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Antioxidant Properties of *Albizia lebbeck* Seed Protein Hydrolysates

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Abstract

Peptide based antioxidants from plant and animal proteins are being identified as food additives and also as potential alternatives in the reduction of oxidative stress. This study investigated the antioxidative potentials of peptide digests of *Albizia lebbeck* seed protein. The proteins were extracted and isolated from *A. lebbeck* by defatting with n-hexane, followed by alkaline solubilization and acid precipitation of the seed meal. The protein isolate was then subjected to enzymatic hydrolysis using four proteases – pepsin, trypsin, papain and chymotrypsin. The resulting hydrolysates were then evaluated for their abilities to reduce ferric ions, as well as their effects on hydroxyl radicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide radicals. Hydrolysates obtained from peptic proteolysis demonstrated the best activities against DPPH radical and ferric ions (57.589 ± 1.286% and 52.000 ± 0.589 mM Fe^{2+}respectively), whereas chymotrypsin hydrolysates scavenged superoxide radicals and hydroxyl radicals better than other protein digests (74.520 ± 0.998% and 36.925 ± 1.880% respectively). The choice of enzyme used and the presence of specific amino acid residues at certain positions of peptides in the hydrolysates influenced their antioxidant capacities. It is concluded that *Albizia lebbeck* seed proteins encode potentially bioactive peptides, which could be harnessed for numerous therapeutic and nutraceutical benefits.

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Keyword
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Introduction

Free radicals have continuously played roles as both toxic and beneficial compounds in cellular respiration, immune response, drug detoxification, among other physicochemical processes (Pham-Huy *et al*., 2008). They are produced from the oxidation of biomolecules in the body through metabolism *in vivo* or from exposure to environmental pollutants, toxicants and other xenobiotics. When there is an apparent shortfall in the ability of the body's detoxification systems to combat an excess of these oxidants, they accumulate in the body, causing a state referred to as oxidative stress (Majhenic *et al.,* 2007). Oxidative stress generally describes a state of imbalance between the systemic generation of reactive oxygen and nitrogen species, and the ability of a biological system to quickly neutralize these reactive compounds or to repair the damage caused (Chandra *et al.,* 2015). Perturbations in the normal cellular redox states can give rise to toxic effects such as DNA strand breakage, nucleotide base damage, erythrocyte lysis, among other deleterious

effects (Chandra *et al.,* 2015). This is thought to occur through the production of free radicals such as singlet oxygen, peroxides, superoxides, that damage cellular components such as lipids, proteins and DNA. Furthermore, some oxidative species act as intracellular messengers in redox signaling; and as a result, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. In humans, oxidative stress is involved directly and/or indirectly in the progression of several diseases, which include diabetes mellitus (Olusola *et al.,* 2018), cancer (Halliwell, 2007), Parkinson's disease, Alzheimers disease (Valko *et al.,* 2007, Pohanka, 2013) atherosclerosis, myocardial infarction (Dean *et al.,* 2011; Ramond et al, 2011) among others. It is important that natural antioxidants are utilized as additives and supplements for the reduction of oxidative stress due to disease progression (Bains and Hall, 2012). As a result, attention has turned to harnessing newer and previously underutilized plant sources for their antioxidant potentials. Specifically, protein hydrolysate preparations and peptides from previously underutilized plants are being investigated for their biological activities. Peptides obtained from hydrolysis of plant and animal proteins have been identified as safer alternatives to synthetic antioxidants. The increased interest of antioxidant peptides obtained from these sources is as a result of their potential roles as dietary supplements (Ajibola *et al.,* 2011: Olusola *et al.,* 2018). Peptide antioxidants have simpler structures than their parent proteins. This enhances greater stability in different situations (heat, protease activities), they elicit no immunoreactions and often exhibit enhanced nutritional and functional properties in addition to their antioxidant activity (Xie, *et al.,* 2008). For most peptides, the ability to scavenge free radicals is mostly dependent on the peptide size, with peptides of low molecular weight being more effective than those of larger molecular weight. (Alashi *et al.,* 2014). One of the plants whose proteins and peptides have not been previously investigated is *Albizia lebbeck. Albizia lebbeck* is a large, deciduous, drought – resistant, leguminous tree belonging to the *Mimosaceae* family. It is commonly known as the Siris tree or Lebbeck tree. It is alternatively known as "women's tongue" because of the rattling sound its mature seed-containing pods make when the wind blows (Verma *et al.,* 2013). It is relatively ubiquitous as it grows well in a wide diversity of regions. It is native to a number of areas especially in the forests of sub – Saharan African and Asian countries. *A. lebbeck* is a plant whose leaves, pods, seeds, stem bark and roots are excellent sources of bioactive phytochemicals and other secondary metabolites of pharmacologic significance (Zia-Ul-Haq *et al.,* 2013; Verma *et al.,* 2013). Its leaves contain flavonoids, saponins, tannins, alkaloids and cyanogenic glycosides (Bobby *et al.,* 2012) just as its roots are reservoirs of lupeol, stigmasterol and echinocystic acid (Verma *et al.,* 2013; Musa *et al.,* 2020). The seeds of *A. lebbeck* are rich sources of minerals such as calcium, iron, magnesium and potassium; phytonutrients which include ascorbic acid and niacin; as well as essential fatty acids (Verma *et al.,* 2013). Zia-Ul-Haq and others (2013) found out that that its seeds contained 34.17% crude protein, 49.07% carbohydrate and 5.62% crude fibre. Its high protein content makes it a good source of potentially bioactive peptides. Proteins of *A. lebbeck* are rich in arginine, lysine, histidine, leucine, isoleucine, phenylalanine, valine, tyrosine, aspartate, glutamate, but limiting in methionine and cysteine (Zia-Ul-Haq *et al.,* 2013). Parts of the plant have been used as feed for ruminant livestock (Hassan *et al.,* 2007) and in traditional medicine (Chulet *et al.,* 2010). Its leaves have been reported to possess antimicrobial, anticancer, anti-diarrheic activities (Bobby *et al.,* 2012) Its stem bark extracts have also been reported to demonstrate anti-parasitic potentials, and its use in treating dental infections have been documented (Qadri *et al.,* 2005; Umar *et al.,* 2009). Despite its numerous biological activities, the antioxidative capacities of hydrolysate preparations of its

proteins have not been evaluated. As a result, this study is geared towards the antioxidant potentials of *Albizia lebbeck* seed protein hydrolysates.

Materials and Methods

Materials

Collection of Seeds

A. lebbeck seeds were obtained from its trees in the botanical garden of Adekunle Ajasin University Akungba Akoko, Ondo State. Nigeria. They were subsequently identified and voucher samples were deposited at the Department of Plant Science and Biotechnology, Adekunle Ajasin University, Akungba Akoko.

Chemicals and Reagents

Enzymes: Pepsin (from porcine gastric mucosa), trypsin (from bovine pancreas), phymotrypsin (from bovine pancreas), papain (from *Carica papaya*) were products of Sigma-Aldrich laboratories, Co-Artrim, United Kingdom.

Other Reagents: 1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, trichloroacetic acid (TCA), potassium ferricyanide, ferric chloride, pyrogallol, hydrogen peroxide, ethylene diamine tetraacetic acid (EDTA) These were also products of Sigma-Aldrich laboratories, Co-Artrim, United Kingdom. All chemicals and reagents used were of analytical grade.

Methods

Isolation of *A. lebbeck* Seed Proteins

The seeds were dried, pulverized and kept in a dry container at 4°C. They were defatted using n-hexane as described by Olusola *et al.,*(2018). The meal was extracted twice with n-hexane using twenty grams (20 g) of seed meal suspended in 200 ml of n-hexane. The meal was then dried at 40°C in a vacuum oven and ground again to obtain a fine powder, termed defatted seed meal, which was stored at -10°C. The protein component of the defatted meal was extracted using the method reported by Ekun *et al.*(2022). Defatted *A. lebbeck* seed meal was suspended in 0.5 M NaOH pH 12.0 at a ratio of 1:10, and stirred for one hour for the purpose of alkaline solubilization, using a magnetic stirrer. This was centrifuged at 18°C and 3000 g for 10 min. One additional extraction of the residue from the centrifugation process was performed with the same volume of 0.5 M NaOH and the supernatants were pooled. The pH of the supernatant was adjusted to 4.0 to facilitate acid-induced protein precipitation using 0.5 M HCl solution; the precipitate formed was recovered by centrifugation. The precipitate was washed with distilled water, adjusted to pH 7.0 using 0.1 M NaOH, freeze-dried and the protein isolate was then stored at -10°C until required for further analysis.

Preparation of *A. lebbeck* Seed Protein Hydrolysates

The protein isolate was hydrolysed using the methods described by Olusola and Ekun (2019) with slight modifications. The conditions for hydrolysis were optimized for each enzyme for maximized activity. Hydrolysis was performed using each of pepsin (pH 2.2, 37ºC), trypsin (pH 8.0, 37ºC), chymotrypsin (pH 8.0, 37ºC and papain (pH 6.0, 60ºC). The protein isolate (5% w/v) was dissolved in the appropriate buffer (phosphate buffer, pH 8.0 for trypsin and chymotrypsin, glycine buffer, pH 2.2 for pepsin, phosphate buffer, pH 6.0 for papain). The enzyme was added to the slurry at an enzyme-substrate ratio (E:S) of 1:50. Digestion was performed at the specified conditions for eight (8) hours. The enzyme was

then inactivated by boiling in water bath (100°C) for fifteen (15) minutes and undigested proteins were precipitated by adjusting the pH to 4.0 with 2 M HCl/2 M NaOH followed by centrifugation at 7000 g for 30 min. The supernatant containing the peptides were then collected. The protein content of samples were determined using biuret assay method with bovine serum albumin (BSA) as standard.

Determination of DPPH Radical Scavenging Activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was measured by using the assay method described by Arise *et al.*(2016a) with slight modifications. 1 mL each protein hydrolysate at different concentrations (0.5 – 2.5 mg/ml) was added to 1 mL 0.1 mM DPPH dissolved in 95 % ethanol. The mixture was shaken vigorously and incubated in the dark and at room temperature for 30 min. The absorbance was read at 517 nm. Ethanol (95 %) was used as a blank. The control solution consisted of 0.1 mL of 95 % ethanol and 2.9 mL of DPPH solution. Analyses were carried out in triplicates. Percentage inhibition of DDPH radical was calculated as follows:

$$\% \text{ DPPH inhibition} = (Abs_{control} - Abs_{sample}) \times 100 / (Abs_{control})$$

EC_{50} values were estimated from percentage inhibition plot, using a non-linear regression plot.

Determination of Ferric Reducing Antioxidant Property

The reducing power of the protein hydrolysates was determined according to the method reported by Olusola *et al.*(2018) with slight modification. An aliquot of 1 ml of different concentrations (0.5 – 2.5 mg/ml) of the hydrolysates (0.2 M PBS, pH 6.6) was mixed with 1 ml of 1% potassium ferric cyanide solution. The mixture was then incubated at 50°C for 30 minutes followed by the addition of 1 ml 10% (w/v) TCA. 1 ml of the incubation mixture was added with 1 ml of distilled water and 0.2 ml of 0.1% (w/v) ferric chloride in test tubes. After a 10 min reaction time, the absorbance of the resulting solution was read at 700 nm. Higher absorbance suggested stronger reducing power. Ascorbic acid was used as the reference antioxidant. An aqueous solution of known Fe(II) concentrations ($FeSO_4 \cdot 7H_2O$; 2.0, 1.0, 0.5, 0.25, 0.125 mM) were used for calibration. Results were expressed as mM Fe^{2+}/mg hydrolysate. All the tests were performed in triplicate.

Determination of Hydroxyl Radical Scavenging Activity

Determination of the ability of the hydrolysates to prevent Fe^{2+}/H_2O_2 induced deoxyribose decomposition was carried out using the method described by Ogunwa *et al.*(2016) with some modifications. The reaction mixture contained 120 µL of 20 mM deoxyribose, 400 µL of 0.1 M phosphate buffer pH 7.4, 40 µL of 20 mM hydrogen peroxide and 40 µL of 0.5M iron (II) tetraoxosulphate (VI). The hydrolysates were added to the mixture. Distilled water was then added to make the mixture volume up to 800 µL. This was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 0.5ml of 2.8% thiobarbituric acid, TBA. The reaction tubes were incubated in boiling water for 20 mins, after which they were centrifuged at 3000g for 10 minutes. The absorbance was taken at 532 nm using a spectrophotometer. Percentage inhibition of the hydroxyl radical was calculated as follows:

$$\% \text{ OH inhibition} = (Abs_{control} - Abs_{sample}) \times 100 / (Abs_{control})$$

Determination of Superoxide Radical Scavenging Activity

The method described by Xie *et al.* (2008) was used to determine the abilities of the hydrolysates to scavenge the superoxide radical. Samples were each dissolved in 50 mM Tris–HCl buffer, pH 8.3 containing 1 mM EDTA and 80 µL was transferred into a clear bottom microplate well; 80 µL of buffer was added to the blank well. This was followed by addition of 40 µL 1.5 mM pyrogallol (dissolved in 10 mM HCl) into each well in the dark and the change in the rate of reaction was measured immediately at room temperature over a period of 4 minutes using a microplate reader at a wavelength of 420 nm. Ascorbic acid was used as control. The superoxide scavenging activity was calculated using the following equation:

$$\text{Superoxide scavenging activity (\%)} = (\Delta Abs/min_b - \Delta Abs/min_s)/\Delta Abs/min_b \times 100$$

where b and s are blank and sample, respectively.

Statistical Analyses

Results were expressed as mean of triplicate observations ± standard error of mean. The data were statistically analyzed using One Way Analysis of Variance (ANOVA) and Duncan's multiple range tests. Differences were considered statistically significant at $p<0.05$ using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

Results and Discussion

DPPH Radical Scavenging Activity

The abilities of *A. lebbeck* seed protein hydrolysates are illustrated in Figure 1 and their values of 50% inhibition are also displayed in Figure 2. All peptide digests demonstrated significantly ($p<0.05$) reduced DPPH scavenging activities when compared to the control, ascorbic acid. Among the hydrolysates, peptide digests obtained from peptic hydrolysis had the highest ($p<0.05$) DPPH scavenging activities at all the studied concentrations, reaching a maximal effect of 57.589 ± 1.286 % at a final concentration of 2.5 mg mL^{-1}. It had the lowest ($p<0.05$) EC_{50} value (1.075 ± 0.021 mg mL^{-1}) among the peptide digests. This was comparable to 56.39 ± 0.45% obtained for peptic hydrolysates of *Citrullus lanatus* seed proteins (Arise *et al.,* 2016a), and higher than 48% for peptic digests of yellow fin protein hydrolysates (Naqash & Nazeer, 2013). The potential bioactivity of a peptide released from enzymatic hydrolysis has been found to be dependent on factors such as nature of proteolytic enzyme utilized, hydrolysis time, as well as identity and position of amino acid residues in the peptide (Udenigwe & Aluko, 2011; Ekun *et al.,* 2022). Pepsin being an enzyme which cleaves polypeptides at C-terminals of hydrophobic and to a lesser extent, acidic residues may have cleaved the proteins into several dissimilar peptides with the required amino acid residues to donate protons to, and consequently scavenge the DPPH radical. At higher concentrations, chymotrypsin hydrolysates demonstrated higher ($p<0.05$) scavenging activities than tryptic and papain digests, displaying an ability of 37.385±0.887 % at a final concentration of 2.5 mg mL^{-1}. This could be that the aromatic aminoacyl residues (tryptophan, tyrosine, phenylalanine) released during proteolysis took part in scavenging the DPPH radical. This is consistent with the report of Wu *et al.* (2011) who reported that, even free aromatic amino acids, tryptophan and tyrosine from egg yolk have antioxidant potentials in foods.

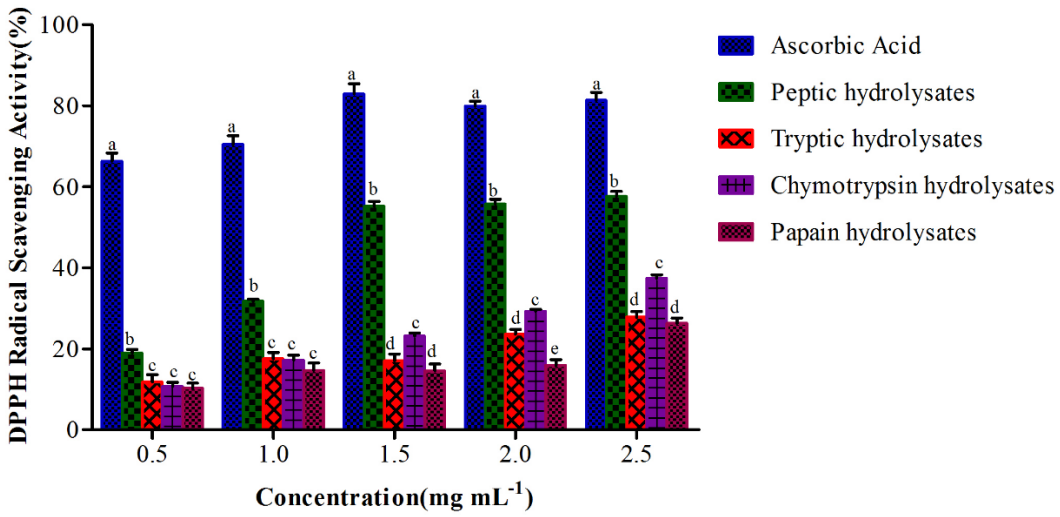

Figure 1. DPPH Radical Scavenging Activity of *A. lebbeck* Seed Protein Hydrolysates

Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Comparisons are made among samples of the same concentration only. Values with different letters are significantly different from one another. Bars carrying the same letter or symbol are not significantly different ($p<0.05$).

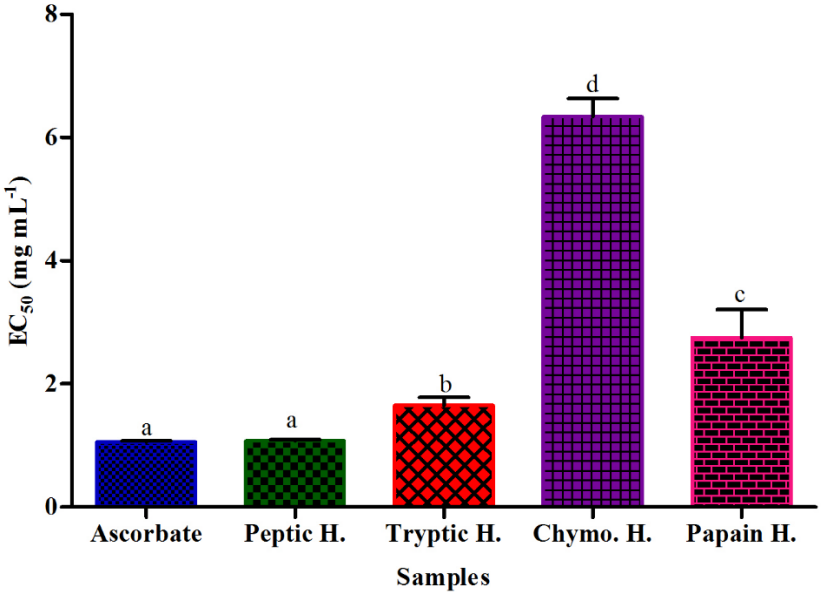

Figure 2. Fifty Percent Effective Concentration Values (EC_{50}) of DPPH Radical Scavenging Activity of *A. lebbeck* Seed Protein Hydrolysates

Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Bars with the same letters do not differ significantly while bars with different letters are significantly different ($p<0.05$) from one another.

Ferric Reducing Antioxidant Property

The relative abilities of *A. lebbeck* seed protein hydrolysates in reducing the ferric ion, in comparison to ascorbate, are depicted in Figure 3. All hydrolysates had significantly lower ($p<0.05$) reducing power when compared to ascorbic acid (control). They also demonstrated a concentration-dependent increase in reducing power. This is consistent with a previous study (Razali *et al.*, 2015) which revealed a similar trend of results. The low ferric-reducing property of *A. lebbeck* seed protein hydrolysates when compared with ascorbate may be attributed to the relatively low amount of sulfur-containing aminoacyl residues in the hydrolysates, which would have otherwise increased antioxidative activity by donating protons to ferric iron in the reaction medium (Lopez-Barrios *et al.,* 2014). However, peptic digests exhibited significantly higher ($p<0.05$) reducing capacities than other hydrolysates, as it converted a maximum of 52.000 ± 0.589% mM of Fe^{3+} to Fe^{2+} at a final concentration of 2.5 mg mL^{-1}. This was comparable to the reducing abilities of peptic digests of *Moringa oleifera* seed proteins (Olusola *et al.,* 2018) but higher than those of peptic hydrolysates obtained from cowpea seed proteins(Olusola and Ekun, 2019). Udenigwe and Aluko (2011) previously reported that acidic amino acid residues are moderately strong contributors to the reduction of ferric ions. This could be the reason why peptic hydrolysates still exhibited high reducing antioxidant power, even with limiting amounts of sulfur – containg aminoacyl residues such as cysteine and methionine. Tryptic hydrolysates also reduced 22.483 ±2.265 mM of Fe^{3+} to $Fe^{2+}$and these were higher ($p<0.05$) than the reducing properties of chymotryptic and papain hydrolysates, and this is consistent with previous studies on unfractionated hydrolysates of watermelon seed proteins (Arise *et al.,* 2016a), and this suggests the presence of some other aminoacyl residues within the peptides, which may have contributed to the reduction of ferric ions.

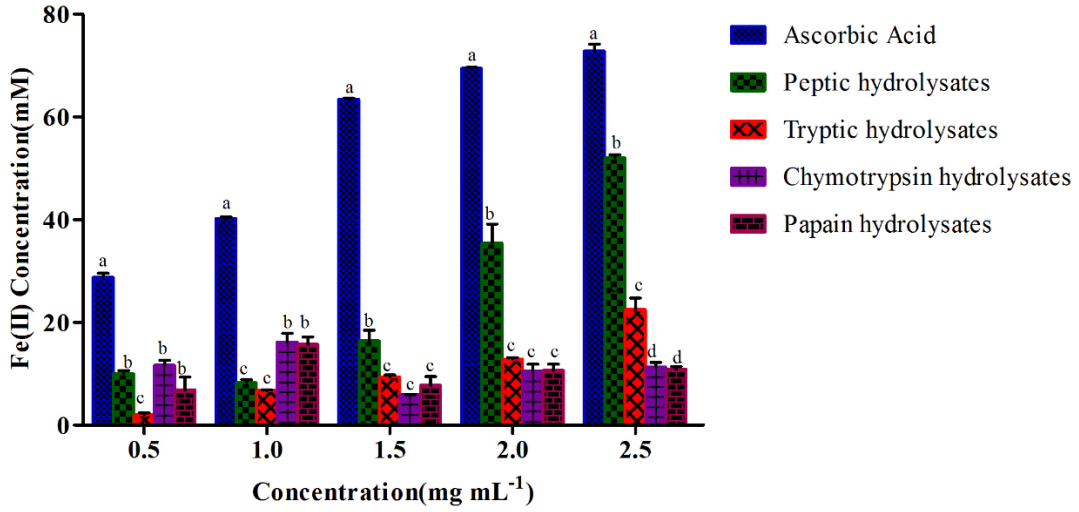

Figure 3. Ferric Reducing Antioxidant Properties of *A. lebbeck* Seed Protein Hydrolysates

Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Comparisons are made among samples of the same concentration only. Values with different letters are significantly different from one another. Bars carrying the same letter or symbol are not significantly different ($p<0.05$).

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging capacities of *A. lebbeck* seed protein hydrolysates are illustrated in Figure 4, while their values of 50% inhibition are displayed in Figure 5. All hydrolysates demonstrated varying hydroxyl radical scavenging activities, such that chymotrypsin hydrolysates displayed significantly higher scavenging property than other digests, attaining a maximum activity of 36.925 ± 1.880 % at 2.5 mg mL^{-1}. There is relative paucity of information relating to the activities of protein hydrolysates on the hydroxyl radical, when compared to reports on their effects on other free radical systems. The effects of *A. lebbeck* seed protein hydrolysates and on the hydroxyl radical indicated that these protein digests had lower OH radical scavenging activities than the standard antioxidant, ascorbic acid. However, chymotrypsin hydrolysates scavenged the OH radical better than the other hydrolysates. Chymotrypsin is a specific endoprotease that cleaves proteins at C-terminal ends of aromatic amino acid residues (Voet *et al.,* 2016). Also, Li & Li (2013) had previously found that the biochemical nature of the amino acid(s) at the C-terminal of peptides is more important to its antioxidative ability than that of its N-terminal. In similar fashion, Siow and Gan (2016) reported that hydrophobic, as well as aromatic aminoacyl residues are strong contributors to the scavenging of free radicals owing to their electron-dense branch chain groups. It therefore follows that the C-terminal aromatic residues arising from chymotrypsin digestion may have contributed strongly to the quenching of the hydroxyl radical. Papain digests and trypsin hydrolysates also recorded maximal scavenging activities of 30.663 ± 0.824 % and 31.544 ± 0.464 % respectively. These activities were significantly higher when compared to the maximum activity of peptic hydrolysates. However, all hydrolysates demonstrated reduced lower ($p<0.05$) hydroxyl radical quenching properties when compared to control. These results translated to EC_{50} values of 0.668 ± 0.114 mg mL^{-1}, 6.148 ± 0.3348 mg mL^{-1}, 3.227 ± 0.288 mg mL^{-1}, 2.697 ± 0.098 mg mL^{-1}, and 1.206 ± 0.045 mg mL^{-1} for ascorbic acid (control), peptic, tryptic, chymotrypsin hydrolysates and papain digests respectively. Among the protein digests, papain hydrolysates had the lowest ($p<0.05$) EC_{50} value. Papain digests also demonstrated hydroxyl radical scavenging activities, but these reduced at higher concentrations and this is exemplified by its low EC_{50} (1.206 ± 0.045 mg mL^{-1}) when compared to other hydrolysates. Papain, being a non-specific enzyme capable of releasing quite a lot of short, dissimilar peptides (Naik, 2012; Voet *et al.,* 2016) could have produced certain peptides which, in turn, might have antagonized the effects of the main biologically active peptides in solution. This phenomenon may have been responsible for the progressive reduction in their activities at higher concentrations. Going by the cleavage specificities of the enzymes used in the hydrolysis, it could be inferred that these hydrolysates contained peptides with these aminoacyl residues which could scavenge the hydroxyl radical, either by virtue of proton donation and/or by their high hydrophobicities.

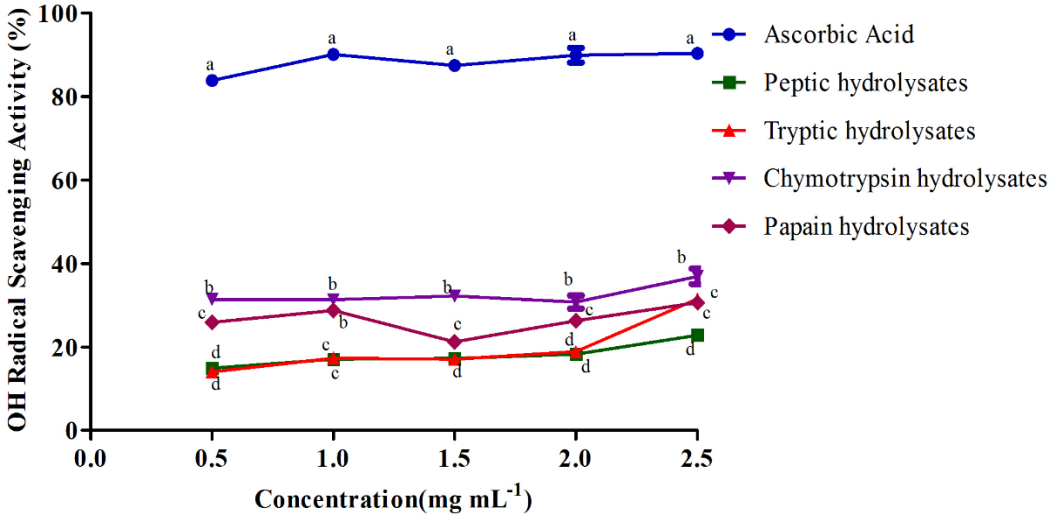

Figure 4. Hydroxyl Radical Scavenging Activities of *A. lebbeck* Seed Protein Hydrolysates

Dots on each line graph are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Dots belonging to different hydrolysate/fractions with different letters at the same concentration are significantly different ($p<0.05$) from one another.

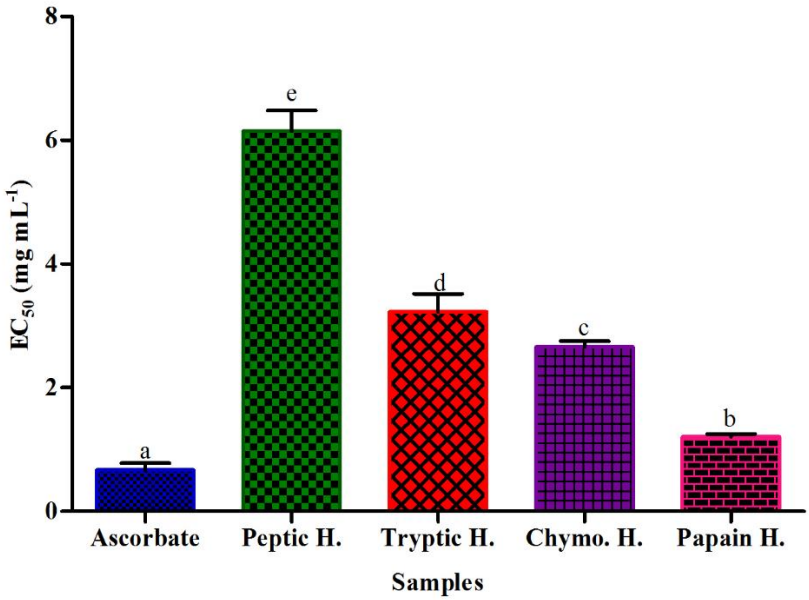

Figure 5. Fifty Percent Effective Concentration Values (EC_{50}) of Hydroxyl Radical Scavenging Activities of *A. lebbeck* Seed Protein Hydrolysates

Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Bars with the same letters do not differ significantly while bars with different letters are significantly different ($p<0.05$) from one another.

Superoxide Radical Scavenging Activity

The effects of *A. lebbeck* seed protein digests on the superoxide radical and their values of 50% inhibition are displayed in Figures 6 and 7 respectively. As with other antioxidant activities, all hydrolysates displayed significantly lower ($p<0.05$) abilities against

the superoxide radical when compared with the control. Peptide digests obtained from chymotrypsin hydrolysis had better ($P<0.05$) capabilities than other hydrolysates in scavenging the superoxide radical, such that it had a maximum activity of 74.520 ± 0.998% at 2.5 mg mL^{-1}. However, there is a relative paucity of information in the literature about the activities of chymotrypsin – hydrolyzed proteins against the superoxide radical. Peptides obtained by chymotrypsin digestion have a number of hydrophobic residues and aromatic residues toward their C-terminals (Voet *et al.,* 2016; Ekun *et al.,* 2022) and these may have contributed to their increased quenching of the superoxide radical as concentrations increased. Tryptic digests were also effective in quenching the superoxide radical (58.659 ± 0.334 % at 2.5 mg mL^{-1}) better than peptic hydrolysates and these results were consistent with the reports of Olusola *et al.*(2018) in their findings with unfractionated *M. oleifera* seed protein hydrolysates. At the same time, it was higher than what was obtained for tryptic digests of watermelon seed proteins (< 20% scavenging activity)(Arise *et al.,* 2016b). Tryptic hydrolysates displayed a concentration – dependent increase in superoxide scavenging activity, attaining a maximum quenching extent of 58.659 ± 0.334 %. Udenigwe and Aluko (2011) earlier stated that lysine residues are especially important contributors to the quenching of the superoxide radicals. This could explain why tryptic hydrolysates were able to scavenge the superoxide radical, owing to the fact that they contain several peptides having lysine residues on their C-terminals. Papain hydrolysates had its maximal scavenging activity of 47.698 ± 1.083 % at a low concentration of 0.5 mg mL^{-1}, and the lowest EC_{50} of 0.970 ± 0.067 mg mL^{-1}. This indicates that the activity of papain digests against the superoxide radical decreased with increasing concentration, and this could be that the continued presence of certain peptides in the solution could have antagonized the activities of peptides that could have contributed positively to the quenching of the superoxide radical.

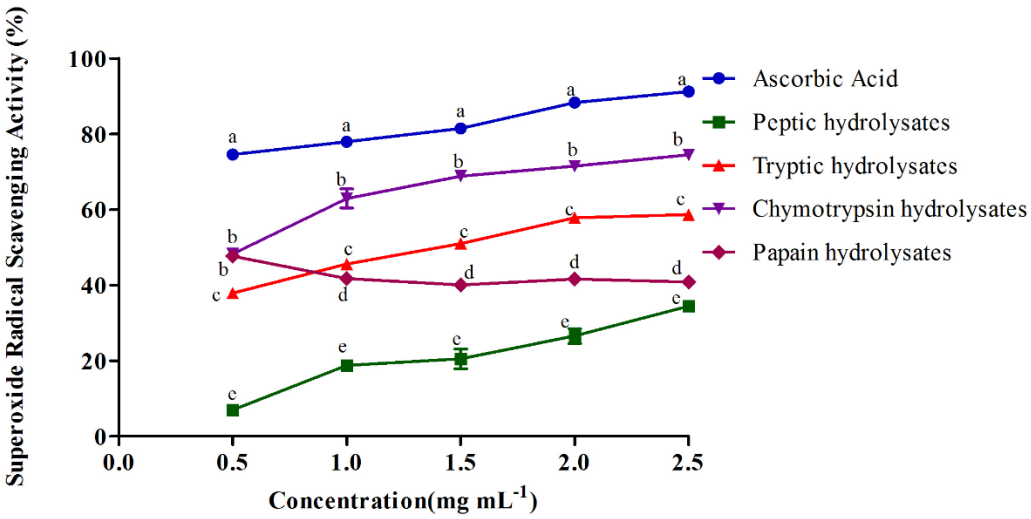

Figure 6. Superoxide Radical Scavenging Activities of *A. lebbeck* Seed Protein Hydrolysates

Dots on each line graph are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Dots belonging to different hydrolysate/fractions with different letters at the same concentration are significantly different ($p<0.05$) from one another.

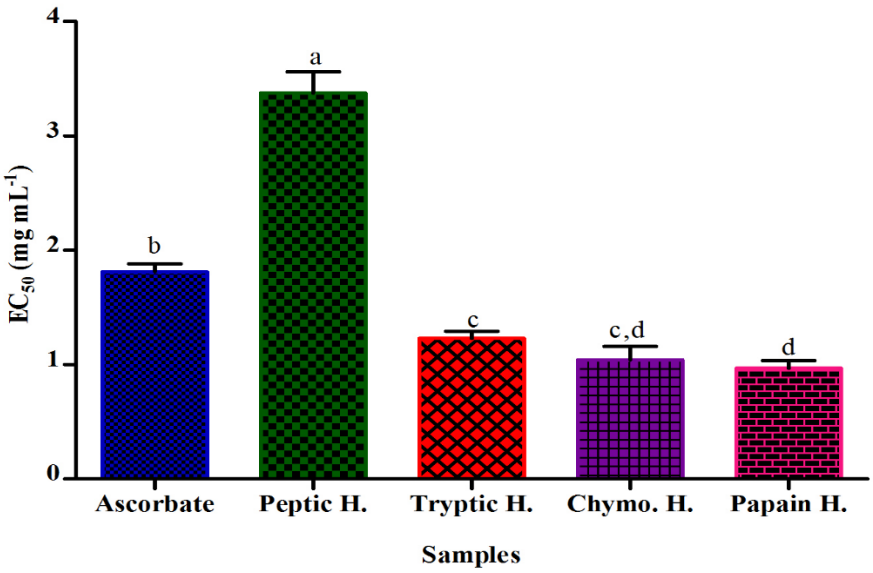

Figure 7. Fifty Percent Effective Concentration Values (EC_{50}) of Superoxide Radical Scavenging Activities of *A. lebbeck* Seed Protein Hydrolysates

Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Bars with the same letters do not differ significantly while bars with different letters are significantly different ($p<0.05$) from one another.

Conclusion

The results of this study demonstrated that the proteins of *A. lebbeck* seed proteins were susceptible to enzymatic hydrolysis and yielded hydrolysate preparations which possessed antioxidant activities against free radical systems and ferric ions *in vitro* howbeit by differing mechanisms. Peptic hydrolysates demonstrated the highest activities against DPPH radical and ferric ions, while chymotrypsin hydrolysates scavenged both hydroxyl and superoxide radicals better than other digests. This implies that, depending on the choice of enzyme used, *A. lebbeck* seed proteins possess biologically active peptides with immense potentials as food additives and therapeutic benefits. This will contribute towards increased value-added use of *A. lebbeck* seeds, which are currently being under-utilized.

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