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Antioxidant and Renin-Angiotensin System Inhibitory Properties of Cashew Nut and Fluted-Pumpkin Protein Hydrolysates

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Antioxidant and renin-angiotensin system (RAS)-inhibitory protein hydrolysates derived from the enzymatic hydrolysis of cashew nut (CNP) and fluted pumpkin (FPP) proteins were investigated. The CNP and FPP hydrolysates (CNPH and FPPH) from pepsin or Alcalase treatments were subjected to membrane ultrafiltration using different MWCOs to obtain <1, 1–3, 3–5, 5–10, and >10 kDa peptide fractions. Hydrolysis of protein isolates at similar enzyme levels allowed obtaining peptic hydrolysates with a lower degree of hydrolysis (46.7–48.0%) when compared to the Alcalase--produced hydrolysates (47.7–50.3%). Amino acid composition revealed that CNPH had 28% hydrophobic residues when compared to higher contents (32–35%) in the 3–10 kDa peptide fractions. In contrast, aromatic residues increased from 8% in the FPPH to 9–13% in the peptide fractions. The *in vitro* •OH and DPPH• scavenging activities were significantly ($p<0.05$) enhanced by ultrafiltration but potency was inversely related to peptide size. The ferric-reducing power was the highest for the <1 kDa CNPH (2.47) when compared to 1.33 for CNPH and other peptide fractions. Metal chelation ability was significantly ($p<0.05$) enhanced by ultrafiltration only for the CNPH with 21% compared to ~96% for the peptide fractions. ACE inhibition was significantly ($p<0.05$) lower for the Alcalase CNPH and peptide fractions (~87%) compared to ~92% of pepsin-CNPH. However, renin inhibition was significantly ($p<0.05$) increased by ultrafiltration from 45.7 and 62.1% to ~82.4 and 96.5% for FPPH and CNPH, respectively. We conclude that the strong antioxidant properties coupled with RAS inhibition make CNPH and FPPH as well as their low molecular weight peptides potential ingredients to formulate health-promoting foods.

INTRODUCTION

Scientific research has focused heavily on evaluating the nutritional value of the underutilized and underexploited food crops. Crops like cashew nut [Liu *et al*., 2018], *Bucchol-zia coriacea* [Ijarotimi *et al*., 2018a], African yam bean [Ajibo-la *et al*., 2016], bambara groundnut [Thammarat *et al*., 2015] and fluted pumpkin seeds [Fagbemi, 2007] have been inves-tigated as functional (foaming and emulsifying) ingredients in industrial applications based on their protein content (15–30%). However, another option is to convert the proteins into value-added bioactive peptides by enzymatic hydrolysis. Bio-active peptides usually contain 2–20 amino acid residues per molecule, and are released upon enzymatic hydrolysis, during food processing or gastrointestinal digestion [Malomo *et al*., 2015]. These peptides may exert diverse physiological effects, such as antihypertensive, antimicrobial, antithrombotic, hypocholesterolaemic, antioxidative, and can also inhibit calmodulin-dependent enzymes [Aderinola *et al*., 2019; Nwa-chukwu *et al*., 2019; Siddeeg *et al*., 2015;Thammarat *et al*., 2015]. These physiological effects are due to their amino acid composition, peptide sequences and molecular weights.

Fluted pumpkin (*Telfairia occidentalis*), commonly known as *fluted gourd*, *Ugu* and *edikangikong* (in Ibo and Efik, Nigeria languages, respectively) is a tropical plant grown in West Africa with its seeds widely consumed in Nigeria as soup condiments [Fagbemi, 2007]. The fermented seeds have high seed pro-tein contents (66–71%), which made them suitable functional ingredients to formulate marmalade and cookies [Fagbemi, 2007]. Cashew nut (*Anarcadium occidentale*) is a heart-like shaped fruit widely grown in Africa, with annual production of about 5–7 metric tonnes, but cultivated mainly as an export crop in Nigeria [Fagbemi, 2009]. Its proteins have a well-bal-anced amino acid composition, which is abundant enough to meet the requirements recommended by the FAO/WHO [Liu *et al*., 2018]. Besides, the SDS-PAGE pattern of cashew nut proteins revealed polypeptides of 21–32 and 53 kDa molecu-lar weights (MW) under reducing and non-reducing condi-tions [Liu *et al*., 2018]. The SDS-PAGE pattern under reduc-ing conditions indicate presence of inter-molecular disulfide bonds in the polypeptide molecules [Liu *et al*., 2018; Malomo

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& Aluko, 2015], which could lead to the production of sulfur-containing peptides with strong antioxidant properties. In addition, the amino acid compositions of fluted pumpkin and cashew nut proteins indicate an abundance of negatively charged amino acids (glutamic and aspartic) that can also contribute to antioxidant properties due to the presence of excess electrons [Udenigwe & Aluko, 2011]. Fluted pumpkin and cashew nut proteins also contain high levels of positively-charged (lysine and arginine) and branched-chain (valine, leucine, isoleucine) amino acids, which are reported to enhance the inhibitory activities of peptides against enzymes involved in hypertension development [Udenigwe *et al*., 2012; Wu *et al*., 2006a, b].

In disease pathogenesis and management systems, free radicals have been implicated to cause oxidative stress, giving rise to unacceptable state of health that reduce the quality of life through development of several chronic diseases. Therefore, it is important to inhibit or scavenge these radicals by using antioxidants [Arise *et al*., 2017]. Although, the body possesses natural self-defense mechanisms (such as antioxidants like glutathione and superoxide dismutase) to fight the free radicals, there exists a concern about the ineffective amounts present during illness and in old age [Thammarat *et al*., 2015]. Therefore, research efforts focused on food protein-derived hydrolysates, and peptides as new sources of natural antioxidants have recently emerged.

Various plant materials including *Linum usitatissimum* [Nwachukwu *et al*., 2019], *Buccholzia coriacea* [Ijarotimi *et al*., 2018b], *Cajanus cajan* [Olagunju *et al*., 2018], *Moringa oleifera* [Aderinola *et al*., 2019], *Vigna subterranean* [Arise *et al*., 2017], *Cucumis melo* [Siddeeg *et al*., 2015], and *Voandzeia subterranea* [Thammarat *et al*., 2015] have been identified as potential antioxidants sources. This was demonstrated by the strong antioxidant activity of their protein hydrolysates, including radical scavenging activity, reducing power and metal ion chelating activities.

Bioactive peptides from food proteins have also exhibited renin-angiotensin system (RAS)-inhibitory activity. Regulation of arterial blood pressure in the human body is mainly achieved through diverse physiological systems [Majumder & Wu, 2015] including the RAS, kinin-nitric oxide system (KNOS), renin-chymase system (RCS) and neutral endopeptidase system (NEPS). The key elements constituting the RAS are both the renin and angiotensin converting enzyme (ACE). The renal renin acts on the zymogen, angiotensinogen from the liver to produce inactive decapeptide, angiotensin-I (AT-I). ACE then, hydrolyzes AT-I by the removal of the dipeptide His-Leu from the C-terminus, to produce angiotensin-II (AT-II), a potent vasoconstrictor octapeptide [Aluko, 2019a; Malomo *et al*., 2015]. High levels of AT-II can lead to excessive vasoconstriction, which causes hypertension, a chronic medical symptom responsible for worldwide health problems because of its ability to trigger cardiovascular complications including peripheral vascular disease and renal dysfunctions [Arise *et al*., 2017]. Thus, RAS inhibitors block the formation of AT-II and potentiate action of the vasodilatory nitric oxide [Malomo *et al*., 2015].

Several potent synthetic ACE inhibitors (captopril, lisinopril, fosinopril, enalapril) are used extensively in the clinical treatment of hypertension but with significant adverse effects (dry cough, skin rashes, headache) on health [Malomo *et al*., 2015]. Recent studies on diverse peptides released from pigeon pea [Olagunju *et al*., 2018], Bambara groundnut [Arise *et al*., 2017], and pistachio [Li *et al*., 2014] proteins have shown ability to modulate RAS functions. Interestingly, studies on food bioactive peptides do not indicate side effects in the hypertension rat model [Udenigwe *et al*., 2017; Girgih *et al*., 2016]. However, previous works have reported on some structural features of potent antioxidant as well as ACE and renin-inhibitory peptides. For example, a quantitative structure-activity relationship (QSAR) study was used to show that amino acids with bulky and hydrophobic side groups are preferred for ACE-inhibitory dipeptides [Wu *et al*., 2006a]. For tripeptides, an aromatic amino acid at the C-terminus with a positively charged amino acid in the middle and a hydrophobic residue at the N-terminus enhanced ACE inhibition. For longer peptides, the ACE-inhibitory activity was dependent on the four amino acid residues present at the C-terminus [Wu *et al*., 2006b]. Meanwhile, the potency of dipeptides that inhibit renin activity was shown to be dependent on a branched-chain amino acid at the N-terminus with tryptophan at the C-terminus [Udenigwe *et al*., 2012]. With respect to antioxidant protein hydrolysates, the presence of sulfur-containing, acidic amino acids, lysine and leucine enhanced free radical scavenging ability [Udenigwe & Aluko, 2011].

Accordingly, more studies are now directed towards understanding how to produce food protein-derived peptides to be used as nutraceuticals for managing chronic diseases. This study, thus aimed to investigate and assess the feasibility of producing enzymatic fluted pumpkin seed and cashew nut protein hydrolysates with *in vitro* antioxidant and RAS-inhibitory activities.

MATERIALS AND METHODS

Materials

Fluted pumpkin seeds and cashew nuts were obtained from the Federal University of Technology, Teaching and Research Farm, Akure, Nigeria, authenticated in the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure, Nigeria. Renin was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) while other enzymes such as Alcalase, pepsin and ACE (rabbit lung) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other analytical-grade reagents were obtained from Fisher Scientific (Oakville, ON, Canada).

Preparation of fluted pumpkin and cashew nuts protein meal

The fluted pumpkin seeds were dehulled and sliced into small pieces according to the previously described method [Fagbemi, 2007]. The sliced seeds were oven dried at 50°C (Plus11 Sanyo Gallenkamp PLC, UK), for 8 h and then pulverized into flour using a coffee grinder. The flour was then defatted continuously for 8 h with a Soxhlet's apparatus using *n*-hexane as a solvent and air-dried for 24 h in a fume hood at room temperature to remove solvent residues. The defatted

flour was then passed through 0.5 mm sieves, packed in plastic containers, labeled as the fluted pumpkin protein flour (FPP), and kept at -20°C.

The cashew nuts were cut into halves using the manual cashew kernel cutter according to the previously described method [Fagbemi, 2009]. After cutting, the nuts were removed and dried in an oven at 100°C for 2 h. The covering testas were removed by squeezing and then winnowed to obtain cream color nuts. The nuts were then broken into smaller pieces and defatted using *n*-hexane solvent. The defatted nuts were air-dried for 24 h in a fume hood at room temperature to remove the residual *n*-hexane, and thereafter milled into flour using a blender (Philips laboratory blender, HR2811 model, The Netherlands). The defatted cashew nut flour (CNP) was passed through a sieve with 0.5 mm mesh size, packed into a polythene bag and then stored at -20°C.

Preparation of fluted pumpkin seed and cashew nuts protein isolates

Fluted pumpkin seed and cashew nuts protein isolates (FPPI and CNPI) were produced from FPP and CNP according to the method described by Malomo *et al*. [2014] with slight modifications. Briefly, FPP and CNP were dispersed in deionized water (1:20, *w/v*) and the dispersion was adjusted to pH 10 using 2 M NaOH to solubilize the proteins while stirring at 37°C for 2 h; this was followed by centrifugation (7,000×*g*, 60 min at 4°C). The precipitate was discarded while the supernatant was filtered and thereafter adjusted to pH 4.0 with 2 M HCl to precipitate the proteins; this was followed by centrifugation (7,000×*g*, 60 min at 4°C). The resultant precipitate was re-dispersed in deionized water, adjusted to pH 7.0 with 2 M NaOH, and freeze-dried to obtain the FPPI and CNPI. Protein contents were determined by the Lowry method [Markwell *et al*., 1978] using 0.1 M NaOH to dissolve the samples.

Preparation of fluted pumpkin and cashew nuts protein hydrolysates and ultrafiltration membrane hydrolysate fractions

Fluted pumpkin seeds and cashew nut protein hydrolysates (FPPH and CNPH) were produced according to the method described by Malomo *et al*. [2015] with slight modifications using Alcalase at 50°C, pH 8 for 4 h and pepsin at 37°C, pH 2 for 2 h. FPPI and CNPI (5%, *w/v*, protein weight basis) were each suspended in deionized water in a glass beaker equipped with a stirrer, and adjusted to the appropriate temperature and pH prior to addition of the proteolytic enzyme. Alcalase (>2.4 U/g) and pepsin (>250 units/mg) were added separately to the FPPI and CNPI slurry at a 1–4:100 ratio (E/S). During hydrolysis, the required pH was maintained constant by addition of NaOH and HCl, respectively after which the enzymes were inactivated by adjusting to pH 4.0 with 2 M HCl followed by immersing the reaction vessel in boiling water bath for 10 min. After cooling to room temperature, the undigested proteins were precipitated by centrifugation (3,500×*g*, 60 min at 4°C) and a portion of each supernatant was freeze-dried as the FPPH and CNPH, which were then stored at -20°C. Protein contents of the freeze–dried FPPH and CNPH were determined using the modified Lowry method [Markwell *et al*., 1978]. The supernatants from Alcalase-produced FPPH and pepsin-produced CNPH at 2:100 and 3:100 ratios (E/S), respectively were also sequentially passed through ultrafiltration membranes with molecular weight cut-off (MWCO) of 1, 3, 5, and 10 kDa in an Amicon stirred ultrafiltration cell (Merck KGaA, Darmstadt, Germany). Thus, the retentate from 1 kDa membrane was passed through 3 kDa membrane whose retentate was passed through 5 kDa and the last retentate was then passed through 10 kDa membrane. The permeate from each MWCO membrane was collected as peptide sizes of <1, 1–3, 3–5, and 5–10 kDa, freeze-dried, and stored at –20°C.

Amino acid composition analysis

The amino acid profiles of the fluted pumpkin and cashew seed protein products were determined using the HPLC Pico-Tag system (Waters, Milford, MA, USA) according to the previously described method after samples were digested with 6 M HCl for 24 h [Bidlingmeyer *et al*., 1984]. The cysteine and methionine contents were determined after performic acid oxidation [Gehrke *et al*., 1985] and the tryptophan content was determined after alkaline hydrolysis [Landry & Delhaye, 1992]. The samples were derivatized for 20 min using a solvent mixture containing 95% ethanol:water:triethylamine:phenylisothiocyanate (7:1:l:1, *v/v/v/v*), dried under vacuum and dissolved in buffer A (940 mL of 0.14 M sodium acetate, pH 6.40, containing 0.05% triethylamine, mixed with 60 mL acetonitrile) prior to HPLC separation on the Pico-Tag column using a flow rate of 0.45 mL/min and detection at 254 nm. The gradient was from 10–50% buffer B (60% acetonitrile and 40% water by volume) in buffer A over 10 min.

Determination of the degree of hydrolysis

The degree of hydrolysis (DH) was defined as percentage of α-amino groups of substrates released during hydrolysis and was determined using the trinitrobenzene sulfonic acid (TNBS) method as previously described [Adler-Nissen, 1979] and modified by Malomo *et al*. [2015]. Briefly, CNPI and FPPI were treated under vacuum with 6 M HCl for 24 h and α-total amino groups as L-leucine equivalent was determined. The DH was calculated as the percentage ratio of the L-leucine equivalent of CNPH and FPPH to that of CNPI and FPPI.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activities of FPPH, CNPH, and peptide fractions were determined according to the method described by Girgih *et al.* [2011]. Experimental samples, GSH and 1,10-phenanthroline (3 mM) were each separately dissolved in 0.1 M phosphate buffer (pH 7.4) while $FeSO_4$ (3.0 mM) and 0.01% hydrogen peroxide were each dissolved in distilled water. An aliquot (50 μL) of samples or GSH (reaction mixture concentration of 1 mg/mL) or buffer (blank) was first added to a clear, flat bottom 96-well plate followed by 50 μL of 1,10-phenanthroline and then 50 μL of $FeSO_4$. To initiate the Fenton reaction in the wells, 50 μL of hydrogen peroxide was added to the mixture, the plate was covered and incubated at 37°C for 1 h with shaking. The change in absorbance values of samples (s) and blank (b)

were measured using Biotex 542 Powerwave XS microplate-reader (Biotek Instruments Inc., Winooski, Vermont, USA) at 536 nm at 10 min intervals for 1 h. The hydroxyl radical scavenging activity was calculated using the reaction rate (ΔA/min) equation below:

$$\text{OH radical scavenging activity (\%)} = = \left(\frac{(\Delta A_{536}/\text{min})b - ((\Delta A_{536}/\text{min})s}{(\Delta A_{536}/\text{min})b}\right) \times 100$$

2,2-Diphenyl-1-picryhydrazyl radical scavenging assay

The scavenging effect of CNPH, FPPH, and their peptide fractions on 2,2-diphenyl-1-picryhydrazyl free radical (DPPH•) was measured according to the method of Aluko & Monu [2003]. FPPH, CNPH and peptide fractions (10 mg) were each dissolved in 1 mL of buffer (0.1 M sodium phosphate buffer, pH 7.0 containing 1% (*w/v*) Triton X-100). DPPH• was dissolved in methanol to a final concentration of 100 μM. A 100 μL aliquot of each sample solution (containing FPPH, CNPH or peptide fractions) was mixed with 100 μL of the DPPH• solution in the 96-well plate to a reaction mixture concentration of 1 mg/mL (0.2 mg/200 μL) and incubated at room temperature in the dark for 30 min. The absorbance values of the blank, reduced glutathione (GSH) and samples were measured at 517 nm using a Biotex 542 Powerwave XS microplate-reader. The blank consisted of sodium phosphate buffer in place of the peptide sample, while GSH (1 mg/mL) was used as the positive control. The percent DPPH• radical scavenging activity of the samples was determined using the following equation:

$$\text{DPPH• radical scavenging activity (\%)} = \left(1 - \frac{A_{517}\ of\ sample}{A_{517}\ of\ blank}\right) \times 100$$

Ferric-reducing power

The ferric-reducing power of the FPPH, CNPH, and membrane fractions was determined according to the modified method of Zhang *et al*. [2008]. Experimental sample or GSH (10 mg) was dissolved in 1 mL of 0.2 M phosphate buffer, pH 6.6; an aliquot (250 μL) was mixed with 250 μL of the buffer and 250 μL of 1% potassium ferricyanide solution. The mixture was thoroughly mixed using a vortex machine and heated at 50°C for 20 min. After incubation, 250 μL of 10% trichloroacetic acid was added followed by 50 μL of 0.1% ferric chloride dissolved in double distilled water and then 200 μL of double distilled water was added to give sample concentration of 1 mg/mL reaction mixture. The solution was allowed to stand for 10 min at room temperature, after which it was centrifuged at 1,000×*g* for 10 min. An aliquot (200 μL) of the supernatant was transferred to a clear bottom 96-well plate and the absorbance was measured at 700 nm using a Biotex 542 Powerwave XS microplate reader. The ferric reducing power was presented as absorbance values at 700 nm.

Chelation of ferrous ions

The ferrous ion chelating activity of the FPPH, CNPH, and membrane fractions was determined using a modified method of Xie *et al*. [2008]. Experimental samples and GSH solution were each combined with 0.05 mL of 2 mM $FeCl_2$ and 1.85 mL double distilled water in a reaction tube to give 1 mg/mL reaction mixture concentration. Ferrozine solution (0.1 mL of 5 mM) was added and mixed thoroughly. The mixture was then allowed to stand at room temperature for 10 min from which an aliquot of 200 μL was removed and added to a clear bottom 96-well plate. A blank experiment was also conducted by replacing the sample with 1 mL of double distilled water. The absorbance of blank and sample at 562 nm was measured using a Biotek microplate reader and the ferrous ion chelating activity of the sample compared to that of GSH. The percentage chelating effect (%) was calculated using the following equation:

$$\text{Ferrous ion chelating activity (\%)} = \left(1 - \frac{A_{562}\ of\ sample}{A_{562}\ of\ blank}\right) \times 100$$

Angiotensin-Converting Enzyme (ACE) inhibition assay

The ability of FPPH, CNPH, and membrane fractions to inhibit *in vitro* ACE activity was measured according to a spectrophotometric method using synthetic *N*-[3-(2-furyl) acryloyl]-l-phenylalanyl-glycyl-glycine (FAPGG) as the substrate (Sigma-Aldrich, St. Louis, MO, USA) as reported by Malomo *et al*. [2015]. Briefly, 1 mL of 0.5 mM FAPGG (dissolved in 50 mM Tris-HCl buffer containing 0.3 M NaCl, pH 7.5) was mixed with 20 μL of ACE (20 mU final reaction activity) and 200 μL sample dissolved in same buffer. The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature using Varian Cary 50-UV/Visible spectrophotometer (Varian Inc., Belrose, NSW, Australia). The buffer was used instead of sample solutions in the blank experiment, while the sample concentration in the reaction mixture was 1 mg/mL. ACE activity was expressed as the rate of reaction (ΔA/min) and inhibitory activity was calculated as:

$$\text{ACE inhibition (\%)} = 1 - \left(\frac{\Delta A/\text{min} - 1(\text{sample})}{\Delta A/\text{min} - 1(\text{blank})}\right) \times 100$$

where: $\Delta A/\text{min}_{(\text{sample})}$ and $\Delta A/\text{min}_{(\text{blank})}$ represent ACE activity in the presence and absence of the samples, respectively.

Renin inhibition assay

In vitro inhibition of human recombinant renin activity by FPPH, CNPH, and membrane fractions was conducted using the Renin Inhibitor Screening Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the previously described method [Malomo *et al*., 2015]. Prior to the assay, renin buffer was diluted in 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20 times with the assay buffer before use and pre-warmed to 37°C prior to initiating the reaction in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA, USA) maintained at 37°C. Before the reaction, (i) 20 μL substrate, 160 μL assay buffer, and 10 μL Milli-Q water were added to the background wells; (ii) 20 μL substrate, 150 μL assay buffer, and 10 μL Milli-Q water were added to the blank wells; and (iii) 20 μL substrate, 150 μL assay buffer, and 10 μL sample (1 mg/mL reaction mixture concentration) were added to the inhibitor wells. The reaction was initiated by adding 10 μL renin to the blank and sample wells. The microplate was shaken for 10 s to mix, incubated at 37°C for 15 min, and the fluorescence intensity (FI)

was recorded using excitation and emission wavelengths of 340 and 490 nm, respectively. The percentage renin inhibition was calculated as follows:

$$\text{Renin inhibition (\%)} = 1 - \left(\frac{\text{FI sample}}{\text{FI (control)}}\right) \times 100$$

Statistical analysis

Triplicate replications were used to obtain mean values and standard deviations. Statistical analysis was performed with SAS (Statistical Analysis Software 9.1) using one-way ANOVA. Duncan's multiple-range test was carried out to compare the mean values for samples with significant differences taken at $p<0.05$.

RESULTS AND DISCUSSION

Amino acid composition

The protein contents of the CNPI and FPPI are 84 and 82.6%, respectively, which fall in the ranges (80–90%) previously reported [Malomo *et al*., 2014] for protein isolates from different plant protein sources. The amino acid compositions of peptides present in CNPH and FPPH as well as their membrane fractions are presented in Table 1. The results clearly showed that Glu, Asp, and Arg were present in the highest amounts of 14.66–23.29, 8.71–11.24, and 5.94–20.89 g/100 g, when compared to 3.32–7.15, 3.20–5.00, and 1.40–2.85 g/100 g obtained for other amino acids such as Val, Ile and Trp, respectively. The potentials of Glu and Asp to donate excessive electrons as free radicals scavengers (strong antioxidant) and Arg to work as NO-precursor, a known vasodilator, made them potential tissue protective agents against oxidative stress [Jakubczyk & Baraniak, 2014] and hypertension [Malomo *et al*., 2015]. Similar levels of Val, Ile, Glu, and Arg have been previously reported for cashew nut albumin and globulin fractions [Liu *et al*., 2018]. In this study, the results (3.27–6.25 g/100 g) obtained for Pro, a crucial amino acid that has been reported to contribute to the effectiveness of ACE-inhibitory peptides, were higher than the 1.33–2.25 g/100 g reported for *Phaseolus lunatus* protein hydrolysates [Magana *et al*., 2015]. The results (Table 1) further confirm the protein hydrolysates as rich sources of hydrophobic (HAA) and aromatic (AAA) amino acids that could contribute to the antioxidant and RAS inhibition activities [Magana *et al*., 2015].

TABLE 1. Amino acid composition (g/100 g) of cashew nut (CNPH) and fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions.

Amino acid	CNPH[1]	CNPH fractions				FPPH[2]	FPPH fractions				Least significant difference ($p<0.05$)
		<1 kDa	1–3 kDa	3–5 kDa	5–10 kDa		<1 kDa	1–3 kDa	3–5 kDa	5–10 kDa	
Asp	11.24	9.10	9.61	10.05	11.12	8.71	10.81	10.62	9.15	11.03	0.97
Thr	3.38	3.14	3.37	4.41	3.91	2.71	3.54	3.59	3.86	3.29	0.03
Ser	4.93	5.37	4.96	5.02	4.99	4.23	5.25	5.74	6.15	4.88	0.87
Glu	18.56	23.29	18.39	18.63	21.42	18.86	21.15	20.15	14.66	18.94	3.42
Pro	5.18	4.12	4.55	3.73	4.38	6.25	4.69	4.89	3.27	4.94	0.77
Gly	4.46	4.21	4.53	3.68	4.36	4.90	4.50	4.42	4.17	4.40	0.84
Ala	4.27	2.98	4.30	4.85	3.94	3.14	3.68	3.41	6.44	4.18	0.06
Cys	0.82	1.95	0.83	0.40	0.40	0.99	0.88	1.05	0.21	0.87	0.11
Val	4.25	5.10	4.57	7.04	7.15	3.32	6.07	5.16	5.97	4.48	0.88
Met	1.37	1.25	1.43	1.38	1.43	1.05	1.49	1.27	1.94	1.41	0.12
Ile	4.24	3.63	4.29	4.50	4.02	3.20	3.75	3.64	5.00	4.33	0.10
Leu	7.90	7.03	7.74	13.56	10.84	6.12	8.76	7.26	10.32	7.96	1.24
Tyr	4.01	3.19	3.88	3.14	3.38	3.45	3.64	3.27	5.00	3.78	0.08
Phe	4.56	4.51	4.65	6.64	5.09	3.10	4.58	4.16	6.02	4.33	0.29
His	2.67	2.39	2.77	1.78	2.01	3.16	2.49	2.78	2.72	2.71	0.12
Lys	3.20	3.96	3.25	2.43	2.64	4.41	3.23	4.41	2.57	3.03	0.14
Arg	13.35	13.44	15.23	5.94	7.10	20.89	9.45	12.28	10.79	13.90	4.21
Trp	1.62	1.40	1.73	2.85	1.84	1.52	2.08	1.91	1.78	1.57	0.08
HAA[3]	28.03	26.06	27.71	35.46	32.16	24.07	29.32	26.68	33.15	28.17	6.20
AAA[4]	10.19	9.10	10.26	12.63	10.31	8.07	10.30	9.34	12.80	9.68	0.94

[1]HAA = Hydrophobic amino acids, [2]AAA = Aromatic amino acids.

Degree of hydrolysis

The bioavailability of peptides released during protein hydrolysis process is related to their structural properties (amino acid composition and sequence), which is dependent on the degree of hydrolysis [Jakubczyk & Baraniak, 2014]. Figure 1 revealed the different degrees of hydrolysis (DH) for the enzymatic treatment of CNPI and FPPI proteins. The CNPI was observed to be more readily hydrolyzed by Alcalase at enzyme to substrate (E/S) ratios of 1–3:100 than the FPPI, which may be due to differences in the primary structure of the two seed proteins. In contrast, similar DH values were obtained for CNPI and FPPI hydrolyzed by pepsin. Generally, for each seed protein, higher DH values were achieved for hydrolysates obtained with Alcalase when compared to pepsin activities. The current outcome is contrary to the findings of Arise *et al*. [2019] where the DH of *Azadirachta indica* seed protein hydrolysates from pepsin activity was higher (27.88%) than the 14.73% obtained for Alcalase hydrolysis. The higher DH observed for the Alcalase-produced hydrolysates (~51%) might be due to the broad and non-specific enzymatic activity when compared to pepsin (47%), which has a narrow proteolytic specificity for peptide bonds formed by amino acids with aromatic or bulky side chains [Aluko, 2019b]. The results are comparable to the 45% DH reported for Alcalase-hydrolyzed horse gram flour [Bhaskar *et al*., 2019]. Decreases in DH with increased E:S may be due to enzyme overcrowding, which could lead to redundancy or antagonistic effects that lowered the rate of proteolysis.

The DH values presented for peptic CNPH (47%) and FPPH (46%) were higher than the values of 14, ~28, and 28% previously reported for hydrolysates obtained by simulated gastrointestinal digestion of pea globulin [Jakubczyk & Baraniak, 2014], neem seed protein [Arise *et al*., 2019], and hemp protein [Malomo *et al*., 2015], all with ACE-inhibitory or RAS-inhibitory activities. The samples preparation, different protein sources, and specificities of the proteases [Jakubczyk & Baraniak, 2014] might have resulted in the differences of DH values obtained for these hydrolysates. The high DH for CNPH and FPPH indicates short peptide lengths, which could enhance inhibition of ACE and renin activities. Overall, the data showed that the CNPI and FPPI were highly susceptible to Alcalase and pepsin proteolysis.

Hydroxyl radical scavenging activity

The hydroxyl radical (•OH) is one of the most damaging reactive oxygen species (ROS) because it reacts with all cellular macromolecules such as proteins, polyunsaturated fatty acids (PUFAs) and nucleic acids to induce severe damages to cells [Jin *et al*., 2016]. Therefore, •OH scavenging is an effective defense strategy of the human body against various diseases elicited by ROS [Zhuang *et al*., 2013]. The ability of CNPH or FPPH and their membrane fractions to scavenge •OH is presented in Figure 2. The •OH scavenging activity (HRSA) of CNPH and FPPH were 58 and 38%, respectively when compared to GSH (57%), a recognized potent physiological antioxidant. However, the HRSA was significantly ($p<0.05$) increased upon membrane filtration and separation into peptides of varying sizes. The results further showed the direct and indirect relationship between the DH (Figure 1) and mo-

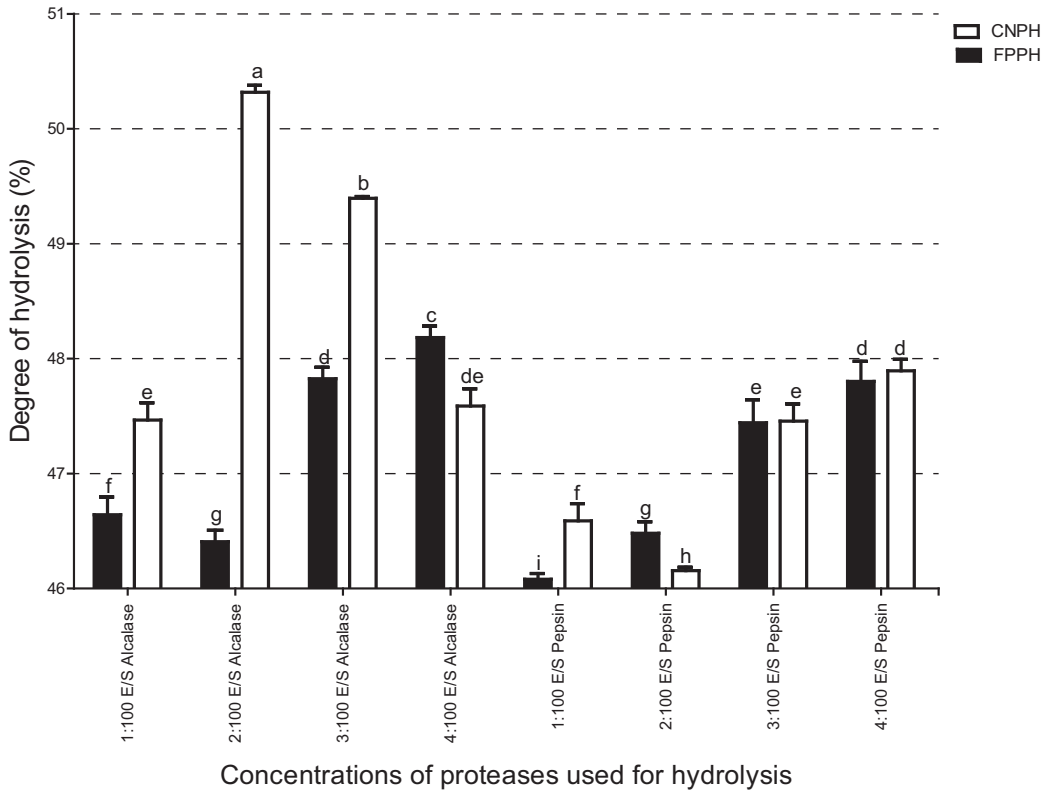

FIGURE 1. Degree of hydrolysis of cashew nut (CNPH) and fluted-pumpkin (FPPH) proteins at different protease concentrations (1–4:100 ratio E/S).

lecular weight sizes, respectively on the HRSA of the protein hydrolysates. For instance, the higher DH of CNPH over FPPH was directly related to their HRSA while the scavenging effect reduced with increase in peptide size. For example, the HRSA values of <1 kDa fractions of CNPH (~99%) and FPPH (95%) were higher than those of the >10 kDa fractions (42 and 38%, respectively). In addition, the CNPH had a higher content of HAA, which may have contributed to the higher HRSA when compared to FPPH.

Previous report [Ketnawa *et al*., 2017] had suggested that the antioxidant activity of peptides is dependent on the molecular mass, hydrophobicity and type of amino acid as well as enzymatic hydrolysis reaction conditions. The current findings (Figure 2) are in agreement with the stronger HRSA for LMW than the HMW peptides of barley glutelin [Xia *et al*., 2012] but are different from the reported trend for *B. coriacea*-derived peptides [Ijarotimi *et al*., 2018b]. Our results are higher than the 23.36, 22.50, 32.3, and 81% HRSA reported for pancreatin-produced pigeon pea [Olagunju *et al*., 2018], black soybean [Ralison *et al*., 2013], 5–10 kDa fractions from *B. coriacea* seed [Ijarotimi *et al*., 2018b], and alfalfa leaf [Xie *et al*., 2008] protein hydrolysates, respectively.

2,2-Diphenyl-1-picryhydrazyl radical scavenging activity

2,2-Diphenyl-1-picryhydrazyl radical scavenging activity (DRSA) is one of the many assays employed to understand the mechanism of antioxidant action of food protein-derived peptides. Figure 3 showed the DRSA of the pepsin-produced (3:100 ratio E/S) CNPH and Alcalase-produced (2:100 ratio E/S) FPPH, which ranged from 40 to 57% when compared to 60% for GSH, a recognized potent physiological antioxidant. Meanwhile, a slightly higher DRSA (41–98%) was observed after the protein hydrolysates were fractionated by ultrafiltration. The improved ability of <1 kDa peptides to scavenge free radicals with maximum efficiency when compared to HMW peptides have been suggested from previous findings on antioxidative potentials of *Parkia speciosa* [Siow & Gan, 2013] and hemp seed [Malomo *et al*., 2015] protein hydrolysates. In addition, the small-size LMW (<1 kDa) peptides may be resistant to endopeptidase degradation in the digestive tract and enhance faster absorption from the gastrointestinal tract into blood circulation, which could promote ability to interact with and scavenge endogenous free radicals [Erdmann *et al*., 2008].

The DRSA values of our peptide fractions (98%) are better than the 32, 56.22, and 53.15% reported for the Alcalase-produced hydrolysates of asparagus extract [Montone *et al*., 2019], *seinat* seed protein albumin, and globulin [Siddeeg *et al*., 2015], respectively. The reason for this might be that CNPH and FPPH peptide fractions probably had higher DPPH affinity and H-atom donating capacity when compared to those of asparagus extract and *seinat* seed protein fractions since DPPH assay involved a reduction mechanism by active compounds [Siddeeg *et al*., 2015]. The high levels of acidic and hydrophobic amino acids may have contributed to the strong DRSA of the protein hydrolysates [Udenigwe & Aluko, 2011].

Ferric reducing power

A reducing power is an effective method for assessing the ability of an antioxidant in electron donation mechanism. Figure 4 shows the reducing power (absorbance) of CNPH, FPPH and their membrane fractions measured at 700 nm. It is noteworthy that an increase in absorbance indicates better reducing power of the test protein sample. The CNPH and its membrane fractions, with the excep-

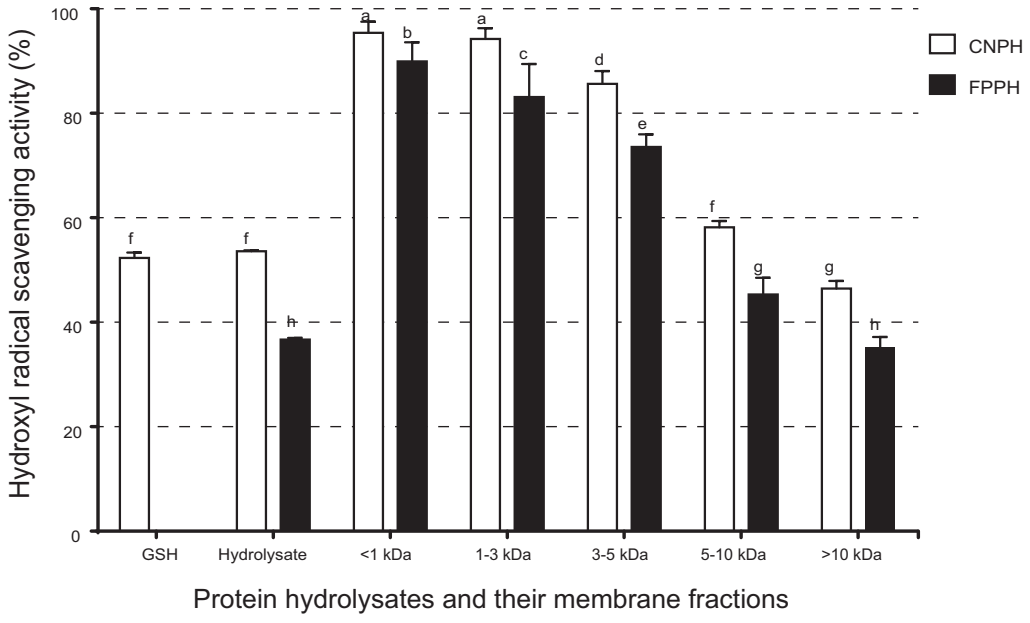

FIGURE 2. Hydroxyl radical scavenging activity of pepsin-produced (3:100 ratio E/S) cashew nut (CNPH) and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions. Columns with the different letters have mean values that are significantly different at $p<0.05$.

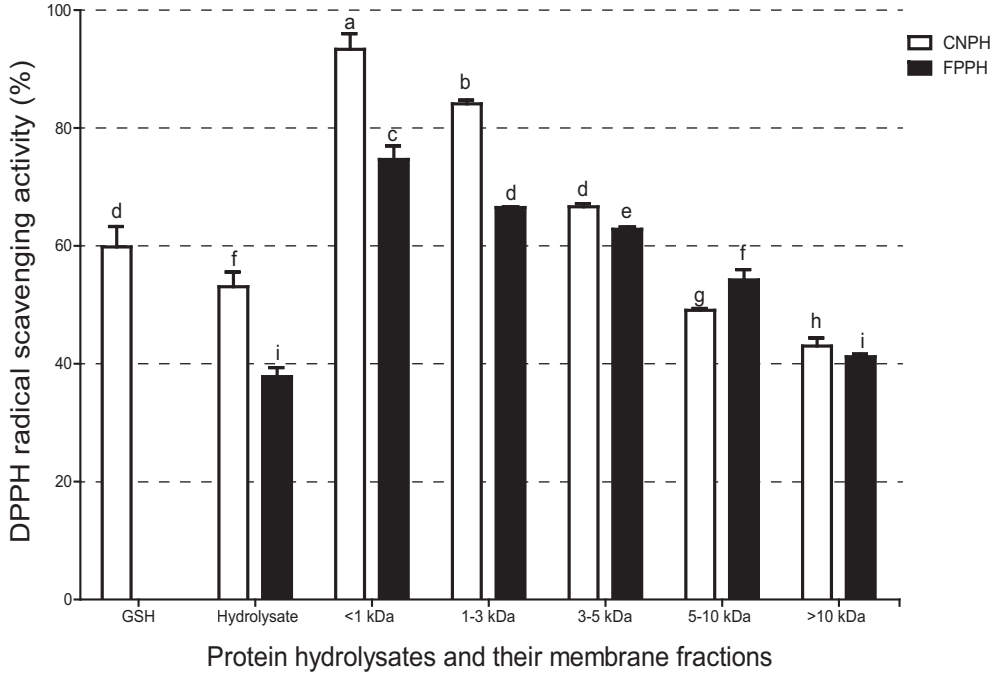

FIGURE 3. DPPH radical scavenging activity pepsin-produced (3:100 ratio E/S) of cashew (CNPH) and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin protein hydrolysates and their membrane fractions. Columns with different letters have mean values that are significantly different at $p<0.05$.

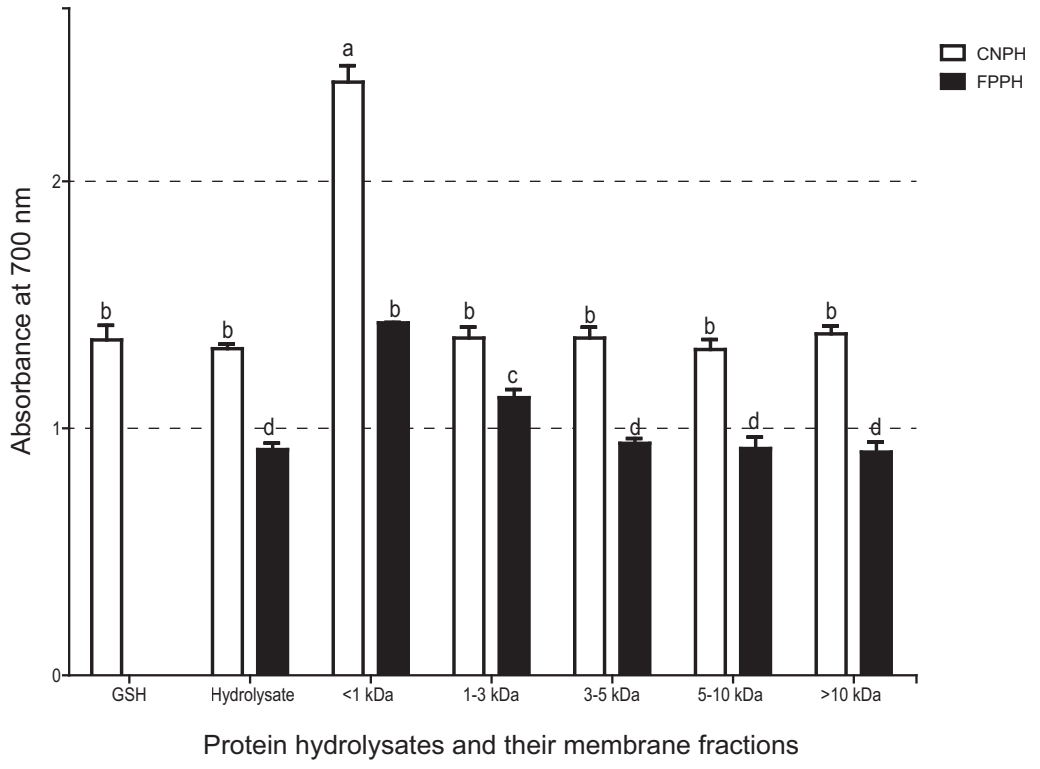

FIGURE 4. Ferric reducing power of pepsin-produced (3:100 ratio E/S) cashew nut (CNPH) and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions. Columns with the different letters have mean values that are significantly different at $p<0.05$.

tion of <1 kDa fraction, exhibited similar absorbance value (~1.4) compared to GSH (depicting same reducing power ability) whereas, the reducing powers of the membrane fractions from FPPH decreased at an increased molecular sizes (Figure 4). The current observation contradicts the previous study [Girgih *et al*., 2011] that reported an increase in reducing power of the hemp protein hydrolysate fractions at increased peptide sizes. The ferric reducing power of the pepsin-produced (3:100 ratio E/S) CNPH and its fractions (1.45–2.47) was higher than those of Alcalase-produced (2:100 ratio E/S) FPPH and its membrane fractions (1.00–1.46), with better reducing ability (2.47 and 1.46) from their respective <1 kDa fractions. The differences in the reducing power exhibited by CNPH and FPPH may be related to the increased availability of hydrogen ions [Kong & Xiong, 2006] produced during CNPI hydrolysis. Notably, the presence of reducing compounds caused reduction of Fe^{3+}/ferricyanide complex to ferrous (Fe^{2+}) through electron donation [Jemil *et al*., 2017], thereby increasing the absorbance of the reaction. This is exemplified by the higher cysteine content of the <1 kDa peptides, which also displayed the highest absorbance value. The results are consistent with previous finding that sulfur-containing amino acids enhance ferric reducing power properties of food protein hydrolysates [Udenigwe & Aluko, 2011].

The current study further supported the findings from a previous study on African yam bean seed protein hydrolysate [Ajibola *et al*., 2013] that the highest reducing power values did not correspond to the higher HAA contents. For instance, the highest reducing power (2.47 and 1.46) but lowest HAA (26.06 and 29.32%) values were obtained for the <1 kDa fractions compared to the lowest reducing power (1.45 and 1.00) but highest HAA (35.46–33.15%) values of 3–5 kDa fractions from CNPH and FPPH, respectively as evident in Figure 4 and Table 1. However, the previous study of Pownall *et al*. [2010] on the dependence of reducing power of pea seed protein hydrolysate fractions on its total HAA contents rather than their peptide sizes is different from present findings.

Ferrous ion chelating activity

Active metals have been suggested to be involved in several key pathogenic processes exhibited in cardio- and/or neurodegenerative diseases in the biological system [Mundi & Aluko, 2014]. For instance, the reaction between redox-active ferrous ion (Fe^{2+}) and H_2O_2 can generate $^{\bullet}OH$, which has been implicated in various oxidant-induced metabolic disorders [Aderinola *et al*., 2018]. Therefore, employing chelating agents such as CNPH and FPPH to inactivate ferrous ions and prevent formation of nontoxic metal complexes could play an important role in preventing oxidative injuries in the body. The results show no effect of peptide size on ferrous ion chelation ability of the samples (Figure 5). However, fractionation of the CNPH led to improved fer-

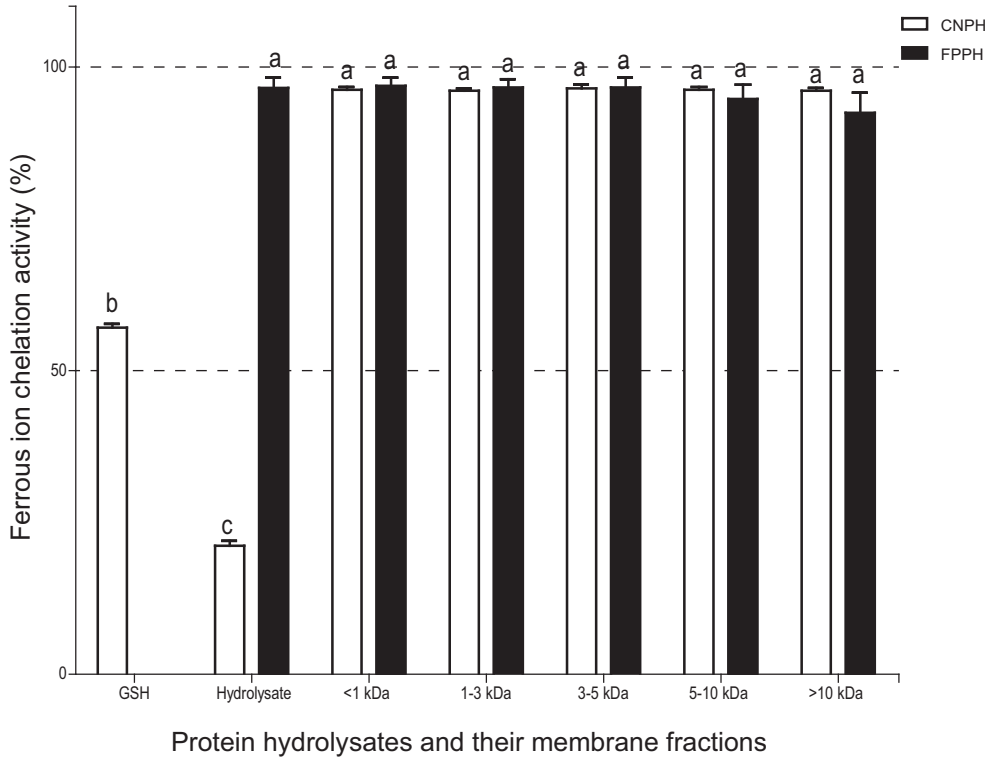

FIGURE 5. Ferrous ion chelation activity of pepsin-produced (3:100 ratio E/S) cashew nut and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions. Columns with the different letters have mean values that are significantly different at $p<0.05$.

rous ion chelation, which suggests stronger synergistic effects for the peptide fractions. The results obtained in this work show stronger ferrous ion chelation for the peptide fractions when compared to the 15.7–38.5% values (also at 1 mg/mL) reported for similar hemp seed peptides peptides [Girgih *et al*., 2011]. The differences may be due to variations in the type of protein substrates and proteolytic enzymes used for each work.

The degree of protein hydrolysis and type of amino acid have been shown to alter ferrous ion chelation activity of isolated peptides [Jamdar *et al*., 2010]. However, the present study is contradictory to the previous report of Jamdar *et al*. [2010] because even though the CNPH had a higher DH, the metal chelating ability was lower than that of FPPH. Moreover, there were no significant ($p>0.05$) differences between the ferrous ion chelation ability of all the peptide fractions.

In vitro activities against the renin-angiotensin converting enzymes system

The CNPI and FPPI were each subjected to enzymatic hydrolysis using Alcalase or pepsin at different concentrations (E/S ratio of 1–4:100) and the hydrolysates evaluated for ACE and renin inhibitions. As shown in Figure 6, the ACE-inhibitory activity of pepsin-CNPH samples was mostly better than the Alcalase-CNPH. In contrast, ACE-inhibitory activity of Alcalase-FPPH was similar to those obtained pepsin-FPPH, except that the 4:100 E/S pepsin-FPPH was significantly lower. The results indicate that intrinsic differences in the primary structure of the proteins may have led to the production of peptides with different amino acid sequences and hence the observed variations in ACE-inhibitory activity of CNPH and FPPH.

The CNPI was hydrolyzed using 3:100 (E/S) pepsin for 2 h, and the hydrolysate passed through membranes of different sizes, and was then tested for ACE-inhibitory activity. The results show that membrane separation did not improve the ACE-inhibitory activity of the CNPH (Figure 7). This is because there were no significant differences ($p > 0.05$) between the CNPH and the <1, 1–3, 3–5 and 5–10 kDa peptide fractions. However, ACE-inhibitory activity was significantly ($p<0.05$) less for >10 kDa peptides when compared to CNPH. The results (Figure 7) are consistent with previous reports [Montone *et al*., 2019; Ratnayani *et al*., 2019] that have showed higher ACE-inhibitory potency of low molecular weight peptides when compared to peptides of bigger sizes.

The FPPI was hydrolyzed with 2:100 (E/S) Alcalase for 4 h and the hydrolysate also passed through membranes of different sizes. As shown in Figure 7, membrane fractionation led to decreased potency of the FPPH peptides against ACE, except 1–3 kDa peptides. Moreover, the smaller-size peptides (<5 kDa) exhibited significantly ($p <0.05$) stronger ACE inhibition than the bigger (>5 kDa) peptides. The results suggest that a strong peptide synergy exists within the protein hydrolysates (pepsin-CNPH and Alcalase-FPPH), which favored a high rate of ACE inhibition. Membrane separation reduced this peptide synergy and hence most of the peptide fractions exhibited weaker ACE inhibition than their respective hydrolysates. Similar findings of reduced peptide potency against ACE as a result of membrane ultrafiltration have also been reported [da Cruz *et al*., 2016]. Results from a previous study support the current observations about the direct dependency of peptide bioactivities on their amino acids content. For instance, highly acidic amino acids (Asp and Glu) led to a net negative charged peptide with improve antioxidant and effective ACE inhibitory action [Nourmohammadi *et al*., 2017]. This was attributed to chelation of the zinc atoms in the active center of ACE, which reduced catalytic activity. Similarly, the presence of aromatic and hydrophobic amino acids has been shown to contribute to potency of ACE-inhibitory peptides [Wu *et al*., 2006a].

In contrast to the ACE inhibition results, Figure 8 shows that the maximum renin inhibition was achieved using 2:100 (E/S) Alcalase (63%) and 4:100 (E/S) pepsin (48%) to prepare CNPH and FPPH, respectively. The present findings showing lower renin-inhibitory activity of the hydrolysates when compared to ACE-inhibitory activity are similar to previous reports on canola [Alashi *et al*., 2014] but contrary to these on hemp [Malomo *et al*., 2015] protein hydrolysates. For the CNPH, the results suggest that initial increase in Alcalase concentration from 1–2:100 (E/S) promoted the production of peptides with structural features that enhanced renin inhibition. However, further increases in Alcalase concentration may have led to hydrolysis and structural inactivation of the active peptides, hence the reductions in renin-inhibitory activity at enzyme levels >2:100 (E/S). The opposite effect was obtained for the FPPH whereby the highest renin inhibition was obtained at 4:100 (E/S) pepsin. Therefore, it is possible that at 1–3:100 (E/S) pepsin, the level of FPPI proteolysis was not sufficient to produce strong renin-inhibitory peptides when compared to the 4:100 (E/S) pepsin. Differences in enzyme specificity (peptide cleavage points) may have contributed to the varied outcome between Alcalase and pepsin digestions. After subjecting the two hydrolysates to membrane filtration process, the renin inhibition activities of their membrane fractions were significantly ($p<0.05$) improved as evident in results presented in Figure 9. However, there were no significant differences between the renin-inhibitory activities of the CNPH peptide fractions. In contrast, the 5–10 kDa FPPH peptide fraction exhibited significantly ($p<0.05$) higher renin-inhibitory activity when compared to the other fractions. The results suggest that for the renin inhibition, peptide composition may be more important than peptide size for the FPPH.

Differences in catalytic mechanisms between ACE and renin might have also contributed to the different results observed for CNPH and FPPH inhibitory activities. For instance, the pepsin-produced CNPH and Alcalase-produced FPPH had higher ACE (Figures 6 and 7) but lower renin (Figures 8 and 9), thus revealing the two peptides to have higher affinity for ACE than renin. A previous study [Olagunju *et al*., 2018] had attributed the low inhibition of renin activity to reduced accessibility to inhibitors as a result of its (renin) more folded protein conformation, which contrasts the more open conformation and easier accessibility of ACE to potential inhibitors.

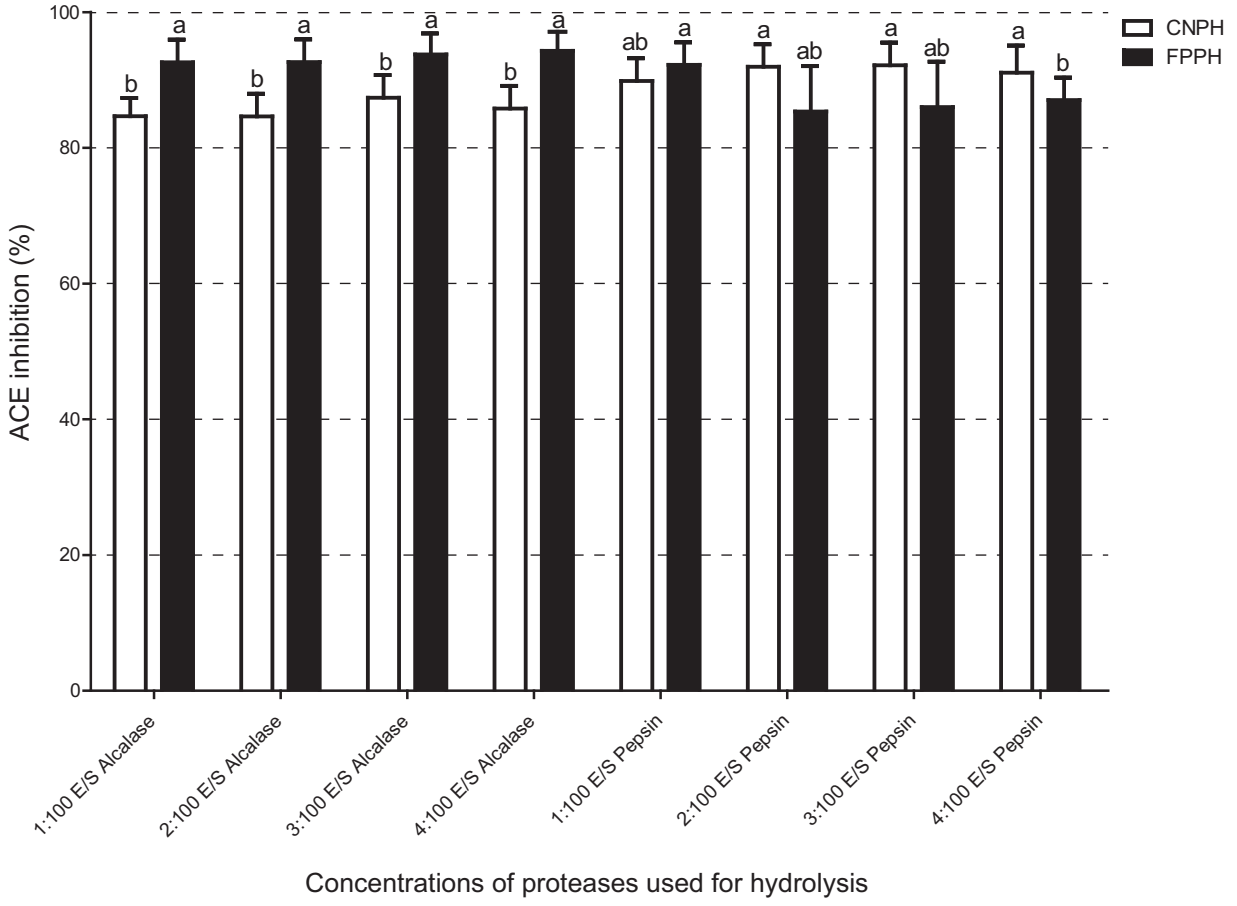

FIGURE 6. ACE inhibition properties of cashew nut (CNPH) and fluted-pumpkin (FPPH) protein hydrolysates produced at different protease concentrations (1–4:100 ratio E/S). Columns with the different letters have mean values that are significantly different at $p<0.05$.

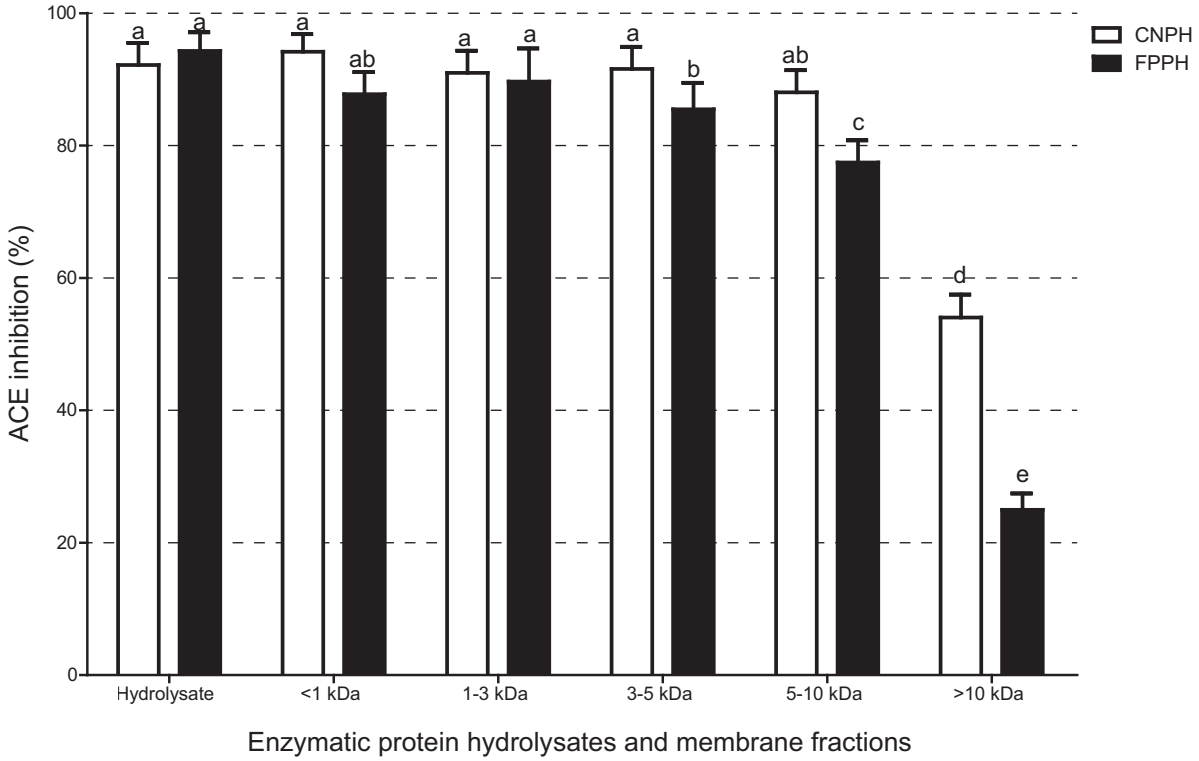

FIGURE 7. ACE inhibition properties of pepsin-produced (3:100 ratio E/S) cashew nut (CNPH) and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions. Columns with the different letters have mean values that are significantly different at $p<0.05$.

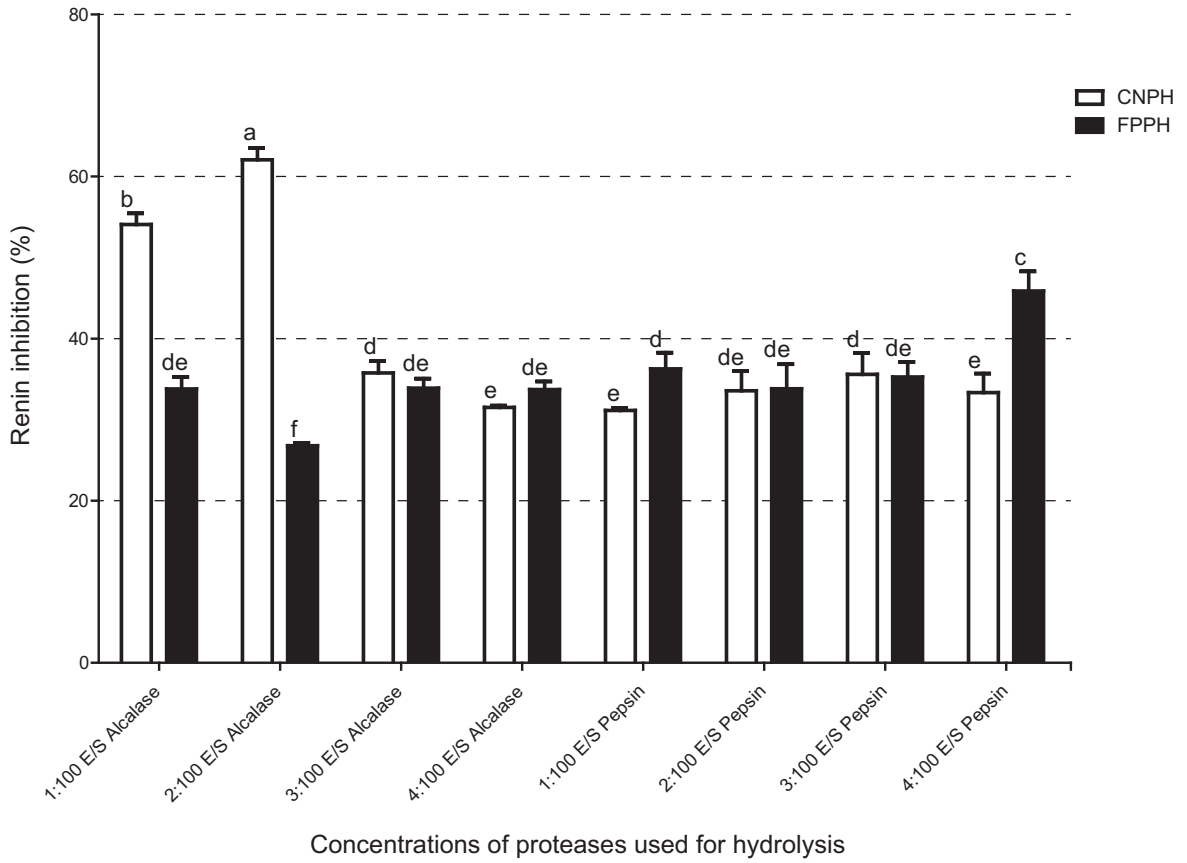

FIGURE 8. Renin inhibition properties of cashew nut (CNPH) and fluted-pumpkin (FPPH) protein hydrolysates produced at different protease concentrations (1–4:100 ratio E/S). Columns with the different letters have mean values that are significantly different at $p<0.05$.

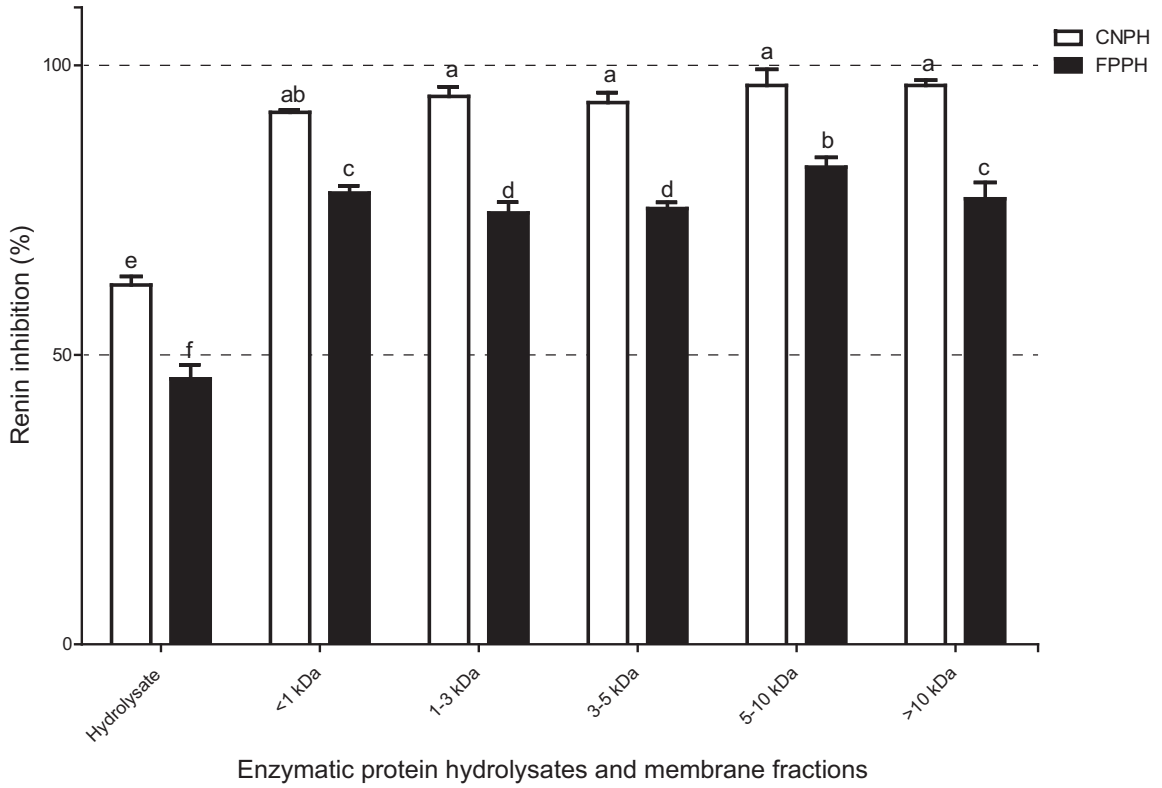

FIGURE 9. Renin inhibition properties of pepsin-produced (3:100 ratio E/S) cashew nut (CNPH) and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions. Columns with the different letters have mean values that are significantly different at $p<0.05$.

CONCLUSIONS

The protease-hydrolysis of CNPI and FPPI yielded peptides with *in vitro* antioxidant and RAS-inhibitory activities, which provides new peptide tools for nutritional improvement of human health. Both CNPH and FPPH contain LMW peptides with potentially fast absorption in the gastrointestinal tract. The strongest scavenging of hydroxyl and DPPH• radicals as well as ferric ion reducing capacity were obtained for the <1 kDa peptide fraction. Stronger ACE inhibition by <1 kDa peptides when compared to bigger peptides was observed only when the CNPI was hydrolyzed by pepsin for 2 h. In contrast, CNPI hydrolysis by pepsin for 2 h yielded <1 kDa peptides with weaker renin inhibitory activity than the bigger peptides. Thus, the potency of peptide fractions may be dependent not only on the type of protease but the length of released peptides. Overall, the strong ACE and renin inhibitions suggest that these protein hydrolysates could be used as suitable ingredients for the formulation of health-promoting functional foods and nutraceuticals.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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