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Research article

Protein hydrolysates of *Moringa oleifera* seed: Antioxidant and antihyperglycaemic potential as ingredient for the management of type-2 diabetes

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ABSTRACT

New plant proteins with high nutritional quality and biological properties are actively searched worldwide. *Moringa oleifera* seed protein isolate was prepared from defatted flour and hydrolyzed using four proteases namely trypsin, pepsin, Alcalase, and thermolysin. Then, antioxidant activity and cellular glucose uptake properties of the hydrolysates were assessed. A high degree of hydrolysis was obtained for hydrolysate prepared using trypsin (60.07%), followed by pepsin (57.14%), Alcalase (50.68%), and thermolysin (45.45%). Thermolysin hydrolysate was the most antioxidant efficient (IC_{50} 0.15 and 0.74 mg/mL for 2,2′-azino-bis(acide 3-ethylbenzothiazoline-6-sulfonique) diammonium salt (ABTS) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, respectively). Trypsin hydrolysate stimulated high glucose uptake by yeast cells (12.34–35.28%). In the absence of insulin, Alcalase hydrolysate was the most efficient for glucose uptake by the muscle, with the rate ranging from 22.03% to 29.93% after 30 min, then from 29.55% to 34.6% after 60 min. The four hydrolysates improved glucose uptake by the muscle in the presence of insulin with the rate ranging from 46.88% to 58.03% after 30 min, and from 50% to 58.18% after 60 min. Therefore, *Moringa oleifera* seed proteins could be used to prepare peptides as components of functional foods for the management of type-2 diabetes.

1. Introduction

Diabetes Mellitus is one of the most prevalent public health issues in industrialized and developing countries. The International Diabetes Federation reported 536.6 million people worldwide with diabetes in 2021. This number would rise up to 783.2 million by 2045 [1]. Diabetes frequently results in high blood glucose levels (hyperglycemia), which can be a result of insufficient insulin (type I) or insulin resistance (type II) [2]. Along with genetic predispositions, poor diet, and inactivity are significant risk factors for type II diabetes. As a result, adequate diet and lifestyle are essential to the management and prevention of diabetes.

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Oxidative stress occurs due to an imbalance between the systemic production of reactive oxygen species and the capability of the organism to detoxify the reactive intermediates or repair resulting damage. Free radicals can harm biological components like lipids, DNA, and proteins while altering the natural redox state of cells [3]. Rahimi-Madiseh et al. (2016) [4] reported a relationship between diabetes and oxidative stress, which releases free radicals that impair tissue function and eventually cause long-term damage to it.

The main mechanisms for managing diabetes include slowing glucose uptake through inhibition of α-glucosidase and α-amylase involved in carbohydrate hydrolysis. The other mechanism consist to inhibit the enzyme dipeptidyl peptidase-IV (DPP-IV), which prolongs the half-life of total circulating GLP-1, peptides that stimulate glucose-dependent insulin secretion in pancreatic beta-cells, by preventing its degradation and inactivation [5]. However, there is a lack of scientific evidence on the effect of protein hydrolysates from legumes on peripheral glucose uptakeby insulin and non-insulin cells. This capture by legume protein hydrolysates constitutes an important mechanism to be explored.

Synthetic drugs used for the management of diabetes and associated complications are very expensive, have side effects due to their long-term use, and in some patients do not lead to the expected positive results [6]. For this reason, it is important to investigate and find new, more potent, less expensive, and significantly safer antioxidants peptides with high glucose uptake properties from natural products. Therefore, a great choice to prevent and manage diabetes and oxidative stress would be to develop functional foods or nutraceuticals from natural resources. Plant proteins and derived peptides are promising materials used for this purpose.

Peptides derived from the digestion of dietary proteins of animal or plant origin have numerous nutritional, technofunctional, and biological functions with beneficial effects on many diseases [7–9]. It has been reported that dietary proteins are key regulators of glucose homeostasis and could therefore be developed in the context of obesity and Type II diabetes [10]. A simple and inexpensive method to convert protein into free amino acids and short-chain peptides is enzymatic hydrolysis. The peptides obtained are more soluble than the whole protein, and the amino acid profile may be enhanced in some hydrolysates or could remain unchanged [11].

Antioxidants are substances that in small quantities. are able to retard the oxidation of materials such as unsaturated fats whilst preventing the excessive accumulation of free radicals or reactive oxygen species. Free radicals are generated either by physiological processes that occur naturally in the body or by external sources such as excessive exposure to sunlight or smoking and have been linked to several diseases [12]. Some diseases such as type-2 diabetes, cardiovascular diseases, hypertension, and cancers have been reported to be linked to oxidative stress. Antioxidant peptides have simpler structures than their parent proteins. This confer greater stability in different situations like heat, exposure to proteases and they have no hazardous immunoreactions and often exhibit enhanced nutritional and technofunctional properties in addition of their antioxidant activity [4,9,11]. Natural antioxidants and more recently plant protein hydrolysates have been reported to be effective for the prevention and treatment of oxidative stress-related diseases [6].

Daily consumption of legume seeds, which are major foodstuffs in most countries and the main economical source of supplementary protein in the diet of developing countries, reduces the occurrence of certain diseases such as diabetes [6,13]. *Moringa oleifera*, a legume from the family of *Moringaceae,* is widely grown in many tropical and subtropical countries as in Africa, with the particularity to be resilient to climate change, poor and averagely dry soils. It is a highly valued plant because every part is useful for food or non-food applications. *Moringa oleifera* seeds are rich in fat, protein, minerals, and vitamins. Their proteins contains all essential amino acids with high nutritional value [14]. Some of the reported health benefits of the *Moringa* tree include antiulcer, antipyretic, anti-inflammatory, antihypertensive, antiepileptic, antidiabetic, hepatoprotective anti-bacterial, anti-fungal, and cholesterol-lowering ability [15]. Recent studies reported that *Moringa oleifera* seeds also showed significant effects in reducing blood lipids and blood pressure, regulating the stomach, protecting the liver from alcohol, and enhancing the body's immunity [16]. More so, *Moringa oleifera* seed protein hydrolysates showed high bioactive properties, such as antioxidant, antidiabetic, and antihypertensive [17,18]. Studies on hypoglycaemic activities of *Moringa oleifera* seed protein hydrolysates focused on evaluation of hemoglobin glycosylation, α-amylase, α-glucosidase, and dipeptidyl peptidase-IV (DPP-IV) inhibitory activities [17,19]. However, there is little or no information on cellular glucose uptake properties of *Moringa oleifera* seeds protein hydrolysates. Therefore, this research aimed to investigate the glucose uptake capacity by yeast and muscle cells, and antioxidant properties of *Moringa oleifera* seeds protein hydrolysates.

2. Materials and methods

2.1. 1. Material and reagents

Moringa oleifera seeds were harvested in June 2023 in the same field in the locality of *Ezezang* (4°15′N and 11°25′E), located in the Centre Region of Cameroon.

Pepsin (from *porcine gastric mucosa*, 3200–4500 U/mg protein), trypsin (from *porcine pancreas*, 1.5 U/g protein), Alcalase (from *Bacillus licheniformis*, ≥2.4 U/g protein), thermolysine (from *Geobacillus stearothermophilus* 0.03–0.17 U/g protein), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ferrozine (3-(2-pyridyl) acid-5,6-bis(4-phenyl-sulfonic)-1,2,4-triazine), Tris buffer, gallic acid, potassium persulfate, catechin, glucose, glucose kit, glucose oxidase (GOP), peroxidase glucose oxidase (PGO), 4-aminophenazone (4-AP), commercial yeast, krebs buffer, human insulin were purchased from Sigma-Aldrich (MO, USA). All the chemicals were of the highest purity available.

2.2. Methods

2.2.1. Preparation of Moringa oleifera seed defatted flour

The Dry seeds were sorted and then ground in a milling machine (Semap, *France*) fitted with a fine sieve of 200 μm mesh size. The

flour obtained was defatted twice using a hexane/ethanol (1:1, v/v) solvent system and a 1:3 (w/v) solid to liquid ratio as described by Mune Mune et al. [20]. The defatted flour was oven-dried at 45 °C and kept in polyethylene bag at 4 °C.

2.2.2. Preparation of Moringa oleifera protein isolate (MOPI) from defatted flour

The defatted flour was suspended in 0.17 M NaCl solution with a flour to liquid ratio 1/20 (w/v). Protein was then extracted twice at pH 2 under constant stirring during 1 h at 65 °C, and recovered at pH 8 by centrifugation at 1290×*g* for 30 min. The extracted protein was washed with distilled water then freeze-dried [21]. Moisture and crude protein content of the isolate was determined according AOAC (1990) methods and were 2.41% and 80.41%, respectively.

2.2.3. Preparation of Moringa oleifera protein hydrolysate (MOPH)

Enzymatic hydrolysis of MOPI was carried out as described by Mune Mune et al. (2018) [6] with slight modifications. Protein solution at 10 mg/mL in distilled water were incubated in the presence of pepsin (enzyme/substrate ration, ESR, 1/50 (w/w), pH 2) at 37 °C; trypsin (ESR, 1/50 (w/w), pH 8) at 37 °C, Alcalase (ESR, 4% (w/v), pH 8) at 55 °C, and thermolysin (ESR, 1% (w/v), pH 8) at 70 °C. The reaction proceeded for 24 h in a shaker water bath closed-top vessel, and the pH was adjusted each 30 min using NaOH 0.5 M. The enzymatic reaction was stopped by heat inactivation at 95 °C for 5 min. The mixture was centrifuged at 1430×*g* for 30 min (4 °C), and the supernatant was freeze-dried and used for further analysis.

2.2.4. Degree of hydrolysis (DH) measurement

DH was determined by measuring the percentage of soluble protein in 10% (w/v) trichloroacetic acid (TCA) to the total protein in the sample, according to Shahi et al. [22]. To an aliquot (3 mL) of MOPH, an equal volume of TCA (20%) was added and incubated at 4 °C for 30 min. The mixture was centrifuged at 1430×*g* for 30 min at 4 °C. The TCA-soluble supernatant and the hydrolysate without addition of TCA were analyzed to determine protein content by the micro-Kjeldahl method. DH was determined based on the following equation:

$$Degree\ of\ hydrolysis\ (DH\ \%) = \frac{Nitrogen\ soluble\ in\ TCA\ (10\ \%)}{Total\ nitrogen\ content\ of\ the\ sample} \times 100$$

2.2.5. Electrophoresis

SDS-PAGE was carried out on *Moringa oleifera* protein isolate and hydrolysates according to the method described by Laemmli (1970) [23], using 4 and 18% (w/v) of acrylamide final concentration for stacking and resolving gels, respectively. About 10 μg of each sample was loaded onto the stacking gel and separated using an ATTO electrophoresis system (Japan) at 150 mV until bromophenol blue reached the bottom of the gel. Molecular weight was calibrated using a FastGene unstained protein marker (10–210 kDa) (Nippon Genetics, Japan). Electrophoresis was carried out under reducing and non-reducing conditions.

2.2.6. Determination of bioactive properties

Different concentrations of protein hydrolysates were prepared in distilled water and used for the evaluation of bioactive properties. For Pepsin hydrolysate: 0.001, 0.005, 0.01, 0.02, 0.04 and 0.06 mg/mL. For trypsin hydrolysate: 5, 7, 9, 11, 13 and 15 mg/mL. For Alcalase hydrolysate: 1, 3, 5, 7, 9 and 11 mg/mL. For thermolysin hydrolysate: 0.1, 0.25, 0.5, 1, 2 and 4 mg/mL.

2.2.6.1. Antioxidant properties of MOPH. Two antioxidant mechanisms were evaluated namely radical scavenging and iron-chelating activity. DPPH radical scavenging activity of MOPHs at different concentrations was measured according to Mune Mune et al. [6]. Briefly, a volume of 1.5 mL of MOPH was mixed with 1.5 mL of an ethanolic (95%) solution of DPPH. The mixture was allowed to stand in the dark at room temperature for 1 h. The absorbance was read at 517 nm against a blank solution. Calibration was done using gallic acid (1–60 μg/mL). DPPH radical scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging activity}(\%) = \frac{Absorbance\ blank - Absorbance\ MOPH}{Absorbance\ blank} \times 100$$

ABTS radical scavenging activity of MOPHs was analyzed according to You et al. [24]. The ABTS solution was prepared at 7.4 mM and mixed with a 140 mM potassium persulfate solution. After 14 h, this reagent was diluted with phosphate buffer (0.1 mM; pH 7.4) to an absorbance of 0.7 ± 0.02 at 734 nm. Gallic acid (0.4–40 μg/mL) was used as standard. Antioxidant activity was measured by mixing 10 μL of MOPH or standard with 1 mL of reagent. The absorbance was measured at 734 nm after 1 min. Activity was calculated as follows:

$$\text{ABTS radical scavenging activity}(\%) = \frac{Absorbance\ MOPH - Absorbance\ blank}{Absorbance\ blank} \times 100$$

The half maximal inhibitory concentration (IC_{50}) was measure to find out the concentration of each MOPH (mg/mL) that can scavenge 50% of DPPH and ABTS *in vitro*.

The reducing power of MOPH was evaluated according to Arise et al. [25]. Briefly, to 1 mL of MOPH were added distilled water (3.7 mL), 2 mM $FeCl_2$ (0.1 mL) and 5 mM ferrozine (0.2 mL) followed by thorough shaking. The mixture was allowed to stand at room temperature for 20 min. The absorbance was read at 562 nm against a blank. The calibration curve was prepared with catechin (5–100 μg/mL), which was used as standard. Reducing power was calculated by the following equation:

$$Reduction\ power\ (\%) = \left(1 - \frac{Absorbance\ sample}{Absrobance\ blank}\right) \times 100$$

2.2.6.2. Hypoglycaemic properties of MOPH. The hypoglycaemic properties of MOPH were assessed through its ability to stimulate peripheral glucose uptake using two cell types: non-insulin-dependent and insulin-dependent cells.

Glucose uptake capacity by yeast cells.

This assay was performed according to the well-defined method of Cirillo [26]. Commercial baker's yeast was dissolved in distilled water to prepare a 1% suspension. The suspension was kept overnight at room temperature (25 °C). On the next day, the yeast cell suspension was centrifuged at 1430×*g* for 10 min. The process was repeated by the addition of distilled water to the pellet until a clear supernatant was obtained. Exactly 10 parts of the clear supernatant fluids were mixed with 90 parts of distilled water to get a 10% (v/v) suspension of the yeast cells.

A 250 μL volume of protein hydrolysates (7, 10 and 12.5 mg/mL) was mixed with 1 mL of glucose (25 mM). The mixture was pre-incubated at 37 °C for 10 min. To initiate the reaction, 25 μL of yeast suspension was added and the mixture was incubated at 37 °C for 1 h. After centrifugation at 1500×*g* for 1 min, glucose was determined using the Fortress kit and the absorbance was read at 505 nm. The control was made up by replacing the sample volume with an equivalent volume of distilled water.

Results were expressed as percentage increase in glucose uptake and calculated as follows:

$$\text{Increase in glucose uptake}(\%) = \frac{Absorbance\ control - Absorbance\ MOPH}{Absorbance\ control} \times 100$$

Glucose Uptake Capacity by Muscle cells.

The Glucose Uptake Capacity by muscle cells was done as described by Hassan et al. [27]. Thin sections of muscle tissue were isolated from a *Wistar* rat that kept fasted for 16–18 h. The rat was then sacrificed by cervical dislocation and dissected to recover the muscle tissue. The muscle tissue from the paw was quickly removed and rinsed in Krebs buffer. Using the blades, the muscle tissue was cut into small pieces as finely as possible (about 1 mm thick). Thereafter, the muscle sections were washed 2–3 times with Krebs buffer and then collected in conical tubes and centrifuged at 1430×*g* for 5 min. The pellet was then recovered, weighed, and introduced into beakers containing 3 g/L glucose Krebs buffer (hyper-glycaemic medium).

Thin sections of muscle tissue were incubated in glucose Krebs buffer in the presence of MOPH at different concentrations (7, 10, and 12.5 mg/mL). Incubation was performed in a shaking incubator at 37 °C with constant agitation for 60 min. Marketed human insulin (100 U/mL) was used as a positive control.

Several aliquots were prepared. Muscle tissue incubated in Krebs buffer containing glucose (11.1 mM); muscle tissue incubated in Krebs buffer containing glucose (11.1 mM) with 100 μL of insulin (100 U/mL); muscle tissue incubated in Krebs buffer containing glucose (11.1 mM) with 100 μL of insulin (50 mIU) and 1 mL of MOPH at different concentrations (7, 10 and 12.5 mg/mL). The remaining glucose was then determined in each aliquot after 30 and, 60 min. Results were expressed as percentage of glucose uptake and calculated as follows:

$$\text{Glucose uptake}(\%) = \frac{Absorbance\ control - Absorbance\ MOPH}{Absorbance\ control} \times 100$$

2.3. Statistical analysis

Results are expressed as mean value ± standard deviation of three different determinations. Means were compared by the one-way ANOVA analysis followed by the Duncan post hoc test. The probability $p < 0.05$ was considered statistically significant. Data analysis and graphic plotting were done using Statistical Package for Social Sciences (SPSS version 26.0, SPSS Inc., USA) and Excel (*version Office*, 2013, MicrosoftInc., USA) software.

3. Results and discussion

3.1. Enzymatic hydrolysis of Moringa oleifera protein isolate

DH is correlated to the amount of bioactive peptides released, depending on the enzyme specificity [17,22]. *Moringa oleifera* seed proteins were hydrolyzed using pepsin, trypsin, Alcalase, and thermolysin. Trypsin had the highest DH (60.07%), followed by pepsin (57.14%), Alcalase (50.68%), and finally thermolysin (45.45%). However, biological activity of protein hydrolysates is highly dependent on the amino acid composition and sequence of peptides released [28]. DH was higher than the ones reported by Olusola et al. [17] using pepsin (29.93%) and trypsin (10.31%) for *Moringa oleifera* seed proteins. In addition, Vaštag et al. [28] reported lower degree of hydrolysis for pumpkin (*Cucurbitaceae Cucurbita Maxima*) protein using Alcalase (26.94%). Also, lower degree of hydrolysis was reported by Mune Mune et al. [6] for the hydrolysis of Bambara bean protein using thermolysin (22%).

3.2. Protein profile analyzed by SDS-PAGE

Moringa oleifera protein isolate and hydrolysates were analyzed under reducing and non-reducing conditions. Results are presented in Fig. 1 and fig. S1. It was reported by Guzman-Albores et al. [29] that *Moringa oleifera* seed protein was rich in albumins (65%),

followed by globulins (13%) and albumins (11%). In addition, Albumins and globulins were characterized by two main bands of low molecular weight (16 and 12 kDa) under non reducing conditions. Similar bands were observed (16 and 18 kDa) for *Moringa oleifera* protein isolate under the same conditions in this study (Fig. 1B). Furthermore, the band at 55 kDa observed for the isolate under non reducing conditions (Fig. 1B) is characteristic of globulins, particularly for 11S globulins. *Moringa oleifera* proteins were probably rich in disulfide bonds because, the band at 55 kDa disappeared under reducing conditions (Fig. 1A) and three new bands appeared at 40, 30 and 20 kDa, and at the same time the intensity of the bands observed at 18 and 16 kDa under non reducing conditions was reduced. It is well known that 11S globulins or legumins consist on basic and acidic subunits linked by disulfide bonds.

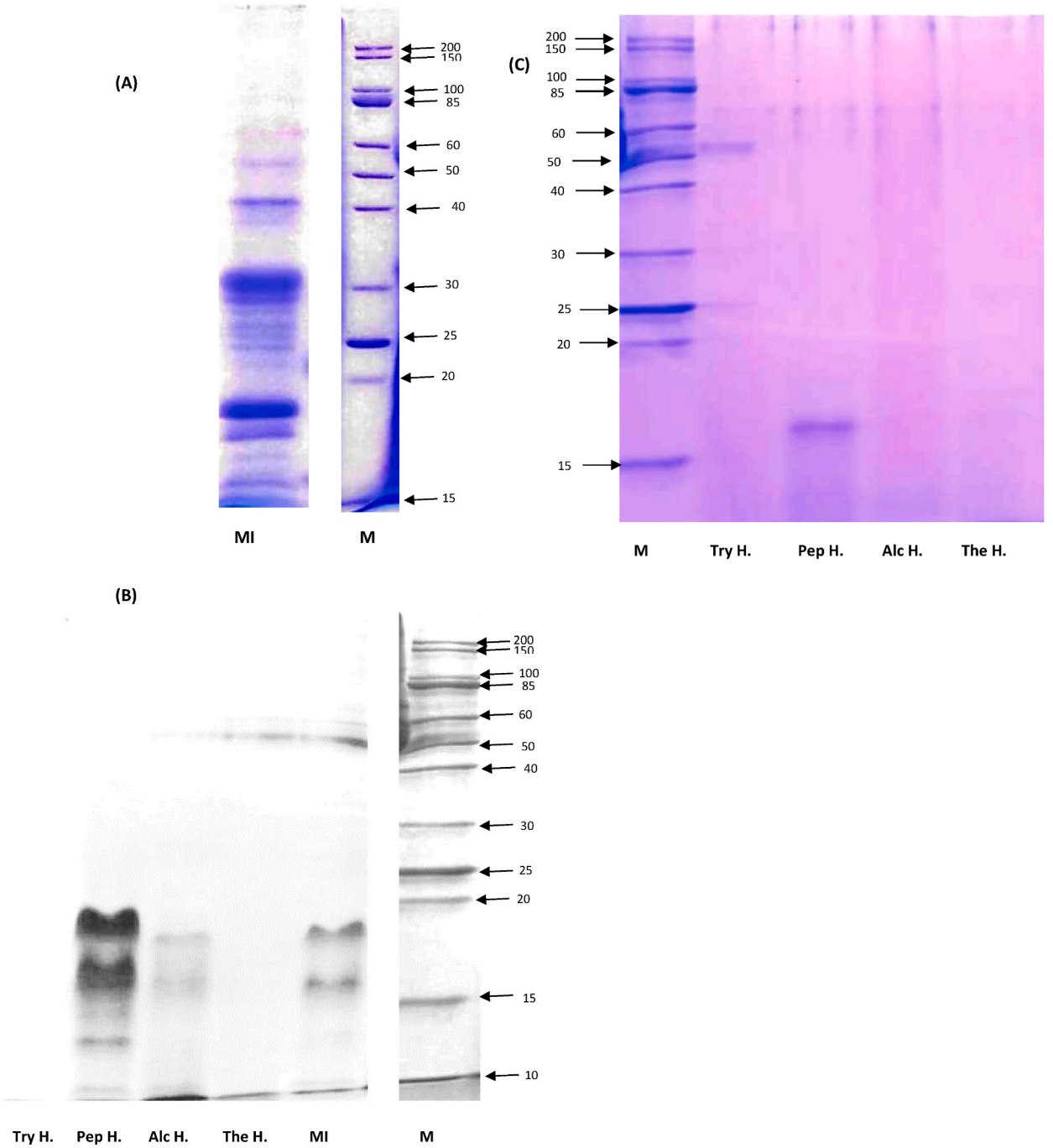

Fig. 1. SDS-PAGE profiles of (A) *Moringa oleifera* protein isolate under reducing conditions, (B) protein isolate and hydrolysates under non reducing conditions, and (C) protein hydrolysates under reducing conditions. M: Molecular weight markers, MI: *Moringa oleifera* protein isolate. H.: Hydrolysate. Try: trypsin. Pep: pepsin. Alc: Alcalase. The: Thermolysin.

Enzymatic hydrolysis of *Moringa oleifera* protein isolate produced different effects on the polypeptide profile of the hydrolysates depending on the protease used. It could be observed in Fig. 1B that albumins and globulins are extensively hydrolyzed by trypsin, while thermolysin partially hydrolyzed globulins as noticed by the band of low intensity at 55 kDa. Hydrolysate produced by Alcalase exhibited residual undigested albumins by the bands of low intensity at 18 and 16 kDa. Pepsin significantly hydrolyzed *Moringa oleifera* globulins, but had less effect on albumins, as observed by intense bands at 18 and 16 kDa for the hydrolysate produced using pepsin (Fig. 1B). Under reducing conditions, no band was observed for hydrolysates produced using trypsin, Alcalase and thermolysin, while a band at 16 kDa was exhibited for pepsin hydrolysate (Fig. 1C). Therefore, enzymatic hydrolysis using proteases did not affect disulfide bonds between polypeptides.

3.3. In vitro antioxidant activities of Moringa oleifera seed protein hydrolysates

3.3.1. DPPH radical scavenging activity

DPPH is a stable free radical that accepts an electron or hydrogen radical to transform into a stable diamagnetic molecule. DPPH radical scavenging activity has been successfully used for assessing antioxidant activity of many plant products [30,31]. DPPH radical scavenging activity of *Moringa oleifera* protein hydrolysates and gallic acid is presented in table S1. It was found that DPPH radical activity increased with the concentration of protein hydrolysates. DPPH activity varied between hydrolysates as follows: pepsin hydrolysate (43.35–78.68%), trypsin hydrolysate (30.97–56.27%), Alcalase hydrolysate (11.10–52.53%), and thermolysin hydrolysate (20.57–74.89%).

The half maximal inhibitory concentration (IC_{50}) of DPPH radical scavenging activity of *Moringa oleifera* protein hydrolysates (Table 1) showed that thermolysin hydrolysate was the most effective (IC_{50} = 0.74 mg/mL), followed by pepsin hydrolysate (IC_{50} = 10.03 mg/mL), Alcalase hydrolysate (IC_{50} = 10.47 mg/mL) and trypsin hydrolysate (IC_{50} = 13.3 mg/mL). However, the hydrolysates were less potent than gallic acid (IC_{50} = 0.0021 mg/mL). Studies related to concentration-dependent DPPH radical scavenging activity for protein hydrolysates from *Moringa oleifera*, cowpea, canola, rapeseed, and soybean seeds have already been reported [17]. In this regard, it was observed that the minimum concentration of hydrolysate required to trap the DPPH radical varied between hydrolysates, with thermolysin hydrolysate being the most effective. Shahi et al. [22] reported that hydrolysates with hydrophobic amino acids reacted more rapidly with DPPH radical compared to hydrolysates consisting mainly on hydrophilic amino acids. This sensitivity could be attributed to an increase in chains containing hydrophobic amino acids, which facilitated reaction with DPPH-free radical. Mune Mune et al. [14] reported that *Moringa oleifera* seed protein contained more hydrophobic amino acids (37.4%) than hydrophilic amino acids. In addition, high DPPH radical scavenging activity of pepsin and thermolysin hydrolysates was probably related to the side chains of amino acids in the released peptides, since these enzymes hydrolyse peptide bonds at hydrophobic and aromatic amino acid positions. And these amino acids have a high reactivity with the DPPH radical.

3.3.2. ABTS radical scavenging assay

ABTS radical scavenging activity of *Moringa oleifera* protein hydrolysates and gallic acid is presented in table S2. As observed for DPPH radical scavenging activity, ABTS radical scavenging of *Moringa oleifera* seed protein hydrolysates increased with the concentration. The following range of activity was observed: pepsin hydrolysate (19.63–35.38%), trypsin hydrolysate (15.29–51.62%), Alcalase hydrolysate (12.32–71.93%), and thermolysin hydrolysate (41.68–93.73%). As presented in Table 1, thermolysin hydrolysate was the most effective (IC_{50} = 0.15 mg/mL), followed by Alcalase hydrolysate (IC_{50} = 0.63 mg/mL) and trypsin hydrolysate (IC_{50} = 4.47 mg/mL). The IC_{50} of pepsin hydrolysate was >5 mg/mL. Similar results were obtained for rice protein. In fact, Zhou et al. [32] reported that rice protein hydrolysates prepared using microbial proteases (*Aspergillusoryzae* and *Bacillus licheniformis*) showed high ABTS radical scavenging capacity compared to hydrolysates prepared using non-microbial proteases. In addition, Alashi et al. (2014) [11] reported that Alcalase hydrolysate has a high ABTS radical scavenging capacity compared to pepsin and trypsin hydrolysates. High ABTS radical scavenging activity for thermolysin and Alcalase was probably related to the specificity of these enzymes. Gómez-Ruiz et al. [33] reported that cysteine was the most active amino acid in ABTS radical scavenging activity, followed by tryptophan, tyrosine, and histidine. Therefore, hydrolysates with high ABTS radical scavenging capacity probably maintained hydrophobic interactions with the hydrophilic ABTS radicals [11].

3.3.3. Ferric reducing antioxidant power (FRAP) of Moringa oleifera seeds protein hydrolysates

The FRAP assay is commonly used to determine the ability of antioxidants to act as reductants by yielding electrons to Fe^{3+} to reduce it into the more stable divalent iron ion, Fe^{2+} [34]. Thermolysin hydrolysate showed significantly high ($p < 0.05$) FRAP activity (74.3 Eq. μg catechin/mg hydrolysate) compared to hydrolysate produced by Alcalase (16.4 Eq. μg catechin/mg hydrolysate), pepsin

Table 1
Half Maximal Inhibitory Concentration (IC_{50}) of DPPH and ABTS free radical scavenging activities for *Moringa oleifera* protein hydrolysates[a].

Sample	Gallic acid	Pepsin hydrolysate	Trypsin hydrolysate	Alcalase hydrolysate	Thermolysin hydrolysate
IC_{50} (mg/mL) DPPH	0.0021^{a1}	10.03^{c1}	13.3^{e2}	10.47^{d2}	0.74^{b2}
IC_{50} (mg/mL) ABTS	0.0035^{a2}	>5	4.47^{d1}	0.63^{c1}	0.15^{b1}

Means followed by different letter (a,b,c,d,e) within the same row are significantly ($p < 0.05$) different. Means followed by different number (1,2) in the same column are significantly ($p < 0.05$) different.

[a] Data are means of triplicate determinations.

(11.79 Eq. μg catechin/mg hydrolysate) and trypsin (3.96 Eq. μg catechin/mg hydrolysate). The low cysteine content (2.11%) in *Moringa oleifera* seed protein [14] probably impacted low FRAP activity of the hydrolysates. In another hand, thermolysin is a metallo-endoprotease that hydrolyses protein bonds on the N-terminal side of hydrophobic amino acid residues, thus producing hydrolysates with acidic amino acid residues able to yield protons to ferric ions [34]. Sulphur containing amino acid residues have the ability to transfer electrons to ferric ions (Fe^{3+}), thereby reducing them into ferrous ion (Fe^{2+}) which promotes the antioxidant activity of the protein hydrolysate. Also, Udenigwe and Aluko [34] reported that acidic and sulphur-containing amino acid residues contribute positively to the reducing properties of protein hydrolysates, while positively charged amino acid residues, such as lysine and arginine, have very weak effects. Hydrophobic amino acids, especially histidine, proline, methionine, cysteine, tyrosine, tryptophan and phenylalanine residues can enhance the antioxidant activity of hydrolysates through interaction with lipid molecules and electron transfer [24].

Similar results were observed by Olusola et al. [17] who reported that pepsin hydrolysates of *Moringa oleifera* seed proteins had a higher iron reducing capacity than trypsin hydrolysates. In contrast, Mune Mune et al. [6] reported that trypsin hydrolysate of Bambara pea protein had a higher iron reducing activity than thermolysin and Alcalase hydrolysates. Then, the amino acid composition of the sample as well as hydrolysis conditions which affected the size of peptides, had an impact on iron reducing capacity of hydrolysates.

3.4. Cellular glucose uptake properties of Moringa oleifera seed protein hydrolysates

Regulation of blood glucose levels is important to prevent the complications associated with diabetes. The hypoglycaemic properties of pepsin, trypsin, Alcalase and thermolysin hydrolysates were assessed by the glucose transport technique across yeast cell membranes and thin sections of tissue isolated from '*Wistar*' rats in the absence or presence of insulin.

3.4.1. Peripheral glucose uptake through non-insulin dependent cells properties

Effect of *Moringa oleifera* seed protein hydrolysates on glucose transport across yeast cell membranes is presented in Fig. 2. The four hydrolysates stimulated glucose uptake across the yeast cell membranes in a concentration-dependent manner, and the activity varied according to the hydrolysate. The percentage of glucose uptake varied from 15.63% to 28.66% for pepsin hydrolysate, 6.27%–34.24% for thermolysin hydrolysate, 12.34%–35.28% for trypsin hydrolysate and 7.36%–32.63% for Alcalase hydrolysate. Generally, peripheral glucose uptake increased with the hydrolysate concentration and trypsin hydrolysate showed higher activity. Peripheral glucose uptake was probably related to peptide size since trypsin hydrolysate showed higher DH, as well as peptide composition. Soybean and flaxseed protein hydrolysates also promoted glucose transport across yeast cell membranes [35,36]. Several factors can influence glucose uptake by yeast cell membranes including the concentration of glucose inside the cells or the subsequent metabolism of glucose. If most of the internal sugar is readily converted to other metabolites, the internal glucose concentration will be low and glucose uptake into the cell will be favoured [37]. Thus, it is possible that increasing glucose transport by yeast cell membranes in the presence of protein hydrolysates from *Moringa oleifera* seeds was due to both the facilitated diffusion and high glucose metabolism. This ability of hydrolysates to stimulate non-insulin-dependent glucose uptake could be due to insulinomimetic effects. Indeed, many authors had already noted the insulinomimetic effect of bioactive peptides [37]. Therefore, the capacity of trypsin hydrolysate to stimulate non-insulin-dependent cellular glucose uptake could be explained by the nature of peptides obtained after hydrolysis by this enzyme, which would be more insulinomimetic than those obtained using the three other enzymes. On the other hand, these effects could also be explained by the capacity of hydrolysates to stimulate the translocation of non-insulin-dependent transporters or to activate the AMPK pathway, which is a cellular glucose capture pathway independent of insulin [35].

3.4.2. Peripheral glucose uptake through insulin-dependent cells properties

In the absence of insulin.

Effect of protein hydrolysates on muscle glucose uptake in the absence of insulin are shown in Fig. 3. The control uptake (muscle

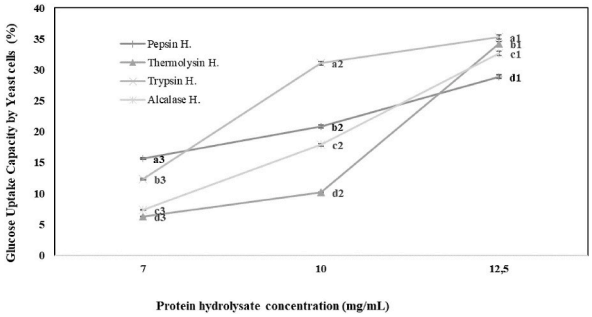

Fig. 2. Glucose Uptake Capacity by yeast cells as a function of the concentration of pepsin, trypsin, Alcalase and thermolysin hydrolysate. H.: Hydrolysate. Means with different letters (a,b,c,d) at the same concentration are significantly ($p < 0.05$) different. Means with different numbers (1; 2;3) for the same protein hydrolysate are significantly ($p < 0.05$) different.

alone) was at about 2%. The hydrolysates strongly stimulated muscle glucose uptake in the absence of insulin, which increased with increasing hydrolysate concentrations. Alcalase hydrolysate was the most effective with uptake rates ranging from 22.03% to 29.93% after 30 min, then from 29.55% to 34.6% after 60 min, followed by pepsin hydrolysate (19.3%–27.9%, and 27.74%–34.51% at 30 and 60 min, respectively); trypsin hydrolysate (15.44%–25.33% and 23.04%–27.97% respectively at 30 and 60 min) and thermolysin hydrolysate (11.11%–21.72% and 21.59%–32.54% respectively at 30 and 60 min). The muscle uptake increased with time, being higher at 60 min than at 30 min for all hydrolysates.

Previous studies have reported a stimulation of glucose uptake by thin sections of muscle tissue in the presence of flaxseed and soybean hydrolysates [35,36]. These authors attributed the glucose uptake properties of hydrolysates to low molecular weight peptides (300–400 kDa) as well as to cationic peptides. This hypothesis may justify the results obtained in this study, because Gonzalez Garza et al. [18] reported the presence of cationic peptides in *Moringa oleifera* seed protein hydrolysates. The ability of hydrolysates to stimulate muscle glucose uptake in the absence of insulin approved the hypothesis of insulin-mimetic effects observed on glucose uptake by yeast cells. Probably, the peptides derived from the hydrolysates, by mimicking the effect of insulin, would be able to stimulate the translocation of GluT4 and thus allow the muscular capture of glucose. These GluTs are involved in the control of cellular glucose metabolism and whole-body energy homeostasis, which are strongly associated with diabetes disorders [38]. The beneficial effects of hydrolysates on GluT4 would therefore support their potential use in the management of diabetes.

In the presence of insulin.

Effect of protein hydrolysates on muscle glucose uptake in the presence of insulin is presented in Fig. 4. It appeared that insulin alone improved muscle glucose uptake, which is greater than those observed with hydrolysates alone, with uptake rates of 42.61% and 47.81% at 30 and 60 min, respectively. The hydrolysates enhanced muscle glucose uptake in the presence of insulin, with effects that increased with increasing hydrolysate concentrations. Uptake rates ranged from 46.88% to 58.03% at 30 min and from 50% to 58.18% at 60 min. Although significant difference was noted between some concentrations when comparing the hydrolysates, they all remained very close in terms of activity at the different concentrations tested. As previously observed in the presence of insulin, muscle uptake of glucose increased with time, being higher at 60 min than at 30 min for all hydrolysates and for all concentrations tested.

Skeletal muscle is known to be the main site of insulin-mediated glucose uptake after carbohydrate consumption in humans. The observed effects of hydrolysates on muscle glucose uptake could be due to a possible additive effect from the previously noted insulinomimetic effects that would combine with those of insulin, or to a potentiating effect of insulin action by increasing insulin sensitivity or the number of insulin receptors stimulating increased GluT4 translocation and consequently glucose uptake. Indeed, it has been shown that translocation of GluT4 from intracellular vesicles to accumulate in the plasma membrane in response to insulin is highly dependent on activation of the insulin substrate 1 receptor [39]. In addition, data from a previous study suggested that acute stimulation on insulin-induced glucose uptake in skeletal muscle was related to the increased phosphorylation and activation of key proteins involved in GluT4 translocation, such as Rac1, AS160 and Akt [39]. Other studies suggested that the increase in glucose uptake was due to increased phosphorylation of AMP-activated protein kinase (AMPK). AMPK functions as an intracellular energy sensor, and pharmacological activation of AMPK has been shown to promote glucose transport. AMPK is thought to be one of the targets of major anti-diabetic drugs, such as thiazolidinediones and biguanides, as well as insulin-sensitive adipokines, although the mechanism appears to be indirect [40]. On the other hand, these insulin-sensitising effects could also be due to a possible beneficial effect on incretins. Indeed, Skuratovskaia et al. [41] have demonstrated positive effects of incretins on insulin sensitivity. Many studies have already shown the beneficial effects of protein hydrolysates on the availability of incretins [6,42].

The increase in glucose uptake under the synergistic effect of insulin and protein hydrolysates showed that protein hydrolysates can

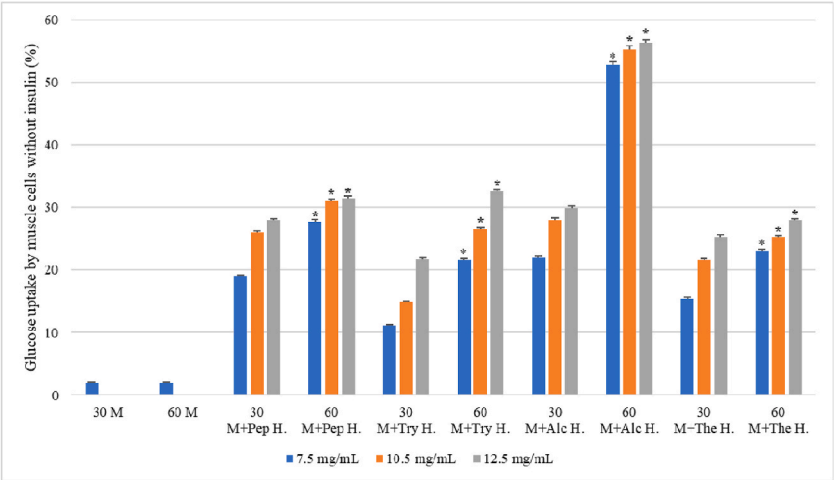

Fig. 3. Glucose Uptake Capacity by muscle cells in the absence of insulin as a function of the concentration of pepsin, trypsin, Alcalase, and thermolysin protein hydrolysates. H.: Hydrolysate. M: Muscle. Pep: Pepsin. Try: Trypsin. Alc: Alcalase. The: Thermolysin. *$p < 0.05$, significantly different at 60 min compared to 30 min for the same sample and concentration.

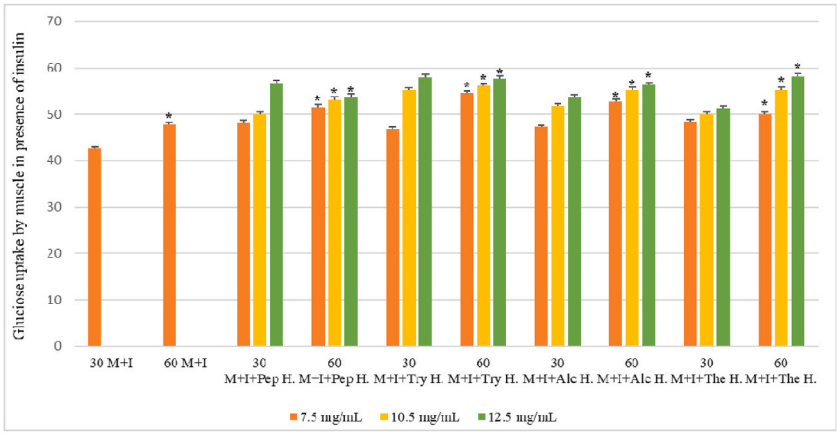

Fig. 4. Glucose Uptake Capacity by muscle cells in the presence of insulin as a function of the concentration of pepsin, trypsin, Alcalase, and thermolysin protein hydrolysates. H.: Hydrolysate. M: Muscle. I: Insulin. Pep: Pepsin. Try: Trypsin. Alc: Alcalase. The: Thermolysin. *$p < 0.05$, significantly different at 60 min compared to 30 min for the same sample and concentration.

counteract the insulin resistance that is one of the main symptoms of type 2 diabetes. However, the great complexity of the mechanisms and receptors involved in cellular glucose uptake in particular the AMPK pathway, requires further investigation to elucidate the effect of protein hydrolysates.

4. Conclusion

To improve application of *Moringa oleifera* protein in food and non-food formulations, especially for the management of type 2 diabetes, antioxidant and antihyperglycaemic potential of *Moringa oleifera* seed protein hydrolysates was analyzed. Results clearly showed that trypsin hydrolysate had the highest degree of hydrolysis followed by pepsin hydrolysate, Alcalase and thermolysin hydrolysate. Protein hydrolysates exhibited antioxidant properties by scavenging DPPH and ABTS radicals and reducing Fe^{3+} to Fe^{2+}. High antioxidant activity was noticed with the thermolysin hydrolysate. Protein hydrolysates also stimulated peripheral glucose uptake in both non-insulin dependent and insulin dependent cells. The trypsin hydrolysate resulted in a better non-insulin-dependent cell uptake with a maximum uptake rate of about 35%. For insulin-dependent cell uptake, the Alcalase hydrolysate showed high activity with maximum glucose uptake rate over 35% in the absence of insulin and 55% in the presence of insulin. Further studies on molecular weight distribution of peptides in relation to bioactivities could be important to better understand the structure-activity relationship. Protein hydrolysates of *Moringa oleifera* seed are therefore good candidates for the management of oxidative stress and hyperglycaemia.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Christian Bernard Bakwo Bassogog: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Carine Emilienne Nyobe:** Writing – original draft, Formal analysis, Data curation. **Fanta Yadang Sabine:** Investigation, Formal analysis. **Ambamba Akamba Bruno Dupon:** Methodology, Investigation, Formal analysis. **Simon Pierre Ngui:** Formal analysis, Data curation. **Samuel René Minka:** Writing – review & editing, Supervision, Methodology. **Ngondi Judith Laure:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Martin Alain Mune Mune:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28368.

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