and other substances in tissues, and is one of the important indicators for evaluating antioxidant capacity. As shown in Fig.3, the scavenging ability of GELNs to hydroxyl radicals increased with the increase of protein concentration, showing a significant dose-dependent manner (P < 0.01), and the maximum scavenging rate was 48.37±1.42% at 2mg/mL. However, the scavenging effect of GEXs on hydroxyl radicals was not obvious, and the maximum scavenging rate was only 4.65±0.33% at 2mg/mL. At the same protein concentration, the hydroxyl radical scavenging ability of GELNs was significantly stronger than that of GEXs (P<0.05). The results showed that GELNs exhibited extremely excellent hydroxyl radical scavenging ability compared with GEXs.

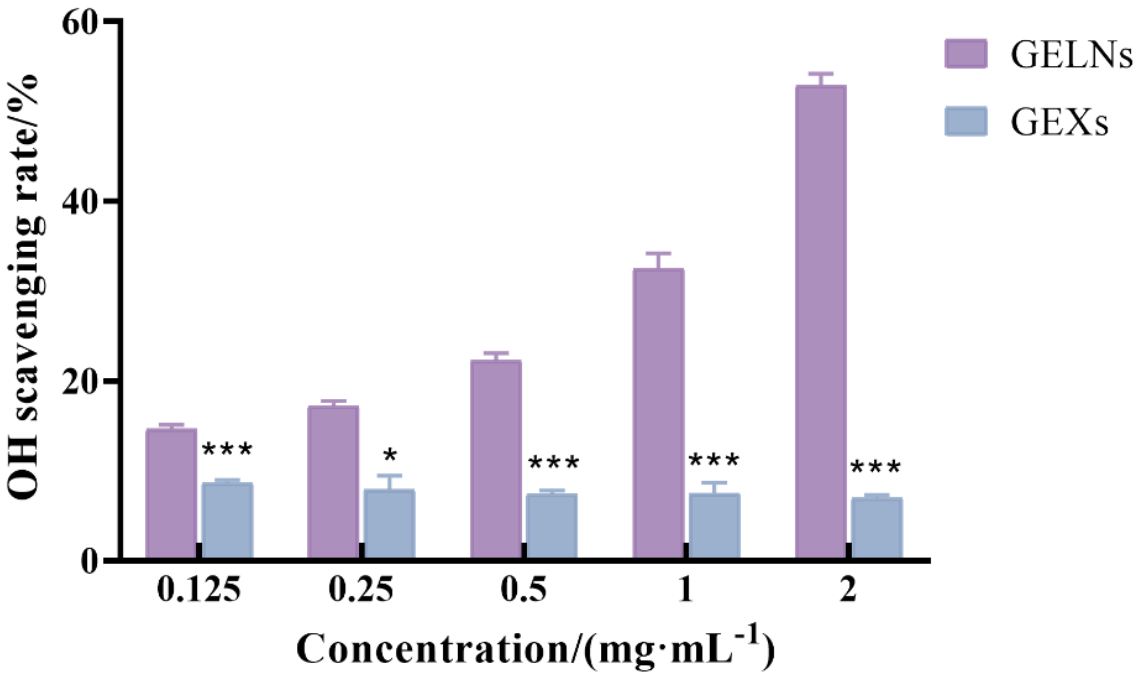

Figure 3. Scavenging ability of hydroxyl radicals by GELNs and GEXs at different concentrations. Values represent the mean±S.D. (n = 3 per group). *P＜0.05, ***P＜0.001 versus GELNs group.

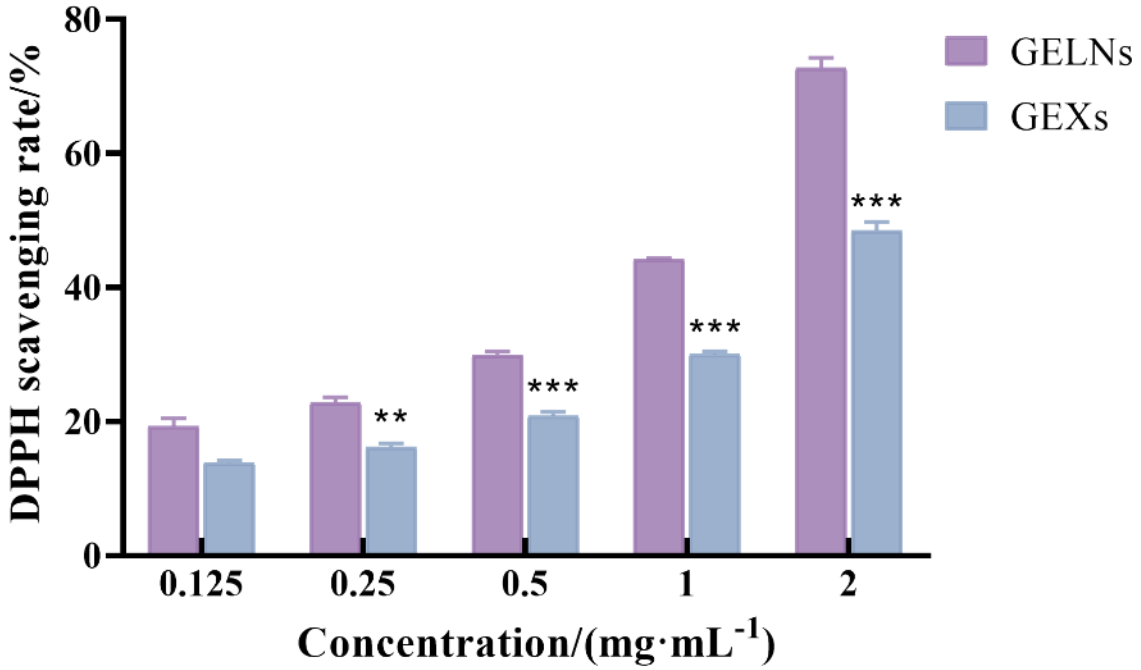

Figure 4. Scavenging ability of DPPH radicals by GELNs and GEXs at different concentrations. Values represent the mean±S.D. (n = 3 per group). **P＜0.01, ***P＜0.001 versus GELNs group.

3.3.2 DPPH Free Radical Scavenging Activity

DPPH method is one of the important indexes to evaluate the antioxidant capacity. As shown in figure 4, the DPPH free radical scavenging ability of GELNs and GEXs increased with the increase of protein concentration, showing a significant dose-effect relationship (P<0.05). The maximum DPPH scavenging rate was 75.17±1.38 % and

66.09±1.45 % at 2mg/mL, respectively. At the same protein concentration, except for low concentration, the DPPH scavenging ability of GELNs was significantly stronger than that of GEXs ($P < 0.01$). The results showed that GELNs showed better DPPH free radical scavenging ability than GEXs.

3.3.3 Reduction ability to Fe^{3+}

The reduction ability of the sample to Fe^{3+} was evaluated by FPAP method. Under acidic conditions, the antioxidant will cause Fe^{3+}(Fe^{3+}-TPTZ) chelated with TPTZ to produce blue Fe^{2+}-TPTZ, which has strong absorbance at 593 nm. The antioxidant capacity of the sample can be obtained by measuring the absorbance of Fe^{2+}-TPTZ. As shown in Fig.5, the reduction ability of GELNs and GEXs to Fe^{3}+ increased with the increase of protein concentration, and showed a significant dose-effect relationship ($P < 0.05$). The maximum FRAP values were 1.63±0.02 and 1.22±0.03 at 2 mg/mL, respectively. At the same protein concentration, except for low concentration, the ability of GELNs to reduce Fe^{3+} was significantly stronger than that of GEXs ($P<0.01$). The results showed that GELNs showed better antioxidant capacity than GEXs.

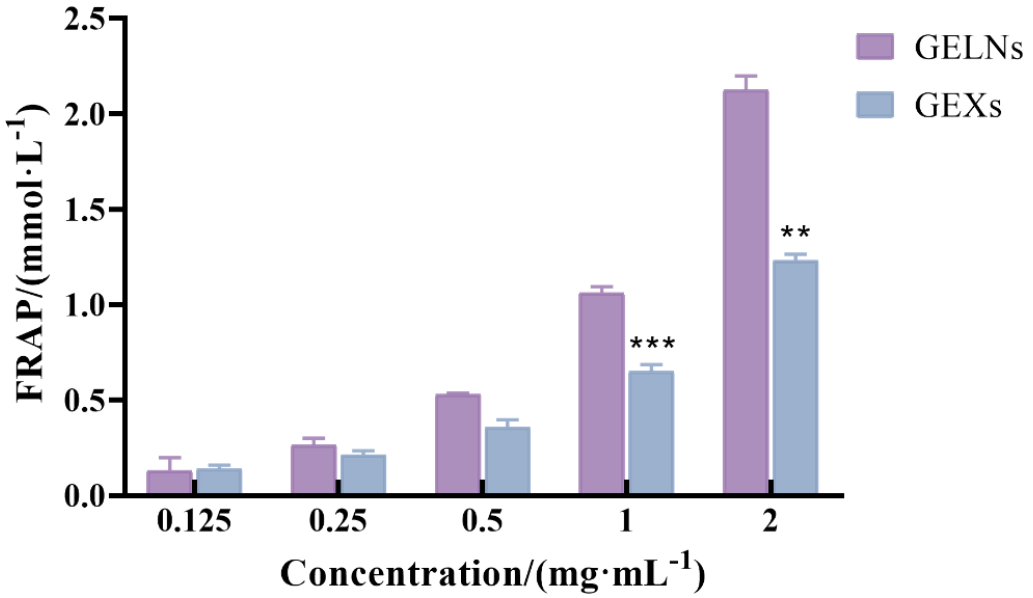

Figure 5. The reduction ability of GELNs and GEXs with different concentrations to Fe^{3+}. Values represent the mean±S.D. (n = 3 per group). **P＜0.01, ***P＜0.001 versus GELNs group.

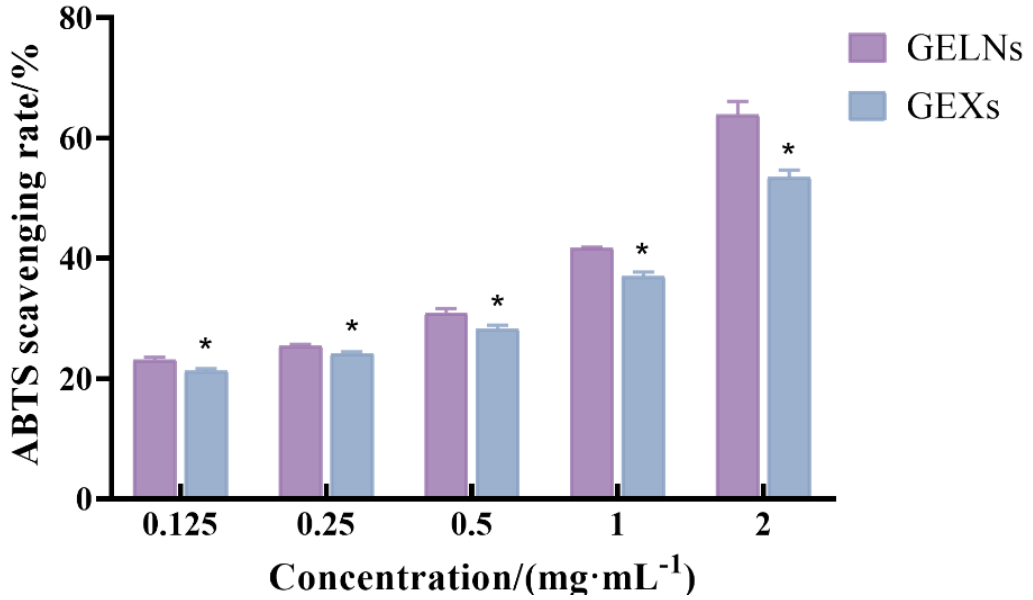

Fig.6 Scavenging ability of ABTS radicals by GELNs and GEXs at different concentrations. Values represent the mean±S.D. (n = 3 per group). *P＜0.05 versus GELNs group.

3.3.4 ABTS Radical Scavenging Ability

ABTS radical is a common chromogenic reagent for evaluating the antioxidant properties of substances. As shown in Fig.6, the scavenging ability of GELNs and GEXs to ABTS free radicals increased with the increase of protein concentration, and had a significant dose-dependent manner ($P < 0.05$). The maximum ABTS scavenging rate was $61.09 \pm 0.91\%$ and 55.75 ± 0.45 % at 2mg/mL, respectively. At the same protein concentration, the ABTS scavenging ability of GELNs was significantly stronger than that of GEXs ($P < 0.05$). The results showed that GELNs showed better ABTS radical scavenging ability than GEXs.

4. Discussion

Studies have shown that oxidative stress can cause damage to the antioxidant defense mechanism in the body, and the system of scavenging free radicals in the body is unbalanced, thereby causing cell damage(Rocca et al., 2020). In this study, GLENs were purified from GEXs by differential centrifugation combined with sucrose density gradient centrifugation. HaCaT cells were used to study the toxic activity of GELNs and GEXs on them and their ability to promote their proliferation. The ability to scavenge DPPH, OH, ABTS free radicals and the ability to reduce Fe^{3+} were selected as indicators to evaluate the antioxidant capacity of GELNs and GEXs. The results showed that compared with GEXs, GELNs not only effectively promoted cell proliferation in vitro, but also showed considerable antioxidant activity, which was a potential natural antioxidant active ingredient.

With the increasing attention to antioxidation, more and more studies have found that oxidative stress injury is closely related to senile and chronic diseases, and can cause a series of complications(Eastman et al., 2020). It is urgent to develop more safe and effective antioxidants. The purified GELNs showed better antioxidant capacity, which proved that the synergistic effect could be achieved by processing and purifying natural active substances. In the future, it is a trend to process a single substance to treat the damage caused by oxidative stress. This study can provide a scientific basis for the treatment of damage caused by oxidative stress.

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