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Research Article

Antioxidant Activities of Protein Hydrolysates from Little Hairtail (*Trichiurus haumela*) of East China Sea

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Abstract: This study investigated antioxidant properties of the little hairtail (*Trichiurus haumela*) protein hydrolysates obtained by commercial protease of *Alcalase* through using various antioxidant assays, including reducing power and free radical scavenging activities. The molecular mass distribution of hydrolysates was also examined to evaluate their relationship with antioxidant activity. The results showed that little hairtail protein hydrolysates had good ability to donate electron or hydrogen and scavenge DPPH, hydroxyl and superoxide anion radicals. The highest value of reducing power and radical scavenging activities was 1.89, 46.15% (DPPH radical), 75.65% (hydroxyl radical) and 82.5% (superoxide anion radical), respectively. The reducing power and free radical scavenging activities of little hairtail protein hydrolysates were related to hydrolysis time to some extent. The molecular mass distribution of hydrolysates showed that their molecular mass was between 337 and 6007Da, which indicated that little hairtail protein hydrolysates were mainly composed of low molecular peptides with antioxidant activity. Conclusively, the little hairtail protein was a good natural source for producing antioxidants, which could be used as antioxidant ingredient with potential applications in various food products.

Keywords: *Alcalase*, antioxidant activity, little hairtail (*Trichiurus haumela*), protein hydrolysates

INTRODUCTION

Reactive Oxygen Species (ROS) including superoxide anion radical, hydroxyl radical, hydrogen peroxide and singlet oxygen are continuously generated within the human body due to the aerobic respiration of organisms (Scalbert *et al.*, 2005). Despite aerobic respiration have significant advantages for the human body, but overproduction of reactive oxygen species has the potential to cause damage because ROS can attack and induce oxidative damage to cellular proteins, lipids, lipoproteins and DNA; finally resulting in cell death (Tuteja *et al*., 2009; Nazeer and Kulandai, 2012). It is well known that some diseases are related to the excessive of free radicals such as neurodegenerative diseases, brain dysfunction, arthrosclerosis, malignancy, diabetes, heart diseases, cancer and declination of the immune system (Aruoma, 1998; Hogg, 1998).

Antioxidants can scavenge redundant free radicals in human body and significantly reduce the damage. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and Propyl Gallate (PG) have been used extensively in the food products (Li *et al*., 2012; Zhou *et al*., 2012). However, the use of synthetic antioxidants in food products in some countries is strictly restricted due to concerns about the potential health risks (Yang *et al*., 2011). Thus, people are increasingly concerned about the development and utilization of more effective antioxidants stem from natural origin. Until now, many studies observed that protein hydrolysates possess antioxidant ability and a wide range of materials are used as protein sources such as soybean protein (Moure *et al*., 2006), wheat germ protein (Zhu *et al*., 2006), fish protein (Ali *et al*., 2009; Je *et al*., 2009), shrimp protein (Guerard *et al*., 2007), etc. It is postulated that the protein hydrolysates contain peptides with antioxidant ability, which can scavenge free radicals and inactivate ROS (Zhou *et al*., 2012; Elias *et al*., 2008). Therefore, maybe it is a good approach to obtaining natural antioxidants from protein hydrolysates.

Hairtail (*Trichiurus haumela*) is a kind of economic fish, distributed extensively in the Indian Ocean and the West Pacific Ocean. The East Sea of China is the main producing area of hairtail in China. The little hairtail refers to hairtail which has small size and low edible value directly and the yield of little hairtail from the East Sea of China increases year and year because of over catch. The protein of little hairtail possess high nutritional value in that it contains eight kinds of essential amino acids of human such as lysine, valine, phenylalanine, methionine, threonine,

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isoleucine, leucine and tryptophan. Thus, the little hairtail is a proper material for producing protein hydrolysates. In despite of many studies on the different kinds of protein hydrolysates with antioxidant activities, there is little research on antioxidant activities of hydrolysates derived from hairtail (*Trichiurus haumela*) protein.

The objective of this study was to use commercial protease of *Alcalase* for the enzymatic hydrolysis of little hairtail (*Trichiurus haumela*) and investigated antioxidant properties of the protein hydrolysates by using various antioxidant assays, including reducing power and radical scavenging activities. In addition, the molecular mass distribution was also examined to evaluate their relationship with antioxidant activity.

MATERIALS AND METHODS

Materials and chemicals: The little hairtail (*Trichiurus haumela*) was captured in East China Sea and kept frozen at -20°C for two weeks before use. *Alcalase* 2.4L^{TM} was purchased from *Novozymes* (Denmark). *Alcalase* 2.4 L was a liquid food grade preparation and has a declared activity of 2.4 Anson U/g (One Anson unit is defined by *Novozymes* as the amount of enzyme which, under specified conditions, digests urea-denatured hemoglobin at an initial rate such that there is liberated an amount of TCA-soluble product per minute which gives the same color with Folin-Ciocalteu Phenol reagent as one milliequivalent of tyrosine at 25°C at pH 7.5). The enzyme was stored at 4°C before used for the hydrolysis experiments. 2, 2-Di (4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Ltd. All other reagents used in this study were of analytical grade, which were purchased from Sinopharm Chemical Reagent Co., Ltd of China.

Preparation of protein hydrolysate: The frozen little hairtail was thawed in 4°C before use. The unfrozen little hairtail of 100 g was cut and mixed with 200 mL deionised water and homogenized in a tissue triturator for 2 min. *Alcalase* 2.4 L was added into the homogenate and the hydrolysis experiments were performed in a shaking incubator at 150 rpm, 55°C, pH 7.5 for 6 h. During the hydrolysis, the sampling was at 1 h, 2 h, 3 h, 4 h, 5 h and 6 h, respectively. The ratio of enzyme to substrate was 0.6%. (The ratio was the proportion (%) of mass of enzymes with respect to mass of raw material of fish.) The hydrolysis reaction was ended by heating the flasks in the thermostat-controlled water bath for 15 min at 95°C to inactivate protease activity. The hydrolysates were centrifuged at 3300×g for 15 min (Himac CR 21G, Japan) and the supernatant of hydrolyzed samples was collected for the determination of antioxidant activity. All experiments were carried out in triplicate.

Reduing power assay: The reducing power of the hydrolysates was determined by the method of Yildirim *et al*. (2001) with some modification. 1 mL sample of different protein concentration was mixed with 2.5 mL phosphate buffer (0.2M, pH 6.6) and 2.5 mL potassium ferricyanide solution (1%, w/v). After the mixtures were incubated at 50°C for 20 min, 2.5 mL trichloroacetic acid (10%, w/v) was added and the reaction mixtures were centrifuged at 3300×g for 10 min. 2.5 mL of the supernatant were mixed with 2.5 mL deionized water and 0.5 mL ferric chloride solution (0.1%, w/v). After 10 min reaction, the absorbance of the solution was measured at 700 nm using a spectrophotometer. The control test was carried out in the same manner, except for using deionized water replacing the sample. All experiments were carried out in triplicate.

DPPH radical scavenging activity assay: The DPPH radical scavenging activity of the hydrolysates was determined according to the method of Shimada *et al*. (1992) with some modification. 2 mL sample solution was mixed with 2 mL of 0.1 mM DPPH dissolved in 95% ethanol. The mixture was shaken equally and incubated at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. The control was conducted in the same manner, except that deionized water was used instead of sample. All experiments were carried out in triplicate. DPPH radical scavenging activity was calculated as:

$$RSA(\%) = \frac{A_C - A_S}{A_C} * 100$$

where,
A_C : Absorbance of control
A_S : Absorbance of sample

Hydroxyl radical scavenging activity assay: The Hydroxyl radical scavenging activity of the hydrolysates was determined according to the method of Li *et al*. (2008) with some modification. After 1 mL 1, 10-phenanthroline (5 mM) was mixed thoroughly with 2 mL PBS (0.2 M, pH7.4) and 1 mL FeSO4 (5 mM), 1 mL H_2O_2 (0.1%, w/v) was added and the mixture were incubated at 37°C for 1 h. The absorbance of the solution was measured at 536 nm using a spectrophotometer and the result was recorded as A_P. The sample or control assay was conducted in the same manner; except that 1mL sample or deionized water was add into the mixture and the absorbance was recorded as A_s and A_c, respectively. All experiments were carried out in triplicate. Hydroxyl radical scavenging activity was calculated as:

$$RSA(\%) = \frac{A_S - A_P}{A_C - A_P} * 100$$

Superoxide anion radical scavenging activity assay: The superoxide anion scavenging activity was measured according to the method described by Yang *et al*. (2011) with some modification. The reaction mixture consisted of 4.5 mL of 50 mM Tris-HCl buffer (pH 8.2) and 4.2 mL deionized water or 3.2 mL deionized water and 1 mL sample. The mixture solutions were preincubated at 25°C for 20 min. The reaction was initiated by the addition of 0.3mL of 3mM 1, 2, 3-trihydroxybenzene (dissolved in 10 mM HCl). The absorbance at 320 nm was recorded every 30s for 4 min. Superoxide anion radical scavenging activity was calculated as:

$$RSA(\%) = \frac{V_0 - V_1}{V_0} * 100$$

V_0 and V_1 are the autoxidation rate of 1, 2, 3-trihydroxybenzene (ΔOD min^{-1}) without or with sample, respectively.

Molecular mass distribution of hydrolysates: Molecular mass distribution of freeze dried hydrolysates with antioxidant activity was determined by size exclusion chromatography. The determination was performed by a Sephadex G-75 column (length 50 cm, diameter 1 cm). The solution of 50 mmol/L PBS (pH 6.98) containing 0.15 mol/L NaCl was chosen as eluent and the flow rate was 0.5 mL/min. The protein concentration of loaded sample was 6.0 mg/mL. Detection was done using a UV monitor at 280 nm. The standards used were bovine serum albumin (M_r = 67000), chymotrypsinogen A (M_r = 25000), lysozyme (M_r = 14400) and vitamin B_{12} (M_r = 1355).

RESULTS AND DISCUSSION

Reduing power: Effect of hydrolysis time on reducing power of hydrolysate was shown in Fig. 1. The reducing power of hydrolysate increased gradually with the time until achieving the maximum value of 1.89 at 5 h and then the reducing power decreased. The reducing power was often used to evaluate the ability of an antioxidant to denote electron or hydrogen[11]. Many researches had revealed that there was a direct correlation between antioxidant activities and reducing power (Li *et al*., 2008; Yu *et al*., 2002). The reducing power of hydrolysate was determined by reducing ferric iron to ferrous iron using colorimetric reaction at 700 nm. It had been widely accepted that the higher the absorbance at 700 nm, the greater the reducing power (Yang *et al*., 2011). This result indicated that the numbers of peptides with strong reducing power produced by cleavage of *Alcalase* was up to maximum at 5 h. However, excessive proteolysis leading to the release of shorter peptides and shorter peptides lost antioxidant activity gradually.

DPPH radical scavenging activity: The ability of little hairtail hydrolysate scavenging DPPH radical was shown in Fig. 2. The result indicated that the DPPH radical scavenging activity of hydrolysate increased gradually with the time and the highest value was up to 46.15% when hydrolysis time was at 4 h. The data was closer that of report by Yust *et al*. (2012), who found that hydrolysis of chickpea protein with *Alcalase* increased the DPPH scavenging effect up to 50%. DPPH was a stable free radical which had been widely used to investigate the scavenging activity of antioxidants had the maximum absorbance at 517 nm. When DPPH radical encountered a proton-donating substrate such as an antioxidant, the radicals were scavenged and the absorbance was reduced (Yust *et al*., 2012). *Alcalase* was a kind of endoprotease of serine type, whose main composition was subtilisin Carlsberg. *Alcalase* could hydrolyze peptide bonds inside the protein and produce peptides. The results obtained suggested that some peptides within little hairtail

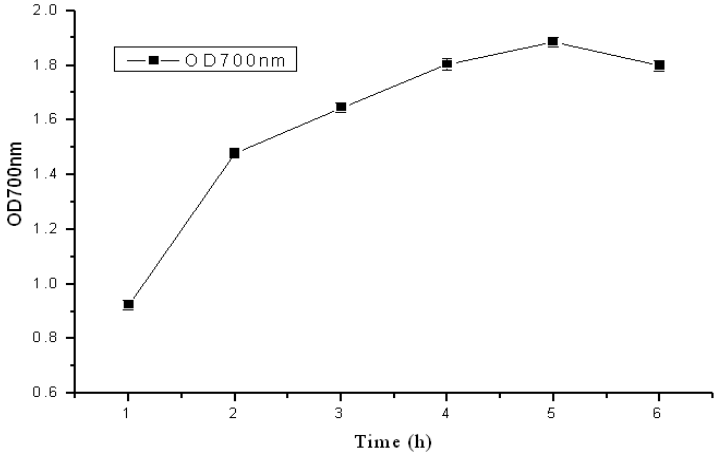

Fig. 1: Reducing power of little hairtail hydrolysate

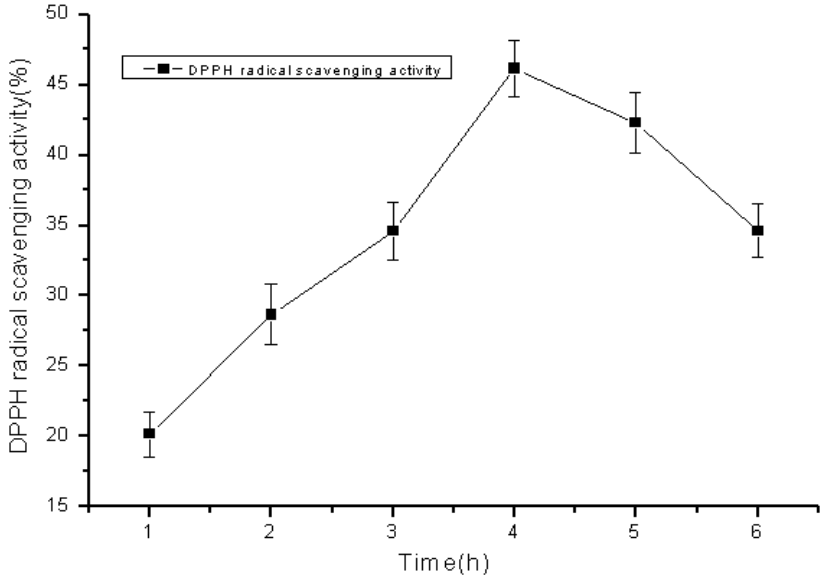

Fig. 2: DPPH radical scavenging activity of little hairtail hydrolysate

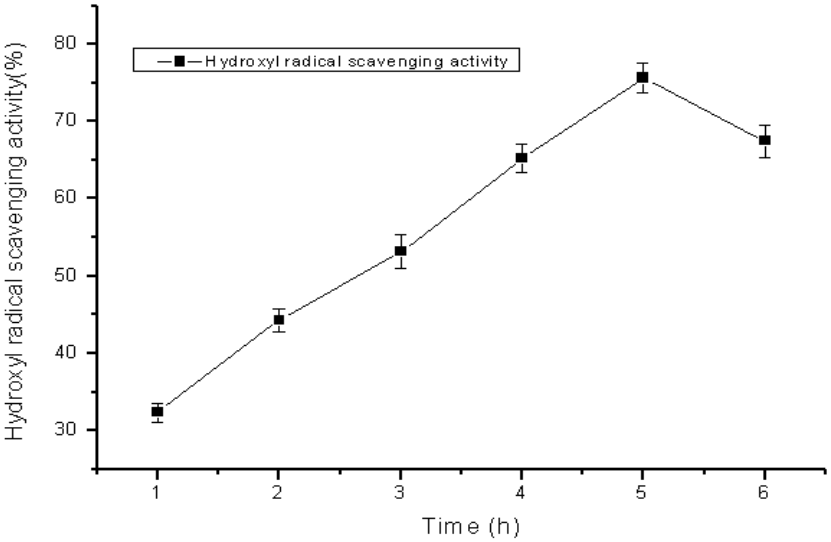

Fig. 3: Hydroxyl radical scavenging activity of little hairtail hydrolysate

protein hydrolysates were significantly strong DPPH radical scavengers.

Hydroxyl radical scavenging activity: The ability of little hairtail hydrolysate scavenging hydroxyl radical was shown in Fig. 3. The result indicated that the hydroxyl radical scavenging activity of hydrolysate increased gradually with the time and the highest value exhibited 75.65% of scavenging activity on hydroxyl radicals when hydrolysis time was at 5 h. The hydroxyl radical scavenging activity of was closer to that of chickpea protein hydrolysate (Fra.IV 81.39%) reported by Li *et al*. (2008). It was well known that the hydroxyl radical was the most reactive among the oxygen radicals and severely damaged biomolecules such as all proteins, DNA, polyunsaturated fatty acid, nucleic acid *et al*., which resulted to aging, cancer and other diseases (Pan *et al*., 2011). Thus, it was necessary to scavenge hydroxyl radical from a living body for defending against diseases.

Superoxide anion radical scavenging activity: The ability of little hairtail hydrolysate scavenging superoxide anion radical was shown in Fig. 4. All samples showed considerable scavenging abilities on superoxide anion radical and the highest value reached 82.5% of scavenging activity. The result was higher than that of Xie *et al*. (2008), who reported that superoxide anion scavenging activity of Alfalfa Leaf Peptides (ALPs) reached 66.96%. Superoxide anion

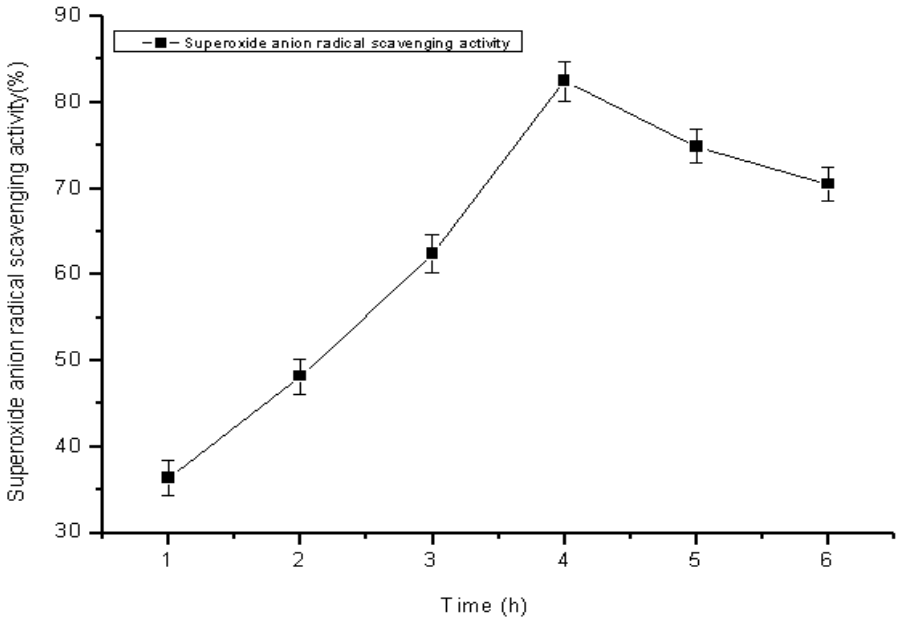

Fig. 4: Superoxide anion radical scavenging activity of little hairtail hydrolysate

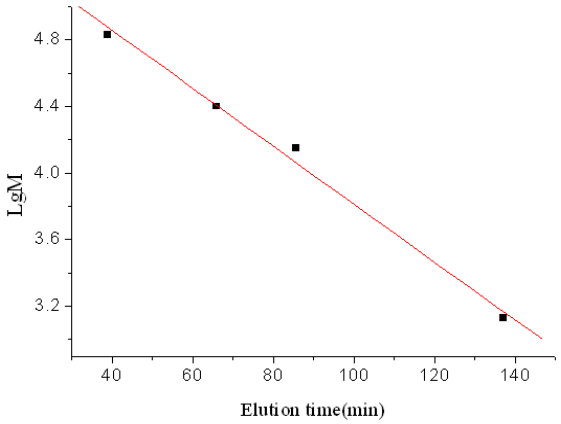

Fig. 5: Relation between l g (molecular mass) and elution time for four standards. Detection was done using a UV monitor at 280 nm.

radical was normally produced first in cellular oxidation reactions. Although superoxide anion radical could not initiate lipid oxidation directly, it caused the formation of hydroxyl radicals and singlet oxygen, which could damage the cells in a living body (Yang *et al*., 2011). Therefore, it was important to evaluate the scavenging effects of an antioxidant on superoxide anion radical.

Since the antioxidant mechanisms are diverse, the antioxidant activities could be measured with different assays. In order to evaluate accurately the antioxidant activity of a selected antioxidant, a system should be better characterized by using different assays based on different mechanisms (Moure *et al*., 2006).

In this research, we have employed one method of reducing power assay and three methods of radical scavenging capacity assays to evaluate the antioxidant activity of little hairtail hydrolysates. The results showed that little hairtail hydrolysates had good reducing power and free radicals scavenging capacities and it could be a potential source of natural antioxidant.

Molecular mass distribution of freeze dried hydrolysates: The relation between l g (molecular weight) and elution time for four standards was shown in Fig. 5 and the equation was as follows:

$$\lg M = 5.55037 - 0.01737t \text{ (R = -0.99648)}$$

where,
M : Molecular mass (Dalton)
T : Elution time (min)

The molecular mass distribution of freeze dried hydrolysates with antioxidant activity was determined by size-exclusion chromatography and the result was shown in Fig. 6. It was observed that the elution time of peak was from 102 to 174 min from Fig. 6. According to the standard curve equation, the molecular mass of freeze-dried hydrolysates was between 337 and 6007Da, which indicated that little hairtail hydrolysates were mainly composed of low molecular peptides with antioxidant activity. Many studies about the fish protein hydrolysate had already shown that the antioxidant activity of hydrolysate was related to their molecular mass distribution. Yang *et al*. (2011) reported that the molecular weight of the main peaks of the tuna head protein hydrolysate (70.5%) was lower than 5000Da. Yang *et al*. (2009) reported that the tilapia retorted skin

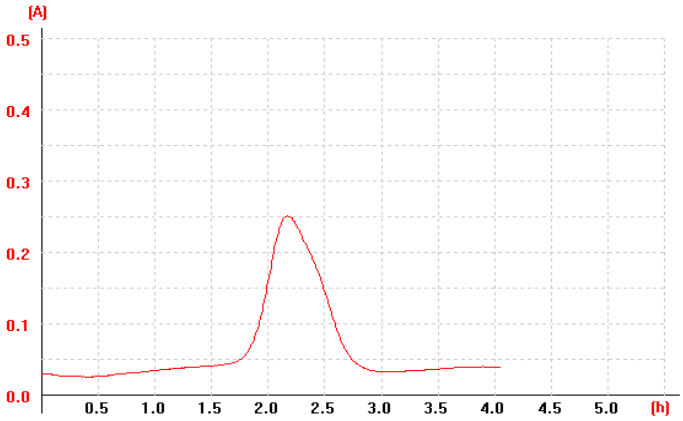

Fig. 6: The chromatogram of size-exclusion chromatography of freeze dried hydrolysates

gelatin hydrolysates whose molecular weights were below 6500Da possessed greater antioxidative activities. These findings indicated that antioxidant activities of protein hydrolysates were highly influenced by their molecular mass.

CONCLUSION

This study examined antioxidant properties of the little hairtail protein hydrolysates obtained by *Alaclase*. The results revealed that little hairtail protein hydrolysates had good ability to donate electron or hydrogen and scavenge DPPH, hydroxyl and superoxide anion radicals. The highest value of reducing power and radical scavenging activities was 1.89, 46.15% (DPPH radical), 75.65% (hydroxyl radical) and 82.5% (superoxide anion radical), respectively. The reducing power and free radical scavenging activities of little hairtail protein hydrolysates were related to hydrolysis time to some extent. The molecular mass distribution of hydrolysates showed that their molecular mass was between 337 and 6007Da, which indicated that little hairtail protein hydrolysates were mainly composed of low molecular peptides with antioxidant activity. The results obtained in this study clearly suggested that little hairtail protein was a good natural source for producing antioxidants, which could be used as antioxidant ingredient with potential applications in various food products. It was necessary that further studies on isolation and purification of antioxidant peptide from little hairtail protein hydrolysates and mechanisms of its antioxidant activities should be carried out.

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