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Research article

Preparation, identification and molecular docking of two novel anti-aging peptides from perilla seed

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ABSTRACT

Perilla seed meal is an important agricultural by-product of perilla oil extraction. The antioxidant and anti-aging activities of perilla seed meal protein hydrolysate were investigated, and the bioactive peptides were identified to maximize the utilization of perilla seed meal resources. Anti-aging peptides were identified using a combination of peptidomics and in silico bioinformatics. Furthermore, the potential molecular mechanism of these peptides was explored through molecular docking and RT-PCR. The results showed a significant anti-aging properties of F2 (MW 3 kDa ~5 kDa) by inhibition of reactive oxygen species (ROS) production and β-galactosidase activity. Nine novel peptides were identified from F2 and subsequently synthesized to explore their bioactivities. The two peptides, NFF and PMR, were found to promote the proliferation of keratinocytes (HaCaT cells) and suppress the level of ROS and the activity of β-galactosidase. Both peptides exhibited a strong binding affinity with the Keap1 protein. Additionally, NFF and PMR downregulated the expression of matrix metalloproteinases (MMPs) and the degradation of collagens (COLs). The potential molecular mechanism underlying the anti-aging properties of perilla seed meal peptides might involve the competitive binding of Keap1 to facilitate the release of Nrf2 and activation of NF-κB signal pathway. This study provides a theoretical basis for the application of perilla seed meal peptides in functional cosmetics and presents a novel perspective for the investigation of additional food-derived peptides.

1. Introduction

Reactive oxygen species (ROS) are chemical compounds generated during the process of respiration that play a crucial role in various physiological processes, including cell cycle regulation, signal transduction, programmed cell death and metabolism [1,2]. However, excessive ROS have a detrimental impact on the nucleophilic centers of biological macromolecules, leading to the degradation of proteins, lipids and genes [3–5]. This process alters tissue function, initiates oxidative stress damage, accelerates the aging process and ultimately contributes to the development of chronic diseases, such as hypertension [3–5]. The incorporation of exogenous antioxidants has demonstrated the ability to alleviate the oxidative damage induced by ROS and reduce the occurrence of diseases [6]. Antioxidants, commonly utilized as supplements for anti-aging purposes, possess the capacity to inhibit or neutralize free radicals [6].

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Antioxidants can eliminate free radicals and mitigate their harmful effects on human cells, thus decelerating aging. Additionally, they can also reduce the activities of elastase, collagenase and the levels of other inflammatory factors, leading to improved skin relaxation and a reduction in aging-related skin manifestations such as wrinkles [7]. Nonetheless, the synthesis of antioxidants is expensive and may involve potential risks of toxicity [1,8]. Natural polyphenols, bioactive compounds and secondary metabolites abundantly present in fruits and vegetables, display remarkable antioxidant characteristics [9,10]. However, the practical application of polyphenols is impeded by their limited solubility and bioavailability [11]. It is worth mentioning that natural peptides obtained from plant proteins, known for their antioxidant properties, have gained considerable international attention for their environmentally friendly attributes, sustainability, cost-effectiveness and absence of adverse effects [8].

Perilla, a member of the Labiatae family, is an annual plant that plays a crucial role in traditional Chinese medicine, as evidenced by its widespread use of stems, seeds and leaves [11]. Perilla seed protein has been widely utilized in the fields of food additives, cosmetics and medicine due to its remarkable nutritional value and efficient protein conversion rate [12]. The protein content of perilla seed meal typically falls within the ranges of 35 %–45 % [12,13]. Perilla seed meals, the primary by-product of oil seed processing, are frequently used as animal feed or discarded, resulting in the loss of valuable functional constituents present in perilla seed meal [12,13]. Previous studies have confirmed the significant antioxidant effectiveness and potential of perilla seed meal protein hydrolysate and perilla peptides [14]. Nevertheless, the precise mechanism of perilla seed peptides (PSPs) has yet to be confirmed. Therefore, it is imperative to investigate PSP in order to enhance the value of perilla and expand its application [12,15].

Protein does not exhibit any pharmaceutical properties in its intrinsic form [16]. Nevertheless, protein can produce active peptides through various processes, including microbial fermentation, gastrointestinal digestion, in vitro treatment with proteases and exposure to extreme pH conditions or high temperature [16]. Enzymatic hydrolysis is commonly used in the production of active peptides to avoid additional organic or toxic reagents, while also ensuring the maintenance of secure and controlled hydrolysis conditions [17]. Previous research frequently utilized membrane filtration and chromatographic techniques to separate peptides [18]. Subsequent to the separation process, the frequent occurrence of multiple peptides remains challenges in the following stages of peptide synthesis and validation [1,19].

The utilization of computer-aided analysis in peptide screening and prediction processes enhances the exploration of potential activities and mechanisms associated with these peptides. This approach incorporates various techniques, including computer simulation screening, safety assessment, activity scoring, toxicity evaluation, and sensitization analysis [1,20]. Ren [1] et al. have identified four novel peptides with favorable water solubility, safety and antioxidant properties from the pool of antioxidant peptides derived from crushed rice protein through computer-assisted screening. In recent years, molecular docking has gained increasingly popularity for its utility in the screening and identification of natural small molecular active compounds, as well as for studying the interaction mechanisms between small molecules and macro-molecules [21,22]. However, there is a dearth of research dedicated to the utilization of computer-based methodologies to accelerate the identification of bioactive peptides from perilla seed. This research gap impedes our capacity to fully comprehend the safety of novel peptides in an efficient and cost-effective manner.

Therefore, the purposes of this study are a) to assess the anti-aging properties of perilla seed hydrolysate through chemical models, HaCaT cell models induced by H_2O_2, and HFF-1 cell models induced by D-gal; b) to identify novel anti-aging peptides by peptidomics and in silico methodologies, subsequently validated by in vitro experimentation; c) to elucidate the molecular mechanism of the identified anti-aging peptides through molecular docking and reverse transcription polymerase chain reaction (RT-PCR) analysis. This study provides a theoretical basis for the high-value utilization of perilla seed by-products in the food and pharmaceutical industries, as well as a novel approach for the identification and investigation the molecular mechanisms of bioactive peptides.

2. Materials and methods

2.1. Materials

Perilla seed meal was purchased from Yunnan. Annexin V-FITC kit and β-gal staining kit were purchased from Beyotime (Jiangsu, China). Cell-countingkit-8 (CCK-8) was obtained from Do Jindo Laboratories (Japan). Alkaline protease, neutral protease and papain are purchased from Yuanye Biological Reagent Co., Ltd (shanghai, China). The following chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, USA): Ophthalaldehyde (OPA), sodium dodecyl sulfate (SDS), 1,1-diphenyl-2- picrylhydrazyl (DPPH) and D-galactose (D-gal). Quantitative real-time polymerase chain reaction (qRT-PCR) primers were purchased from Generay Biotech Co., Ltd (Shanghai, CHINA). GeneJET RNA purification kit was bought from Iermo Fisher Scientific (USA). RNA Reverse Transcription Kit was obtained from PrimeScript™ RT reagent Kit (Takara, Japan).

2.1.1. Production of perilla seed crude protein (PSCP)

Hu's [23] method was used to extract crude protein from perilla seeds and was adjusted. In brief, perilla seed powder solution (1 g:10 mL) was continuously stirred at 60 °C until the pH of the solution remained unchanged at 9.0. The mixture was centrifuged (ST16, Thermo Fisher Scientific Co, Ltd., American) at 5411 $\times g$ for 10 min and the pH of the supernatant was adjusted to the isoelectric point of 4.0. After standing overnight at 4 °C, the precipitate was collected by centrifugation and washed with distilled water for desalination. The precipitate was freeze-dried (LyovaporL-200, Buchi Laboratory Equipment Trading Ltd. Co., Ltd., Switzerland) and the powder was the PSCP. Protein content and moisture content of perilla seed meal and PSCP were determined by moisture analyzer (PAL-3, Shanghai Jing Ning Scientific Instrument Co., Ltd., Shanghai, China) and Kjeldahl nitrogen analyzer (DI110C, Beijing Jia sheng Xingye Technology Co., Ltd., Beijing, China) respectively.

2.2. Preparation and separation of perilla seed peptides (PSP)

2.2.1. Screening of proteases

Three enzymes (alkaline protease, neutral protease and pepsin) were used to hydrolyze PSCP. Table S1 lists the optimum temperature and PH of the three enzymes respectively. The ratio of solid to liquid was 1:30 (g: mL) and the amount of enzyme was 3000 u/g PSCP. After hydrolysis for 4 h, the enzyme was inactivated at 100 °C for 10 min and the supernatant was centrifuged at 5411×*g* for 10 min. The free radical scavenging ability of DPPH and the degree of hydrolysis (DH) of supernatant were measured.

2.2.2. Determination of DPPH free radical scavenging activity

The DPPH test was modified to align with Fu's [24] methodology. Briefly, 150 μL of DPPH solution (0.05 mg/mL in ethanol) was added to 150 μL of sample solution to be tested, incubated in the dark for 30 min and the absorbance of the sample was measured at 517 nm (Multiskan SkyHigh, Thermo Fisher Scientific Co, Ltd., American). The DPPH radical scavenging rate of the test sample was calculated using the formula below:

$$\text{DPPH radical scavenging rate} = \left(1 - \frac{A1 - A2}{A3}\right) \times 100\%$$

Where, A1: the absorbance of the DPPH-containing sample;
A2: the ethanol-containing sample's absorption value;
A3: the DPPH-in-ethanol absorption value.

2.2.3. Determination of hydrolysis degree (DH)

DH was determined by an *o*-phthaldialdehyde (OPA) method of Wang [25]. 400 μL of hydrolysate and 3 mL of OPA reagent were combined and after 2 min of room temperature incubation, the mixture's absorbance (VS3200, Agilent Technologies Co. Ltd., American) was measured at 340 nm. The standard curve of leucine is used to calculate the concentration of free amino in the sample to be measured. The DH of the test sample was calculated using the formula below:

$$\text{DH} = \frac{A1}{A2} \times 100\%$$

Where, A1: the concentration of free amino in the sample to be tested (mol/L);
A2: the concentration of nitrogen in the sample to be tested (mol/L).

2.2.4. Ultrafiltration separation of PSP

The supernatant of method 2.2.1 passes through ultrafiltration membranes (Master Flex F/L, Pall Corporation Co., Ltd., American) with molecular weight cut-off (MWCO) of 5 and 3 kDa in turn. Therefore, the retentate in the concentration step of 5 kDa membrane (<5 kDa) was collected and lyophilized, while the permeate was packaged by 3 kDa membrane, collecting retentate (3~5 kDa) and permeate (<3 kDa). Until additional analysis was required, the final permeate and retentate were freeze-dried and stored at −20 °C.

2.3. Identification of PSP

Fraction F2 was loaded from an autosampler into a Zorb ax 300SB-C18 peptide Trap (Agilent Technologies, Wilmington, DE) and then separated by liquid chromatography column (0.15 mm × 150 mm, RP-C18, DE). 0.1 % formic acid aqueous solution was employed as solution A in the liquid phase and 0.1 % ethyl formic acid aqueous solution (ethyl formic acid 84 %), was utilized as solution B.Q Exactive Mass Spectrometer (Themo Fisher) was used for mass spectrometry analysis and the corresponding database was searched by MaxQuant 1.5.5.1 software to obtain the results of peptide identification and quantitative analysis.

http://districtdeep.ucd.ie/PeptideRanker/ was used to predict the activity of each peptide (between 0 and 1) [26]. The closer the calculated value is to 1, the higher the activity the fragment shows. Values of peptide sequences above 0.8 were considered for further analysis. service.php? AnOxPePred 1.0 was used to predict the free radical scavenging activity of PSP [26]. Values of peptide sequences above 0.4 were considered for further analysis. The allergenicity of PSP were analyzed online using Allergen FP v.1.0 (http://www.ddg-pharmfac.net/A llergenFP). The toxicity of PSP was analyzed online using Toxin Pred (http://crdd.osdd.net/raghava/toxinpred/). The novelty of PCSP were analyzed online using https://www.uwm.edu.pl/biochemia/php/en/biopep.

2.4. Molecular docking of PSP with Keap1

Discovery 2019 calculated the semi-flexible molecular docking between PSP and Keap1. The crystal structure of Keap1 (PDB ID: 2FLU, 1.50 Å, PDB DOI: https://doi.org/10.2210/pdb2FLU/pdb) was obtained from the RCSB PDB database (http://www.rcsb.org/) prior to docking. Then, the ligand (peptide from perilla seed) required for molecular docking was prepared using Discovery 2019. For the target protein, the crystal structure required pretreatment, including removing water molecules, supplementing hydrogen atoms and charges. The binding energy (kcal/mol) value represented their binding capacity. Lower binding capacity represents more stable ligand-receptor binding.

2.5. Synthesis of polypeptide

GL Biochem Ltd. (Shanghai, China) produced the screened peptides using the traditional 9-fluorenylmethyloxycarbonyl solid phase synthesis method, yielding a purity of above 95 %.

2.6. Cell culture

Human skin fibroblast HFF-1 and immortal human keratinocyte HaCaT (Chinese Academy of Science, Shanghai, China) were cultivated at 37 °C and 0.5 % CO_2 (China Academy of Sciences) in Dulbecco's modified Eagle medium (DMEM) with 10 % (v/v) fetal bovine serum. 0.25 % trypsin was used to digest HaCaT and HFF-1.

2.7. Cell viability

(a) 2×10^4 cells per well were put onto a 96-well plate, cultured for 24 h, then replaced with test sample DMEM medium and treated for another 24 h. Then it was replaced by DMEM medium containing 10 % CCK-8. After 2 h of incubation, the absorbance at 450 nm was measured. The following formula was used to determine the cell viability:

$$\text{Cell viability} = \frac{A2 - A1}{A3 - A1} \times 100\%$$

Where, A1: the absorbance in the absence of the cell group;
A2: the test sample's absorbance in the presence of the cell group;
A3: the absorbance of the cell group that has not been treated.

(b)In a 96-well plate, 5×10^3 cells were added to each well, cultured for 24 h and then replenished with DMEM medium containing 0.4 % serum for an additional 24 h. After that, it was replaced by 100 μL DMEM (containing 0.4 % serum) containing the test sample and cultured for 72 h. 100 μL of 10 % CCK-8 solution was used to replace the test solution for 2 h and then the absorbance was read at 450 nm. The following formula was used to calculate the sample's proliferative activity:

$$\text{Proliferative activity} = \frac{A2 - A1}{A3 - A1} \times 100\%$$

Where, A1: the absorbance in the absence of the cell group;
A2: the test sample's absorbance in the presence of the cell group;
A3: the absorbance of the cell group that has not been treated.

2.8. Storage stability test of polypeptide

Polypeptide was prepared into 1 mg/mL solution and stored at 5 °C, 25 °C and 40 °C (under illumination). The changes of appearance, pH and peak area of peptides during storage were observed. The pH and peak area of polypeptide were determined by pH meter (FE28, Mettler Toledo, Switzerland) and HPLC (1260, Agilent Technologies Co. Ltd., American) respectively.

$$\text{Polypeptide degradation rate} = \frac{A1 - A2}{A1} \times 100\%$$

Where, A_1 represent Peak areas at day 0 of PSP storage;
A_2 represent Peak areas at day 0 of PSP storage.

2.9. Effect of PSPs on the ROS concentration in HaCaT cells with H_2O_2-induced premature aging

The intracellular ROS levels in HaCaT cells were detected using DCFH-DA as a fluorescent probe, in accordance with the methodology of He [27] et al. In 24-well plates, 1×10^5 HaCaT cells were seeded and cultured for 24h. Afterwards, the test samples (in DMEM) were added to the wells and incubated for an additional 4h. After the test samples were incubated with 800 μM H_2O_2 for 2 h, the relative amount of ROS was determined by measuring the fluorescence intensity of DCF using a reactive oxygen species (ROS) detection kit (Beyotime) and flow cytometry (FCM) (BD FACSCanto, Becton Dickinson Medical Devices (Shanghai) Co.,Ltd., Shanghai, China).

2.10. Effect of PSPs on the activity of SA-β-gal in premature aging of HFF-1 cells mediated by D-gal

The senescence degree of HFF-1 cells was determined using the experimental approach of He [27] et al. by measuring senescence-associated-β-galactosidase (SA-β-gal) activity. After inoculating HFF-1 cells at a density of 2×10^4 cells per well in a 48-well plate after 24 h, the test sample solution containing a final concentration of 20 mg/mL D-gal was employed instead of the growth medium for 72 h. SA-gal enzyme activity was measured using an X-gal staining kit (Beyotime). The number of cells that β-gal positive

shows the degree of cellular senescence.

2.11. Real-time polymerase chain reaction method for detecting relative expression of MMP-1, MMP-3, MMP-9, COL-I and COL-II mRNA

The cells were seeded at a density of 10^5 cells/well in a twelve-well plate and divided into control group (NC), H_2O_2 group and H_2O_2+ peptides group. The H_2O_2+peptide group was treated with different concentrations of NFF (10, 20 μg/mL) and PMR (100, 200 μg/mL), while the control group and H_2O_2 group were treated with DMEM medium. After the sample was treated for 4 h, the culture medium was taken out and treated with 400 μM H_2O_2 for 2 h. Polypeptide samples with different concentrations were added to each well and incubated for 24 h in a 5 % CO_2 incubator at 37 °C.

After 24 h of culture, the cells were collected by trypsinization and total RNA was extracted, purified and quantified. The RNA was reverse transcribed into complementary DNA (cDNA) according to the instructions of the reverse transcription kit, pre-denatured at 95 °C for 5 min, denatured at 95 °C for 15 s, annealed at 60 °C for 30 s, extended at 72 °C for 30 s, 40 cycles and maintained at 72 °C for 10 min. β-actin was served as the internal control and the expression levels of MMP-1, MMP-3, MMP-9, COL-I and COL-III genes were calculated. The experiment was repeated three times separately.

2.12. Data statistics

The mean standard deviation of at least two independent repeated experiments done in duplicate or triplicate was used to represent the data. Differences were considered at NS, $p > 0.05$, *$p < 0.05$, **$p < 0.01$, * * * $p < 0.001$, #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ using a two-tailed Student's t-test analysis of variance.

3. Result and discussion

3.1. Preparation of perilla seed meal protein and its protein hydrolysate

The most popular method of protein extraction in commercial production is thought to be alkaline extraction and acid precipitation because of its high yield and low cost [28]. 41.79 g perilla seed crude protein was extracted from 100 g perilla seed meal powder by alkali extraction and acid precipitation. Table S2 showed the comparison of protein content and moisture content of perilla seed meal crude protein (PSCP) with that of original perilla seed meal powder. The protein content of the isolated protein was 58.82 %, which is better than the previously reported 41.83 % [23].

The type of protease is very important to the biological activity of protein hydrolysate [29]. In this study, alkaline protease, neutral protease and papain were used to hydrolyze perilla protein to screen the most suitable enzyme. It is widely recognized that the extent of hydrolysis directly impacts the composition, length and structure of polypeptides, consequently influencing their functional properties [30]. Therefore, DH and DPPH free radical scavenging activities were used as indicators to evaluate the hydrolysis ability of each enzyme. The result was shown in Fig. 1a, alkaline protease and neutral protease were the most widely hydrolyzed, followed by papain. The DPPH free radical scavenging activities of the three types of enzymatic hydrolysates, which were 76.67 % (alkaline protease), 79.79 % (neutral protease) and 76.20 % (papain), were not significantly different (Fig. 1b).

These findings should be emphasized due to the lack of research on the multifunctional characteristics of neutral protease hydrolysate [31]. The hydrolysis process of alkaline protease needs to be maintained under alkaline conditions. After the protein is treated with alkali, the amino acids in it undergo racemization, which reduces the L-enantiomer of essential amino acids and their digestibility and produces toxic D-amino acids [32].

In addition, Guo et al. found that an increase in DH does not necessarily correlate with enhanced biological activity [33]. Neutral

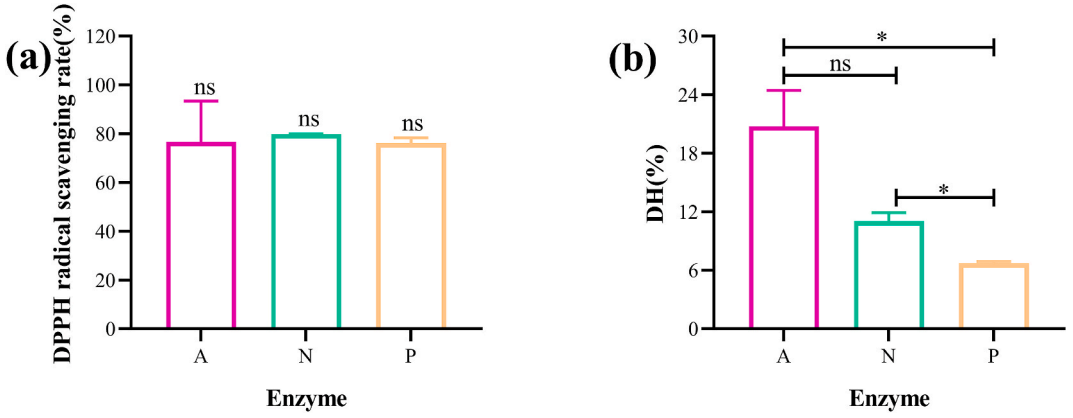


Fig. 1. DH and DPPH Radical Scavenging activity of enzymatic hydrolysates from different proteases. Values are shown as mean ± SD (n = 2). Alkaline protease: A, Neutral protease: N and Papain: P. * represents $P < 0.05$, ns represents no significant difference.

protease, an endoenzyme [31] predominantly catalyzes the hydrolysis of peptide bonds containing aromatic amino acid residues on the carboxyl side, namely tryptophan, tyrosine and alanine. Notably, the abundance of these amino acids is positively associated with antioxidant activity [34]. Therefore, neutral protease was used as hydrolase in the follow-up study.

3.2. Separation and characterization of PSP

3.2.1. Ultrafiltration separation of perilla seed crude protein hydrolysate (PSCPH)

Protein hydrolysate refers to a mixture comprising unhydrolyzed proteins, peptides exhibiting varying hydrophobicity, chain lengths and net charges, as well as free amino acids [8]. Appropriate separation methods are helpful to more accurate structure and activity of peptides [35]. Ultrafiltration is a commonly used separation technology for peptides, which can be divided into different components based on molecular weight [1]. To separate antioxidant peptides, PSCPH was divided into three fractions: F1 (MW $<$ 3 kDa), F2 (3 kDa $<$ MW $<$ 5 kDa) and F3 (MW $>$ 5 kDa), using an ultrafiltration membrane. The DPPH radical scavenging ability was then determined for these three fractions.

3.2.2. Determination of DPPH radical scavenging activity of PSCPH ultrafiltration components

The DPPH radical clearance capacity of each fraction, as depicted in Fig. 2, exhibited a clear dose-dependent relationship. The ic_{50} value of F1(0.15 mg/mL) is much lower than that of F3 (0.31 mg/mL) and similar to F2 (0.18 mg/mL) ($P > 0.05$). Furthermore, the molecular weight distribution of component F2 was determined. As illustrated in Fig. S1, all the constituents in F2 were polypeptide fragments with a molecular weight of less than 5000 Da, with 49.37 % accounting for the range of 3000~5000 Da and 50.63 % accounting for less than 3000 Da. The component with a MW of less than 5000 Da demonstrated stronger DPPH radical scavenging activity, which is consistent with the results of the prior investigation. Because of its higher fluidity and diffusivity, peptides with a molecular weight below 3000 Da exhibit a greater propensity to interact with target molecules, thereby yielding a more potent antioxidant effect [29]. As a result, component F2 was chosen to undergo subsequent cell-level efficacy testing.

3.2.3. Antioxidant activity of F2 in HaCaT

The safe concentration of F2 for HaCaT cells was detected by the CCK-8. In short, during the 24 h treatment with F2 of 50, 100, 200 and 400 μg/mL, the survival rate of HaCaT cells in the F2 group exceeded 80 %, which indicated that F2 had no obvious cytotoxic effect on HaCaT cells at the concentration of $<$400 μg/mL (Fig. 3a).

Hydrogen peroxide (H_2O_2) holds significant prominence as a reactive oxygen species, capable of directly instigating cellular oxidative stress and senescence [36]. The level of oxidative damage to cells can be assessed by measuring the levels of ROS in the cells [37]. The intensity of fluorescent DCF produced by ROS oxidation can serve as an indicator of intracellular ROS levels [38]. As shown in Fig. 3b, the relative expression of ROS in cells from the H_2O_2-injured group was 7.82 ± 0.49, which was substantially different from that in the blank control group ($p < 0.001$), indicating that H_2O_2 incubation raised the intracellular ROS level. The level of ROS in HaCaT cells pretreated with F2 at 200 and 400 μg/mL concentrations was considerably lower than in the H_2O_2 damage group ($p < 0.01$). The results suggested that pre-treating HaCaT cells with F2 effectively mitigated cellular oxidative damage. Previous studies have demonstrated that antioxidant peptides derived from ultrafiltration components of watermelon seeds, eggshell membranes and broken rice protein hydrolysates possess the ability to safeguard cells against oxidative damage by reducing excessive levels of ROS [1, 38,39]. Therefore, F2 was regarded as a reliable source of peptides that are antioxidants.

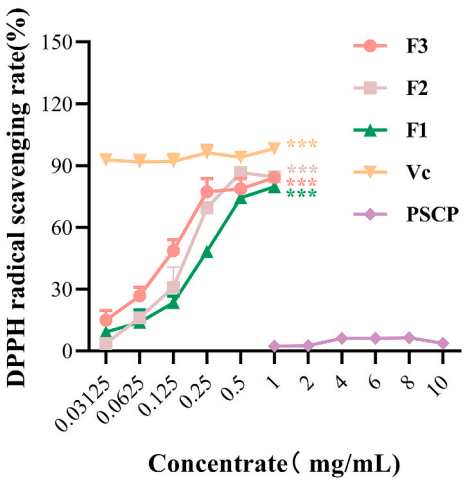
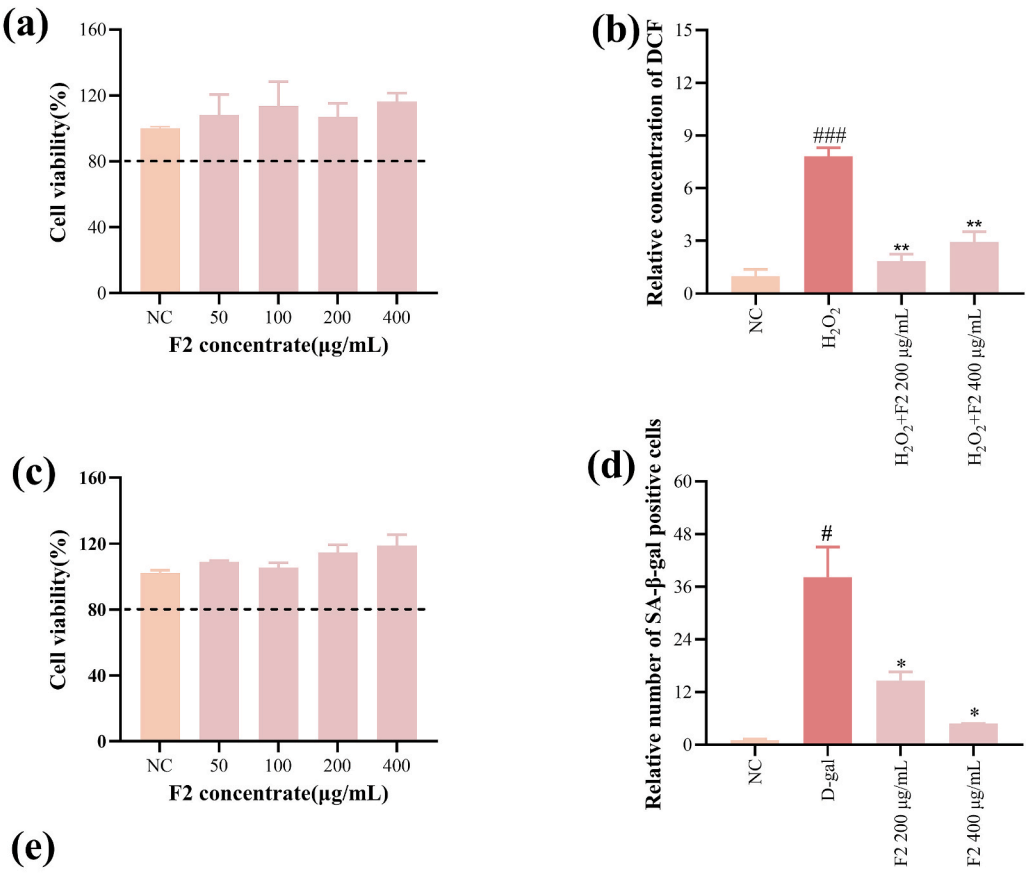
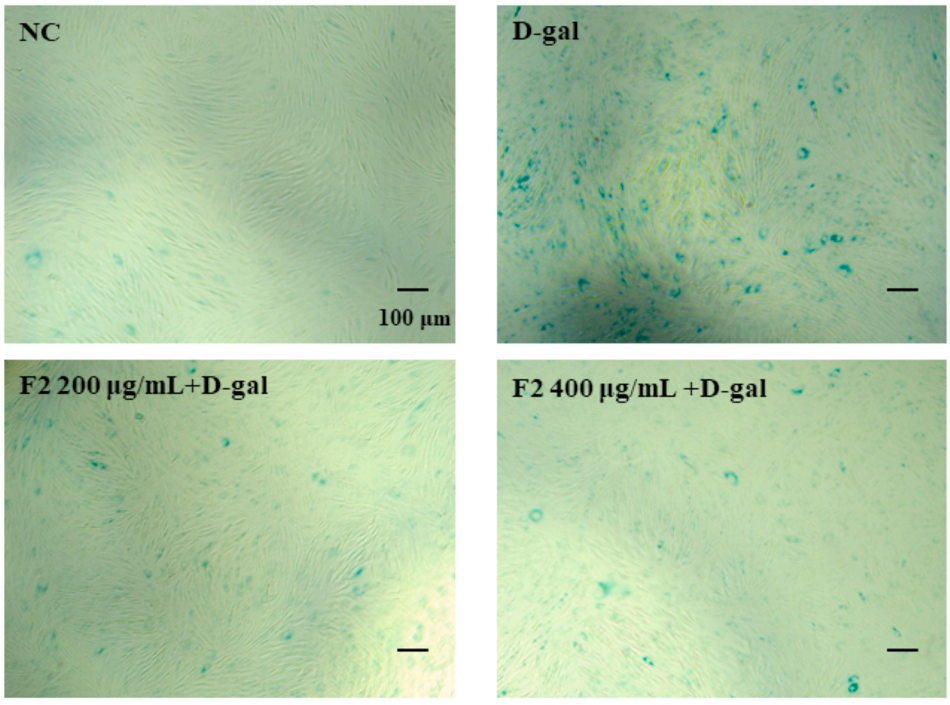


Fig. 2. DPPH radical scavenging activity of ultrafiltration components of PSCPH. * * * indicates that the DPPH radical scavenging activity of ultrafiltration components is significantly different from that of perilla protein ($p < 0.001$).



(e)



(caption on next page)

Fig. 3. Protective effect of F2 on HaCaT cells damaged by H_2O_2 and protective effect of F2 on HFF-1 cells damaged by D-gal: (a) The cell viability of HaCaT cells treated by F2, (b) The ROS of HaCat cells induced by H_2O_2 under the protection of F2, (c) The cell viability of HFF-1 cells treated by F2, β-gal positive cells (in blue) were observed through microscopy (e) and counted (d), #$p < 0.05$, ###$p < 0.001$ versus the blank control group (NC), *$p < 0.05$; **$p < 0.01$ versus the D-gal injured group.

3.2.4. *Anti-senescent activity of PSPs in human skin fibroblast (HFF-1)*

The safe concentration of F2 for HFF-1 cells was detected by the CCK-8 method. To put it briefly, after a 24 h treatment with F2 at concentrations of 50, 100, 200 and 400 μg/mL, the viability of HFF-1 cells in F2 group was more than 80 %, suggesting that F2 did not appear to have any discernible cytotoxic effect on HFF-1 cells at concentrations lower than 400 μg/mL (Fig. 3c).

D-galactose (D-gal) is a model widely used to explore aging [40]. The conversion of elevated levels of D-gal by galactose oxidase into aldose and hydroperoxide leads to the generation of reactive oxygen species (ROS), triggering inflammation, oxidative stress, impairment of mitochondrial function and ultimately, apoptosis [41]. Therefore, the D-gal-induced anti-aging model was utilized to assess the anti-aging effectiveness of F2. In Fig. 3e, senescent cells exhibiting positive SA-β-gal expression were observed as cells with a dark blue appearance. Treatment with D-gal at a concentration of 20 mg/mL for a duration of 72 h resulted in an elevation of β-galactosidase activity when compared to the control group (NC), consequently augmenting the number of senescent cells ($p < 0.05$, Fig. 3d). Senescent cell formation may be inhibited during F2 incubation, as evidenced by the significant dose-dependent decrease ($P < 0.05$) in the number of SA-β-gal positive cells in the F2 group as compared to the D-gal group. These results indicated that pre-treatment of HFF-1 cells with F2 effectively prevented cell senescence. In the previous research, the monomer polypeptide with anti-aging effect was successfully separated and purified from the enzymatic hydrolysate of Agaricus blaze [42]. As a result, F2 can be regarded as a good source of anti-aging peptides.

3.2.5. *Identification of potential PS derived antioxidant peptides and in silico analysis*

Based on prior experimental findings, the identification of the F2 structure was achieved. Conventional techniques for separation and purification are intricate and may result in the depletion of bioactive compounds. Conversely, computer informatics enables swift identification of potential bioactive peptides within protein hydrolysate [43]. Consequently, this investigation employed computer prediction methods, encompassing peptide activity score, free radical scavenging activity, toxicity and sensitization, to discern antioxidant peptides exhibiting promising properties. The mass spectrum obtained is depicted in Fig. S2. A comprehensive total of 1696 peptide sequences were deciphered using LC-MS/MS. The evaluation of polypeptide screening was illustrated in Fig. 4. Ultimately, a selection of 9 novel peptides (RAW, FGRL, NFF, PMR, WGRP, MYF, FAGR, WFL and GEMF), which exhibited non-toxic and non-allergenic properties and demonstrated successful docking with the Keap1 protein.

3.2.6. *Molecular docking simulation of antioxidant mechanisms*

The human antioxidant defense system possesses the capability to counteract the harmful effects of free radicals [8]. The Nrf2/Keap1 pathway governs the synthesis of antioxidants and defense proteins via Nrf2, thereby safeguarding cells against oxidative stress [44]. In a state of normal physiological conditions, the dissociation of Nrf2 from Keap1 occurs within the cellular environment, followed by its degradation through ubiquitination [1,44]. Under stress conditions, Nrf2 and Keap1 dissociate into the nucleus and

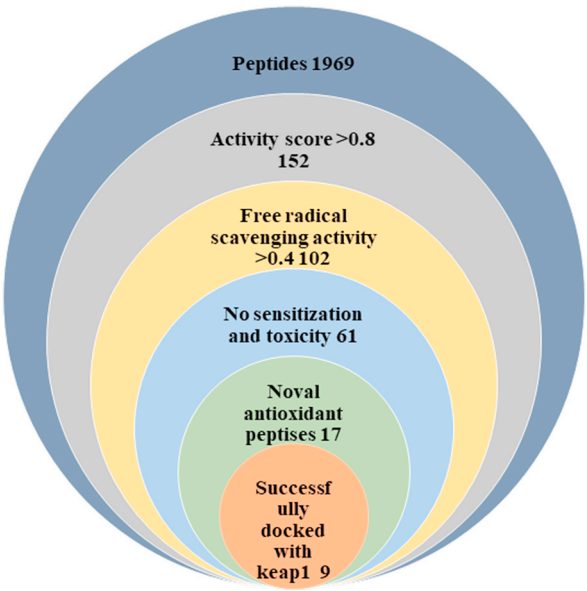


Fig. 4. Screening method and quantity of peptides.

Table 1
Activity scores of PSPs and their molecular docking sites with Keap1.

Number	Peptide	Activity score	Free radical scavenging activity	Keap1 Binding Energy (kcal/mol)	Effort	
					Hydrogen bond	Hydrophobic
1	RAW	0.884907	0.43946666	−271.793	Gly367、Val606、Ile559、Val418、Val465、Gly417、Ile416、Ser602、Glu82	Cys368
2	FGRL	0.947393	0.40923873	−210.62	Ala510、Val512、Val465、Cys513、Leu365、Val604、Val418、Leu557、Val463、Ile416	Arg415、Ala556、Ala607
3	NFF	0.986707	0.40036428	−195.146	Gly367、Ile559、Val418、Val467、Val465、Val512、Gly464、Val463	Ala366、Ala607
4	PMR	0.903736	0.43705025	−185.843	Gly462、Ile416、Gly464、Val512、Val465、Val418、Ala510、Leu557	Val606
5	WGRP	0.969705	0.5153805	−176.725	Val369、Val606、Gly367、Val465、Val512、Ile559、Val418、Ala510	Ala607
6	MYF	0.981789	0.50796968	−154.298	Gly511、Val512、Leu365、Arg415、Gly367、Val606、Val465、Ile559、Leu557	Ala366、Ala556
7	FAGR	0.849376	0.41867596	−130.957	Ile559、Val465、Val463、Val604、Val606、Arg415、Gly509、Gly462、Ala510、Val512	-
8	WFL	0.995533	0.46672744	−112.677	Val463、Ile559、Gly464、Ala510、Leu557、Gly558、Gly605、Val606、Gly367	Ala366、Val465、Val418、Val512、Arg415
9	GEMF	0.843572	0.42946669	−99.5078	Ile416、Val420、Val606、Ile559、Val512、Gly367、Val465、Gly464、Val418、Val463、Ile416	Ala556、Val606

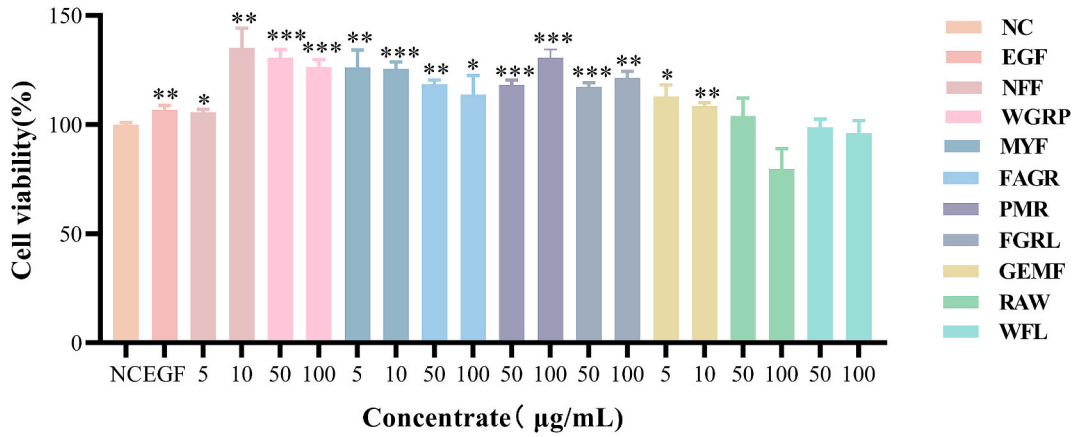


Fig. 5. Effect of PSPs on proliferation of Hacat, Values are expressed as mean ± SD (n = 3). Differences were considered at * $p < 0.05$, ** $p < 0.01$, * * * $p < 0.001$ versus the blank control group (NC).

Nrf2 transcriptional and translational activities increase, thereby upregulating the expression of antioxidant enzymes [45,46]. As one of the main defense pathways of oxidative damage, Keap1-Nrf2-ARE can reduce the exogenous oxidative damage of human cells [35]. The stimulation of cytoprotective protein-related genes and antioxidant enzymes downstream of the pathway is observed when the interaction between keap1 and Nrf2 is inhibited, leading to antioxidant effects [47]. The Discovery Studio 2019 CDOCKER application was used to perform molecular docking research on the PSPs and Keap1 proteins.

The results of molecular docking, as depicted in Fig. S3 and Table 1, provided visual representation of the binding interactions between PSPs and Keap1. The extent of binding is determined by factors such as the fraction of binding energy, the active site of the receptor and the number of amino acid residues involved in the interaction [6]. These findings suggested that the PSPs have the potential to independently attach to the Keap1 protein, indicating the spontaneous binding capability of the PSPs to the Keap1. The left side of Fig. S3 showed the 3D structure of Keap1 protein with the lowest docking energy to the nine PSPs and the right side showed the corresponding theoretical binding mode. The findings demonstrated that these nine PSPs established a stable docking conformation by integrating within the active cavity of Keap1 [48].

Nine PSPs (RAW, FGRL, NFF, PMR, WARP, MYF, FAGR, WFL and GEMF) have been observed to bind to keap1 via hydrogen bonding and hydrophobic interactions, which is consistent with the research reported in the literature [35,49]. Antioxidant peptides engage with the active site of the Keap1 protein, fostering a stable conformation that triggers the Keap1-Nrf2 signaling pathway and thereby exerts antioxidant activity [35,49].

3.3. Effect of synthetic peptide on proliferation of HaCaT cells and storage stability

Nine screened peptides were synthesized, and their efficacy was verified. Among them, RAW, FGRL, PMR, WGRP, FAGR and WFL are water-soluble peptides, so the test concentration is set at 0–400 μg/mL. NFF, MYF and GEMF are water-insoluble peptides, which can be dissolved in 50 % DMSO. Research finding indicate that maintaining a DMSO volume fraction below 0.1 % in the Sigma

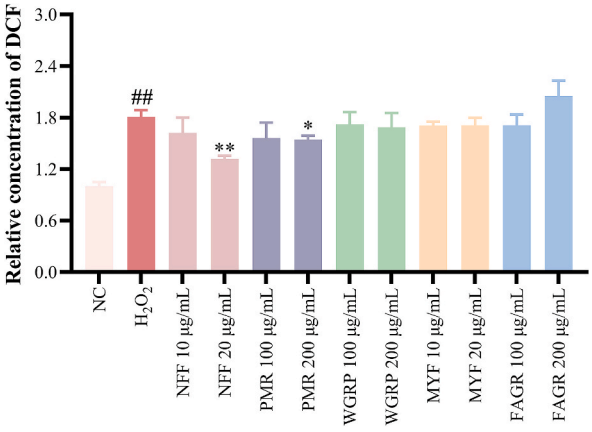


Fig. 6. Effects of PSPs on the ROS level of HaCaT stimulated by H_2O_2. Values are expressed as mean ± SD (n = 2). #$p < 0.05$, ###$p < 0.001$ versus the blank control group (NC); *$p < 0.05$, **$p < 0.01$ versus the H_2O_2injured group.

preserves the cellular morphology, allowing for normal growth and reproduction. Consequently, the recommended test concentration range for NFF, MYF and GEMF was 0–20 μg/mL [50]. The CCK-8 method was employed to determine the non-toxic concentration of the synthetic peptide on HaCaT cells. After 24 h, the viability of HaCaT cells in the synthetic peptide group surpassed 80 %, signifying the lack of significant cytotoxicity of the synthetic peptide on HaCaT cells at the tested concentration (Fig. S4). According to the report, natural antioxidants have the ability to induce cellular proliferation [51]. Epidermal growth factor (EGF) has been observed to

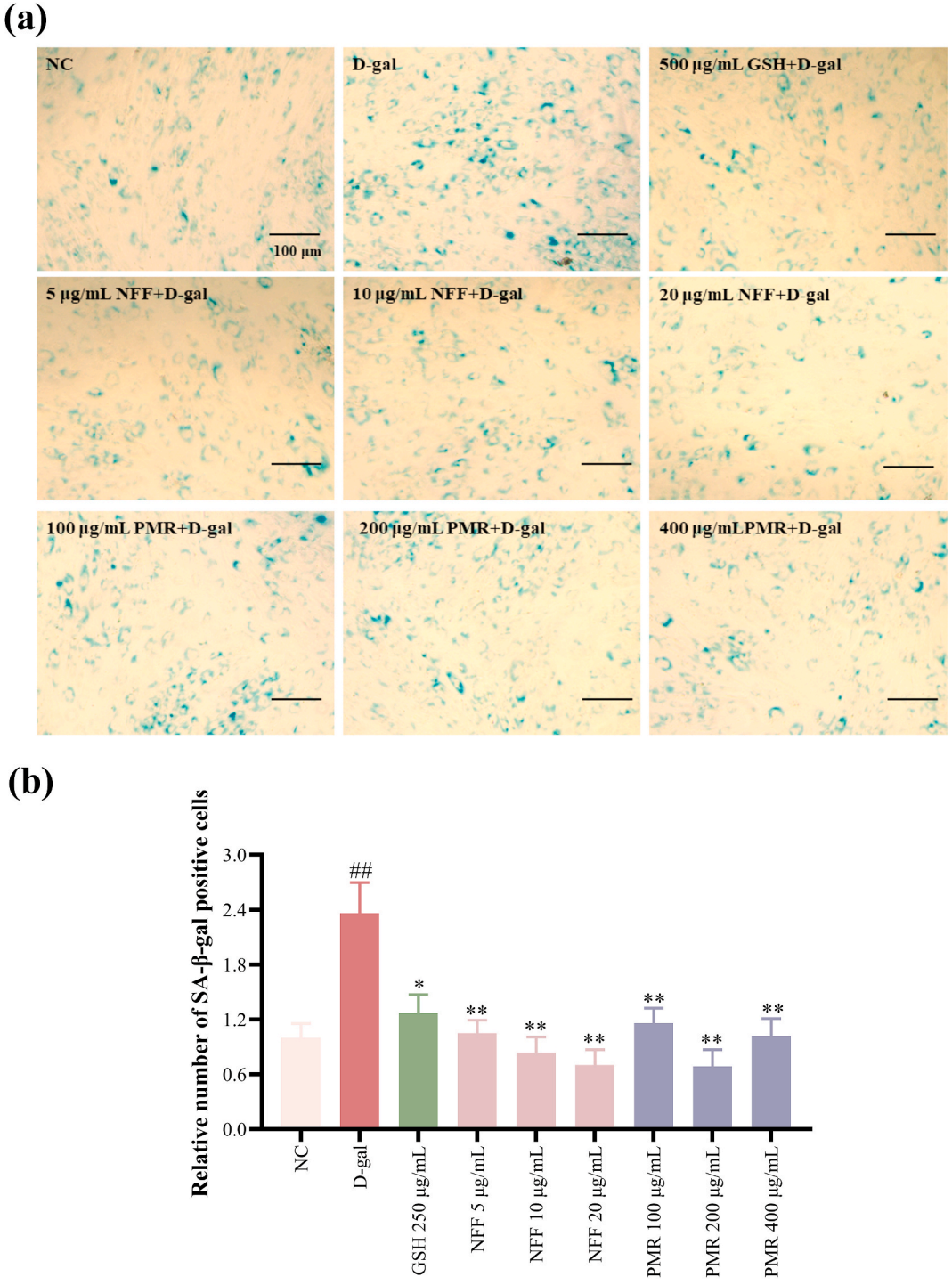


Fig. 7. Protective effect of PSPs on HFF-1 cells damaged by D-gal：β-gal positive cells (in blue) were observed through microscopy (a) and counted (b), ###p < 0.001 versus the blank control group (NC); **p < 0.01 versus the D-gal injured group; *p < 0.05 versus the D-gal injured group.

facilitate the morphogenesis of epithelial cells and branches of exocrine glands [52], thus making it a suitable candidate for utilization as a positive control. Specifically, the concentration of 100 μM EGF has been found to enhance the proliferation of HaCaT keratinocytes, resulting in a cell viability of 106.76 %.

As shown in Fig. 5, NFF, WGRP, MYF, FAGR, PMR, FGRL and GEMF promoted the proliferation of HaCaT keratinocytes and the cell viability were as follows135.16 ± 9.06 % (10 μg/mL), 126.35 ± 3.39 % (100 μg/mL), 125.51 ± 3.23 % (10 μg/mL), 113.74 ± 8.7 % (100 μg/mL), 130.678 ± 3.74 % (100 μg/mL), 121.37 ± 3.06 % (100 μg/mL) and 108.54 ± 1.47 % (10 μg/mL). It has been suggested that the promotion of cell proliferation is associated with the prevention of oxidative stress during cell culture [51]. Therefore, the first five peptides (NFF, WGRP, MYF, FAGR, PMR) were screened for antioxidant verification. The pH and peak area of NFF and PMR during storage were shown in Fig. S5. The degradation of NFF, PMR, WGRP, FAGR and MYF was investigated under different temperature and light conditions for 60 days. At 25 °C and 40 °C (light), NFF exhibited degradation rates of 15.40 % and 31.87 % respectively. PMR experienced degradation when stored at 40 °C (light), with a degradation rate of 83.04 %. WGRP showed degradation rates of 2.33 % and 93.06 % when exposed to 25 °C and 40 °C (light) respectively. Similarly, MYF exhibited degradation rates of 5.57 % and 83.21 % at 25 °C and 40 °C (light) respectively. The peak area of FAGR remained unchanged under the storage conditions of 5 °C, 25 °C and 40 °C (light). The visual characteristics of NFF, PMR, MYF and FAGR remained consistent throughout the storage period. However, the color of WGRP exhibited a yellowing effect after 30 days of storage at 40 °C (light). Consequently, it is advisable to store peptides at low temperatures in a light-free environment.

3.4. Antioxidant activity of synthetic peptide in HaCaT

The utilization of the membrane permeation probe DCFH-DA, which undergoes oxidation by intracellular ROS resulting in the production of intensely fluorescent DCF in proportion to ROS levels, was employed to evaluate the efficacy of synthetic peptide in inhibiting ROS generation [53]. Following treatment with H_2O_2, the level of reactive oxygen species (ROS) in HaCaT cells exhibited a statistically significant increase of 1.81 ± 0.08 times compared to the control group ($p < 0.001$). In the NFF (20 μg/mL) and PMR (200 μg/mL) groups, ROS generation was observed to be 1.32 ± 0.04 times and 1.54 ± 0.05 times higher than in the control group, respectively. Additionally, these values demonstrated a reduction of 25.40 % and 12.80 % when compared to the H_2O_2 group, as illustrated in Fig. 6. The increase in antioxidant enzymes brought on by the Keap1-Nrf2 pathway's activation may be the reason for the decrease in ROS in the NFF and PMR treatment groups [53]. However, cellular senescence is closely related to ROS accumulation [53]. This discovery implies that NFF and PMR possess the potential to mitigate the detrimental effects of reactive oxygen species (ROS) on aging and injury. Consequently, we investigated to assess the anti-aging effectiveness of NFF and PMR, aiming to validate our hypothesis.

3.5. Anti-senescent activity of synthetic peptide in HFF-1

The viability of HFF-1 cells in the synthetic peptide group surpassed 80 %, suggesting that the synthetic peptide did not exhibit significant cytotoxicity towards HFF-1 cells at the tested concentration (Fig. S6). Fig. 7a depicted the impact of NFF and PMR on SA-β-gal in D-Gal-induced premature senescent cells. The dark blue cells in the figure signify senescent cells exhibiting positive SA-β-gal expression. Following D-gal treatment, the rate of positive staining was observed to be 2.36 ± 0.03 times greater than that of the control group ($p < 0.01$), suggesting that D-gal treatment induces senescence in HFF-1 cells. As expected, the pretreatment of HFF-1 by NFF and PMR decreased the activity of β -galactosidase induced by D-gal (reducing the number of senescent cells and the blue depth). As shown in Fig. 7b, the positive staining rates of NFF (20 μg/mL) and PMR (400 μg/mL) were 0.70 ± 0.17 and 0.69 ± 0.18 times that of the control group, respectively, which decreased by 70.25 % and 70.87 % compared with the D-gal group, and the effect was better than that of GSH (50.61 %) of 500 μg/mL. Therefore, the results of SA-β-gal activity showed that NFF and PMR can be used as anti-aging substances [54].

3.6. Effects of synthetic peptide on mRNA and proteins expression of MMP-1, MMP-3, MMP-9, COL-I and COL-III in HFF-1 cells irradiated by H_2O_2

Oxidative stress promotes the expression of Matrix metalloproteinases (MMPs) in cells by activating NF-κB signaling pathway [55,56]. Matrix metalloproteinases (MMPs) are involved in extracellular matrix remodeling through the degradation of extracellular matrix components and are also involved in the inflammatory response by regulating the pro-inflammatory cytokines TNF-α and IL-1β [57,58]. Overexpression of MMP-1 will destroy type I and III collagen, while MMP-3 can degrade proteoglycan and type IV collagen fiber [59]. MMPS gradually destroy the integrity of human dermis, and long-term action can seriously damage skin collagen, thus making human skin aging [60]. MMP-1 and MMP-3 expression are often used to represent collagenase activity in human skin. Therefore, plant extracts or compounds that can inhibit the activities of collagenase enzymes might have the potential to be used as cosmetics to prevent skin aging [61]. As shown in Fig. 8a–e, compared with the control (NC) cells, the mRNA levels of MMP-1, MMP-3 and MMP-9 in H_2O_2-induced group were significantly increased, while the levels of COL-I and COL-III were significantly decreased. In comparison to the H_2O_2 group, NFF (20 μg/mL) substantially down-regulated the expression of MMP-9 while up-regulating the expression of COL-1 and COL-III. Additionally, PMR (200 μg/mL) significantly decreased the expression of MMP-1, MMP-3 and MMP-9 and inhibited the degradation of Col-1. Nrf2 signaling pathway plays an important role in anti-oxidative stress response and it can effectively restore redox homeostasis in cells after being activated [62]. The activation of Nrf2 inhibits the activation of NF-κB signaling pathway, thus protecting extracellular matrix [63]. Therefore, it is speculated that the anti-aging matrix of perilla peptide is

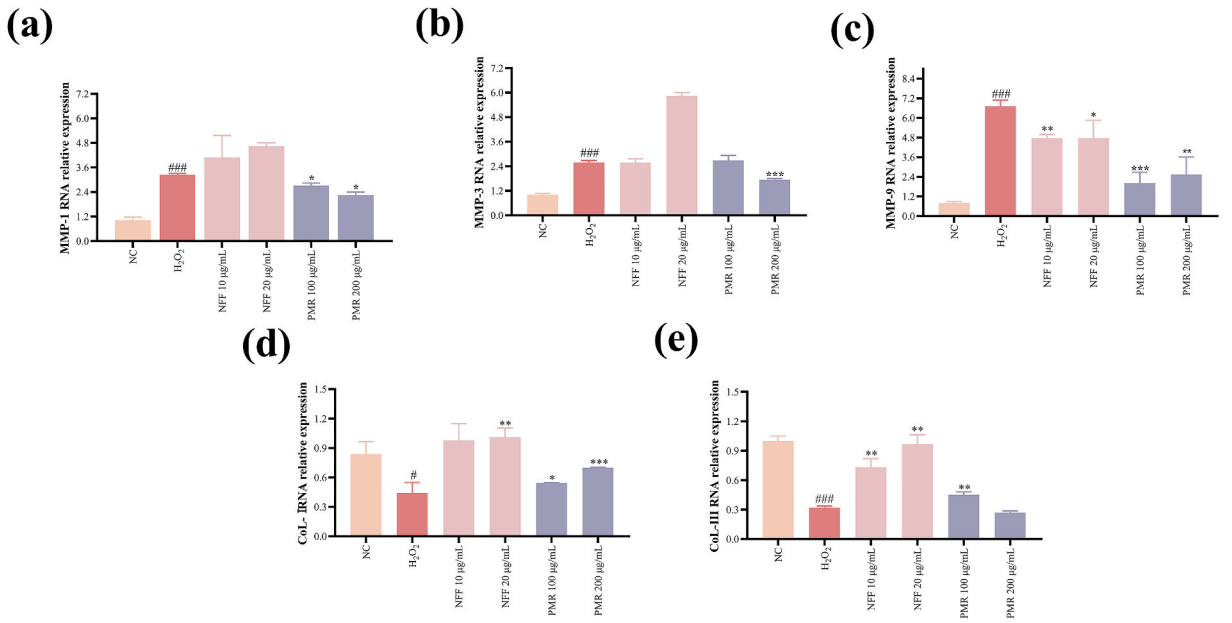


Fig. 8. Anti-aging effect of peptide on HFF-1 cells stimulated by H_2O_2. Ie cells were collected and lysed, and the relative expression levels of immune response related genes MMP-1, MMP-3, MMP-9, COL-I and COL-III were determined by qRT-PCR (β-Actin expression was the internal control). Each experiment is made in triplicate. #$p < 0.05$, ###$p < 0.001$ versus the blank control group (NC); ***$p < 0.001$, **$p < 0.01$; *$p < 0.05$ versus the H_2O_2 injured group.

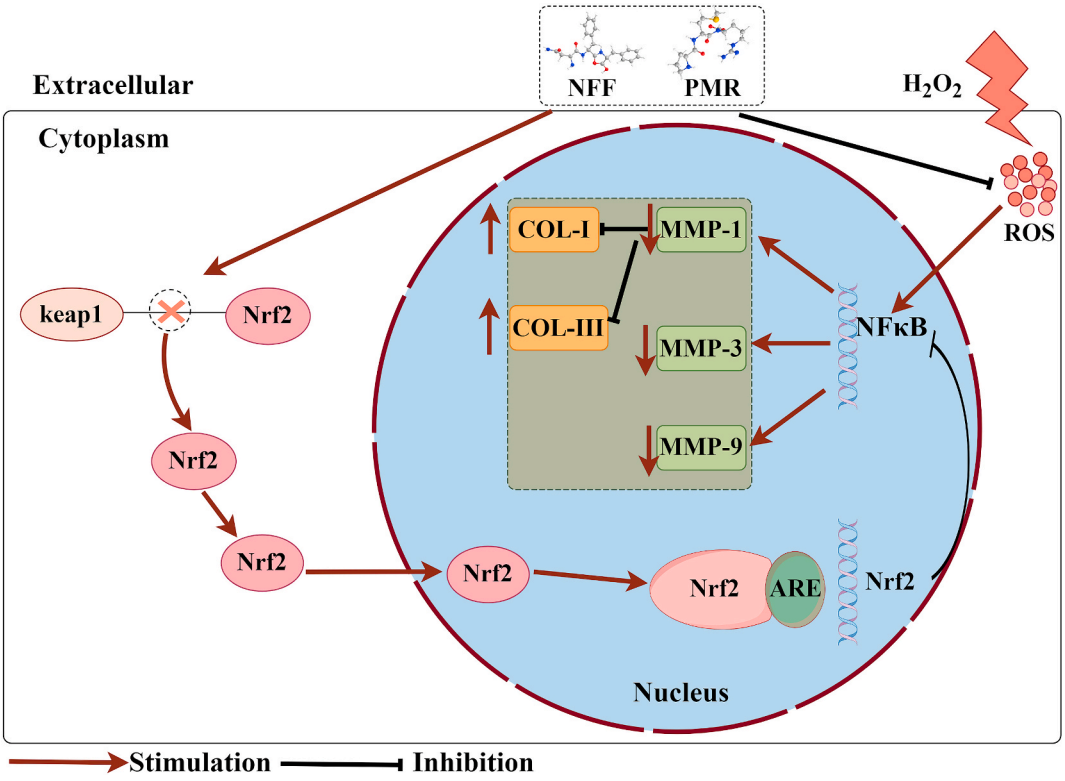


Fig. 9. Role of peptides in aging induced by oxidative stress (By Figdraw).

shown in Fig. 9. The synthetic peptide interacted with Keap1 and inhibits the formation of Keap1-Nrf2, which means that the free Nrf2

is dissociated from its inhibitor Keap1 and transported to the nucleus to combine with ARE. The activation of Nrf2 signal pathway inhibits the activation of NF-κB signal pathway, reduces the expression of MMPs and increases the expression of COLs.

4. Conclusion

In summary, perilla seed meal has been proved to be a good source of anti-aging peptides. The hydrolysate fraction 2 (F2) of perilla seed meal with molecular weight of 3 kDa < MW < 5 kDa has the activities of scavenging free radicals, inhibiting intracellular ROS production and β-gal galactosidase activity. Nine novel anti-aging peptides were identified by the combination of peptidomics and silicon analysis. The two peptides, NFF and PMR, were found to promote the proliferation of keratinocytes (HaCaT cells) and suppress the level of ROS and the activity of β-galactosidase. Both peptides exhibited a strong binding affinity with the Keap1 protein. Additionally, NFF attenuated the expression of senescence marker SA-β-gal and inflammatory-related enzyme MMP-9 in HFF-1 and inhibited the degradation of COL-I and COL-III. Similarly, PMR reduced the expression of inflammatory-related enzymes MMP-1, MMP-3, and MMP-9 in HFF-1, and inhibited the degradation of COL-I and COL-III. These results indicated the interdependence between inflammation and aging. The potential molecular mechanism of anti-aging of peptide derived from perilla seed meal may be related to the activation of Nrf2 pathway, which may attribute to the fact that the peptide might involve the competitive binding of Keap1 to facilitate the release of Nrf2 and activation of NF-κB signal pathway. This study provides a theoretical basis for the application of perilla seed anti-aging hydrolysates and peptides in cosmetics and food industries, a way to increase the value of agricultural by-products, and a reference for the efficient identification and molecular mechanism research of more food-borne peptides. This work identified two novel peptides derived from Perilla Seed and exhibited potential application in cosmetic and pharmaceutical application. However, molecular dynamics simulation and western blotting should be conducted to explore the expression of downstream antioxidant enzymes of Nrf2 signal pathway. The stability and compatibility in cosmetic formulations should be investigated for the application of perilla peptide in cosmetics.

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Lingling Wang: Writing – original draft, Validation, Supervision, Software, Methodology, Investigation, Conceptualization. **Liping Qu:** Writing – review & editing, Validation, Conceptualization. **Bingwei He:** Writing – review & editing, Supervision, Methodology, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33604.

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