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Novel antioxidant peptides from tiger nut (Cyperus esculentus L.): Isolation, characterization, and cytoprotective functions on HepG2 and Caco-2 cells

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Abstract

The antioxidant activity of the peptides from tiger nut was investigated for the first time. The tiger nut peptides (TNP) were found to perform excellent antioxidant activity supported by their chemical and cell antioxidant behaviors, amino acid composition, and morphological observation. Higher 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity and ferric ion reducing antioxidant power were observed in the TNP with more hydrophobic amino acids compared with traditional soybean and peanut peptides. Furthermore, the peptides from tiger nut could effectively protect H_2O_2-induced HepG2 and Caco-2 cells from oxidative damage by enhancing endogenous antioxidant enzyme activities and reducing oxidative stress levels, especially the T3 peptides purified from the fraction less than 1 kD molecular weight. And increased catalase, superoxide dismutase, and glutathione peroxidase activities, while high intracellular reactive oxygen species and malondialdehyde inhibition occurred. This study could provide a new strategy for the development of promising antioxidant peptides for food applications.

Introduction

Normal physiological metabolism is invariably accompanied by the transition from inhaled oxygen into reactive oxygen species (ROS). The ROS level can increase in the body under an unhealthy external environment and/or abnormal body metabolism[1]. Oxidative stress by surplus ROS causes cellular oxidative damage, which has been a significant risk factor for chronic illnesses, such as cardiovascular disease and diabetes[2,3]. To mitigate the negative impact, antioxidants are developed to capture and neutralize the free radicals. However, the current synthetic antioxidants in food are subjected to strict supervision due to safety, high cost, and limited activities[4]. Some bioactive peptides have been demonstrated to be capable of scavenging free radicals and reducing peroxidation, being well-tolerated and safe[5].

Antioxidant peptides are the oligopeptides or polypeptides with antioxidant properties, as a kind of bioactive peptide composed of amino acids[6]. They can perform biomolecular synergistic effects when the equilibrium between intracellular oxidant production and antioxidant content is compromised, exhibiting electronic provisioning capability, chelating metal ions, and scavenging excessive free radicals[7]. Antioxidant peptides have received significant attention in recent years because of their great potency, natural safety, and efficient absorption[8, 9]. For instance, the peptides obtained from soybeans[5] and peanuts[10] have been found to act antioxidant activities. The peptides derived from hydrolyzed proteins possess much greater antioxidant activity compared to their parent proteins[11]. And the antioxidant capacities of peptides can be further affected by various factors including their molecular weight and amino acid composition[12,13,14].

Tiger nut (*Cyperus esculentus L.*) is a tuber of the sedge family, widely cultivated in Europe, Africa, and Asia[15]. It is rich in vital nutrients and bioactive molecules including fats, proteins, vitamins, and phenols, seen as a valuable material for the food industry. Most of the tiger nut studies focus on the extraction or

application of its oil, but the defatted tiger nut contributes to significant waste[16]. The increasing global population and living standards have led to increased demand for quality protein products. And there is a heightened emphasis on the creation and utilization of novel plant protein resources. It is noted that the protein from defatted tiger nut has a high content of essential amino acids (46.03%), beyond the WHO/FAO standard of 36% and greater than soybean protein (41%)[17]. Moreover, the tiger nut protein has been reported to facilitate blood circulation and immune regulation, and reduce cardiovascular disease risk[15,18]. Tiger nut is expected to be a promising and underutilized source of available excellent proteins and/or bioactive peptides.

Currently, the research on tiger nut protein has the primary focus on its extraction optimization and properties, but few studies were further conducted on their peptides. As far as our knowledge, there is no research focused on the antioxidant potential of tiger nut peptides (TNP). The direct insight into the integral processes of intracellular absorption and substance metabolism is provided by cell experiments. And combining the strengths of chemical and cell experiments yields a comprehensive and accurate assessment of the antioxidation potential of peptides[19]. In this work, peptides were obtained from tiger nut by enzymatic hydrolysis, ultrafiltration classification, and Sephadex G-15 fractionation, and their antioxidant activities were further investigated by a combined analysis of chemical antioxidant behavior, amino acid composition, cellular antioxidant properties on H_2O_2-mediated oxidative injured HepG2 and Caco-2 cells, and morphological observation for the first time. This study could provide fresh insights and guidance for the development and potential application of tiger nut peptides, and a promising new source of antioxidant peptides.

Results and discussion

Preparation and Experimental Optimization of TNP

The effect of enzyme-substrate ratios, temperatures, and times on the DH and DPPH RSA of the enzymatically hydrolyzed samples of tiger nut is illustrated in Figure S1. As the enzyme-substrate ratio increased, the DH initially increased and then remained constant, reaching its maximum value of 38.94% at a ratio of 10000 U/g (Figure S1 A). A similar trend was also observed in the preparation of novel antioxidant peptides from camel bone proteins by Wang et al[20]. This might be attributed to the substrate saturation by enzyme excess at high enzyme-substrate ratios. Consequently, the addition of more enzymes does not increase the rate of the hydrolysis reaction, resulting in no further increase in DH. For DPPH RSA, there was an increase from 8000 U/g to 11000 U/g of enzyme-substrate ratio, indicating the stronger antioxidant activity, followed by a decrease at higher ratio.

The DH and DPPH RSA of the samples with hydrolysis temperature are presented in Figure S1 B. The DH significantly increased as temperature from 45 ℃ to 55 ℃ ($p < 0.05$), related to a deeper hydrolysis. However, the DH decreased with increased temperature beyond 55 ℃. Temperature is a critical factor for enzyme stability. And a suitable hydrolysis temperature could contribute to an efficient enzyme

reaction, ultimately resulting in increased DH. However, dissociation of the secondary bonds of the enzyme might occur under higher temperatures, and there was a decrease in enzyme activity[21]. Interestingly, a maximum DPPH RSA of the sample also showed the strongest antioxidant capacity of 71.03 ± 0.53% at 55 ℃.

As shown in Figure S1 C, the DH and DPPH RSA during TNP preparation were significantly impacted by the hydrolysis time. As the hydrolysis proceeded, the DH rapidly increased with time, followed by a decrease. A peak of the value of 51.48 ± 0.16% was found at 3 h. It is consistent with the time single factor of enzymatic hydrolysis of wheat germ protein by Tian et al[22]. Over the period of 3–5 h, the polypeptide chain of protein broke down, resulting in fewer specific sites for the protease to act on in the substrate[23]. Additionally, similar trend was observed in the DPPH RSA (70.67 ± 0.47%) of TNP with hydrolysis time. At the initial stage (1–3 h), the enzymatic breakdown of the tiger nut protein resulted in the production of peptides with antioxidant activity, and the DPPH RSA increased. However, some peptides were disrupted to smaller peptides with decreased antioxidant activity by further enzymatic hydrolysis, resulting in a decrease in the DPPH RSA of TNP. Consequently, the peptides generated from enzymatic hydrolysis of tiger nut exhibited antioxidant activity. And the optimal preparation conditions for TNP were determined to be the enzyme-substrate ratio of 10000 U/g, hydrolysis temperature of 55°C, and hydrolysis time of 3 h.

Basic Functional Properties

Water/Oil Holding Capacity and Solubility

The water/oil holding capacity and solubility of tiger nut protein and TNP are summarized in Table 1. The WHC and OHC of protein and peptide are related to functional properties, texture, mouthfeel, and storage stability of food system. After enzymatic hydrolysis, the TNP presented decreased WHC and OHC, and dramatically increased solubility compared with those of tiger nut protein. This was consistent with the fact that decreased WHC and OHC were considered higher solubility. This could be because enzymatic hydrolysis broke down the peptide chain in protein structure, resulting in smaller molecular weight and carboxyl and amino-terminal groups exposed. And the peptides produced became more hydrophilic, and the solubility increased, with decreased water and oil absorption. The peptides have a good solubility and the mass loss upon disposal, resulting in a negative value.

Table 1
Water/oil holding capacity, solubility, and foaming and emulsifying properties of tiger nut protein and TNP.

Function	Tiger nut protein	TNP
WHC (g/g)	2.86 ± 0.01[a]	—
OHC (g/g)	4.18 ± 0.01[a]	1.99 ± 0.01[b]
Solubility (%)	6.08 ± 0.12[a]	31.92 ± 2.25[b]
FC (%)	31.67 ± 2.89[a]	15.00 ± 0.00[b]
FS (%)	63.49 ± 5.50[a]	80.00 ± 0.00[b]
Emulsibility (%)	50.98 ± 0.00[a]	41.32 ± 0.00[b]
Emulsion stability (%)	76.92 ± 0.01[a]	60.00 ± 0.00[b]
Different letters in the same amino acid group indicate significant differences ($p < 0.05$).		
WHC, water-holding capacity; OHC, oil-holding capacity; FC, foaming capacity; FS, foaming stability.		

Foaming and Emulsifying Properties

Foaming and emulsifying properties are essential functional properties of protein and peptide products used in food industry applications. And the FC, FS, emulsibility, and emulsion stability of tiger nut protein and TNP are shown in Table 1. Good solubility is the critical factor for excellent foam properties[24]. The TNP performed higher FS compared with tiger nut protein. And there was a lower value in the viscosity of TNP and a rise in exposure of hydrophobic groups of the system caused by protein hydrolysis, which could be responsible for the decreased foaming performance. Exposure to hydrophobic groups could lead to an increase in insoluble proteins or peptides, and the insoluble proteins and peptides were adsorbed to the interface, increasing the thickness of the interface layer due to protein-protein interaction. The insoluble protein particles played a beneficial role in FS properties[25].

Proteins and peptides are characterized by presenting hydrophilic and hydrophobic functional groups. Therefore, they can adsorb oil at the oil-water interface to form emulsions and ensure their stability without flocculation for some time[26]. The TNP performed inferior emulsifying performance compared with protein, with decreased emulsibility and emulsion stability as shown in Table 1. The lower emulsification performance may be attributed to the small molecular weight of TNP, and the formed adsorption film was not enough to maintain oil-water stability, thus decreasing the emulsion stability.

Antioxidant Activity Analysis

Antioxidant Activity of Crude Peptides

The antioxidant activities of the peptides were determined by measuring DPPH RSA, ABTS RSA, hydroxyl RSA, and FRAP in this study (Fig. 1A), and their amino acid composition was summarized in Table 2. The DPPH RSA, ABTS RSA, hydroxyl RSA, and FRAP of TNP increased with concentration increasing, consistent with the trend for camel bone peptides reported by Wang et al[14]. It is widely accepted that the free radical DPPH• can be transform into stable molecule DPPH-H when absorbing an electron or a proton from compounds such as antioxidants[27], and antioxidants can also inhibit the formation of blue-green $ABTS^{\bullet+}$, causing the color to fade. The DPPH radical scavenging rate of TNP was 64.05-124.07% over a concentration range of 1–20 mg/mL, and it reached 76.16 ± 1.33% at 5 mg/mL, significantly greater than that of soybean peptides (49.43 ± 0.51%) and peanut peptides (71.89 ± 0.60%) ($p < 0.05$). Additionally, the ABTS radical scavenging rate of TNP reached 44.00 ± 0.49% at 5 mg/mL, more than that of soybean peptides (47.80 ± 0.88%) ($p < 0.05$). These indicated that the TNP is effective in scavenging both DPPH and ABTS free radicals, meaning it could provide many electrons.

Table 2
Amino acid analysis of proteins and TNP.

Amino acids (g/100g)	Tiger nut protein	TNP	Soybean peptides	Peanut peptides
Aspartic (Asp)	6.62 ± 0.08^{a}	9.02 ± 0.13^{b}	12.17 ± 0.29^{d}	12.71 ± 0.17^{c}
Threonine (Thr)	4.82 ± 0.16^{a}	4.65 ± 0.26^{a}	3.99 ± 0.18^{c}	2.59 ± 0.13^{b}
Serine (Ser)	4.38 ± 0.40^{a}	4.05 ± 0.45^{a}	4.22 ± 0.86^{a}	4.30 ± 0.65^{a}
Glutamic (Glu)	18.48 ± 0.91^{a}	17.40 ± 0.64^{a}	18.97 ± 0.70^{ab}	20.73 ± 0.75^{b}
Glycine (Gly)	5.75 ± 0.04^{a}	3.05 ± 0.06^{b}	4.29 ± 0.25^{c}	6.08 ± 0.13^{a}
Alanine (Ala)	5.61 ± 0.08^{a}	7.47 ± 0.13^{b}	4.67 ± 0.25^{c}	4.26 ± 0.16^{c}
Cysteine (Cys)	1.54 ± 0.12^{a}	0.86 ± 0.06^{b}	1.16 ± 0.11^{ab}	1.23 ± 0.17^{ac}
Valine (Val)	6.06 ± 0.24^{a}	6.74 ± 0.39^{a}	5.10 ± 0.25^{c}	4.21 ± 0.23^{b}
Methionine (Met)	1.65 ± 0.04^{a}	2.00 ± 0.00^{a}	1.12 ± 0.23^{b}	0.98 ± 0.29^{b}
Isoleucine (Ile)	4.52 ± 0.52^{a}	4.74 ± 0.26^{a}	4.71 ± 0.27^{a}	3.54 ± 0.20^{b}
Leucine (Leu)	8.44 ± 0.04^{a}	8.47 ± 0.00^{a}	8.11 ± 0.20^{a}	7.02 ± 0.19^{b}
Tyrosine (Tyr)	2.36 ± 0.16^{a}	2.46 ± 0.26^{a}	3.36 ± 0.14^{b}	3.42 ± 0.04^{b}
Phenylalanine (Phe)	4.01 ± 0.12^{a}	4.56 ± 0.39^{a}	5.31 ± 0.12^{b}	5.48 ± 0.09^{b}
Lysine (Lys)	6.34 ± 0.08^{a}	4.51 ± 0.06^{b}	6.78 ± 0.12^{d}	3.72 ± 0.03^{c}
Histidine (His)	3.42 ± 0.08^{a}	2.78 ± 0.06^{b}	2.88 ± 0.14^{b}	2.44 ± 0.01^{c}
Arginine (Arg)	8.25 ± 0.16^{a}	10.30 ± 0.26^{b}	7.61 ± 0.02^{d}	12.63 ± 0.06^{c}
Proline (Pro)	7.74 ± 0.32^{a}	6.93 ± 0.26^{b}	5.57 ± 0.05^{d}	4.64 ± 0.03^{c}
Acidic amino acids (NCAA)	25.1 ± 0.99^{a}	26.42 ± 0.77^{a}	33.44 ± 0.58^{b}	31.13 ± 0.41^{c}
Basic Amino Acids (PCA)	18.01 ± 0.32^{a}	17.59 ± 0.26^{ab}	18.79 ± 0.01^{c}	17.26 ± 0.25^{b}

Different letters in the same amino acid group indicate significant differences ($p < 0.05$).

Amino acids (g/100g)	Tiger nut protein	TNP	Soybean peptides	Peanut peptides
Hydrophobic amino acids (HAA)	39.58 ± 0.99[a]	41.77 ± 1.36[a]	31.35 ± 0.64[b]	35.75 ± 0.66[c]
Different letters in the same amino acid group indicate significant differences ($p < 0.05$).				

The hydroxyl RSA of TNP was 5.04–38.82% at 1–20 mg/mL, and exhibited a low value of 11.71 ± 1.31% at 5 mg/mL compared with other peptides. This showed relatively poor ability of TNP to convert hydroxyl radicals and stop the radical chain reaction. It was widely acknowledged that the presence of acidic amino acids such as Glu and Asp contributed to strong capacity of peptides to scavenge hydroxyl radicals. And the lower hydroxyl RSA of TNP presented could be supported by their amino acids analysis results with low content of the acidic amino acids (Table 2).

Antioxidants can also reduce $Fe3^{+}$-tripyridyltriacridazine (Fe^{3+}-TPTZ) to produce Fe^{2+}-TPTZ (blue color). The FRAP of TNP was 0.17–1.78 at 1–20 mg/mL. And it reached 0.60 ± 0.03 at 5 mg/mL, higher than that of soybean peptides (0.22 ± 0.02) and peanut peptides (0.29 ± 0.01) ($p < 0.05$). Some sulphur-containing amino acids as the significant contributors to FRAP, so it may be possible that the high FRAP value due to the sulphur-containing amino acid (such as Met), because its sulfhydryl group is prone to oxidation by reactive species (Table 2) [28]. These indicated that the peptides from tiger nut (TNP) had higher antioxidant activities with strong DPPH and ABTS RSA and FRAP than soybean and/or peanut antioxidant peptides.

The amino acid composition of peptide plays an important role in its antioxidant activity. The antioxidant activity of protein hydrolysates depends on the contents of hydrophobic amino acids[29]. This is because the hydrophobicity of the antioxidant peptides allows them to readily interface hydrophobic interactions with the lipid bilayer of the membrane and enter the target organ, thereby exhibiting excellent free radical scavenging properties[30]. It was observed in the amino acid composition results that TNP had high hydrophobic amino acid content, particularly Ile, Ala, Met, Val, Pro, and Leu (Table 2), supporting the higher antioxidant indexes of TNP compared with the soybean and/or peanut antioxidant peptides. In conclusion, these results indicated that the peptides obtained from tiger nut had strong antioxidant activities, considered a potential antioxidant peptide.

Antioxidant Activity of TNPs by Ultrafiltration Classification

The SDS-PAGE profiles of tiger nut protein, soybean protein, peanut protein, and TNP are depicted in Figure S2. As shown in the profiles, the tiger nut protein had a smaller molecular weight compared with the soybean and peanut proteins. And the tiger nut protein contained six main bands distributed at 27.472 kD, 24.468 kD, 18.677 kD, 16.815 kD, 12.170 kD, and 10.376 kD molecular weights. Accordingly, it was expected that there might be low molecular weight structure less than 10 kD for TNP, but it was difficult to separate in 15% polyacrylamide gel and SDS-PAGE system. Therefore, the ultrafiltration was

further performed to separate TNP. Four fractions with different molecular weights were obtained after ultrafiltration: TNP-1 (> 10 kD), TNP-2 (3–10 kD), TNP-3 (1–3 kD), and TNP-4 (< 1 kD), and their antioxidant activities were assessed (Fig. 1B). The DPPH RSA, ABTS RSA, hydroxyl RSA, and FRAP of the peptides increased with the molecular weight decreasing, showing stronger antioxidant capacity. Small peptide with lower molecular weight tends to have great potential for bioactivity[12,31]. Therefore, the TNP-4 fraction with low molecular weight (< 1 kD) was used for further purification in this study.

Antioxidant Activity Analysis of TNP-4 by Sephadex G-15 Fractionation

Peptides can be separated by dextran gel column based on their molecular mass weight. The substance with larger molecular weight would be eluted first, while smaller molecule has a long retention time due to its tight combination with the gel. As presented in Fig. 2A, the TNP-4 was separated into three fractions by Sephadex G-15 gel column with elution time increasing, and they were sequentially named components T1, T2, and T3. It was indicated that T1 has the largest molecular weight, and T3 was the smallest peptide fragment. The three fractions were further collected separately, and tested for antioxidant indexes. As shown in Fig. 2B, the T3 exhibited the highest DPPH radical scavenging rate, ABTS radical scavenging rate, and FRAP with the values of 46.49 ± 0.07%, 37.45 ± 0.15%, and 0.87 ± 0.00 at 0.5 mg/mL, respectively, suggesting strong antioxidant capacities. However, the T2 has the highest hydroxyl radical scavenging rate of 26.53 ± 0.14%. This was because the biological activity of peptides was also correlated to its amino acid composition and hydrophobic property, except for peptide size[11].

Cytoprotective Functions on HepG2 and Caco-2 Cells

Cell Cytotoxicity

Cell experiments provide a promising alternative apart from the *in vitro* chemical technique to evaluate antioxidant activity. The cytotoxic effect of TNP, TNP-4, T1, T2, and T3 on HepG2 cells and Caco-2 cells were shown in Fig. 3, respectively. All the viabilities of the tiger nut peptide-treated HepG2 and Caco-2 cells increased, and they presented dose-dependence, showing increased viabilities with the augmentation of the TNPs at 0.1–0.5 mg/mL. These indicated that the TNPs were non-toxic to HepG2 and Caco-2 cells between 0.1–0.5 mg/mL, and promoted their proliferation to some extent. In addition, the cell viability of the T1, T2, and T3 groups was significantly greater than GSH ($p < 0.05$). Particularly, the viability of the cell group treated by T3 had the highest cell viability among the samples of TNP, TNP-4, T1, T2, and T3, reaching 152.87 ± 1.74% for HepG2 cells and 139.82 ± 3.60% for Caco-2 cells at 0.5 mg/mL, efficiently promotional cell proliferation.

Cytoprotective Effects on Cell Damage Induced by H_2O_2. Protection of TNPs Against Oxidative Stress in Cells

The cytoprotective potentials of TNPs was further demonstrated by testing the viability of HepG2 and Caco-2 cells cultured in various H_2O_2 concentrations. The results showed that the cell viability reduced

to 52.05 ± 2.33% and 52.29 ± 1.15% for HepG2 and Caco-2 cells, respectively, after treatment with 1 mM H_2O_2 for 2 h, which were close to 50%. And it was indicated that the cell viability of HepG2 cells and Caco-2 cells damage groups were 50.99 ± 2.02% and 50.49 ± 1.37%, respectively (Fig. 4A and B). The oxidative damage model induced by H_2O_2 was successfully established.

As shown in Fig. 4A and B, the cell viability of the tiger nut peptide-treated groups, (especially TNP-4, T1, T2, and T3) increased compared with the damage groups. This demonstrated that the peptides obtained from tiger nut could protect the HepG2 and Caco-2 cells against oxidative stress, and the cytoprotective effect enhanced with peptide concentration increasing, showing a dose-dependent pattern. The TNP had the weakest protective effect among the tiger nut peptide samples, similar to GSH. After ultrafiltration, gel separation, and purification, the purity of peptides increased, accompanied by decreased molecular weight, and the protective effect of peptide samples on the cells enhanced. This trend was consistent with the results of cytotoxicity assay in Fig. 3. And the T3 exhibited the strongest cytoprotective effect, significantly higher than that of GSH, TNP, TNP-4, T1, and T2 ($p < 0.05$), increasing the viability of HepG2 cells and Caco-2 cells by 17.87% and 15.67% at 0.5 mg/mL, respectively. The protective effect of TNPs (TNP-4, T1, T2, T3) against oxidative damage was more pronounced than that of GSH, probably as a consequence of the difficulty of GSH in crossing the cell membrane[32]. These results suggested that the novel peptides obtained from tiger nut could adequately protect cells against oxidative damage.

Morphological Observation

The morphology of H_2O_2-induced HepG2 cells and Caco-2 cells models treated by TNP, TNP-4, T1, T2, T3, and GSH was presented in Fig. 4C and D. The damage group showed few surviving cells by H_2O_2, and most of the cells disintegrated and floated in the medium. However, it was obvious that the peptides from tiger nut and GSH efficiently protected the HepG2 cells and Caco-2 cells against H_2O_2-stimulated oxidative damage. Particularly, the cells by TNP-4, T1, T2, and T3 showed low apoptosis, and presented similar morphology to those in the control group including clear cell outlines and similar shapes and sizes, suggesting an excellent cytoprotective effect of the peptides. These were consistent with their protective effect results of cellular oxidative stress.

CAT, SOD, GSH-PX Activities, and MDA, ATP, ROS Contents.

The H_2O_2 is a common inducer of oxidative stress and can cause damage through the Fenton reaction[33]. MDA, a lipid peroxidation product, can indirectly assist in demonstrating the degree of oxidative damage triggered by ROS. The previous researches have demonstrated that intracellular SOD, GSH-PX, and CAT activities can be enhanced by bioactive peptides derived from food-sourced proteins, hence providing cell protection[31,34], with increased enzyme activity, increased cellular activity, and increased energy production. Consequently, the present study sought to evaluate cellular ROS levels, SOD, GSH-PX, and CAT activities, and MDA and ATP contents.

As illustrated in Fig. 5, the activity of HepG2 and Caco-2 cells decreased to 50.98 ± 2.02% and 50.49 ± 1.3%, respectively, in comparison to the control group. The activities of SOD, GSH-PX, and CAT were significantly reduced after treatment with 1 mM H_2O_2 for 2 h, the ROS level and MDA content were significantly increased ($p < 0.05$). This suggested that the cells were severely damaged due to intense oxidation by H_2O_2. In comparison to the damage group, after preincubation of the cells with 0.1–0.5 mg/mL TNPs and GSH for 24 h and then oxidatively damaged, the enzyme activities of cells increased gradually, showing a dose-dependent pattern, while ROS level and MDA content decreased gradually. These intracellular oxidative stress indicators correspond to cell viability as described in section 3.4.2.1, indicating that cell viability is regulated by these endogenous antioxidant enzymes. The phenomenon wherein T1, T2 and T3 outperformed TNP-4 confirmed the characteristics of G-15 purification. These results implied that the peptides from tiger nut, especially T3, has excellent protective effects on both cells, could perform cytoprotective effects against oxidative damage by enhancing intracellular enzyme activity and lowering oxidative stress levels.

In this study, novel antioxidant peptides were obtained from tiger nut, and their chemical antioxidant activity and cytoprotective functions on H_2O_2-mediated oxidative injured HepG2 and Caco-2 cells were investigated for the first time. The crude peptides obtained by Alcalase hydrolysis of tiger nut were separated by ultrafiltration into four fractions with different molecular weights of TNP-1 (> 10 kD), TNP-2 (3–10 kD), TNP-3 (1–3 kD), and TNP-4 (< 1 kD) which was further classified into three molecular weight classes (T1, T2, and T3) by fractionation. The tiger nut peptides performed strong antioxidant activity compared with traditional soybean and peanut peptides. And the antioxidant activity of the peptides improved greatly by gradual ultrafiltration and fractionation, showing a negative correlation with their molecular weight. Among the tiger nut peptides, the T3 fraction demonstrated excellent antioxidant capacity at both *in vitro* and cellular levels, displaying strong DPPH RSA and FRAP, and promoting and protective effects on H_2O_2-injured HepG2 and Caco-2 cells by augmenting endogenous antioxidant enzyme activities and reducing oxidative stress levels. The findings provided new insights into tiger nut peptides, and confirmed their free radical-scavenging and cellular-antioxidant activity. This study demonstrated the potential of tiger nut as a promising new source of antioxidant peptides for food industry.

Materials and methods

Materials

Tiger nuts were obtained from a local market in Hengshui City (Hebei, China). Alcalase (200U/mg) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). DPPH was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). ABTS and MTT kit were purchased from Beijing Solarbio Technology Co., Ltd. (Beijing, China). HepG2 cells, Caco-2 cells, FBS (10% [v/v]), and MEM were purchased from Changsha Abiowell Biotechnology Co., Ltd. (Changsha,

China). Penicillin–streptomycin (1% [v/v]) were purchased from Shanghai Beyotime Biotechnology Co., Ltd. (Shanghai, China). Other chemicals used were analytical grade.

Preparation and Experimental Optimization of Tiger Nut Peptides (TNP)

Preparation of TNP

TNP was prepared from tiger nuts according to the method described by Alemán et al[35]. with modification. Briefly, the defatted tiger nut meal was filtered through a 100-mesh standard sieve, combined 1:6 (powder:solution, w/v) with deionized water, and the protein solubility was determined using the Coomassie Brilliant Blue between pH 7.0–11.0. The pH was determined to be 9.0 in conjunction with the enzymatic conditions for Alcalase. Hydrolysis was then carried out, then centrifuged by a centrifuge (HC-3018, Anhui USTC Zonkia, China) at 7156×g for 20 min at 25 ℃ to collect the aqueous phase, which was freeze-dried. The degree of hydrolysis (DH) and DPPH radical scavenging activity (RSA) are crucial parameters for evaluating the efficiency of TNP preparation. And a single factor experimental design was conducted to optimize the preparation process (Table S1), with DH and DPPH RSA as dependent variables.

Degree of Hydrolysis (DH)

The pH-stat method was employed to assess the DH of bean meal. The DH of TNP was calculated by Eqs. (1) and (2). The initial pH of enzymatic hydrolyzed was 9.0. The pH was kept stable by adding 1 mol/L NaOH solution dropwise every hour. The DH of bean flour was calculated according to the equation by recording the amount of alkali consumed throughout the process (mL)[23].

$$\mathrm{DH}\backslash:(\%)=\frac{\mathrm{V}\times\mathrm{C}}{\mathrm{M_P}\times\mathrm{H_{tot}}\times\alpha}\times100\%$$

1

$$\alpha=\frac{10^{\mathrm{pH\text{-}pK}}}{10^{\mathrm{pH\text{-}pK}}+1}$$

2

where V is the NaOH volume (mL), C is the concentration of NaOH (mol/L), M_p is the mass (g) of the protein (N×6.25), a means the average level of amino group dissociation; H_{tot} means the number of peptide bonds per gram of protein substrate (mmol), and pK was determined using the 7.0.

DPPH Radical Scavenging Activity (RSA)

The samples (0.5 mL) were mixed with 1.0 mL DPPH solution (0.2 mmol in absolute ethanol) and 0.5 mL deionized water, and kept in the dark for 30 min. The DPPH solution was replaced by anhydrous ethanol as the blank control. The absorbance was measured at 517 nm, and the DPPH RSA was calculated by Eq. (3)[3].

DPPH RSA (%) = $\left(1-\frac{A_1-A_2}{A_0}\right)\times 100$ (3)

where A_1 represents the value of samples, A_2 represents the value of samples mixed with absolute ethanol, and A_0 represents the value of DPPH solution mixed with absolute ethanol.

Basic Functional Properties

Preparation of Tiger Nut Protein

The tiger nut meal (100 mesh) was dispersed with deionized water with pH adjusted to 9.0 at a ratio of 1:8 (powder: solution, w/v). The obtained mixture was stirred by a magnetic stirrer (DF-101S, Gongyi Yuhua, China) at 40 ℃ for 1 h, centrifuged at 4025×g for 10 min, and the supernatant was precipitated at pH 4.5 for 1 h. The precipitate was then mixed with deionized water and adjusted to pH 7.0 after centrifuging, and freeze-dried to obtain tiger nut protein.

Water and Oil Holding Capacity

The samples (0.1 g) were mixed with 1 mL of deionized water or soybean oil, with the mass as m_1 and the centrifuge tube mass as m_0, standing at room temperature for 10 min, then centrifuged at 1789×g for 10 min to collect the precipitate. The total weight was recorded as m_2. Water-holding capacity (WHC) and oil-holding capacity (OHC) were expressed as grams of water or oil retained per gram of protein and calculated by Eq. (4)[36].

WHC/OHC (g/g) = $\frac{m_2-m_1-m_0}{m_1}$ (4)

Solubility

The samples (20 mg/mL) were mixed thoroughly and shaken for 1 h. The protein content of the supernatant was determined with the Biuret method after centrifuging at 7156×g for 5 min at 25 ℃. The solubility was calculated by Eq. (5).

Solubility (%) = $\frac{C_1}{C_2}\times 100\%$ (5)

where C_1 is the content of protein in the supernatant, C_2 is the content of protein in the original solution.

Foaming Properties

The foaming capacity (FC) and foaming stability (FS) were measured according to the method described by Wang et al[20]. The samples (10 mg/mL) were transferred 20 mL into measuring cylinders and homogenized in a homogenizer (S10, Shanghai Zigui, China) for 2 min. FC and FS were calculated by Eqs. (6) and (7).

FC (%) = $\frac{V_1-V_0}{V_0}$ (6)

FS (%) = $\frac{V_1-V_2}{V_1-V_0}$ (7)

where V_0 (mL) is the volume of sample before foam, V_1 (mL) and V_2 (mL) are the foam volumes at 0 min and 30 min, respectively.

Emulsifying Properties

The samples (10 mg) were dissolved with 5 mL of deionized water in a tube, and 5 mL of soybean oil was then added, with the height recorded as H_0 (cm). After homogenizing for 30 min, the samples were centrifuged at 1789×g for 5 min at 25°C, and the emulsion layer height was recorded as H_1 (cm). And the emulsion layer height of the sample treated by heating at 80°C for 30 min and centrifuging was recorded as H_2 (cm). The emulsibility and emulsion stability were determined by Eqs. (8) and (9), respectively.

Emulsibility (%) = $\frac{H_1}{H_0}$ (8)

Emulsion stability (%) = $\frac{H_2}{H_1}$ (9)

SDS-PAGE

The SDS-PAGE was performed using a 15% separating gel and 4% stacking gel according to the method described by Sharma et al.[37] and Liu et al.[23] with modification. Peanut protein, Soybean protein, tiger nut protein, and TNP (10 mg/mL) were mixed with the loading buffer (2×) using a vortex (VORTEX-5, Haimen Kylin-Bell Lab, China). Using the Bicinchoninicacid (BCA) method to control the protein loads on SDS-polyacrylamide gels between 30–40 μg. The molecular mass (MW) marker (10–235 kDa, Shanghai Epizyme Biotech Co., Ltd., China) was utilized in the gel. Electrophoresis was performed with 30 min concentrate at 80 V, and followed by 120 V for 1 h. The log (MW) as a function of Rf (migration distance of the protein/migration distance of the dye front) was plotted to calculate the molecular mass of proteins and TNP.

Antioxidant Activity Measurements

The antioxidant activities of the tiger nut peptides were studied by a combined analysis of DPPH radical scavenging abilities (RSA), ABTS RSA, hydroxyl RSA, and ferric ion reducing antioxidant power (FRAP). The TNP was configured into a 20 mg/mL solution and diluted to 1, 5, and 10 mg/mL, respectively. The DPPH RSA measurement was the same as section 2.2.3. The ABTS RSA was assayed following the method of Zhu et al.[7] Their hydroxyl RSA and FRAP were determined using the corresponding kits (Grace

Biotechnology Co. Ltd., China). Ascorbic acid (5 mg/ml) was used as the standard. And the soybean peptides and peanut peptides were used as the control concentration groups, and performed the same treatment to evaluate their antioxidant activities.

Amino Acid Analysis

Amino acid analysis was performed using an automated amino acid analyzer (LA8080, Hitachi High-Tech Co, Ltd., Japan). The samples were weighed, hydrolyzed with hydrochloric acid, fixed, and passed through a 0.22 μm microporous filter membrane.

Ultrafiltration Membrane Classification

The TNP was configured into a 50 mg/mL solution and dialyzed for 24 h using a dialysis bag with a cutoff molecular weight of 100 Da. To ensure the separation effect of ultrafiltration was not obstructed, the treated peptide solution was filtered through a 0.8 μm microporous membrane filtration to remove remaining particles and contaminants. Three ultrafiltration membranes of the ultrafiltration centrifuge tube with molecular weight cutoffs of 1, 3, and 10 kDa (Millipore, USA) were used to divide the solution into four fractions, > 10 kDa (TNP-1), 3–10 kDa (TNP-2), 1–3 kDa (TNP-3), and < 1 kDa (TNP-4), by centrifuging at 1789×g for 30 min at 25 ℃.

Isolation and Purification

The empty column (2.6×70 cm, Ruida Henghui, China) was filled with Sephadex G-15 (HA-0220, Ruida Henghui, China) by wet-natural sedimentation method. The peptides were dissolved, and the column was equilibrated with ultrapure water. The flow rate was maintained at 1.5 mL/min. The absorbance was measured at 220 nm, and elution peaks were collected for antioxidant activity measurement[38].

Cytoprotective Effects on HepG2 and Caco-2 Cells Damage Induced by H_2O_2

Fetal bovine serum (FBS, 10% [v/v]), penicillin-streptomycin (1% [v/v]), and MEM were the ingredients of the culture medium used to produce HepG2 cells, Caco-2 cells needed 20% FBS. The culture conditions were maintained at 5% CO_2 and 37 ℃.

Cytotoxicity Assay

The cell density was kept at 4.5×10^3 cells/well in the 96-well plate and cultured until adherent. The supernatant was then removed, and 100 μL of 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL samples were added for another 24 h cultivation, respectively. Cell viability was measured by MTT solution. The 100 μL DMSO was added after removing the supernatant, and the absorption of the sample was measured at 490 nm to determine the cytotoxicity of the peptides[39].

H_2O_2-Induced Oxidative Stress Assay and Indicator Measurement

The cell culture process was the same as section 2.9.1. And 100 μL of 0.2, 0.5, 1, 1.2, and 1.5 mM H_2O_2 were added into the cells for 2 h, respectively, to establish the oxidative damage model with the cell viability around 50%. The cells were preincubated with the peptide samples for 24 h before adding H_2O_2 to investigate their cytoprotective effects. The ROS (Shanghai Beyotime Biotech. Inc., China), SOD, CAT, GSH-Px, MDA, and ATP content (Nanjing Jiancheng Co., China) were determined by kits detection.

Microscopic Observation of Morphological Change

The cell morphology was observed by an inverted biomicroscope (DSZ2000X, Beijing Cnmicro Hengye, China) at 10× magnification.

Statistical Analysis

All the experiments were measured three times, and the data were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) by Duncan's multiple tests ($p < 0.05$) was conducted via SPSS 26.0 software (SPSS Inc., Chicago, USA).

Abbreviations

TNP: tiger nut peptide; ROS: reactive oxygen species; H2O2: hydrogen peroxide; DH: degree of hydrolysis; pI: isoelectric point; DPPH: 1,1-diphenyl-2-picroyl; ABTS: 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; •OH: hydroxyl radical; FRAP: ferric ion reducing antioxidant power; RSA: radical scavenging ability; WHC: water-holding capacity; OHC: oil-holding capacity; FC: foaming capacity; FS: foaming stability; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase; GSH: glutathione; GSH-PX: glutathione peroxidase; ATP: adenosine triphosphate; MEM: minimum essential medium; FBS: fetal bovine serum; MTT: 3- (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; DMSO: dimethyl sulfoxide.

Declarations

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Author contributions

Yu Quan: Formal analysis, Investigation, Data curation, Visualization, Writing-original draft. Lin Chen: Conceptualization, Writing-review & editing. Meiqi Fan: Software, Writing-review & editing. Xia Zhao: Visualization, Writing-review & editing. Jianxiong Hao: Writing-review & editing, Project administration, Funding acquisition.

Competing interests

The authors declare no competing financial interest.

Data availability

Data will be made available on request.

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Figures

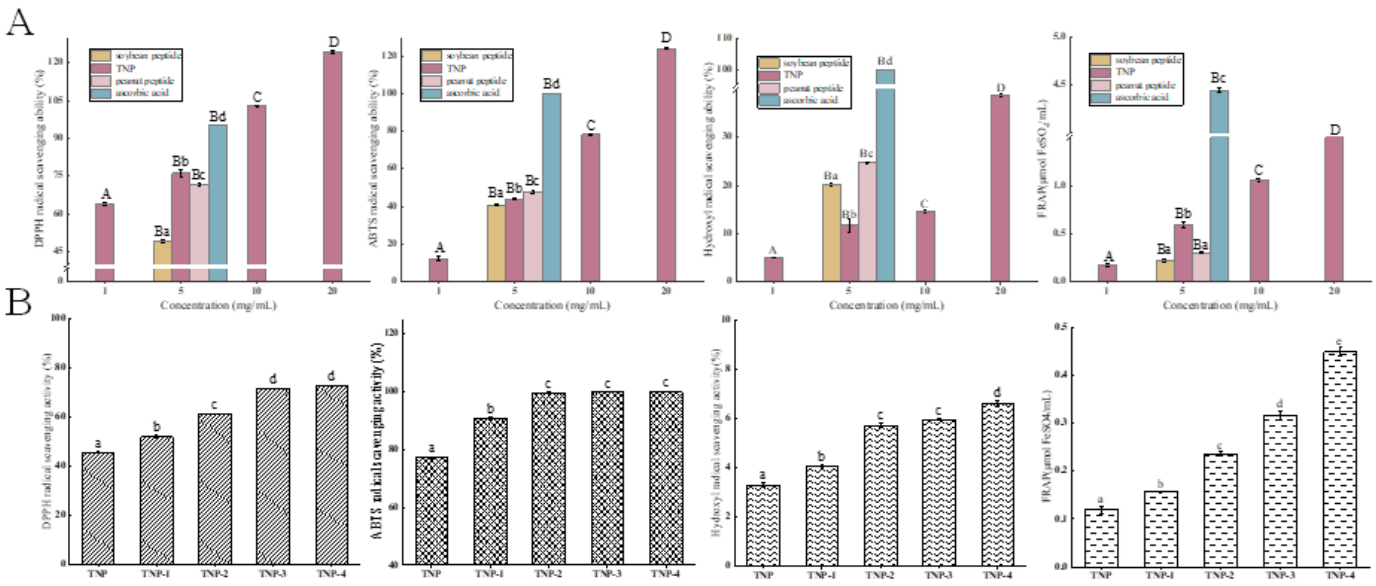


Figure 1

The DPPH radical scavenging activity, ABTS radical scavenging activity, hydroxyl radical scavenging activity, and FRAP of peptides at different concentrations. (A) Antioxidant properties of TNP, soybean peptides, and peanut peptides at the concentrations of 1-20 mg/mL. (B) Antioxidant properties of TNP-1, TNP-2, TNP-3, and TNP-4 at the concentration of 0.8 mg/mL. Different uppercase letters and lowercase letters represent statistically significant differences, respectively ($p < 0.05$).

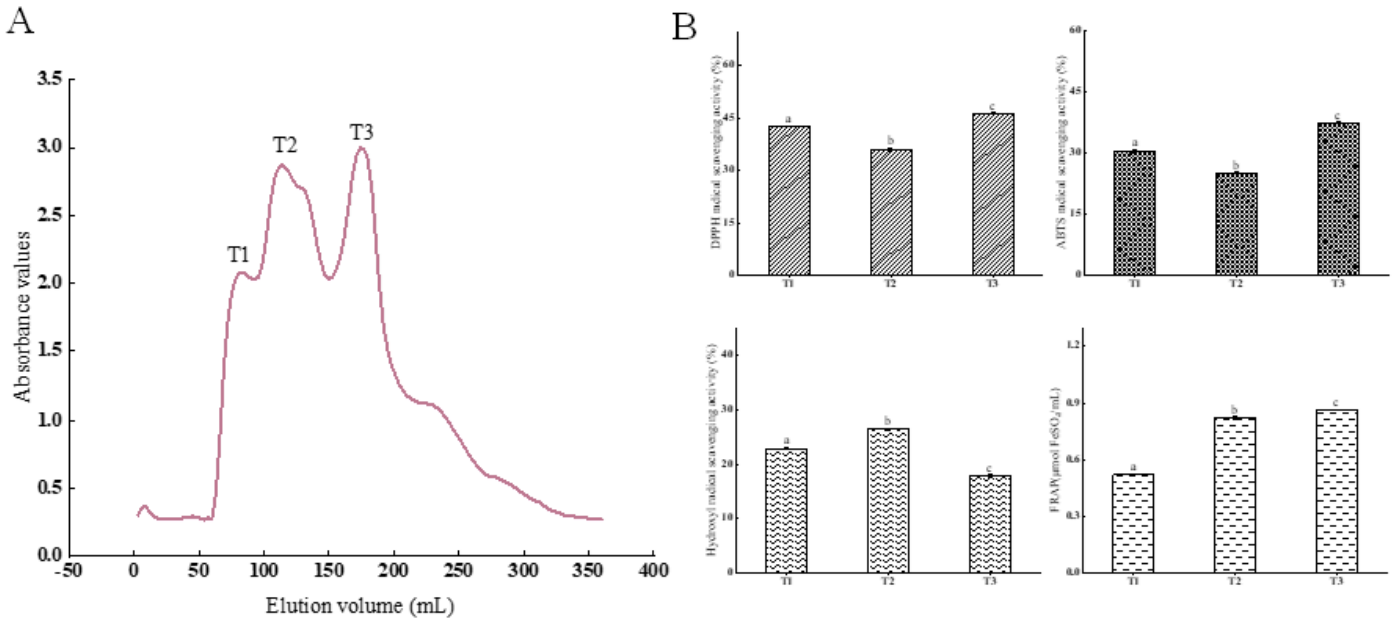


Figure 2

(A) Elution profile of TNP-4 in Sephadex G-15 column. (B) Antioxidant properties of T1, T2, and T3 at the concentration of 0.5 mg/mL. T1, T2, and T3 represent three elution peaks. Different letters represent statistically significant differences ($p < 0.05$).

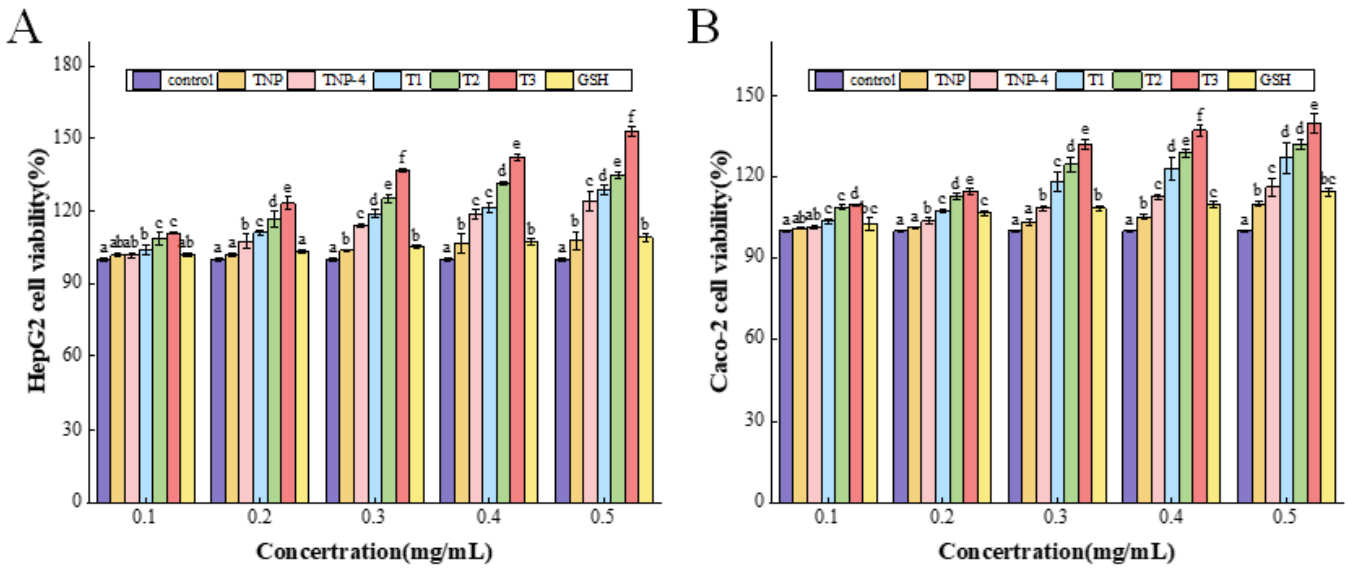


Figure 3

The cytotoxic effect of TNP, TNP-4, T1, T2, and T3 on HepG2 cells (A) and Caco-2 cells (B). Different letters represent significant differences between different samples in the same group ($p < 0.05$).

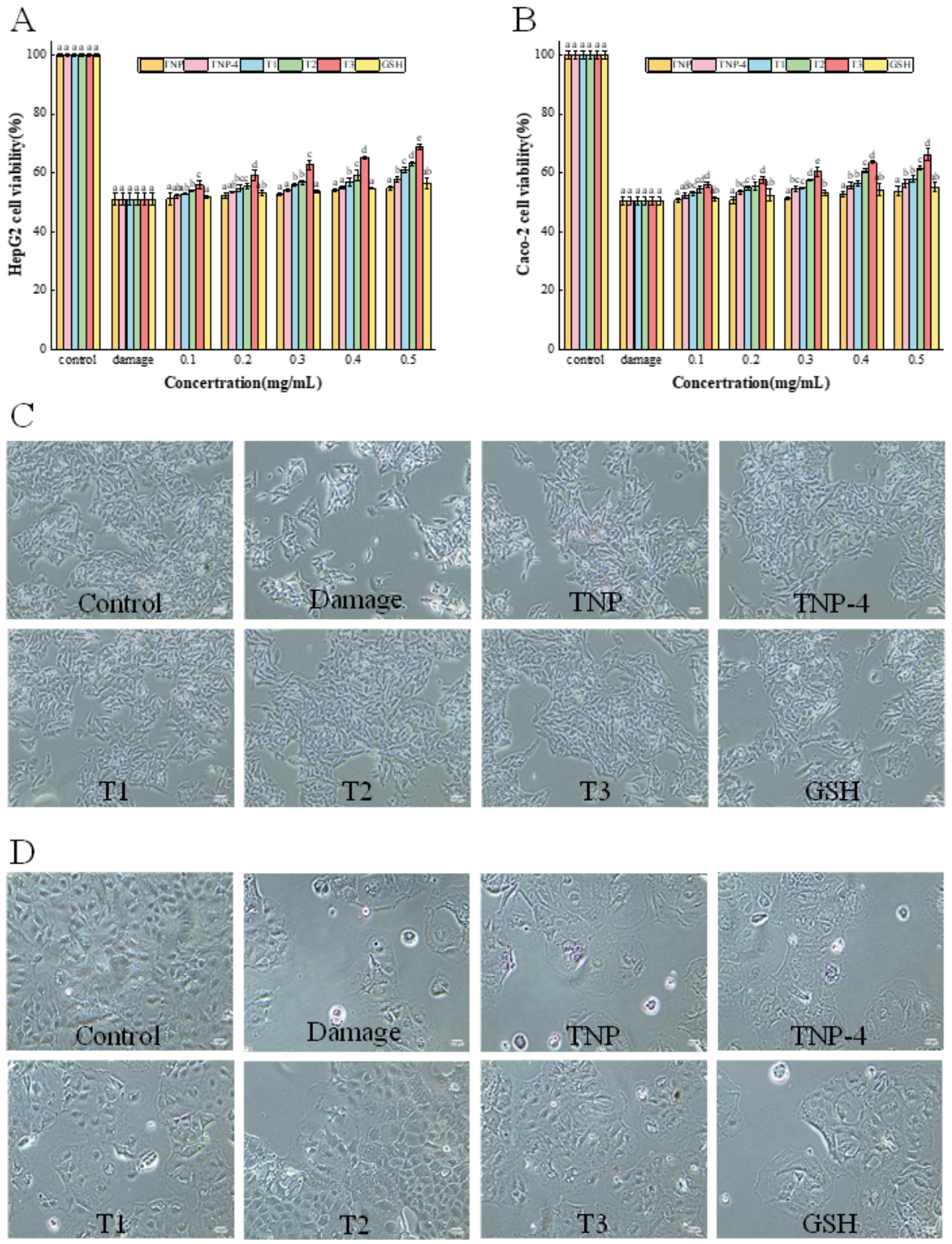


Figure 4

Effect of TNPs on cell viability of HepG2 cells (A) and Caco-2 cells (B) in H_2O_2 induced oxidative stress with the treatment of TNP, TNP-4, T1, T2, T3, and GSH, and their morphological changes (C, HepG2 cells; D, Caco-2 cells). Control group means normally growing cells. Damage group means H_2O_2-induced cells. The remaining groups were treated with peptides at 0.5 mg/mL and induced by H_2O_2. Different letters represent significant differences between different samples in the same group ($p < 0.05$).

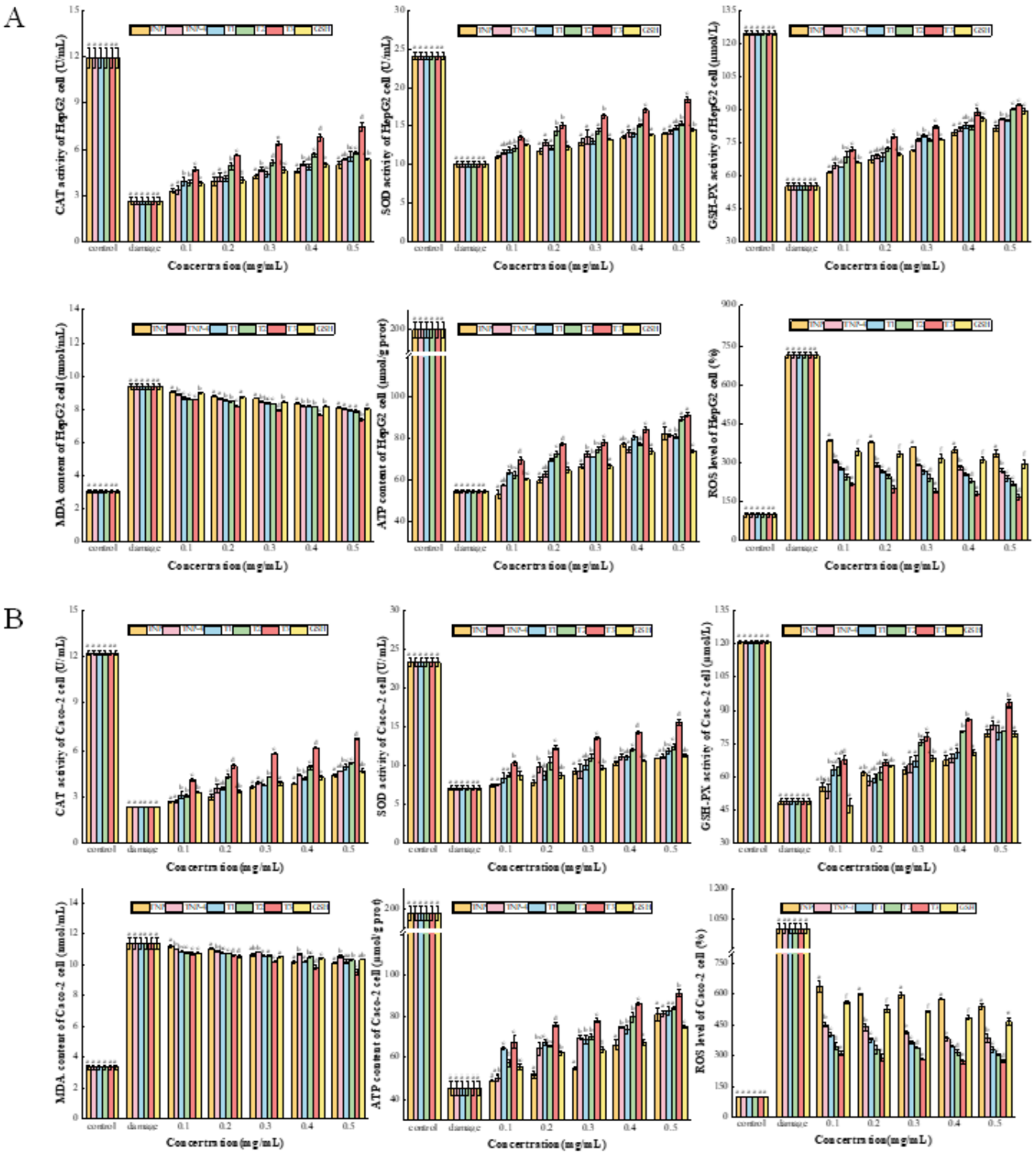


Figure 5

The cellular antioxidant activity analysis containing CAT, SOD, GSH-PX activities and MDA, ATP, ROS contents of H_2O_2-oxidized HepG2 cells (A) and Caco-2 cells (B) with the treatment of TNP, TNP-4, T1, T2, T3, and GSH. Different letters represent significant differences between different samples in the same group ($p < 0.05$).

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