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Original Article

Antioxidant activity of the simulated gastro-intestinal digestion hydrolysate of two edible Nigerian marine molluscs: *Tympanatonus fuscatus* var radula (L.) and *Pachymelania aurita* (M.)

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Abstract: The multifunctional nature of antioxidant peptides makes them more attractive candidates as dietary ingredients in health maintenance. Therefore, food protein-derived antioxidant peptides are continuously investigated. This study investigated the *in vitro* antioxidant properties of hydrolysate and ultrafiltered peptide fractions of *Pachymelania aurita* and *Tympanatonus fuscatus* var radula-two commonly consumed marine molluscs known as periwinkles in southern Nigeria. Simulated gastrointestinal digestion (SGID) of soluble proteins of *T. fuscatus* and *P. aurita* was carried out using pepsin, trypsin and chymotrypsin, and the SGID hydrolysates were fractionated using a 3 kDa membrane filter. The hydrolysates and their fractions were investigated for anti-lipid peroxidation, hydroxyl radical scavenging activity (HRSA), ferric reducing antioxidant property (FRAP) and metal chelation activity, and they demonstrated clear antioxidant properties in all the assay models used. Low molecular weight fractions of the hydrolysates demonstrated more potent antioxidant activity than higher molecular weight fractions. This is profound in the metal chelation assay, where low molecular weight peptide fractions, T ≤ 3 kDa and P ≤ 3 kDa (IC_{50} values of 8.10 ± 0.011 and 5.56 ± 0.50 µg/ml respectively) had activity that is similar to that of EDTA (11.84 ± 0.89 µg/ml). Similar activity effects were observed in other assays where there was about 3-fold higher activity in low molecular weight fractions. These results demonstrate the presence of antioxidant peptide(s) in the protein hydrolysates of the periwinkles.

Keywords: Antioxidant peptides, Nigerian periwinkle, *Tympanatonus fuscatus*, *Pachymelania aurita*, hydrolysate

Introduction

Free radicals are defined as atoms, molecules or ions that have unpaired valence electron [1]. The majority of free radicals encountered within the body are: reactive oxygen species (ROS) such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$) and non-free-radical species such as H_2O_2 and singlet oxygen ($^1O_2$); and reactive nitrogen species (RNS) such as nitrite and peroxynitrite. At a physiological concentration, and in appropriate sub-cellular location, ROS/RNS can act as potent signaling molecules to facilitate myriads of biological events. However, when the levels of free radicals surpass the tolerance of the cellular antioxidant defense system, so much so that, the free radicals are not efficiently detoxified, then oxidative stress results, leading to the progress of multiple metabolic diseases, such as hypertension, respiratory cardiovascular disease, type-2 diabetes, neurodegenerative diseases, and certain types of cancer [2].

Usually, there are panels of both enzymatic and non-enzymatic antioxidant mechanisms that maintain a normal physiological oxidative state in the body, whose activities can be augmented by the dietary antioxidants. As such, food and food supplements with excellent antioxidant properties are continuously being evaluated for their potentials in health maintenance, in ame-

liorating the harmful effects of oxidative stress. Although synthetic chemical antioxidants have shown strong antioxidant capacity, and profound application in industries to retard lipid oxidation [3], they are not recommended for consumption as food additives without restriction, because of the associated safety concerns relating to their use [4]. Therefore, screening of antioxidant compounds from natural sources, particularly, from food products has become imperative, and has since continued to attract significant research efforts. Dietary antioxidant peptides have quite attractive features such as: high membrane permeability, easy absorption, no tissue accumulation and fast clearance from the blood, that make them promising antioxidant agents [5]. These bioactive peptides are most often than none, latently encrypted within protein structure, which may be released during *in vivo* digestion, *in vitro* enzymatic hydrolysis or food processing steps, including microbial fermentation, germination, and ripening [6]. Indeed, enzymatic protein hydrolysis is a preferred means of producing bioactive peptides, because of the specificity of the enzyme(s) involved, and also the ability of the investigator to exert control over the degree of hydrolysis, thereby making the process reproducible. Classical metabolism of protein suggests that food-derived proteins and peptides are susceptible to arrays of proteolytic modifications during gastrointestinal digestion, before they get absorbed and transported to the site(s) where they can perform their physiological roles. Therefore, biologically active peptides, such as antioxidant peptides, must be able to survive gastrointestinal proteolytic degradation before they can exhibit any *in vivo* physiological activity. It was on this premise that the crude protein extracts in this study were subjected to the simulated gastrointestinal digestion (SGID), using the three prominent gastrointestinal tract proteases, and after which the resulting hydrolysates were separated based on size, using membrane filter. There is quite an increasing body of evidence revealing the production of antioxidant peptides from the enzymatic hydrolysis of food proteins. Antioxidants have been characterized from fish [7], marine rotifer, *Brachionus rotundiformis* [6], bambara bean protein hydrolysates [8], marine bivalve mollusc *Tergillarca granosa* [9] and Irish brown macroalgae [10]. More specifically, an exhaustive review of bioactive peptides from marine molluscs has also been published [11].

Tympanotonus fuscatus var radula and *Pachymelania aurita* are two species of marine molluscs referred to as mudwhelks, commonly called Nigerian periwinkles, which are consumed as staple protein source in south southern Nigeria. The antimicrobial [12], antiproliferative [13] and angiotensin I converting-enzyme inhibitory potentials of the extracts of these molluscs have been reported. Two main chemical mechanisms that are used to assess antioxidant capacity of natural antioxidants are the inhibition of: electron migration (EM) and hydrogen atom transfer (HAT). Some HAT-based reactions include; the Oxygen Radical Absorbance Capacity (ORAC) assay; the Total Radical Trapping Antioxidant Parameter (TRAP) assay; and the Lipid Peroxidation Assay (LPA), while EM-based redox reactions include; 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity; Ferric reduction antioxidant property (FRAP); transient metal ion chelation (which can inhibit Fenton reaction that produces hydroxyl radical) and hydroxyl radical scavenging assay [14]. Our preliminary studies on 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging potentials of the extracts of these molluscs, revealed a robust DPPH scavenging ability of the extracts (data not shown). This necessitated the need to investigate the antioxidant potentials of the simulated gastrointestinal digestion hydrolysates and their ultra-filtered fractions obtained from the two marine molluscs-*Tympanotonus fuscatus* var radula and *Pachymelania aurita*.

Materials and methods

Sample collection

Live *Tympanotonus fuscatus* var radula and *Pachymelania aurita* were purchased from the Oron Beach Market, Oron, Akwa Ibom State, Nigeria (GPS coordinates: 4°49'37.6'' N 8°14'04.4'' E).

Sample collection and extract preparation

The flesh and hemolymph of each mollusc were homogenised in 0.1 M phosphate buffered saline (pH 7.2) at a ratio of 1:3 (w/v). The homogenates were left at 4°C to extract for 24 hr with intermittent swirling (at 2 hr interval) after which the supernatants were collected by centrifugation using a tabletop centrifuge (Microfield Instruments, England, model 800D), at

3,500 rpm for 15 min. The pellets were re-extracted in like manner as described and the supernatants were separately pooled according to the species and freeze-dried.

Determination of protein concentration in crude samples and hydrolysates

The protein content of the hydrolysate and fractions were determined by the protein binding assay method of Bradford described by [15] using bovine serum albumin (BSA) as standard protein and Coomassie brilliant blue G-250 as dye.

Simulated gastrointestinal digestion

Simulated gastrointestinal digestion was carried out according to the method described by [8]. Crude protein extract solutions (25 mg/ml) were prepared in 0.01N HCl and the pH was adjusted to 2.10. Pepsin was added to the protein solutions at an enzyme-substrate ratio (ESR) of 8% (w/w) of the crude protein and incubated at 37°C for 60 minutes, after which the pH was adjusted to 7.50 and then trypsin and chymotrypsin were added at the ESR of 4 and 2% (w/w) respectively and incubated for another 90 minutes. The hydrolysis was terminated by heating the hydrolysates on a water bath at 98°C for 10 min. The hydrolysates were centrifuged using a tabletop centrifuge at 4,000 rpm for 15 min and the supernatants were collected as the crude hydrolysate of *P. aurita* (PAH) and crude hydrolysate of *T. fuscatus* (TFH), freeze-dried and stored at -20°C until required.

Fractionation of antioxidant peptides from the SGID hydrolysates

The SGID hydrolysates were ultra-filtered using a hydrophilic 3 kDa molecular weight cut-off membrane centrifugal filter (Millipore Corporation, Bedford, MA, USA) to obtain two fractions. The hydrolysate was introduced into the vials and centrifuged at 10,000× g for 20 min at 4°C according to the manufacturer's instruction. The permeate (peptides with MW ≤ 3 kDa) and the retentate (peptides with MW > 3 kDa) are referred to as P ≤ 3 kDa and P > 3 kDa for *P. aurita* and T ≤ 3 kDa and T > 3 kDa for *T. fuscatus* respectively and were collectively referred to as UF fractions. The fractions were kept at -20°C until required. Protein content and antioxidant of these fractions were determined.

Ferrous ion-chelating ability assay

The ferrous ion-chelating (FIC) assay was carried out according to [16] with some modifications. Briefly, 1 ml of sequentially diluted concentrations of the UF fractions of *P. aurita* and *T. fuscatus* were each mixed with 1 ml of 50 µM $FeCl_2 \cdot 4H_2O$ and incubated for 5 min at room temperature, after which the reaction was initiated by adding 1 ml of 250 µM ferrozine and mixed. The resulting solutions were incubated at room temperature for 10 min and the absorbance was measured at 562 nm. A control experiment was conducted in the absence of sample or standard chelator.

$$\text{Chelating effect (\%)} = \frac{\text{Absorbance of control-Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

Ferric ion reductive potential

The assay was according to [17]. To 1 ml of the hydrolysates, UF fractions or standard ascorbic acid, 1 ml of phosphate buffer (pH 6.6, 0.2 M) was added and vortexed, after which 2.5 ml of 1% potassium ferricyanide was added. The resulting mixture was incubated in a water bath at 50°C for 20 min. After the incubation, 2.5 ml of 10% trichloroacetic acid was added, centrifuged at 4000 rpm for 10 minutes, and the supernatant was collected. Distilled water (2.5 ml) and 0.5 ml of 0.1% ferric chloride were added to 2.5 ml of the supernatant and vortexed and the absorbance was measured at 700 nm. The absorbance was plotted against the various concentrations.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured using the method of Halliwell [18]. In brief, the reaction system contained 1.0 ml of Fenton reagent (containing 20 mM deoxyribose, 0.3 mM EDTA, 20 mM H_2O_2, 1 mM L-Ascorbic acid, 0.5 mM $FeCl_3.6H_2O$ in 0.1 M phosphate buffer, pH 7.4) and 0.1 ml of serially diluted concentrations of the hydrolysates and UF fractions of *T. fuscatus* and *P. aurita*, which was incubated at 37°C for 1 h followed by the addition of 1.0 ml of 1% (w/v) thiobarbituric acid (TBA) and 1.0 ml 10% (w/v) trichloroacetic acid. The resulting mixtures were further incubated in water bath at 95°C for 20 min

and allowed to cool. The malondialdehyde-TBA adduct formed was extracted into 1.0 ml of butan-1-ol and quantified spectrophotometrically at 532 nm. Similarly, a control assay was conducted in the absence of either sample or standard.

The percentage inhibition was calculated using the expression:

$$\text{Percentage inhibition} = \frac{\text{Absorbance (control)-Absorbance (sample)}}{\text{Absorbance (control)}} \times 100\%$$

Anti-lipid peroxidation activity

The assay of anti-lipid peroxidation activity was carried out according to the thiobarbituric acid reaction method [19] using 1% ascorbic acid as positive control. Ten percent (10%) egg yolk (0.25 ml) was added to test tubes containing 0.1 ml of 150 mM Tris-HCl buffer, pH 7.2, 0.05 ml of 0.07 M $FeSO_4$ and serially diluted concentrations of the ultrafiltered peptide fractions of *T. fuscatus* and *P. aurita*. The reaction mixtures were incubated at 37°C for 1 h after which 0.5 ml of 0.1 N HCl, 0.2 ml of 9.8% SDS, 0.9 ml of distilled water and 2.0 ml of 0.8% thiobarbituric acid were added sequentially. The reaction mixtures were incubated in boiling water at 100°C for 30 min and then cooled, followed by the addition of 2.0 ml of butan-1-ol. The butanol layer was collected after centrifugation and the absorbance was measured at 532 nm. Similarly, a control assay was conducted in the absence of either sample or standard antioxidant.

The percentage inhibition was estimated using the expression below:

$$\text{Inhibition of Lipid Peroxidation} = \frac{\text{Absorbance of (control)-Absorbance of (sample)}}{\text{Absorbance of (control)}} \times 100\%$$

Statistical analysis

All experimental data were expressed as mean values (means ± SD). All experimental results were analyzed using the GraphPad Prism7 software program (GraphPad Software Inc., San Diego, California, USA). One-way analysis of variance (ANOVA) was used to analyze the data, and difference between means of the activities of samples and their corresponding standard antioxidants used for each assay were separated by Tukey's post-hoc test. The results were considered statistically significant at $P < 0.05$ and were indicated with asterisk.

Results

Ferric reducing antioxidant property (FRAP)

The reductive antioxidant potential is a measure of the ability of compounds to reduce ferric ion to ferrous ion which has characteristic Perl Prussian blue colour which is measured at 700 nm and a higher absorbance at this wavelength indicates a higher reducing power. The SGID hydrolysates and their fractions displayed varying degrees of reductive ability which were similar to the result produced by ascorbic acid standard. Lower molecular weight fractions (≤ 3 kDa) displayed highest reductive potential with least concentration of sample producing highest absorbance of all sample investigated (**Figure 1**). The reductive ability of the crude hydrolysate and low molecular weight fractions of *T. fuscatus* and *P. aurita* are comparable with that of a standard antioxidant (Ascorbic acid) used in this experiment.

Metal chelation activity

Metal ion chelation ability is an important antioxidant property because it can prevent/terminate the further propagation of free radicals in the body. Ferric ion chelation (FIC) activity of the hydrolysate and ultra-filtered peptide fractions of *T. fuscatus* and *P. aurita* and a standard metal chelator (EDTA) is shown in **Figure 2**. Low molecular weight fraction ($P \leq 3$ kDa) had the highest chelation activity with the IC_{50} (concentration that produced 50% inhibition) value of 5.56 ± 0.065 µg/ml. This activity is higher than the corresponding activity displayed by the standard EDTA (IC_{50} was 11.84 ± 0.89 µg/ml). Interestingly, low and high molecular weight fractions of *P. aurita* and *T. fuscatus* behaved alike and showed significantly ($P < 0.05$) higher activity than EDTA but the difference between crude hydrolysates and EDTA was not significant. This could mean that separation plays an important role in the chelation activity of the peptides.

Hydroxyl radical scavenging activity

Hydroxyl radical (OH) is extremely reactive, more toxic than other radical species and can attack macromolecules such as DNA, proteins and lipids. OH is believed to be generated by Fenton reaction from Fe^{2+} (Cu^{+})/H_2O_2. Thus, OH

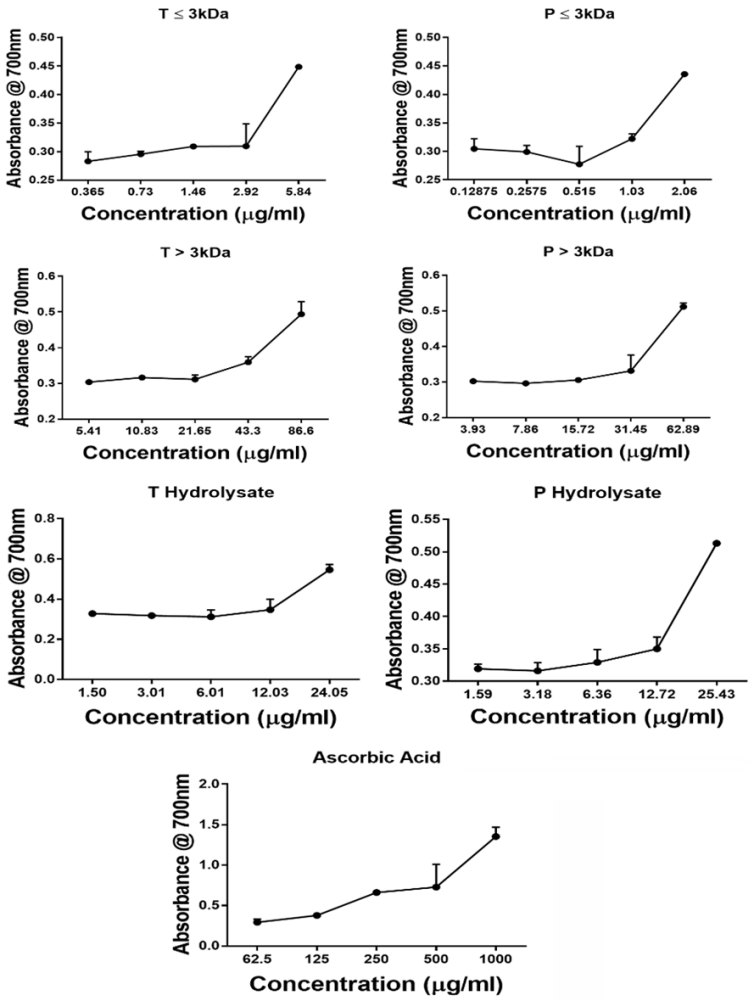

Figure 1. Ferric Reducing Antioxidant Property (FRAP) of hydrolysate and ultrafiltered fractions of *T. fuscatus*, *P. aurita* and Standard Ascorbic acid. Where P and T represents *P. aurita* and *T. fuscatus* respectively. P ≤ 3 kDa and P > 3 kDa are ultrafiltration permeate and retentate of *P. aurita* respectively while T ≤ 3 kDa and T > 3 kDa are ultrafiltration permeate and retentate of *T. fuscatus* respectively.

scavenging activity of an antioxidant can be achieved by direct scavenging of OH or preventing the formation of •OH by chelating the free metal ions. In the result of the •OH scavenging-ability of the SGID hydrolysate, ultrafiltered (UF) peptide fractions and Mannitol, mannitol gave the highest scavenging activity expressed in percentage (47.92 ± 1.67%). However, when the result was expressed as percentage per milligram, low molecular weight fraction of *P. aurita* (P ≤ 3 kDa) produced the highest (2415.36 ± 49.75%/mg) activity (**Figure 3**) followed by T ≤ 3 kDa fraction (1342.45 ± 60.42%/mg). The difference in the activity is significant at $P < 0.05$.

Lipid peroxidation inhibitory activity

Lipid peroxidation refers to oxidative deterioration of lipids containing carbon-carbon double bond(s) as found in cholesterol, phospholipids and unsaturated fatty acids. Lipid peroxidation and DNA damage are associated with many chronic diseases. The anti-lipid peroxidation activity (**Figure 4**) of the hydrolysate and UF peptide fractions revealed substantial activity in the low molecular weight peptide fractions, T ≤ 3 kDa and P ≤ 3 kDa activity of 860.66 ± 55.16 and 1971.30 ± 286.62%/mg respectively.

Discussion

The major defense against oxidative stress involves the mechanisms that can "mop up" the free radicals, preventing them from causing harm, and the mechanisms that can repair or ameliorate the damage. The aim of this study was to investigate the antioxidant potentials of peptides obtained from the protein extracts of *Pachymelania aurita* and *Tympanatonus fuscatus* var *radula*. The ferric ion reducing antioxidant power (FRAP) assay, revealed a dose-dependent reductive potential in both the SGID hydrolysates and UF peptide fractions of proteins obtained from *T. fuscatus* and *P. aurita* (**Figure 1**). The UF (low molecular weight) peptide fractions displayed higher reductive capability, having a correspondingly higher absorbance and slope when the resulting ferrous-ferrozine complex formed was quantified spectrophotometrically at 700 nm. This observation was not different from what was observed in previous studies involving the enzymatic hydrolysate of algae, where the FRAP increased with the increasing degrees of hydrolysis, so that the highest FRAP activity was in the low molecular weight peptide fractions [10, 20]. This suggests

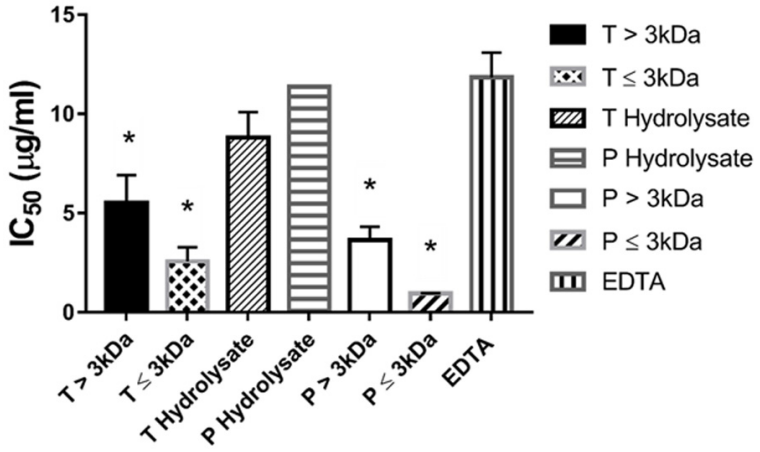

Figure 2. Metal Chelation Activity of hydrolysate and ultrafiltered fractions of *P. aurita* and *T. fuscatus*. The results are mean values of experiments carried out in duplicate. Values with asterisk are statistically different from EDTA at $P < 0.05$. Where P and T represents *P. aurita* and *T. fuscatus* respectively. P ≤ 3 kDa and P > 3 kDa are ultrafiltration permeate and retentate of *P. aurita* respectively while T ≤ 3 kDa and T > 3 kDa are ultrafiltration permeate and retentate of *T. fuscatus* respectively.

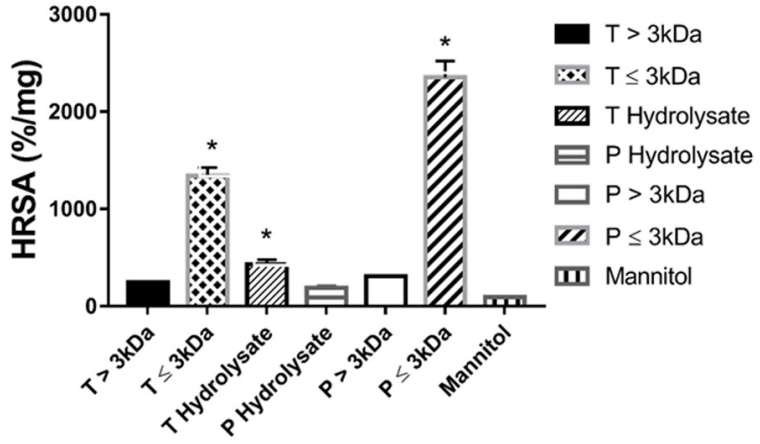

Figure 3. Hydroxyl Radical Scavenging Activity (HRSA) of Hydrolysate, Mannitol (Standard) and Ultrafiltered peptide of *P. aurita* and *T. fuscatus*. The results are mean values of experiments carried out in duplicate. Values with asterisk are statistically different from Mannitol at $P < 0.05$. Where P and T represents *P. aurita* and *T. fuscatus* respectively. P ≤ 3 kDa and P > 3 kDa are ultrafiltration permeate and retentate of *P. aurita* respectively while T ≤ 3 kDa and T > 3 kDa are ultrafiltration permeate and retentate of *T. fuscatus* respectively.

that gastrointestinal digestion of theses molluscs can lead to the release of short antioxidant peptide(s) that can alleviate oxidative stress. The peptides displaying this property can contribute to the *in vivo* antioxidant system by possibly interrupting the generation of OH radical through the reduction of the Fe^{3+} (required in the Fenton reaction) into Fe^{2+} ion. The ferric reducing capacity of compounds may show the potential antioxidant ability to terminate the free radical's chain reaction [21, 22]. In fact, there is speculation that FRAP may be due to a hydrogen-donating ability of the peptides [23]. Transition metals such as iron and copper are known to aggravate oxidative stress, as they can react with hydrogen peroxide to produce a toxic reactive hydroxyl radical. Thus, chelation of transition metals is an important antioxidant property, as it holds potential to intercept lipid peroxidation and also formation of hydroxyl radical in a Fenton reaction. In this study, the metal ion chelation activity of the hydrolysate and fractions were appreciable, and some were not significantly different when compared with that of standard metal chelator, EDTA (**Figure 2**), while low molecular weight peptides (≤ 3 kDa) gave the highest chelating effect, showing relatively lower IC_{50} values. This was in line with previous reports that had observed potent metal chelation activity in lower molecular weight peptide fractions of protein hydrolysate of chickpea [24] and *Fucus spiralis* [25]. It is well established that antioxidants, particularly metal chelators, have found important application in the management of neurodegenerative diseases such as Alzheimer's disease and other chronic diseases [2, 26]. Another EM-based antioxidant assay carried out in this study was hydroxyl radicals (OH) scavenging activity (HRSA). Hydroxyl radicals which are formed by the Fenton reaction are considered the most reactive oxygen radicals which can destroy biological macromolecules easily, such as proteins, carbohydrates, lipids, and nucleic acids. Therefore, removing hydroxyl radicals is an effective protection of the body against several

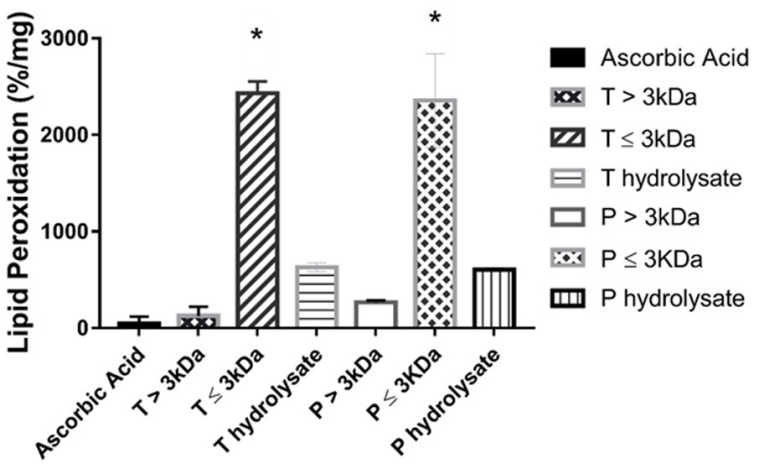

Figure 4. Lipid Peroxidation Inhibitory Activity of Hydrolysate and Ultrafiltered peptide of *P. aurita* and *T. fuscatus.* The results are mean values of experiments carried out in duplicate. Values with asterisk are statistically different from Ascorbic Acid at $P < 0.05$. Where P and T represents *P. aurita* and *T. fuscatus* respectively. $P \leq 3$ kDa and $P > 3$ kDa are ultrafiltration permeate and retentate of *P. aurita* respectively while $T \leq 3$ kDa and $T > 3$ kDa are ultrafiltration permeate and retentate of *T. fuscatus* respectively.

diseases [27]. Hydroxyl radical scavenging activity, as with other antioxidant activities evaluated in this study revealed the highest percentage scavenging activity (when divided by peptide concentration) in low molecular weight fractions. Thus, peptides from *T. fuscatus* and *P. aurita* upon digestion can serve as a scavenger to reduce or eliminate damages induced by hydroxyl radicals in food and biological systems to improve human health as shown in some studies [28]. Similar findings have been reported with casein-derived peptides [29] and with *Olea europaea* peptides [30], where highest activity was attributed to low molecular weight peptides. The OH scavenging activity of antioxidants can be by any of the following mechanisms: direct scavenging of OH, prevention of OH formation through the chelation of free metal ions or by converting H_2O_2 (precursor of OH) to other harmless compounds in a living organism [31]. Lipid peroxidation and DNA damage are implicated in a variety of chronic health challenges such as cancer, atherosclerosis and aging [32]. The anti-lipid peroxidation (HAT-based antioxidant reaction) assay of the hydrolysates and fractions of *P. aurita* and *T. fuscatus* revealed higher activity in smaller weight fractions which was similar to the activity produced by the ascorbic acid standard. This observation supports the previous findings on the lipid peroxidation inhibitory activity of hydrolysates obtained from bivalve molluscs, where small-sized peptides obtained from these molluscs displayed the most potent activity [9]. Studies have shown that the hydrophobic property of antioxidant peptides play an important role in quenching lipid-derived radicals and so hydrophobicity is thought to be a critical property of lipid peroxidation inhibitors [33]. Generally, molecular size, amino acid sequence and composition [27, 34] have been implicated to play a key role in the antioxidant capacities of antioxidant peptides, and in most cases, low molecular weight peptides are reputed to be the most potent [34, 35] and which also are expected to evade gastrointestinal proteolytic degradation. Low molecular weight antioxidant peptides have been isolated from *Conger myriaster* [36] and *Tergillarca granosa* [9]. The antioxidant activity of the hydrolysates could be because of the disruption of the tertiary structures that hitherto limit the antioxidants capacities of the amino acid residues that have been buried in the core of the protein structure and as such remained inaccessible to pro-oxidants. In conclusion, the periwinkle protein hydrolysates demonstrated clear antioxidant properties in all the assay models used. Low molecular weight fractions of the hydrolysates demonstrated more potent antioxidant activity than higher molecular weight fractions.

Disclosure of conflict of interest

None.

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