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# Extraction And Characterization of Bioactive Peptides Derived from the Hydrolysates of Total Soluble Proteins of Pistachio Nuts (*Pistacia vera* L.)

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This study explored the potential of pistachio nut (*Pistacia vera* L.) total soluble proteins to release bioactive peptides exhibiting angiotensin-converting enzyme (ACE) inhibition, antioxidative, and antibacterial activities. The total soluble proteins were extracted from the ground, defatted nut using 0.010 M phosphate buffered saline, pH 6.8. The extracted proteins were hydrolyzed at different time intervals using trypsin and chymotrypsin. Hydrolysates of the total soluble proteins at 24 h digestion time exhibited the highest ACE-inhibition activity of 76.67 ± 0.10% and 70.83 ± 0.00% for chymotrypsin and trypsin digestion, respectively. The 24 h enzymatic hydrolysates were further fractionated in RP-HPLC using a C18 Vydac column. The C1 fraction from the 24 h chymotryptic hydrolysates and T2 from the 24 h tryptic hydrolysates exhibited the highest ACE-inhibition activities with an $IC_{50}$ value of 147.7 ± 0.8 and 148.7 ± 0.6 μg/mL, respectively. The 24 h chymotryptic and tryptic hydrolysates also exhibited a DPPH radical scavenging activity of 83.7 ± 1.1% ($EC_{50}$ = 356.5 ± 1.0 μg/mL) and 80.4 ± 0.2% ($EC_{50}$ = 402.7 ± 1.1 μg/mL), respectively. The hydroxyl radical scavenging activities of the 24 h chymotryptic and tryptic hydrolysates were found to be 22.8 ± 1.0 and 16.6 ± 3.7%, respectively. However, the 24 h tryptic and chymotryptic hydrolysates did not exhibit any antibacterial activity against the gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus*. Therefore, the total soluble proteins of pistachio nuts were found to contain peptides exhibiting ACE-inhibition and antioxidative activities upon hydrolysis with trypsin and chymotrypsin.

***Keywords:*** *Pistachio, antioxidative, antibacterial, angiotensin-converting, bioactive peptides*

## INTRODUCTION

Pistachio nuts (*Pistacia vera* L.) was ranked as one of the food products to contain the highest antioxidant potential due to its phenolic compounds (Halvorsen *et al.,* 2006). Pistachio extracts have also been shown to exhibit anti-inflammatory, antinociceptive, and antimicrobial activities (Orhan *et al.*, 2006; Ozcelik *et al.* 2005), as well as cardioprotective

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and vasoprotective capacities (Rajaei *et al.,* 2010).

Aside from the high phenolic content, pistachio nuts also contain high amounts of dietary proteins (Ros*,* 2010). En route to the human system, protein hydrolysates have been found to exhibit drug- or hormone-like activities that promote health. Proteins derived from plant food sources have more potent bioactivities such as antihypertensive, antioxidative, antimicrobial, opoid, modulatory, and other functionalities (Korhonen and Pihlanto, 2006).

In the Philippines, cardiovascular diseases and those caused by pathogens are among the leading causes of mortality (NSO, 2013). Thus, the utilization of natural food protein sources with health-promoting activities is significant. Hypertension or increased blood pressure is one of the most controllable risk factors associated with cardiovascular diseases and can be regulated through antihypertensive peptides with Angiotensin I-converting enzyme (ACE) inhibitory activity. ACE is an enzyme that converts angiotensin I to angiotensin II and is responsible for increase in blood pressure. Such ACE inhibiting peptides have the capability to minimize production of angiotensin II which in turn exhibits antihypertensive property (Natesh *et al.,* 2003; Unger, 2002).

Cardiovascular diseases are also associated to chronic degenerative stresses invoked by many oxidative species such as reactive oxygen, nitrogen, and free radicals (Bowler and Crapo, 2002; Dhalla *et al*., 2000; Park *et al.,* 2009). Antioxidative peptides contain a unique amino acid sequence that act as electron donors to free radicals resulting to a peptide radical that do not initiate or propagate further oxidation reactions (Elias *et al.,* 2008).

The interest in antibacterial peptides as potential antibiotic pharmaceuticals is still in top priority due to their rapid and broad-spectrum antibacterial property against antibiotic-resistant bacteria (Brogden and Brogden, 2011). These peptides perform their bioactivity by disrupting the cellular matrix of bacteria leading to cell death.

With these evidences that peptides have the effect of lowering blood pressure, reduce the risk of oxidative stress, and provide antagonistic role against bacterial strains, the exploitation of different plant proteins can be a means of screening for potential peptides that exhibit antihypertensive, antioxidative, and antibacterial activities. Hence, the main focus of this study is to screen for potential ACE-inhibiting, antioxidative, and antibacterial peptides present in pistachio nut (*Pistacia vera* L.) total soluble proteins.

## METHODOLOGY

***Sample Preparation.*** Fifty grams of decorticated pistachio nuts were ground to a fine paste using a mortar and pestle. The resulting meal was then defatted with n-hexane in a 5:1 ratio of solvent volume to meal weight at 4 °C for 3 h. The defatted meal was stored in freezer until use.

***Extraction of Total Soluble Proteins.*** The total soluble proteins of pistachio nuts were extracted using 0.010 M phosphate buffered saline (pH 6.8) in a 10:1 ratio of solvent volume to meal weight at 4°C for 3 h. The resulting mixture was then filtered and centrifuged at 10,000 $\times g$ for 15 min at 4°C using an Allegra X-30R refrigerated centrifuge. The clarified crude extract was collected and stored at 4°C until use.

***Protein Content Determination.*** The protein content of the sample was determined using Bradford (1976) with bovine serum albumin (BSA) as protein standard.

***Enzymatic Hydrolysis of the Total Soluble Proteins.*** Four hundred microliters of the total soluble proteins (1 mg/mL) were digested separately with chymotrypsin and trypsin (1 mg/mL) using an enzyme to substrate ratio (v/v) of 1:8. Hydrolysis was performed at different time intervals (0 min, 5 min, 15 min, 30 min, 1 h, 5 h, 12 h, and 24 h) at 37°C and pH 7.4. Enzymatic activity was stopped by heating the digests at 100 °C for 10 min. After heating, the mixture was immediately kept in ice. The progress of enzymatic hydrolysis was monitored using SDS-PAGE.

***Extraction of Angiotensin-Converting Enzyme (ACE) from Pig Lungs.*** *Preparation of Acetone Lung Powder*. The angiotensin-converting enzyme was extracted from the acetone pig lung powder according to the method of Folk *et al.* (1960) with slight modifications. The pig lungs were suspended in two volumes of acetone and then homogenized using a blender. The resulting mixture was filtered through cheesecloth and the residual solvent was evaporated from the tissue residue under the hood. The tissue residue was then resuspended in four volumes of diethyl ether, followed by homogenization using a blender. The solvent was then removed by filtration in cheesecloth while the residue was dried under the hood. The dried residue powder was used for ACE extraction.

*ACE Extraction from Acetone Lung Powder.* A modified method of Cushman and Cheung (1971) was used to extract ACE from acetone lung powder. The crude ACE was extracted from the acetone lung powder using 0.10 M sodium borate buffer (pH 8.0) with 0.30 M NaCl for 3 h at 4°C. The mixture was then clarified at 10,000 *x g* for 40 min at 4°C. The resulting clear, reddish supernatant was collected, labeled as the crude ACE extract and stored at 4°C.

***Reverse-Phase High Performance Liquid Chromatography (RP-HPLC).*** Fractionation of enzymatic hydrolysates was performed on Agilent Technologies 1200 Series HPLC system using a Vydac C18 column (4.6x250 mm, 5 μm bead, 30 mm pore size). A 25 μL sample was injected into the column and eluted at 25°C via a mobile phase using water and acetonitrile as solvents. Gradient elution was done starting from 2 to 60% water for 45 min, 60 to 100% acetonitrile for 5 min and 98 to 2% acetonitrile for 2 min ending with 2% acetonitrile for 2 min. The flow rate was set to 0.50 mL/min and the peptide fractions were detected using a UV detector at 242 nm. All samples were clarified using 0.45 μL microfilter prior to analysis. Each fraction was collected manually and tested for ACE inhibitory activity. Fractions with high ACE inhibition were subjected to $IC_{50}$ determination.

***Characterization of Bioactive Peptides.*** *Spectrophotometric Angiotensin Converting Enzyme (ACE) Inhibition Assay*. The method of Cheung *et al.* (1980) with slight modifications was used to determine the ACE-inhibition activity of pistachio nut protein hydrolysates. A 250 μL assay mixture containing 100 mM phosphate buffer, 300 mM NaCl, 5 mM hippuryl-L-histidine-L-leucine (HHL) and 50 μL protein hydrolysate was pre-incubated at 37°C for 5 min. Fifty microliter ACE from pig lung was added to the assay mixture to initiate the reaction. For the control, 250 μL 1 N HCl was added first before adding the ACE. A blank solution was done by replacing the 50 μL protein hydrolysate with 100 mM phosphate buffer. The assay mixtures were incubated at 37°C for 30 min. The enzymatic reaction was stopped by adding 250 μL 1 N HCl (except for the control) to each assay mixture. The hippuric acid formed was extracted with 1.0 mL ethyl acetate by vortex mixing for 15 s. An aliquot of 0.70 mL of ethyl acetate layer from each mixture was collected and evaporated to dryness. The hippuric acid that remained was redissolved in 1.30 mL water and the absorbance of the resulting solution was read at 228 nm. Pulverized Captopril served as positive control.

The percent inhibition of the protein hydrolysates was calculated using the equation adopted from Li *et al.* (2005):

$$\text{ACE inhibition (\%)} = \frac{B-A}{B-C} \times 100 \quad \text{(Equation 1)}$$

where A is the absorbance of hippuric acid generated in the presence of ACE inhibitor (sample); B is the absorbance of the hippuric acid generated without the ACE inhibitor (blank); and C is the absorbance of hippuric acid generated without ACE (control).

The $IC_{50}$ value is defined as the concentration of peptide inhibitor required to inhibit 50% of the ACE activity under assayed conditions. The value was determined using GraphPad® Prism 6 software.

*Antioxidative Activity Assay. Hydroxyl Radical Scavenging Activity.* The hydroxyl radical scavenging activity of the 24 h trypsin and chymotrypsin hydrolysates was determined according to the method of Tanzadehpanah *et al.* (2012) with slight modifications. A mixture of 5.0 mM 1,10-phenantroline, 5.0 mM $FeSO_4$, and 15.0 mM EDTA were mixed with 10 mM PBS (pH 6.8). Then, 150 μL of protein hydrolysate (1 mg/mL) and 210 μL of 0.03% $H_2O_2$ were added. The resulting mixture was incubated at 37°C for 60 min and the absorbance was read at 536 nm. Ascorbic acid (1 mg/mL) was used as a positive control. The hydroxyl radical scavenging activity was calculated using the equation:

$$\text{Hydroxyl Radical Scavenging Activity (\%)} = \frac{A_c - A_s}{A_c - A_o} \times 100 \qquad \text{(Equation 2)}$$

where $A_c$ is the absorbance of the control solution in the absence of $H_2O_2$; $A_s$ is the absorbance of the sample; $A_o$ is the absorbance of the blank solution using distilled water instead of sample.

*2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity.* A modified method of Saha *et al.* (2008) was used to assess the antioxidant capacity of the protein hydrolysates to quench a stable DPPH radical. Two hundred fifty microliters of protein hydrolysates at different concentrations (0-1 mg/mL) was mixed with 1.10 mL 0.004% DPPH solution (in 95% ethanol). The mixture was shaken vigorously using vortex mixer. The reaction mixture was incubated for 30 min in the dark at room temperature. The absorbance was then measured at 519 nm. Ethanol and ascorbic acid was used as blank and positive control, respectively. The radical scavenging activity of the sample was calculated using the equation:

$$\text{DPPH Radical Scavenging Activity (\%)} = \frac{A_o - A_s}{A_o} \times 100 \qquad \text{(Equation 3)}$$

where $A_o$ is the absorbance of the blank solution using 95% ethanol instead of sample and $A_s$ is the absorbance of the sample.

The $EC_{50}$ value is defined as an effective concentration of protein hydrolysates that is required to scavenge 50% of DPPH radical activity under assayed conditions and determined using GraphPad® Prism 6 software.

*Antimicrobial Susceptibility Assay.* The antibacterial activity of the protein hydrolysates against *Escherichia coli* (BIOTECH 1634) and *Staphylococcus aureus* (BIOTECH 1582) was done by disc diffusion method. Mueller Hinton Agar (MHA) was used as the medium for the two test microbial stocks.

Molten MHA was inoculated via pour plate method with 1.0 mL of the 24 h old test microbial stocks dissolved in saline solution. The inoculated medium was then poured into a plate at around 4 mm in depth and was allowed to solidify at room temperature.

Filter paper discs of approximately 6 mm in diameter were soaked with 15 μL of the protein digests and were placed in the inoculated MHA plate. Each disc was pressed down to ensure complete contact with the agar surface and distributed evenly. The agar plates were incubated for 12 h at 37°C. Sterile water discs were used as negative control and 10% phenol discs as positive control. The diameter of zone of complete inhibition (in mm) of the protein hydrolysates against the two microbial stocks was measured and compared with that of the diluent (0.010 M PBS, pH 6.8) and the controls.

***Densitometric Analysis.*** The electrophoretograms were scanned and the resulting images were analyzed using ImageJ software (Abramoff *et al.*, 2004) to determine the degree of hydrolysis of the proteins.

***Statistical Analysis.*** All analyses were done in duplicates and subjected to ANOVA followed by Tukey's test at $p<0.05$ level using GraphPad® Prism 6 software. The activity for each assay is expressed as mean activity ± standard deviation.

## RESULTS AND DISCUSSION

***Extraction of the Total Soluble Proteins from Pistachio Nuts.*** The total soluble proteins of pistachio nuts (TSPPN) were extracted using 0.010 M phosphate buffered saline (pH 6.8). The protein content of this extract was found to be 1.23 mg/mL. SDS-PAGE profile of TSPPN reveals 5 major protein bands with approximate molecular weights of 56, 31, 26, 24, and 13 kDa (Figure 1). This protein profile was consistent with those obtained by Noorbakhsh *et al.* (2011). According to Shokraii and Esen (1988), majority of the proteins from pistachio nut are globulins amounting to 66.0%, followed by albumins, glutelins, and prolamins, amounting to 25.0, 7.3, and 2.0%, respectively.

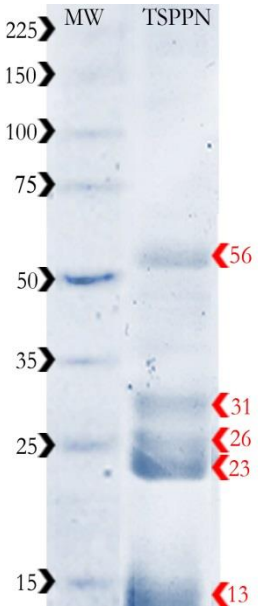



***Figure 1.*** *SDS-PAGE Profile of the Total Soluble Proteins of Pistachio Nuts (TSPPN). MW: Molecular Weight Markers; TSPPN: Total Soluble Proteins of Pistachio Nuts.*

***Enzymatic Hydrolysis of TSPPN.*** The enzymatic digestibility of TSPPN using chymotrypsin (Figure 2a) and trypsin (Figure 2b) were evaluated at different time intervals from 0 min to 24 h (Figure 2). It was observed that the 56 kDa polypeptide of TSPPN was the most susceptible to digestion for both enzymes. Even at 0 min, the short contact time between the proteins and enzyme prior to immediate boiling was enough to cleave the protein into smaller fragments. Moreover, it also possible that 56 kDa polypeptide contains more cleavage sites for trypsin and chymotrypsin than the other polypeptides which explains the ease of its hydrolysis (Fastrez and Fersht, 1973). Using densitometric analysis, 73% and 60% of the 56 kDa polypeptide were hydrolyzed by chymotrypsin and trypsin, respectively.

On the contrary, three polypeptides were fairly resistant to chymotrypsin and trypsin. It can be noted that chymotrypsin was able to digest completely the 31 kDa and 26 kDa polypeptides only after 5 h of incubation. However, the 23 kDa polypeptide was fairly resistant to chymotrypsin digestion such that 60% of the polypeptide still remained after 24 h of digestion.

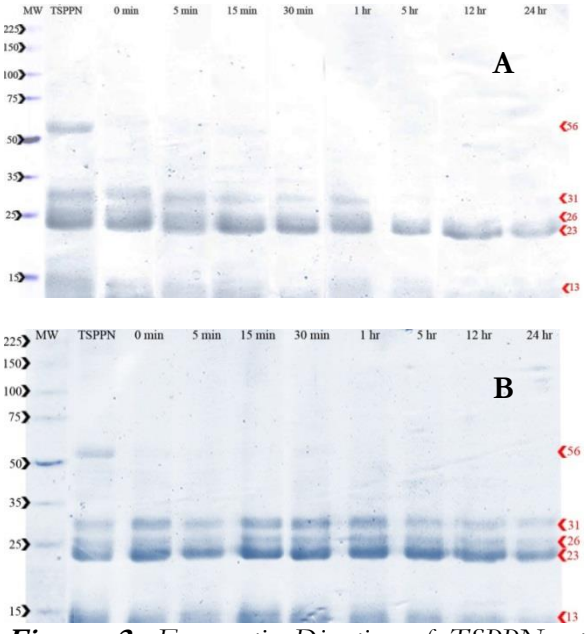



***Figure 2.*** *Enzymatic Digestion of TSPPN at Different Time Intervals using (A) Chymotrypsin and (B) Trypsin. Lane MW: Molecular Weight Markers; TSPPN: Total Soluble Proteins of Pistachio Nuts.*

In general, TSPPN showed more resistance to digestion using trypsin. After 24 h digestion, 75, 76 and 90% remained for the 31, 26, and 23 kDa polypeptides, respectively. The differences in the susceptibility of the polypeptides to enzymatic hydrolysis may be attributed to differences in amino acid content and sequences. Likewise these three polypeptides may contain greater number of cleavage sites for chymotrypsin than trypsin.

***Angiotensin-Converting Enzyme (ACE) Inhibition Assay.*** Preliminary ACE inhibition assay shows that both tryptic and chymotryptic hydrolysates yielded ACE inhibition activities at different digestion times as shown in Figure 3. However, the highest ACE inhibition activity of 76.67% and 70.83% was observed at 24 h for chymotrypsin and trypsin, respectively. Although no significant differences in terms of ACE inhibition activity were obtained at different time intervals for chymotryptic digestion, still the 24 h digestion time was chosen for RP-HPLC fractionation due to the extent of hydrolysis whereby the concentration of smaller peptides was expected to be the highest. According to Hernandez-Ledesma *et al.* (2011), majority of the ACE inhibitory peptides have relatively short sequences, ranging from 2 to 12 amino acids, since the inhibition site for ACE cannot accommodate much larger peptide molecules. Hyun and Shin (2000) also observed that ACE inhibitory peptides are effectively released after 24 h of digestion or when the hydrolysis of the protein is almost complete.

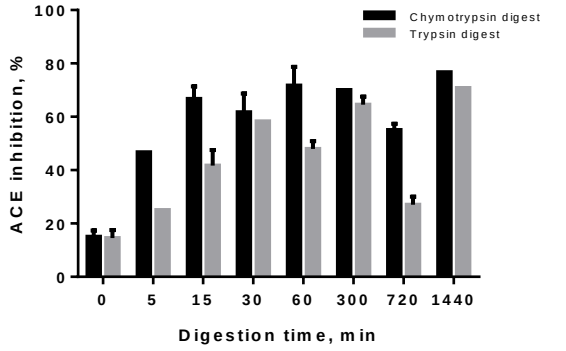



***Figure 3.*** *Percent ACE inhibition of chymotryptic and tryptic hydrolysates of TSPPN digested at different time intervals (60 min = 1 h; 300 min = 5 h; 720 min = 12 h; 1440 min = 24 h).*

***Fractionation of 24 h Chymotryptic and Tryptic Hydrolysates using RP-HPLC.*** Figure 4 shows the RP-HPLC profile of the 24 h chymotryptic (Figure 4a) and tryptic (Figure 4b) hydrolysates. Both profiles shows several peaks but only three peaks were chosen for ACE assay for the chymotryptic hydrolysates (labelled C1, C2, and C3) while two were selected for the tryptic hydrolysates (labelled T1 and T2). Table 1 shows the ACE inhibitory activities of the selected peaks and their corresponding $IC_{50}$ values.

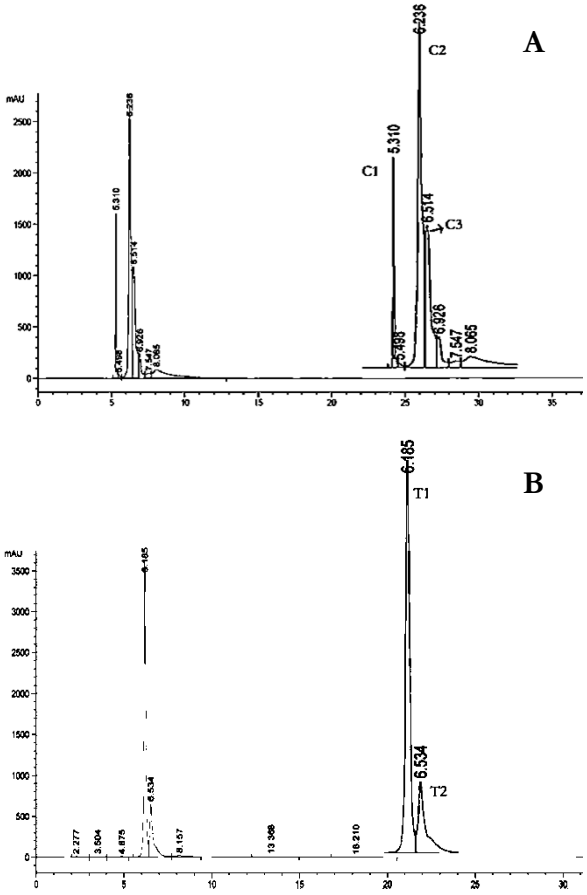



***Figure 4.*** *RP-HPLC profiles of the 24 h (A) chymotryptic (B) tryptic hydrolysates. Inset: magnified chromatograms.*

**Table 1. ACE Inhibition Activities of the 24 h Hydrolysates from RP-HPLC Fractionation.**

| Fraction No. | ACE inhibition (%) | $IC_{50}$ (µg/mL) |
|---|---|---|
| C1 | 65.04 ± 4.38 | 147.7 ± 0.8 |
| C2 | 27.88 ± 1.19 | nd |
| C3 | 23.01 ± 0.00 | nd |
| T1 | 58.41 ± 1.25 | 236.4 ± 3.0 |
| T2 | 71.24 ± 6.88 | 148.7 ± 0.6 |
| Captopril (positive control) | 94.53 ± 2.21 | 67.01 ± 2.21 |

**nd: not determined*

It can be noted that from among the chymotryptic hydrolysates, C1 exhibited the highest ACE inhibition activity of 65.04% while C3 has the lowest activity with a value of 23.01%. On the other hand, for the tryptic hydrolysates, T2 showed a relatively higher activity than T1, with a value of 71.24%. However, the individual inhibitory activity of the fractions does not surpass that of captopril, a known ACE inhibitor and a prescribed medicine for hypertension. But, the synergistic activities of all these peptide fractions may ultimately have an impact in controlling and alleviating hypertension.

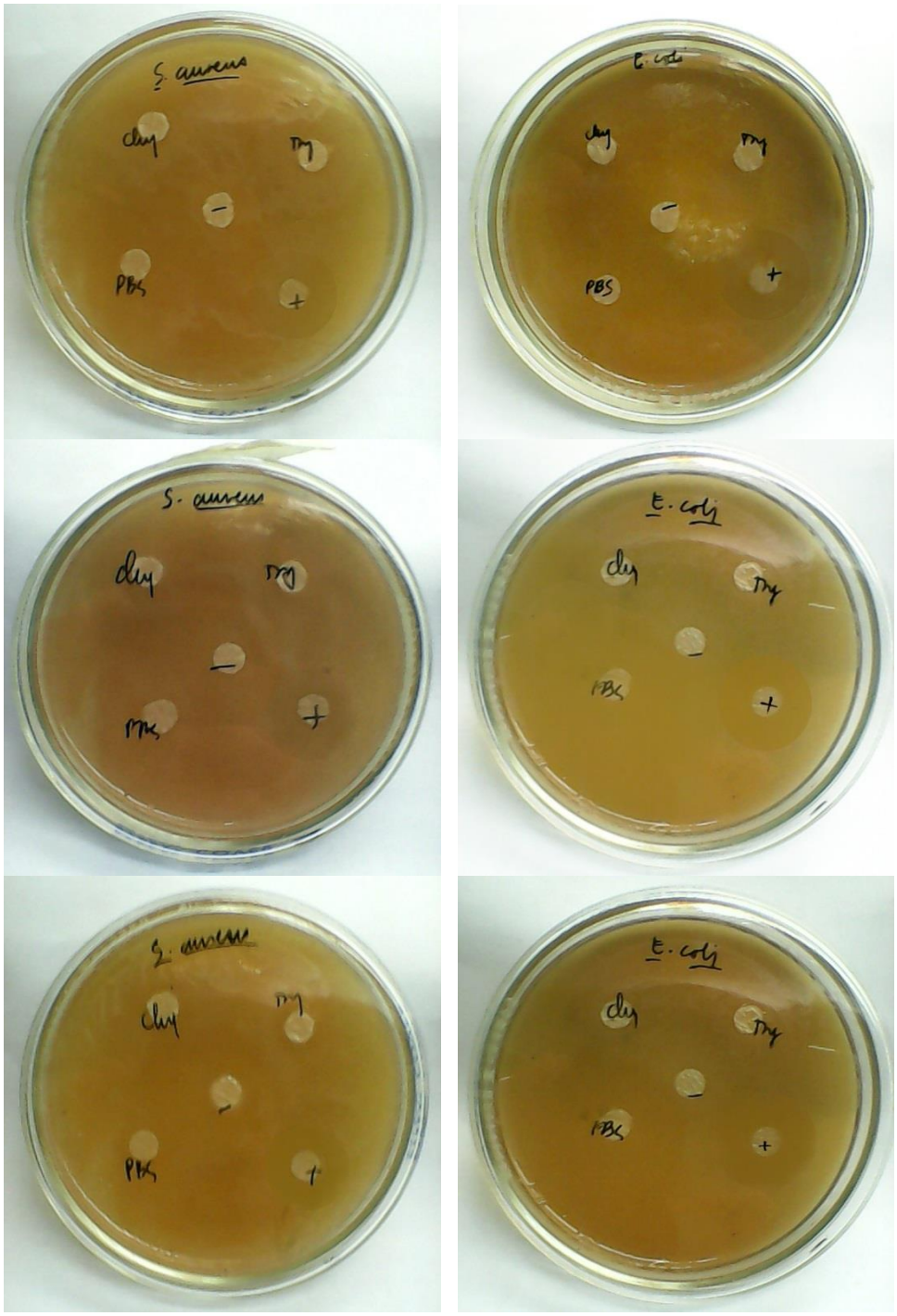
***Hydroxyl and DPPH Radical Scavenging Assays of the 24 h Hydrolysates.*** The 24 h chymotryptic and tryptic hydrolysates, which have the highest degree of protein hydrolysis, were also screened for any antioxidant activity. As shown in Table 2, both tryptic and chymotryptic hydrolysates contained peptides that were able to scavenge the generated hydroxyl radical. Between the two, the chymotryptic hydrolysates have consistently exhibited a greater antioxidant effect of 22.802% and $EC_{50}$ 356.451, for hydroxyl and DPPH radical scavenging activities, respectively. On the other hand, the tryptic hydrolysates exhibited a lower antioxidant ability of 16.616% and $EC_{50}$ 402.717 for hydroxyl and DPPH radical scavenging activities, respectively. However, when compared with ascorbic acid which is a known natural antioxidant, the radical scavenging activities of both hydrolysates were significantly lower and were less effective. In a similar study by Amsa *et al.* (2013), they noted that the radical scavenging activity was strongly correlated to the degree of hydrolysis polypeptide. Hence, the use of other proteolytic enzymes or longer digestion time may be necessary to hydrolyze the remaining undigested polypeptides and release more effective antioxidants. Abu-Salem *et al.* (2013) also reported that low molecular peptides generally exhibit higher antioxidant properties. Hydrolysis of proteins is also favored in generating and releasing potent antioxidant peptides (Bamdad *et al.*, 2011; Chanput *et al.*, 2009). Other studies also indicate that peptides released from protease digestion of nut proteins, such as peanut and walnut, have potent DPPH and hydroxyl radical scavenging activities (Chen *et al.* 2012; Ji *et al.* 2014).

**Table 2. Antioxidant activities of the 24 h chymotryptic and tryptic hydrolysates.**

| Sample | Hydroxyl Radical Scavenging Activity (%) | DPPH Radical Scavenging Activity, $EC_{50}$ (µg/mL) |
|---|---|---|
| Chymotryptic Hydrolysates | 22.802 ± 1.029 | 356.451 ± 1.047 |
| Tryptic Hydrolysates | 16.616 ± 3.688 | 402.717 ± 1.065 |
| Positive Control (Ascorbic acid) | 97.968 ± 0.129 | 125.893 ± 1.050 |

### 3.6 Antimicrobial Susceptibility Assay

The presence of antimicrobial peptides in the 24 h chymotryptic and tryptic hydrolysates were also determined using disc diffusion method. As shown in Figure 5, there were no zones of inhibition observed for both 24 h hydrolysates against the gram-positive *Staphylococcus aureus* (Figure 5a) and gram-negative *Escherichia coli* (Figure 5b). The results imply that the hydrolysates were devoid of biocidal peptides generated by the cleavage specificities of chymotrypsin and trypsin. It is also possible that peptides released upon hydrolysis with these proteases do not conform to an overall secondary structure with biocidal activity since there is a strong correlation between peptide structure and function of antimicrobial peptides (Epand and Vogel, 1999). The use of other proteolytic enzymes or a combination of such may produce peptides with antimicrobial and antiviral activities (Florisa *et al.*, 2003; Pellegrini, 2003; Zeng *et al.*, 2008).




**A** **B**

***Figure 5.*** *Antimicrobial susceptibility assay of the 24 h hydrolysates against (a) Staphylococcus aureus and (b) Escherichia coli. (Chy: 24 h chymotryptic hydrolysate; Try: 24 h tryptic hydrolysate; PBS: diluent control; (-): negative control; (+): positive control).*

## CONCLUSION

The total soluble proteins of pistachio nuts were found to contain bioactive peptides exhibiting ACE inhibition and antioxidant activities. These peptides were released from their parent proteins through enzymatic hydrolysis using chymotrypsin and trypsin. The 24 h chymotryptic and tryptic hydrolysates showed the highest ACE inhibition activities and the same hydrolysates also displayed hydroxyl and DPPH radical

scavenging activities. However, the 24 h hydrolysates from both enzymes were devoid of antimicrobial activities against the gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus*.

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