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Purification and identification of an antioxidant peptide from *Pinctada fucata* muscle

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**ABSTRACT**

*Pinctada fucata* muscles were hydrolysed by alcalase and filtered using ultrafiltration membranes to obtain peptides with molecular weights (MWs) less than 5 kDa. The percolate was then freeze-dried and named crude antioxidant peptides mixture from *Pinctada fucata* muscles (AOP). In this study, AOP was purified sequentially using sephadex gel chromatography, next reversed-phase high-performance liquid chromatography. The MW of the antioxidant peptide from *P. fucata* muscle was 1039.56 Da. The amino acid sequence was Gly–Ala–Gly–Leu–Pro–Gly–Lys–Arg–Glu–Arg based on matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF). The natural peptide exhibits good scavenging capacity against free radicals; the IC50 on 2,2-diphenyl-1-picyrylhydrazyl and -OH of FC2 were closer to vitamin C and butylated hydroxytoluence; however, the IC50 values of O2− of FC2 were a bit poor. Its antioxidant activity was attributed to the hydrophobic amino acid residues enriched in the N-terminal and electrophilic ability mediated by Glu and electron acceptors such as Lys and Arg. The synthesized peptide exhibited a reduced ability to scavenge free radicals compared to the natural peptide. The proposed method is a feasible technique to prepare antioxidant peptides from *P. fucata* and could be useful to obtain ingredients in nutraceutical and cosmetic applications.

**INTRODUCTION**

Natural antioxidant peptides have shown unrivalled strong antioxidant capacity and safety given their excellent physical and chemical properties, including thermal stability and solubility, which differ from synthetic antioxidants (Xie, Huang, Xu, & Jin, 2008). Peptide fragments with activity and function could be released from protein chain by specific protease enzymes, whereas the long protein chain does not exhibit these activities. However, the enzymatic hydrolysate was a mixture, and the antioxidant activity of different peptide fragments varied widely based on the amino acid composition, sequence and structure (Zou, He, Li, Tang, & Xia, 2016). Recent studies have focused on plant proteins, especially soybean proteins. For example, soybean antioxidant peptides ranging in size from 600 to 1700 Da were obtained by purification, and their sequence structures were explored using mass spectrometry (Chen, Muramoto, & Yamauchi, 1995; Liu et al., 2014). In addition, numerous antioxidant peptides were purified from milk proteins and aquacultures, and the active sequence was usually different.

At present, there are many researchers studying and explaining the antioxidant properties of peptides. However, the relationship between antioxidant properties and structure has not been fully clarified. However, most researchers agree that antioxidant peptides that contain 2–16 amino...
acid residues have strong antioxidant effects and are easily absorbed (Sarmadia & Ismaila, 2010; Tang et al., 2010).

Currently, numerous methods have been applied to the separation and purification of peptides, such as gel filtration chromatography, ion exchange chromatography, reversed-phase high-performance liquid chromatography (RP-HPLC) and high-performance capillary electrophoresis. Gel filtration chromatography (Pan, Cao, Guo, & Zhao, 2012) and high-performance liquid chromatography (HPLC) (Parente, Patel, Caldeo, Piraino, & McSweeney, 2012) are the most widely used techniques for isolation and identification of bioactive peptides. Mass spectrometry (MS) and protein sequence measuring are commonly used. Compared with electron spray ionization mass spectrometry (ESI-MS) (Shriv, Kailasa, & Wu, 2009), matrix-assisted laser desorption ionization MS (MALDI-TOF–TOF) (Sheng, Covey, Shew, Winger, & Campana, 1994; Zhong, Zhang, Wen, & Li, 2004) is a more suitable structural analysis method for biological molecules, including proteins, oligosaccharides and peptides. Further studies on purification, structures, functions and mechanisms of peptides will be beneficial to the development and application of bioactive peptide in pharmaceutical, food and cosmetics industries (Li, Jia, & Yao, 2009).

Pinctada fucata is an important pearl-culturing mollusc shell. After the pearl is taken, the meat of P. fucata not only has high nutrition value but also contains rich functional active substances. Wu et al. (2012) optimized alcalase to hydrolyse P. fucata muscle and found that the hydrolysate product has high antioxidant activity. The aim of this study was to purify antioxidant peptides mainly using sephadex gel chromatography and RP-HPLC. Moreover, sequence of antioxidant peptides was characterized via MALDI-TOF–TOF, amino acid composition analysis, infrared scans and chemical synthesis. Finally, the method for synthesis of peptides was described to explain the structure–activity relationship of antioxidant peptides from P. fucata muscle.

Materials and methods

Materials

P. fucata muscle was purchased from Hainan Aquaculture Base, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences. Alcalase was purchased from Novozymes Biotechnology Co., Ltd. (Beijing, China). Potassium bromide, cinnamylate (cyno-4-hydroxy-cinnamic acid [CHCA]), Fmoc-amino acids and Kaiser Reagents were of guaranteed reagents and purchased from Sigma-Aldrich. In addition, 2,2-diphenyl-1-picrylhydrazyl (DPPH), salicylic acid and Sephadel G-25 were purchased from Sigma-Aldrich. Acetonitrile and trifluoroacetic acid (TFA) was of LC grade and purchased from Anpel Scientific Co., Ltd. (Shanghai, China). All other reagents used in the experiment were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Guangzhou, China).

Preparation of enzymatic hydrolysates of P. fucata muscle

P. fucata muscle was separated manually and homogenized using homogenizer (DS-1, Shanghai Specimen and Model Factory, Shanghai, China). Then, the homogenate was treated according to the previously described method (Pramanik et al., 2002) with a slight modification. The homogenate was mixed with phosphate buffer solution (PBS, 0.2 mol/L) at pH 6.0 at a ratio of 3.2 (w/v), and 5000 U/g alcalase was added. The mixture was subjected to ultrasonic wave treatment at 58 ± 2°C and 300 W for 17 min and then placed into water bath at 58 ± 2°C for 1.5 h. Samples were removed. Next, samples were heated in boiling water bath at 100°C for 10 min to deactivate enzyme. The hydrolysates were centrifuged at 10,000 r/min for 15 min.

The supernatant was filtered using two ultrafiltration (UF) membranes (Millipore ultrafiltration device, Dongrui Technology Co., Ltd., Guangdong, China) with 2-μm pores and a 5-kDa molecular weight (MW) cut-off to obtain peptides with MWs less than 5 kDa (Wu et al., 2013). The percolate was then freeze-dried (3K30 high-speed refrigerated centrifuge, Sigma, Germany) and stored at −20°C. The precipitate powder was dissolved in distilled water, diluted to 50 mg/mL and named AOP.

Gel filtration chromatography

AOP was purified using a Sephadex G-25 gel filtration column (1.6 × 40 cm, GE Ltd., USA) on a semi-preparative Zorbax SB C-18 column (9.4 × 250 mm, 5 μm, Agilent, USA). Peptides were collected with an automatic fraction collector from Waters Technologies. The detection was performed at 268 and 280 nm. The optimized conditions were as follows: mobile phase A, Milli-Q water/0.1% (v/v) TFA; mobile phase B, MeOH; flow rate, 2.0 mL/min; temperature, 30°C; and injection volume, 2.0 mL. The elution programme was as follows: 100% A (0–2 min), 100–90% A (2–5 min), 90–50% A (5–11 min), 50% A (11–13 min), 50–30% A (13–14 min) and 30% A (14–30 min). Five purified peptides named FC1, FC2, FC3, FC4 and FC5 were isolated, collected and lyophilized.

The fraction purity was identified by RP-HPLC (1100 Series, Agilent Ltd., USA). In total, 20 μL of fractions was analysed in a Phenomenex column (4.6 × 250 mm, 5 μm) at 268 and 280 nm using a gradient elution of Milli-Q water/0.1% (v/v) TFA and MeOH at 1.0 mL/min.

MALDI-TOF determination

The desirable antioxidant peptide fractions of FC2 after RP-HPLC purification were directed into the mass spectrometer (Ultraflex III TOF/TOF, Bruker Daltonics, Bremen, DE). On stainless steel plates of MALDI, 0.5 μL of the sample of matrix was added and dried. The matrix of α-CHCA (0.5 mg/mL) was dissolved in 50% ACN and 0.1% TFA (Sigma Chemical Co., St. Louis, MO, USA) with final concentration of 0.5 g/L in the solution. The spectra were obtained in reflectron mode with an ion source voltage and reflected voltage of 8 and 29.5 kV, respectively, at a frequency...
of 200 Hz. The mass range for MS was between 800 and 1800 m/z. Multiple replicates of bands from different gels of the same strain were generally analysed (Li, Sadqi, Liu, Chen, & He, 2015). The software flexcontrol 3.3.85.0 was used for control and analysis (Lin et al., 2007). Observed experimental values for peptide masses were consistent with theoretical calculated values.

### Amino acid composition analysis

Briefly, 0.3 g of FC2 was accurately weighed in a hydrolysis tube, and 10 mL of HCl (6 mol/L) was added for acidolysis. The tube was sealed after nitrogen filling three times and stored at 110°C for 22 h. After cooling and evaporating the excess of hydrochloric acid, the sample was repeatedly washed with pH 2.2 sodium citrate buffer solutions and subjected to instrumental analysis on an automatic amino acid analyser (835-50, Hitachi Ltd., Japan). The sample was treated in no. 2619 (52051) ion exchange column (2.6 × 150 mm) at a flow of 0.225 mL/min at 53°C for 72 min.

### Synthesize of peptide

The novel antioxidant peptide was synthesized by Fmoc solid phase peptide synthesis using ASP48S (Peptron Inc.) (Domingues et al., 2013; Domingues, Riske, & Miranda, 2010) and identified using a Gemini-NX C18 column (4.6 × 250 mm, 5 μm). Elution was performed with a linear gradient of acetonitrile containing 0.1% (v/v) TFA and 0.1% (v/v) TFA in water. The molecular mass of the peptide was 1039.56 Da, and the amino acid sequence of the peptide was Gly- Ala- Gly- Leu- Pro- Gly- Lys- Arg- Glu- Arg based on electron spray ionization.

### Reducing power

The reducing power was measured according to the previously reported method (Oyaizu, 1988; H.-C. Wu, Chen, & Shiau, 2003). A volume of 1 mL of the sample was added to 1 mL of 0.2 M phosphate buffer (pH 6.6) and the same volume of 1% potassium ferricyanide. The sample was incubated in a water bath at 50°C for 20 min. Then, 1 mL of 10% TCA was added to the reaction mixture. After centrifugation, 2 mL of the supernatant fluid was taken and mixed with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride, the mixture solution incubated for 10 min at 50°C in a water bath. The absorbance was measured at 700 nm. High absorbance indicated high reducing power.

### DPPH radical scavenging activity

The DPPH assay was performed according to the previously described method (Wang, Li, Chi, Zhang, & Luo, 2012) with some appropriate modifications. Briefly, 0.5 mL of the sample was added to 0.5 mL of distilled water and 0.2 mM DPPH in 95% ethanol. The solution was incubated for 30 min at room temperature in the dark. This group was treated as the experimental group. The control sample, including DPPH solution without sample, was also prepared. In addition, 95% ethanol was substituted for DPPH solution in the blank sample. The absorbance was measured at 540 nm. In addition, the samples with different concentration were also measured. The antioxidant activity of the sample was evaluated by the half inhibition concentration (IC50). DPPH radical was calculated with the following equation:

\[
\text{DPPH radical scavenging activity(%) = } \left( \frac{1 - (A_j - A_i)}{A_0} \right) \times 100\%
\]

where \(A_i\) was sample absorbance rate, \(A_0\) and \(A_j\) were the absorbance of control group and the blank, respectively.

### Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured according to the reported method (Ajibola, Fashakin, Fagbemi, & Aluko, 2011) with slight modifications. In the assay, hydroxyl radicals are briefly generated by the Fenton reaction (Richmond, Halliwell, Chauhan, & Darbre, 1981). \(A_i\): 0.5 mL of the sample was mixed with 1, 10-phenanthroline solution (0.5 mL, 0.75 mM) and pH 7.4 PBS (1 mL, 0.15 M). FeSO₄ solution (0.5 mL, 0.75 mM) was added into the mixture. The hydroxyl radicals were generated by 0.01% H₂O₂ (v/v, 0.5 mL). The solution was incubated for 60 min at 37°C in a water bath, next, measured under wavelength of 536 nm. Deionized water instead of the sample and repeat the operation H₂O₂ solution at 536 nm is measured for absorbance \(A_0\), deionized water instead of the sample solution to repeat the above operation, the absorbance measured at 536 nm \(A_j\). The hydroxyl radical scavenging activity was calculated by the following formula:

\[
\text{Radical scavenging activity(%) = } \left( \frac{(A_j - A_i)}{(A_0 - A_i)} \right) \times 100\%
\]

### Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity was measured by means of improved pyrogallol autoxidation (Marklund & Marklund, 1974) with some modifications. Briefly, the reaction was initiated by 0.1 mL of the sample, 2.8 mL of Tris- HCl buffer (pH 8.2, 0.1 M). The mixture was incubated in a 25°C water bath for 10 min, 0.1 mL of pyrogallic acid (3 mM) was added and absorbance was then measured at 325 nm every 30 s per 5 min. The blank contained an equal volume of deionized water instead of sample. The absorbance regression was drawn based on time, and its slope was marked as \(V\), which indicated pyrogallol autoxidation rate. The capacity of scavenging the superoxide anion radical was calculated using the following equation:

\[
\text{Superoxide anions scavenging activity(%) = } \left( 1 - \frac{V_{\text{sample}}}{V_{\text{control}}} \right) \times 100\%
\]

where \(V_{\text{control}}\) and \(V_{\text{sample}}\) were the absorbances of the control group without sample and the sample group, respectively.

### Statistical analysis

Statistical analysis was performed using SPSS Statistic 17.0 (SPSS, Inc., Chicago, IL). Analysis of variance was applied, and every sample was analysed in triplicate to identify statistically significant differences using Duncan’s multiple range test (\(P < 0.05\)).
Results and discussion

Gel filtration chromatography of AOP and the antioxidant activity

As shown in Figure 1, AOP was separated into five fractions (F1–F5). Four standards were used to indicate the MWs. The MW of AOP was primarily under 1 kDa. The MWs of F1–F5 were 1345.19, 989.13, 499.10, 309.91 and 102.14 Da, respectively.

The results of the reducing power, DPPH, hydroxyl and superoxide anion-free radicals scavenging ability expressed as IC_{50} were presented in Table 1. The DPPH IC_{50} of F2 at 4.64 mg/mL was slightly higher than F5 at 4.21 mg/mL. However, the ∙OH and ·O_{2} IC_{50} values of F2 with the maximum reducing power were the minimum values, indicating that F2 was the strongest antioxidant component.

As shown in Figure 2, the purity analysis of F2 was performed via HPLC. The diagram clearly showed that F2 was still a mixture and required further purification. According to the conditions of gel separation, the F2 was enriched as much as possible before being evaporated, freeze-dried and resolved to 50 mg/mL for the next RP-HPLC purification.

Isolation of F2 peptides by RP-HPLC and their antioxidant activity

Using a 0.2-μm UF membrane, F2 was further separated by semi-preparative RP-HPLC on a Zorbax, SB C-18 column, and

Table 1. Antioxidant capacity of F series of separations.

| Indexes          | F1   | F2   | F3   | F4   | F5   |
|------------------|------|------|------|------|------|
| Reducing power   | 0.61 ± 0.03 | 0.80 ± 0.09 | 0.57 ± 0.01 | 0.54 ± 0.04 | 0.69 ± 0.01 |
| DPPH IC_{50} (mg/mL) | 5.38  | 4.64  | 4.83  | 5.40  | 4.21  |
| ·OH IC_{50} (mg/mL) | 5.67  | 4.31  | 4.58  | 5.43  | 4.69  |
| ·O_{2} IC_{50} (mg/mL) | 11.50  | 10.32  | 11.93  | 14.60  | 12.16  |

DPPH: 2,2-Diphenyl-1-picrylhydrazyl.
Five purified peptides named FC1, FC2, FC3, FC4 and FC5 were separated as shown in Figure 3. A variety of peptides were eluted on RP-HPLC given their different polarities, and this technique was popularly applied in the isolation and purification of small peptide segments (Dan, Ganesan, Flood, Tsai, & Reif, 2000), especially those less than 5000 Da (Sanz-Nebot, Benavente, Toro, & Barbosa, 2001).

The antioxidant activities are presented in Table 2. Reducing power, DPPH, hydroxyl and superoxide anion-free radical scavenging ability presented as IC\textsubscript{50} values of the FC series were compared with ascorbic acid (vitamin C [Vc]), which served as a positive control. The DPPH and \textbullet\text{OH} IC\textsubscript{50} of FC2 were the lowest and similar to those of Vc. The \textbullet\text{O}_2 IC\textsubscript{50} value of FC4 (0.658 mg/mL) was the minimum value. Taking all indicators into consideration, the antioxidant ability of FC2 was the highest.

FC2 purity analysis was performed (Figure 4). The area of the peak at 17 min reached 65.4%, indicating that FC2 was considered to be of high purity.

**Characterization and analysis of the structure–activity relationship**

**MALDI-TOF characterization**

MALDI-TOF was used to characterize the amino acid sequence of FC2. The mass spectrum and the secondary
mass spectrum are presented in Figures 5 and 6, respectively. In Figure 5, the mass-to-charge ratio \((m/z)\) of the fragment was 1040.56 Da, indicating that the MW of FC2 was 1039.56 Da.

The molecular ion peak with the strongest signal was chosen for the secondary time of flight MS analysis. Using flexanalysis to analyse the spectrum, sequence information was inferred from the fragments, which was matched and compared with Blast sequence databases for proteins and polypeptides. The following sequences were obtained: GAGLPGKRER and GAGLPGKVWR. The corresponding amino acid sequences were Gly–Ala–Gly–Leu–Pro–Gly–Lys–Arg–Glu–Arg and Gly–Ala–Gly–Leu–Pro–Gly–Lys–Val–Trp–Arg, respectively.

Given the affinity of a basic amino acid for protons, these amino acids play a special role in peptide bond cleavage. The MW of the *P. fucata* antioxidant peptide was in the range of 750–1700 Da, which was consistent with the results of Wu et al., (2003). In addition, the antioxidant peptides that were isolated contained the acidic amino acid Glu and the alkaline amino acids Lys and Arg, which are in line with Dr. Je, Qian and Kim (2007) research results. Some researchers (Rival, Fornaroli, Boeriu, & Wichers, 2001; Suetsuna, Ukeda, & Ochi, 2000) found that the antioxidant activity was dependent not only on the content of hydrophobic amino acids but also on the position, especially in the C-terminus. It was inferred that FC2 with strong antioxidant activity was attributed to its electrophilic ability through Glu and the electron acceptors Lys and Arg. Moreover, a large amount of hydrophobic amino acids preferred to combine with hydrophobic groups, preventing the chain reaction by competing with substrate and performing its antioxidant activity (Chen, Muramoto, Yamauchi, & Nokihara, 1996). Combined with amino acid composition analysis, comprehensive analysis was necessary to determine the species and amounts of amino acids in a peptide.

**Amino acid composition analysis**

Some amino acids and their derivatives exhibited antioxidant capability, such as cysteine, tryptophan, tyrosine, leucine and so on. Some researchers found that amino acids containing sulphur, including cysteine and methionine (Qian, Jung, & Kim, 2008), could be directly related to free radicals or enzyme action, as an effective antioxidant *in vivo* (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005).
Togashi et al. (2002) also extracted antioxidant peptides from collagen, which is enriched with hydroxyproline and proline. Parts of the amino acid composition of the oligopeptide are presented in Table 3.

As shown in Table 3, Trp was not included in FC2. The result also showed that FC2 contained high levels of Glu, Leu, Lys and Arg, which was consistent with the result of the amino acid sequence. In addition, there were many active amino acids in the FC2, such as γ-aminobutyric acid, ornithine and taurine. γ-Aminobutyric acid is an important inhibitory neurotransmitter in the central nervous system. It is considered as a natural and non-protein amino acid that is beneficial for sleep, skin beauty and exhibits anti-epilepsy properties. Ornithine participates in urea cycle metabolism, transforming ammonia to urea for detoxification. Taurine plays a key role in infant growth and intellectual development. It also has biological functions of protecting eyesight and heart.

Identification of the synthetic peptide (Gly–Ala–Gly–Leu–Pro–Gly–Lys–Arg–Glu–Arg)
The peptide synthesized in a solid phase was identified by RP-HPLC and ESI-MS, and the chromatogram is presented in Figure 7. The main peak of the total area occupied 98.35% at 10.85 min. The Fmoc-protected amino acid was a synthetic peptide of high purity under these conditions of identification and purification. In addition, the Kaise test was exacted under 180°C. The MW of synthetic peptide was 1039.33 Da, and the synthetic requirements were met.

Comparison of antioxidant activity between FC2 and the synthetic peptide
Synthetic peptides easily formed β-folding structures when the N-terminus was enriched in hydrophobic amino acid residues. The synthetic peptide in this article contained Gly and Pro, which unfolded the structure to eliminate the effect of the hydrophobic amino acids. Table 4 presents the antioxidant activity of the natural antioxidant peptide (Gly–Ala–Gly–Leu–...
Antioxidant capacity of different components.

| Indexes     | FC2     | Synthetic peptide | GSH     | Vc      | BHT      |
|-------------|---------|-------------------|---------|---------|----------|
| Reducing power (2.5 mg/mL) | 0.43 ± 0.03 | 0.57 ± 0.01 | 2.33 ± 0.01 | 1.43 ± 0.01 | 1.33 ± 0.01 |
| DPPH IC₅₀ (mg/mL)     | 0.032   | 0.39             | 0.001   | 0.03    | 0.025    |
| -OH IC₅₀ (mg/mL)      | 0.27    | 0.98             | 0.06    | 0.22    | 0.27     |
| O₂⁻ IC₅₀ (mg/mL)      | 1.05    | 1.18             | 0.05    | 0.26    | 0.30     |

DPPH: 2,2-Diphenyl-1-picrylhydrazyl; Vc: vitamin C; BHT: butylated hydroxytoluene.

Pro–Gly–Lys–Arg–Glu–Arg and synthetic peptide compared with reduced glutathione (GSH), Vc and butylated hydroxytoluene (BHT). The results showed that GSH is the strongest antioxidant capacity, followed by the Vc, BHT, FC2 and synthetic peptide in Table 4. Compared with FC2, synthetic peptide IC₅₀ of scavening DPPH and -OH were 12 times and 3 times, respectively. However, the IC₅₀ values of ·O₂⁻ clearance and reducing power were similar, which potentially resulted from its possible secondary structure of natural sequences (Kashirin, Sibilev, Beloborodov, & Deigin, 2000). Although the chemical synthesis of linear peptide when dissolved N-terminal hydroporphic amino acid residues tends to β-folding structure, it contains Gly and Pro which can weaken fold and open the peptide structure. This may affect its oxidation resistance different from natural peptide. Compared with Vc and BHT, the IC₅₀ of scavening DPPH and -OH of FC2 were closer to Vc and BHT, but the IC₅₀ values of ·O₂⁻ of FC2 were a bit poor, accounting for 23.81% of Vc and 28.57% of BHT, respectively.

Conclusion

In the present study, a new antioxidant peptide was isolated, purified and identified from P. fucata muscle. Its MW was 1039.56 Da, and the amino acid sequence was Gly–Ala–Gly–Leu–Pro–Gly–Lys–Arg–Glu–Arg. The antioxidant peptide from P. fucata had good scavenging capacity against free radicals, such as the IC₅₀ of scavening DPPH and -OH of nature antioxidant were closer to Vc and BHT, although the IC₅₀ values of ·O₂⁻ of FC2 were a bit poor. In addition, its antioxidant activity was attributed to its hydroporphic amino acid residues enriched in the N-terminus and its electrophilic ability mediated by Glu and electron acceptors, such as Lys and Arg. It also presumed that the synthesized peptide was inferior to the natural corresponding peptide regarding scavenging free radicals, but it was a feasible method to prepare antioxidant peptides. This research might provide the basis for further cloning expression research. The results indicated that the antioxidant peptides of P. fucata might be useful ingredients in nutraceutical and cosmetic applications.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Table 4. Antioxidant capacity of different components.

| Indexes     | FC2     | Synthetic peptide | GSH     | Vc      | BHT      |
|-------------|---------|-------------------|---------|---------|----------|
| Reducing power (2.5 mg/mL) | 0.43 ± 0.03 | 0.57 ± 0.01 | 2.33 ± 0.01 | 1.43 ± 0.01 | 1.33 ± 0.01 |
| DPPH IC₅₀ (mg/mL)     | 0.032   | 0.39             | 0.001   | 0.03    | 0.025    |
| -OH IC₅₀ (mg/mL)      | 0.27    | 0.98             | 0.06    | 0.22    | 0.27     |
| O₂⁻ IC₅₀ (mg/mL)      | 1.05    | 1.18             | 0.05    | 0.26    | 0.30     |

DPPH: 2,2-Diphenyl-1-picrylhydrazyl; Vc: vitamin C; BHT: butylated hydroxytoluene.

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