Antioxidant and anti-inflammatory properties of novel peptides from Moringa oleifera Lam. leaves

Article *in* South African Journal of Botany · June 2021

DOI: 10.1016/j.sajb.2021.05.033

South African Journal of Botany 141 (2021) 466–473

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Antioxidant and anti-inflammatory properties of novel peptides from *Moringa oleifera* Lam. leaves

Sara Avilés-Gaxiola[a], Josefina León-Félix[a], Yazmín B. Jiménez-Nevárez[a], Miguel A. Angulo-Escalante[a], Rosalio Ramos-Payán[b], Juventino Colado-Velázquez III[c], J. Basilio Heredia[a,*]

[a] Centro de Investigación en Alimentación y Desarrollo, A.C. Nutraceutical and Functional Foods. Carretera a El Dorado Km 5.5, Col. Campo el Diez, Culiacán, CP 80110, México
[b] Universidad Autónoma de Sinaloa. College of Chemical-Biological Sciences. Calzada de las Américas Norte 2771, Col. Burócrata, Culiacán, CP 80030, México
[c] Universidad Autónoma de Occidente. Health Sciences Department. Blvd. Lola Beltrán and Blvd. Rolando Arjona. Culiacán, CP 80020, México

ARTICLE INFO

Article History:
Received 21 January 2021
Revised 30 March 2021
Accepted 30 May 2021
Available online xxx

Edited by AR Ndhlala

Keywords:
Moringa oleifera
Hydrolysate
Plant peptides
Antioxidants
Nitric oxide
Macrophages

ABSTRACT

Moringa oleifera is a tree used as a medicinal herb by several populations. Due to their curative and preventive properties, all parts have been studied, especially the leaves. They have been found to act as antithrombotic, antihypertensive, anticancer, immunomodulating, and antioxidant agents. This study was aimed to characterize and evaluate the antioxidant and anti-inflammation activities of *Moringa oleifera* leaves protein hydrolysate obtained by *in vitro* gastrointestinal digestion. The antioxidant activity of these peptides was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and Oxygen Radical Absorbance Capacity (ORAC) assays. RAW 264.7 macrophages were stimulated with lipopolysaccharides, and the effect of the peptides over nitric oxide production was measured to assess the anti-inflammatory effect. Furthermore, peptides were identified by nanoscale liquid chromatography coupled to tandem mass spectrometry. *Moringa oleifera* leaves peptides (1.33 mg/ml) inhibited DPPH and ABTS radicals by 45.70 and 93.09%, respectively, and had an ORAC activity of 3.27 mM Trolox equivalent/g. They were not cytotoxic to lipopolysaccharides-induced RAW 264.7 macrophages and, at a concentration of 100 μg/ml, inhibited the nitric oxide production by 30.51%. The sequences of 14 novel peptides were identified. Our findings suggest that *Moringa oleifera* leaves peptides released by *in vitro* gastrointestinal digestion might be a potential resource for natural antioxidant and anti-inflammatory components. However, further *in vivo* experiments are needed.

1. Introduction

Moringa oleifera Lam. (MO), also known as "the miracle tree," is a tree species belonging to the Moringaceae family, within the order Brassicales. The Moringaceae family has 13 species, with MO as the most cultivated and studied one (Tshabalala et al., 2019). MO is native to sub-Himalayan tracts of Northern India, where it was first described as a medicinal herb. Later, it was distributed to Africa and Ethiopia. Nowadays, since the tree can grow in various conditions, it is also found in distant places such as Latin America and the Pacific Islands, among other countries (Bartíková et al., 2020). In Africa particularly, folk medicine uses MO (a.k.a. panacea) to treat and prevent more than 300 diseases such as cancer, diabetes, malaria, dementia, hypercholesterolemia, Parkinson's, and asthma, among others. Also, MO has been used to combat child malnutrition (Granella et al., 2021; Matic et al., 2018).

Due to their preventive and curative properties, all MO parts have been studied, particularly leaves, which are an important dietary source for humans (Nouman et al., 2020). Consumption of MO leaves may enrich the human diet in bioactive components, for example, biologically active peptides. Bioactive peptides are released during gastrointestinal digestion and are small compounds with biological activity, for example, antithrombotic, antihypertensive, immunomodulating, anticancer, and antioxidant agents (Görgüç et al., 2020; Lin et al., 2019; Paula et al., 2017; Yun et al., 2020).

There is increased interest in the use of plant peptides for the treatment of oxidative stress. Overproduction of free radicals and oxidants can cause oxidative stress and oxidative damage to biological molecules promoting cancer, cardiovascular disease, and neurological

Abbreviations: MO, Moringa oleifera; MOP, Moringa oleifera leaves protein; MOPH, Moringa oleifera leaves protein hydrolysate; NO, nitric oxide; LPS, lipopolysaccharides; DH, degree of hydrolysis; SDS-PAGE, sodium dodecyl sulfate gel electrophoresis; DPPH, 2,2-diphenyl-1-picrylhydrazyl assay; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid assay; ORAC, Oxygen radical absorbance capacity assay

* Corresponding author.
E-mail address: jbheredia@ciad.mx (J.B. Heredia).

https://doi.org/10.1016/j.sajb.2021.05.033
0254-6299/

diseases (Pizzino et al., 2017). Natural antioxidants are the subject of increasing interest for their role in preventing diseases. Several studies have shown that antioxidant and anti-inflammatory peptides have a protective effect against ROS, contributing to reducing oxidative stress (Saenjum et al., 2012).

MO leaves have high amounts of crude protein (23.0 to 30.3%) composed of the essential amino acids methionine, phenylalanine, threonine, leucine, valine, histidine, isoleucine, lysine, and tryptophan (Aderinola et al., 2018; Granella et al., 2021; Su and Chen, 2020). Therefore, they may be a good source of bioactive peptides. MO leaves protein hydrolysate (MOPH) is hypoglycemic; nevertheless, its full bioactive potential is unknown (Paula et al., 2017).

For this reason, this study aimed to evaluate the antioxidant and anti-inflammation activities of MOPH obtained by *in vitro* gastrointestinal digestion. The antioxidant activity of MOPH was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and Oxygen Radical Absorbance Capacity (ORAC) assays. RAW 264.7 macrophages were stimulated with lipopolysaccharides (LPS), and the effect of MOPH over nitric oxide (NO) production was measured to assess the anti-inflammatory effect. Furthermore, novel peptides were identified within MOPH by nanoscale liquid chromatography-coupled to tandem mass spectrometry (nano LC-MS/MS).

2. Material and methods

2.1. Chemical reagents

Protein extraction, hydrolysis, hydrolysis degree, antioxidant assays, peptide identification, and Griess reagents: pepsin, tris (hydroxymethyl)aminomethane hydrochloride (TRIS-HCl), NaCl, poly (vinylpolypyrrolidone) (PVPP), phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), ammonium sulfate, pepsin, HCl, $NaHCO_3$, NaOH, pancreatin, phenolphthalein, formaldehyde, ABTS, Trolox, $K_2S_2O_8$, DPPH, fluorescein, 2,2'-azobis-(2-amidino-propane) dihydrochloride (AAPH), K_2HPO_4, KH_2PO_4, H_3PO_4, formic acid, acetonitrile, sulfanilamide, N(1-naphthyl) ethylenediamine dihydrochloride, indomethacin, and LPS were purchased from Sigma-Aldrich (MO, USA). Electrophoresis reagents were purchased from Bio-Rad (CA, USA). Murine macrophage cell line RAW 246.7 was purchased from American Type Culture Collection (VA, USA). Cell culture reagents were purchased from GIBCO (MA, USA). [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) was purchased from Promega Co (WI, USA).

2.2. Plant material

The present investigation was conducted on 20 plants of *Moringa oleifera* Lam. marked from a larger number of plants cultivated in a family-owned Farm of Culiacán, Sinaloa, México (24° 51′37.0'' N / 107°12′59,6'' W and 86 m above sea level). The farm has sandy-loam soil, which was irrigated at regular intervals as required. The selected plants were three years old, and after harvest, samples were taken into the laboratory. They were first soaked in gentle commercial detergent for 15 min and washed in running tap water, followed by washing them in double-distilled water. Samples were further disinfected with chlorine (150 ppm), and right after, they were double washed in double-distilled water. The fresh young leaves were excised from the plants. After cleaning, leaves were dried at a temperature of 40 °C for 72 h in an Excalibur 3526T food dehydrator (Ca, USA). The dried leaves were milled in a KRUPS Gx41011 coffee grinder (CDMX, Mexico). MO leaves flour was stored at 4 °C in sealed containers until further use.

2.3. Characterization of Moringa oleifera leaves flour

Moisture, ash, crude protein content (N x 6.25), crude fiber, and crude fat were performed according to AOAC official methods 925.10, 923.03, 978.02, 962.09 and 963.15, respectively (AOAC, 1992). Nitrogen free extract was calculated by difference: 100-(% moisture + % ash + % protein + % fiber + % fat).

2.4. Moringa oleifera leaves protein extraction

MO leaves flour protein was extracted in 0.05 M Tris-HCl buffer pH 8.0 containing 2% (W/V) PVPP, 0.15 M NaCl, 0.01 M EDTA, and 0.001 M PMSF, 1:5 (w/v). The solution was agitated at 4 °C for 30 min and subsequently filtered through a cheesecloth layer. The solid retained fraction was discarded, and the filtrate was centrifugated at 10000 rpm and 4 °C for 30 min. The supernatant was precipitated at 90% saturation of $(NH_4)_2SO_4$ at 4 °C for 18 h. The precipitate was centrifugated at 10000 rpm and 4 °C for 30 min. The pellet was recovered and dissolved in double distilled water and dialyzed with a membrane of molecular weight cut-off (MWCO) of 2 kDa against double distilled water at 4 °C. The dialysate was further lyophilized, and MO leaves protein (MOP) was stored at -20 °C until further analyses.

2.5. Digestion of Moringa oleifera leaves protein

The simulated gastrointestinal digestion of MOP was done based on the modified method from Minekus et al. (2014). The protein isolate was suspended in pepsin solution (2000 units per ml) 1:20 (w/v) at 37 °C and pH 3 during 2 h of continuous stirring to simulate passage through the stomach. pH was revised and adjusted with HCl 1 M every 30 min. Afterward, pH was adjusted to 7.0 with NaOH 1 M, and subsequently, $NaHCO_3$ and pancreatin were added at a concentration of 0.1 M and 100 units per ml, respectively, to simulate intestinal fluid. The solution was stirred for 2 h at 37 °C, while pH was revised and adjusted with NaOH 1 M every 30 min until it was placed on ice to stop the reaction. The enzymes were inactivated by heating the hydrolysate for 20 min at 80 °C. Later, the hydrolysate was centrifuged at 4000 rpm, and 37 °C for 20 min and the supernatant was recovered and ultrafiltered with a 5 kDa MWCO to remove enzymes and undigested protein. The permeated was recovered, lyophilized and stored at -20 °C until further analyses.

2.6. Degree of hydrolysis of Moringa oleifera leaves protein after gastrointestinal digestion

The degree of hydrolysis (DH) of the MOP after *in vitro* gastrointestinal digestion was calculated using the relationship between α-amino nitrogen and total nitrogen according to the following equation:

$$DH\ (\%) = \frac{\alpha - amino\ nitrogen}{Total\ nitrogen}\ x\ 100$$

The α-amino nitrogen represents the proportion of nitrogen that was once part of a peptide bond and then is found in the free state; it was quantified by the Sorensen formol titration technique (Silva and Silveira, 2013). Total nitrogen represents bound nitrogen within peptide bonds, as determined by the AOAC official method 978.02 (AOAC, 1995).

2.7. Electrophoretic profile of Moringa oleifera leaves protein and protein hydrolysate

Electrophoretic profile was determined for MOP and MOPH. MOP was dissolved in double distilled water to a concentration of 0.9 mg/ml. This aliquot (aliquot #1A) was subsequently combined with denaturing buffer (1:1) (aliquot #1B). MOPH was dissolved in

double distilled water to a concentration of 10 mg/ml (aliquot #2A). Subsequently, aliquot #2A was combined with denaturing buffer (3:1) (aliquot #2B). The denaturing buffer was prepared with 5% β-mercaptoethanol (v/v), 2% sodium dodecyl sulfate w/v, 10% glycerol v/v, and 0.025% bromophenol blue w/v in 62.5 mM Tris-HCl pH 6.8. 20 μl of both aliquots, #1B and #2B, were boiled at 100 °C for 5 min and loaded on a discontinuous sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (stacking section at 6% and the resolving section at 15%) according to the protocol described by Laemmli (1970) using a Mini electrophoresis cell Protean II from Bio-Rad (CA, USA). Protein separation was carried out at 120 V until the dye reached the lower edge of the gel before staining with Coomassie Brilliant Blue R-250, according to Neuhoff et al. (1985). The gel picture was obtained by using an Axygen gel documentation system BL-Imagen (MA, USA).

2.8. Antioxidant activity of Moringa oleifera leaves protein and protein hydrolysate

ORAC assay: the oxygen radical absorbance capacity was measured for MOP and MOPH. They were dissolved in phosphate buffer according to the protocol developed by Huang et al. (2002). AAPH was used as the peroxyl radical generator and fluorescein as the fluorescent probe. Trolox was used as a standard, and its curve ranged from 6.5 to 125 μM Trolox equivalent (TE)/g. The reaction mixture contained 25 μl of the sample/blank (phosphate buffer)/ Trolox standard curve, 75 μl of 0.8 M AAPH, and 200 μl of 0.106 μM fluorescein. The sample/ blank (phosphate buffer)/ Trolox standard curve and fluorescein were pre-incubated at 37 °C for 15 min. APPH was added to start the reaction. The fluorescence was measured every 70 s for 70 min (485 nm excitation and 580 nm emission) in a Synergy HT multi-detection microplate reader from BioTek (VT, USA). The values were calculated using the regression equation that relates Trolox concentration and the net area under the fluorescein decay curve. The results are expressed as mM TE/g.

DPPH scavenging capacity: DPPH radical scavenging assay was carried out according to Karadag et al. (2009) for MOP and MOPH. They were dissolved in double-distilled water. Briefly, 20 μl of the samples were placed in a 96-well microplate. Then, 280 μl of DPPH was added and left to incubate for 30 min in the dark. Absorbance was measured at 540 nm in an Epoch microplate spectrophotometer from Biotek (VT, USA). Water was used as a blank, and the results were expressed as the percentage of DPPH inhibition at a concentration of 1.33 mg of the sample per ml.

ABTS assay: the assay was carried out according to Thaipong et al. (2006) for MOP and MOPH. They were dissolved in double-distilled water. The ABTS was first dissolved in double distilled water to 7.4 mM. Subsequently, the ABTS stock solution reacted in the dark with a 2.6 mM potassium persulfate solution (1:1, v/v) for 16 h at room temperature. 100 μl of the resulting solution were diluted in 2900 μl of methanol (reaction solution). 15 μl of the samples were placed in a 96-well microplate. Then, 285 μl of the reaction solution was added and left to incubate for 2 h in the dark. Absorbance was measured at 734 nm in an Epoch microplate spectrophotometer from Biotek (VT, USA). Water was used as a blank, and the results were expressed as the percentage of ABTS inhibition at a concentration of 1.33 mg of the sample per ml.

2.9. In vitro anti-inflammatory activity of Moringa oleifera leaves protein hydrolysate

Cell culture: RAW 264.7 macrophages were grown in Dulbecco's Modified Eagle's medium (DMEM)/F12 supplemented with 7.5% heat-inactivated fetal bovine serum (FBS) and GlutaMax. No antibiotics were used. RAW 264.7 macrophages were plated and incubated in a humidified atmosphere (5% CO_2 at 37 °C). Cells were subcultured by scraping and seeding them in 75 cm^2 flasks.

Treatment of RAW 264.7 macrophages with LPS: macrophages were plated into 96-well plates at a density of 2.5×10^4 cells per well and incubated for 24 h at 37 °C and 5% CO_2 in 200 μl of DMEM/F12 medium supplemented with 7.5% heat-inactivated FBS. Subsequently, macrophages were incubated for 2 h either with the extracts at various concentrations (1-100 μg/ml), vehicle (dimethyl sulfoxide, 0.5%, V/V) or indomethacin (30 ug/mL). After that, macrophages were incubated with LPS at a concentration of 10 μg/ml (20 h at 37 °C). Finally, the cell-free supernatants were collected and stored at -20 °C until NO quantification.

Determination of NO production: nitrite was used as an indicator of NO production in the culture medium. It was measured according to the Griess reaction. 50 μl of each cell culture supernatants were mixed with 100 μl of the Griess reagent (50 μl of 0.1% N(1-naphthyl) ethylenediamine dihydrochloride in 2.5% of phosphoric acid and by 50 μl of 1% sulfanilamide) during 10 min at room temperature. Subsequently, the optical density was measured (540 nm) using a microplate reader (Yang et al., 2012). Nitrite concentrations were calculated by comparison with a standard curve of $NaNO_2$ prepared in a fresh culture medium.

Cell viability: macrophages were plated in 96-well plates at a density of 1.2 × 104 cells/well for 24 h. Subsequently, cells were treated with MOPH (0-100 μg/ml) and later incubated for 24 h. MTS assay was used in order to determine cell viability. 20 μl of MTS was added to each well, and macrophages were then incubated (4 h, 5% CO_2 at 37 °C). Optical density was measured on a microplate reader at 490 nm.

2.10. Characterization of Moringa oleifera leaves protein hydrolysate

MOPH peptides identification by nano LC-MS/MS: ultrapure water was prepared from a Millipore purification system (MA, USA). MOPH was dissolved and desalted using Ziptip C18 resin and further lyophilized to near dryness. MOPH was resuspended in 20 μl of 0.1% formic acid before LC-MS/MS analysis. For peptides resolution, the Ultimate 3000 nano UHPLC system (ThermoFisher Scientific, USA) was used; the trapping column was a PepMap C18, 100Å, 100 μm × 2 cm, 5 μm and the analytical column a PepMap C18, 100Å, 75 μm × 50 cm, 2 μm. The loaded sample amount was 2 μg. Mobile phase A was composed of 0.1% formic acid in water; mobile phase B was composed of 0.1% formic acid in acetonitrile. The LC linear gradient was as follow: from 6% to 9% B for 8 min, from 9% to 14% B for 16 min, from 14% to 30% B for 36 min, from 30% to 40% B for 15 min, and from 40% to 95% B for 3 min, eluting with 95% B for 7 min. The total flow rate was 250 nl/min. For mass spectrometry, the full scan was performed between 300−1,650 m/z at the resolution of 60,000 at 200 m/z, the automatic gain control target for the full scan was set to 3e6. The MS/MS scan was operated in Top 20 mode using the following settings: resolution 15,000 at 200 m/z; automatic gain control target 1e5; maximum injection time 19 ms; normalized collision energy at 28%; isolation window of 1.4 Th; charge state exclusion: unassigned, 1, > 6; dynamic exclusion 30 s. For data analysis, MS raw file was analyzed with PEAKS STUDIO X. The parameters were set as follows: the protein variable modifications were dioxidation (M), oxidation (HW), and deamination (variable); the enzyme specificity was set to as none. The precursor ion mass tolerance was set to 10 ppm, and MS/MS tolerance was 0.05 Da. Only high confident identified peptides were chosen for downstream peptide sequencing analysis.

Peptide structures and physicochemical properties were predicted using the PepDraw tool. The potential biological activity of the peptides was predicted by using the BIOPEP database. Swisstarget algorithm was used to predict targets of the peptides.

2.11. Statistical analysis

Each experiment was conducted in triplicate, and the standard deviations were calculated. Data were reported as mean ± standard

Table 1
Proximate composition of *Moringa oleifera* leaves flour.

Component	Content (%)
Protein	27.77 ± 0.31
Fat	8.09 ± 0.02
Ash	11.49 ± 0.07
Moisture	2.46 ± 0.03
Crude fiber	23.34 ± 0.05
Nitrogen free extract	1.%2 ± 0.31

deviations. Data were subjected to analysis of variance (ANOVA) using the statistical software Minitab from MiniTab Inc. (PA, USA). Means were compared using Tukey's multiple comparison test at $p \leq 0.05$.

3. Results

3.1. Characterization of Moringa oleifera leaves flour

The proximate composition of MO leaves flour is presented in Table 1. The main component was protein, while moisture showed the lowest content.

3.2. Degree of hydrolysis of Moringa oleifera leaves protein

The DH for MOP after enzymatic digestion has not been reported before. The DH of MOP by Sorensen formol titration technique and the AOAC total nitrogen official method was 3.53 ± 0.29%. This result confirms that MOP underwent hydrolysis by pancreatin and pepsin.

3.3. Electrophoretic profile of Moringa oleifera leaves protein and of Moringa oleifera leaves protein hydrolysate

Electrophoresis was performed to identify the characteristic protein pattern of MOP and determine whether peptides were produced after the hydrolysis process. SDS-PAGE analysis revealed that MOP is composed of several protein bands (Fig. 1a). The majority of them showed a mass of over 20 kDa, and two strong bands between 10 and 15 kDa and 5 and 10 kDa were identified. From what is shown, MOP was susceptible to pepsin and pancreatin digestion (Fig. 1b).

Table 2
Antioxidant activity of *Moringa oleifera* leaves protein, and *Moringa oleifera* leaves protein hydrolysate.

Assay	MOP	MOPH
DPPH (%)*	36.68 ± 4.86[a]	45.70 ± 3.57[a]
ABTS (%)*	67.18 ± 0.67[b]	93.09 ± 0.07[a]
ORAC (mM TE/g)	1.81± 0.21[b]	3.27 ± 0.24[a]

* At a concentration of 1.33 mg/ml

3.4. Antioxidant activity of Moringa oleifera leaves protein and of Moringa oleifera leaves protein hydrolysate

The percentage of DDPH and ABTS inhibition and mM of TE/g of MOP and MOPH values are given in Table 2. The results showed that both MOP and MOPH could scavenge ABTS radical cation and exhibited strong ORAC assay activity. However, in both assays, MOPH presented significantly greater antioxidant activity. As for DPPH radical scavenging, no significant differences were found between MOP and MOPH.

3.5. In vitro anti-inflammatory activity of Moringa oleifera leaves protein hydrolysate

3.5.1. Cell viability *as affected by* Moringa oleifera *leaves protein hydrolysate*

The potential toxic effect of MOPH was evaluated with the MTT assay. The results are shown in Fig. 2, where the cell viability of RAW264.7 macrophages was not affected by MOPH in any

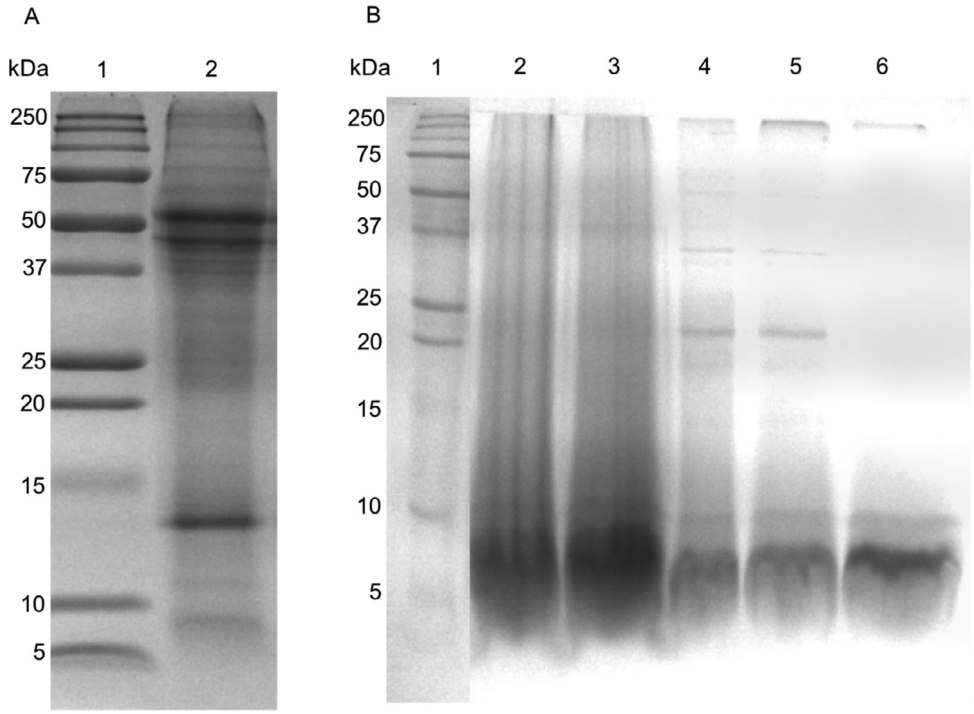

Fig. 1. Assessment of the *in vitro* digestibility of *Moringa oleifera* leaves protein (MOP) by electrophoresis SDS-PAGE 15%. A. 1) Marker, 2) MOP. B. 1) Marker, 2) MOP after 1 h of pepsin digestion, 3) MOP after 2 h of pepsin digestion, 4) MOP after 2 h of pepsin digestion and 1 h of pancreatin digestion, 5) MOP after 2 h of pepsin digestion and 2 h of pancreatin digestion, 6) ultrafiltered and centrifuged MOP hydrolysate.

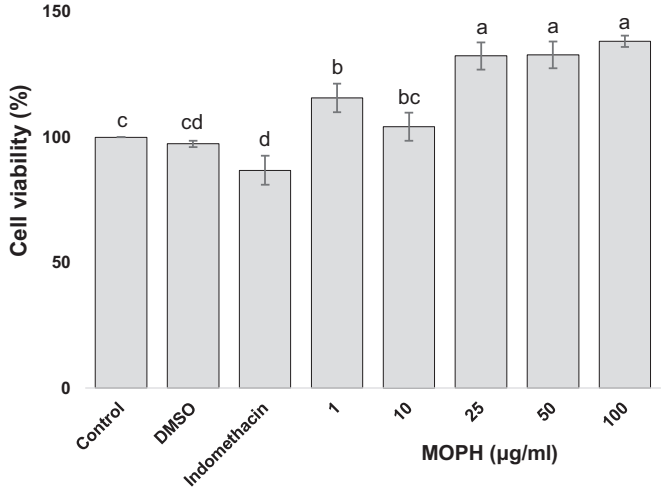

Fig. 2. Effect of *Moringa oleifera* leaves protein hydrolysate on the viability of LPS-stimulated RAW 264.7 cells. The values are expressed as the mean ± SD.

concentration (from 1 to 100 μg/ml). Therefore, these concentrations were used in the subsequent experiment.

3.5.2. Determination of Nitric oxide production by LPS stimulated RAW264.7 macrophages after Moringa oleifera leaves protein hydrolysate exposure

The effect of different concentrations of MOPH (0 to 100 μg/ml) on NO production by LPS stimulated RAW264.7 macrophages is shown in Fig. 3. A minimum amount of NO was produced by macrophages when cultured in medium alone (control). LPS-stimulated macrophages released NO at a level of 31.93±0.27 μM. Only the concentrations of 50 and 100 μg/ml of MOPH significantly reduced by 15.68 and 30.51%, respectively, the NO that resulted from LPS exposure.

3.6. Moringa oleifera leaves protein hydrolysate characterization

The sequences, physicochemical properties, and biological potential of the main peptides present in MOPH are listed in Table 3. The identified sequences of 14 peptides showed a mass of 726.31 to 1520.63 Da, a pI between 3.01 to 9.84, hydrophobicity that ranks between 5.14 to17.14 Kcal * mol^{-1}, and a net charge from -2 to +1. The main bioactivities predicted for the identified peptides are angiotensin I-converting enzyme and dipeptidyl peptidase IV inhibitor, as well as an antioxidant. The chemical structures of the 14 peptides are presented in Fig. 4.

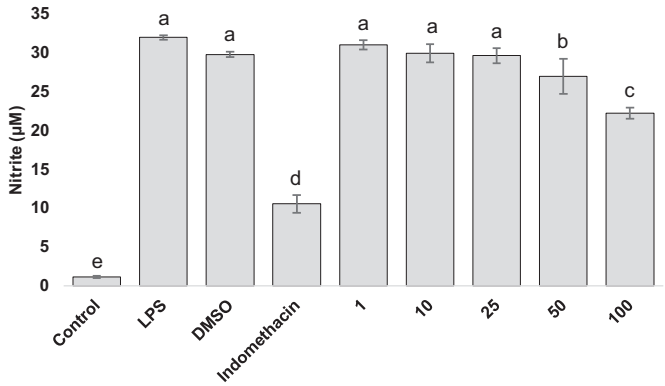

Fig. 3. Effect of *Moringa oleifera* leaves protein hydrolysate on NO production in LPS-stimulated RAW 264.7 cells. The values are expressed as the mean ± SD.

4. Discussion

4.1. Moringa oleifera *leaves proximate composition*

Wide ranges of values have been reported in the literature for the different constituents of MO leaves. These values depend on the solar radiation, humidity, type of soil, harvest time, and geographic area (Firenzuoli and Gori, 2007). Protein, fat, ash, crude fiber, and nitrogen-free extract contents in MO leaves are near or within the range of values previously reported (Alves et al., 2017; Evum and Sansthan, 2014; Leone et al., 2018; Leone et al., 2016; Su and Chen, 2020; Sultana, 2020). Protein, carbohydrate, high fiber, and low-fat content of MO leaves makes MO leaves flour a great source of nutrients. The ash content in MO leaves flour is considered a measure of the mineral content (Sultana, 2020). As for fiber, it is mainly cellulose with small amounts of lignin (Su and Chen, 2020).

4.2. Degree of hydrolysis of Moringa oleifera *leaves protein after pepsin and pancreatin digestion*

The DH is defined as the proportion of cleaved peptide bonds within a protein hydrolysate (El et al., 2015). The degree of hydrolysis by proteolytic enzymes of the gastrointestinal tract usually varies from 3.26 to 36.41% (Silvestre et al., 2013). The value here reported for MOP after pepsin and pancreatin digestion is near the DH reported for other plant sources of protein when using these enzymes, such as lupin, with a value of 3.37% (Schlegel et al., 2019). Other sources, such as whey protein concentrates, showed 2-25% DH by pepsin and trypsin (proteolytic enzymes), depending on the process duration (Kim et al., 2007). Regarding the time used in this research, the result is similar to what has already been reported for digestive enzymes (Schlegel et al., 2019; Kim et al., 2007; Shu et al., 2018). Moreover, when the food is a protein concentrate or isolate, the DH is around 5%, close to the MOPH value (Vidal et al., 2018).

The DH for MOPH is considered low; it may depend upon *Moringa oleifera* leaves protein being poor in certain bonds such as those of trypsin and pepsin cleavage (C-terminal of lysine and arginine and after phenylalanine and leucine, respectively) or that these bonds are unavailable for the digestive enzymes (Silvestre et al., 2013). A low value of the DH indicates protein that is not highly nutritious since it suggests the formation of peptides greater than three amino acids. However, this does not compromise the peptides' bioactivity (Silvestre et al., 2013). For example, high DH does not always translate into increases in the antioxidant capacity of the hydrolysates since hydrolysates with low DH values have shown high antioxidant capacity (Vidal et al., 2018).

4.3. Electrophoretic profile of Moringa oleifera *leaves protein and of* Moringa oleifera *leaves protein hydrolysate*

SDS-PAGE analysis showed that protein bands around 50-55 and 10-15 kDa in size were predominant in MOP. These bands may be the constituent of Rubisco, the most abundant soluble protein in chloroplasts, making up to 50% or more of all proteins in plant leaves. Rubisco is composed of eight small subunits of around 14 kDa and eight large subunits of around 56 kDa (Ma et al., 2009). The MOPH electrophoretic profile shows that almost all MOP underwent hydrolysis since its characteristic protein bands disappeared. Also, the centrifugation and ultrafiltration processes removed undigested protein.

4.4. Antioxidant activity of Moringa oleifera *leaves protein, and* Moringa oleifera *leaves protein hydrolysate*

MOPH has a higher antioxidant activity than MOP in terms of ABTS, DPPH, and ORAC assays. These suggest that the enzymatic hydrolysis may have caused an increase in exposed amino acid

Table 3
Main peptide sequences in *Moringa oleifera* leaves protein hydrolysate identified by nanoLC-MS/MS.

Sequence	Mass (Da)	pI	HydrophobicityKcal * mol^{-1}	Net Charge	BIOPEP	Swiss Target
1 LAYKPPG	744.42	9.78	+10.67	+1	Dipeptidyl peptidase IV inhibitor and angiotensin-converting enzyme inhibitor	Neurotensin receptor 1: Family A G protein-coupled receptor
2 YHSEVPV	829.40	5.06	+12.83	-1	-	Cystinyl aminopeptidase; protease
3 WPPTFEQPK	1000.46	3.01	+9.17	-1	Antioxidant	HLA class I histocompatibility antigen A-3; Surface antigen
4 LLGFDNR	833.44	6.74	+11.14	0	-	Integrin alpha-IIb / beta-3; Receptor membrane
5 QVWPTPGLK	1020.57	9.84	+9.35	+1	Angiotensin-converting enzyme inhibitor	Angiotensin-converting enzyme; protease
6 FTKDDEWSCFPF	1520.63	3.68	+15.22	-2	-	Neurokinin 1 receptor; Family A G protein-coupled receptor
7 VEQNLVPGLK	1095.63	6.81	+13.82	0	Angiotensin-converting enzyme inhibitor	Cathepsin D; protease
8 TMMLMT	726.31	5.28	+5.14	0	Antioxidant	HLA class I histocompatibility antigen A-3; Surface antigen
9 VQLPGWRVFP	1197.66	11.11	+5.94	+1	Angiotensin-converting enzyme inhibitor	Neuropilin-1 (by homology); Secreted protein
10 SYLPPLSAEVTAK	1374.74	6.57	+13.11	0	Dipeptidyl peptidase IV inhibitor and angiotensin-converting enzyme inhibitor	HLA class I histocompatibility antigen A-3; Surface antigen
11 TMKGPPDTLQ	1086.54	6.46	+15.12	0	Angiotensin-converting enzyme inhibitor	HLA class I histocompatibility antigen A-3; Surface antigen
12 MPWHEQ	826.34	5.06	+12.01	-1	Antioxidant	Gastrin releasing peptide receptor; Family A G protein-coupled receptor
13 LTAPGQATLPT	1068.58	5.36	+9.35	0	Dipeptidyl peptidase IV inhibitor. angiotensin-converting enzyme inhibitor and anticancerogenic	HLA class I histocompatibility antigen A-3; Surface antigen
14 LLTPEGPKE	982.53	4.08	+17.14	-1	-	HLA class I histocompatibility antigen A-3; surface antigen

residues, promoting an increase in scavenging activity (Bamdad et al., 2017). Most antioxidant peptides have been reported to contain a high proportion of hydrophobic amino acids, which is the case with most of the identified peptides (Zou et al., 2016). Hydrophobic amino acids increase peptides solubility in non-polar environments, and therefore a better interaction with free radicals is promoted (Saisavoey et al., 2019). There are different amino acids with known antioxidant activity, such as tyrosine and glycine, which act through hydrogen atom transfer. ORAC is a hydrogen atom transfer-based method that measures peptides' ability to quench peroxyl radical by H-donation (Esfandi et al., 2019; Li et al., 2017). Peptides containing one of these amino acids are 1, 2, 4, 5, 7, 9, 10, 11, 13, 14 (Table 3). As for peptide 1, it contains both. The antioxidant activity of any MOPH has not yet been reported before. As for ORAC, MOPH had a value of 3.27 $\pm$ 0.24 mM TE/g. Other plant sources such as defatted peanut meal hydrolysate and hemp seed hydrolysate have shown values of 1.35 and 0.7 mM TE/g, respectively (Zheng et al., 2012; Logarušić et al., 2019).

On the other hand, the amino acids cysteine, tryptophan, histidine, phenylalanine, and tyrosine acts mainly by single electron transfer. DPPH and ABTS are single electron transfer-based methods (Esfandi et al., 2019; Li et al., 2017). Peptides containing one or more of these amino acids are 1, 2, 3, 4, 5, 6, 9, 10 and 12 (Table 3). As for DPPH, MOPH at a concentration of 1.33 mg/ml inhibited in 45.70% the DPPH radical. Similar values have been reported for other plant sources such as oat, barley, and tomato hydrolysates, which DPPH radical quenching at the same concentration was 46.55, 53.2 and 33.25%, respectively (Bamdad and Chen, 2013; Bamdad et al., 2011; Esfandi et al., 2019; Meshginfar et al., 2018; Tsopmo et al., 2010).

As for ABTS radical scavenging activity, MOPH had a value of 93.09%. Hydrolysates for other plant sources such as sorghum and gluten meal for the same amount of hydrolysate had ABTS radical scavenging activity values of 66 and 93%, respectively (Xu et al., 2019) (Hu et al., 2020).

4.5. Anti-inflammatory activity of Moringa oleifera *leaves protein hydrolysate*

4.5.1. Cell viability as affected by Moringa oleifera *leaves protein hydrolysate*

Activated macrophages are important for humoral immunity as they remove necrotic debris and cells via phagocytosis to alleviate an injury or kill a pathogen (Hirayama et al., 2018). MOPH hydrolysate showed no cytotoxicity in LPS-induced RAW 264.7 macrophages. Instead, they promoted their growth. As for this, peptides have been considered low or nontoxic molecules since their degradation products are amino acids, which may be used as nutrients by the cells, promoting their growth (Avilés-Gaxiola et al., 2020; Gupta et al., 2013).

4.5.2. Nitric oxide production as affected by Moringa oleifera *leaves protein hydrolysate*

Inhibition of NO production is important in the therapeutic management of inflammatory diseases because it is an inflammatory response to oxidative stress by cells such as macrophages, endothelial cells, and neurons. When it is overproduced, it may play a critical role in diseases such as neurodegenerative disorders and diabetes mellitus due to DNA damage (Sharma et al., 2007). NO production was increased when macrophages were stimulated with LPS. MOPH in the higher concentration (100 μg/ml) significantly diminished NO production. A molecule that reduces NO production can suppress oxidative signaling and alleviate inflammatory response (Bamdad et al., 2017). Other peptides have been shown to inhibit NO by directly scavenging and modulating cellular inflammatory pathways (Saisavoey et al., 2019). One of the amino acids associated with a NO inhibitory effect is arginine, which inhibits nitric oxide synthase (Víteček et al., 2012). Peptides 4 and 9 contain arginine (Table 3). Other amino acids that have been found in common anti-inflammatory peptide structures are leucine and glycine (Saisavoey et al., 2019). At least one of these amino acids was found in peptides 1,4, 5,

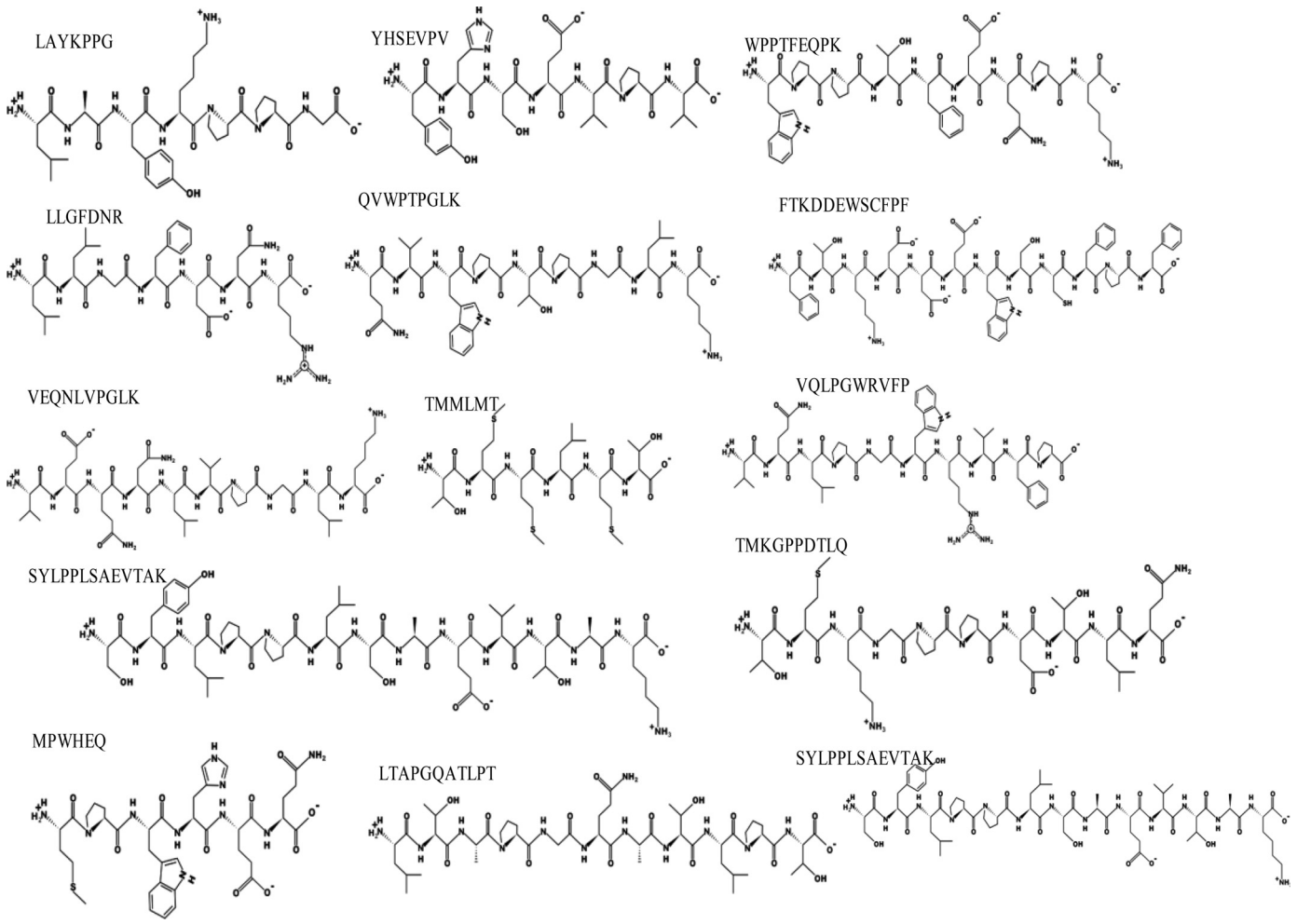

Fig. 4. *Moringa oleifera* peptides chemical structures.

7, 8, 9, 10, 11, 13 and 14 (Table 3); while both of them were detected in peptides 1, 4, 5, 7, 9, 11, 13 and 14 (Table 3).

4.6. *Moringa oleifera* leaves protein hydrolysate characterization

The sequence of peptides from MO leaves had not been previously reported. The analysis of the main peptides of the MOPH using the BIOPEP database showed the presence of angiotensin I-converting enzyme and dipeptidyl peptidase IV inhibitory peptide sequences. These peptides could be further evaluated for their preventive potential against chronic diseases. The presence of amino acids associated with antioxidant and anti-inflammatory activity was expected; this is widely described in sections 4.4 and 4.5.2.

5. Conclusions

MO leaves are a source of bioactive compounds such as peptides produced by enzymatic hydrolysis. The present study demonstrated, for the first time, the antioxidant and anti-inflammatory activities of MOPH. With the information reported in this article, it can be concluded that the MOPH obtained by the use of digestive enzymes may be potentially used as foods/nutraceuticals for improving human health and for treating oxidative and inflammatory-related disorders. However, further *in vitro* and *in vivo* experiments are needed to confirm their benefits and safety before being used by humans.

Funding sources

Thanks to CONACYT for providing a scholarship to Aviles-Gaxiola (597872)

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

We would like to thank the Office of Graduate Studies from the Centro de Investigación, Alimentación y Desarrollo (CIAD-CONACYT-México) for their support.

References

Aderinola, T.A., Fagbemi, T.N., Enujiugha, V.N., Alashi, A.M., Aluko, R.E., 2018. Amino acid composition and antioxidant properties of Moringa oleifera seed protein isolate and enzymatic hydrolysates. Heliyon 4, e00877. https://doi.org/10.1016/j.heliyon.2018.e00877.

Alves, A.d.S., Teixeira, E., Oliveira, G., Arantes-Pereira, L., Oliveira, C., Costa, L., 2017. Physicochemical characterization and antioxidant activity of decolorized *Moringa oleifera* Lam. leaf flour. Nutrire 42, 31. https://doi.org/10.1186/s41110-017-0058-6.

AOAC, 1992. American Organization of Analytical Chemists International. Official methods 962.09, 978.02, 925.10, 923.03, 963.15. Moisture, ash, crude protein, crude fiber, and crude fat determination. Washington, DC.

Avilés-Gaxiola, S., Gutiérrez-Grijalva, E.P., León-Felix, J., Angulo-Escalante, M.A., Heredia, J.B., 2020. Peptides in Colorectal Cancer: Current State of Knowledge.

Plant Foods Hum. Nutr. 75, 467–476. https://doi.org/10.1007/s11130-020-00856-6.
Bamdad, F., Bark, S., Kwon, C.H., Suh, J.-W., Sunwoo, H., 2017. Anti-inflammatory and antioxidant properties of peptides released from β-lactoglobulin by high hydrostatic pressure-assisted enzymatic hydrolysis. Molecules 22, 949. https://doi.org/10.3390/molecules22060949.
Bamdad, F., Chen, L., 2013. Antioxidant capacities of fractionated barley hordein hydrolysates in relation to peptide structures. Mol. Nutr. Food Res. 57, 493–503. https://doi.org/10.1002/mnfr.201200252.
Bamdad, F., Wu, J., Chen, L., 2011. Effects of enzymatic hydrolysis on molecular structure and antioxidant activity of barley hordein. J. Cereal Sci. 54, 20–28. https://doi.org/10.1016/j.jcs.2011.01.006.
Bartíková, M., Holková, L., Šafránková, I., Němec, P., 2020. Causal agents of powdery mildew on *Moringa stenopetala* (Baker f.) Luf. and *Moringa oleifera* Lam. in Ethiopia. S. Afr. J. Bot. 129, 457–462. https://doi.org/10.1016/j.sajb.2019.12.002.
El, S.N., Karakaya, S., Simsek, S., Dupont, D., Menfaatli, E., Eker, A.T., 2015. In vitro digestibility of goat milk and kefir with a new standardised static digestion method (INFOGEST cost action) and bioactivities of the resultant peptides. Food Funct. 6, 2322–2330. https://doi.org/10.1039/C5FO00357A.
Esfandi, R., Walters, M.E., Tsopmo, A., 2019. Antioxidant properties and potential mechanisms of hydrolyzed proteins and peptides from cereals. Heliyon 5, e01538. https://doi.org/10.1016/j.heliyon.2019.e01538.
Evum, U.P.C.V.V., Sansthan, G.A., 2014. Nutritional Potentials of *Moringa oleifera* Leaves in Uttar Pradesh, India. Res. J. Med. Plant 8, 288–289. https://doi.org/10.3923/rjmp.2014.283.289.
Firenzuoli, F, Gori, L., 2007. Herbal medicine today: clinical and research issues. Evid.-Based Complement. Alternat. Med. 4, 37–40. https://doi.org/10.1093/ecam/nem096.
Görgüç, A., Gençdağ, E., Yılmaz, F.M., 2020. Bioactive peptides derived from plant origin by-products: Biological activities and techno-functional utilizations in food developments–A review. Food Res. Int. 136, 109504. https://doi.org/10.1016/j.foodres.2020.109504.
Granella, S.J., Bechlin, T.R., Christ, D., Coelho, S.R.M., de Oliveira Paz, C.H., 2021. An approach to recent applications of *Moringa oleifera* in the agricultural and biofuel industries. S. Afr. J. Bot. 137, 110–116. https://doi.org/10.1016/j.sajb.2020.10.006.
Gupta, S., Kapoor, P., Chaudhary, K., Gautam, A., Kumar, R., Raghava, G.P., Consortium, O.S.D.D., 2013. In silico approach for predicting toxicity of peptides and proteins. PLoS One 8, e73957. https://doi.org/10.1371/journal.pone.0073957.
Hirayama, D., Iida, T., Nakase, H., 2018. The phagocytic function of macrophage-enforcing innate immunity and tissue homeostasis. Int. J. Mol. Sci. 19, 92. https://doi.org/10.3390/ijms19010092.
Hu, R., Chen, G., Li, Y., 2020. Production and Characterization of Antioxidative Hydrolysates and Peptides from Corn Gluten Meal Using Papain, Ficin, and Bromelain. Molecules 25, 4091. https://doi.org/10.3390/molecules25184091.
Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J.A., Prior, R.L., 2002. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J. Agric. Food Chem. 50, 4437–4444. https://doi.org/10.1021/jf0201529.
Karadag, A., Ozcelik, B., Saner, S., 2009. Review of methods to determine antioxidant capacities. Food Anal. Methods 2, 41–60. https://doi.org/10.1007/s12161-008-9067-7.
Kim, S., Ki, K., Khan, M., Lee, W., Lee, H., Ahn, B., Kim, H., 2007. Peptic and tryptic hydrolysis of native and heated whey protein to reduce its antigenicity. J. Dairy Sci. 90, 4043–4050. https://doi.org/10.3168/jds.2007-0169.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685. https://doi.org/10.1038/227680a0.
Leone, A., Bertoli, S., Di Lello, S., Bassoli, A., Ravasenghi, S., Borgonovo, G., Battezzati, A., 2018. Effect of *Moringa oleifera* leaf powder on postprandial blood glucose response: In vivo study on Saharawi people living in refugee camps. Nutrients 1, 1494. https://doi.org/10.3390/nu10101494.
Leone, A., Spada, A., Battezzati, A., Schiraldi, A., Aristil, J., Bertoli, S., 2016. *Moringa oleifera* seeds and oil: Characteristics and uses for human health. Int. J. Mol. Sci. 17, 2141. https://doi.org/10.3390/ijms17122141.
Li, X.R., Chi, C.-F., Li, L., Wang, B., 2017. Purification and identification of antioxidant peptides from protein hydrolysate of scalloped hammerhead (*Sphyrna lewini*) cartilage. Marine Drugs 15, 61. https://doi.org/10.3390/md15030061.
Lin, L., Zhu, Q., Zhao, M., Zhao, K., Tian, Y., Yang, Y., 2019. Purification of peptide fraction with antioxidant activity from *Moringa oleifera* leaf hydrolysate and protective effect of its in vitro gastrointestinal digest on oxidatively damaged erythrocytes. Int. J. Food Sci. Technol. 54, 84–91. https://doi.org/10.1111/ijfs.13907.
Logarušić, M., Slivac, I., Radošević, K., Bagović, M., Redovniković, I.R., Srček, V.G., 2019. Hempseed protein hydrolysates' effects on the proliferation and induced oxidative stress in normal and cancer cell lines. Mol. Biol. Rep. 4, 6079–6085. https://doi.org/10.1007/s11033-019-05043-8.
Ma, Z., Cooper, C., Kim, H. J., Janick-Buckner, D., 2009. A study of Rubisco through western blotting and tissue printing techniques. CBE—Life Sciences Education, 8, 140-146. https://doi.org/10.1187/cbe.09-01-0003
Matic, I., Guidi, A., Kenzo, M., Mattei, M., Galgani, A., 2018. Investigation of medicinal plants traditionally used as dietary supplements: A review on *Moringa oleifera*. J. Public Health Africa 9, 841. http://doi.org/10.4081/jphia.2018.841.
Meshginfar, N., Mahoonak, A.S., Hosseinian, F., Ghorbani, M., Tsopmo, A., 2018. Production of antioxidant peptide fractions from a by-product of tomato processing: mass spectrometry identification of peptides and stability to gastrointestinal digestion. J. Food Sci. Technol. 55, 3498–3507. https://doi.org/10.1007/s13197-018-3274-z.
Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Dupont, D., 2014. A standardised static in vitro digestion method suitable for food–an international consensus. Food Funct. 5, 1113–1124. https://doi.org/10.1039/C3FO60702J.
Neuhoff, V., Stamm, R., Eibl, H., 1985. Clear background and highly sensitive protein staining with Coomassie Blue dyes in polyacrylamide gels: a systematic analysis. Electrophoresis 6, 427–448. https://doi.org/10.1002/elps.1150060905.
Nouman, W., Bashir, T., Gul, R., Gull, T., Olson, M.E., Shaheen, M., Rosa, E., Domínguez-Perles, R., Soliman, W.S., 2020. Metalliferous conditions induce regulation in antioxidant activities, polyphenolics and nutritional quality of *Moringa oleifera* L. Int. J. Phytoremediation 22, 1348–1361. https://doi.org/10.1080/15226514.2020.1775547.
Paula, P.C., Sousa, D.O., Oliveira, J.T., Carvalho, A.F., Alves, B.G., Pereira, M.L., Vasconcelos, I.M., 2017. A protein isolate from *Moringa oleifera* leaves has hypoglycemic and antioxidant effects in alloxan-induced diabetic mice. Molecules 22, 271. https://doi.org/10.3390/molecules22020271.
Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Bitto, A., 2017. Oxidative stress: harms and benefits for human health. Oxidat. Med. Cell. Longevity 2017, 8416763. https://doi.org/10.1155/2017/8416763.
Saenjum, C., Chaiyasut, C., Chansakaow, S., Suttajit, M., Sirithunyalug, B., 2012. Antioxidant and anti-inflammatory activities of gamma-oryzanol rich extracts from Thai purple rice bran. J. Med. Plants Res. 6, 1070–1077. https://doi.org/10.5897/JMPR11.1247.
Saisavoey, T., Sangtanoo, P., Reamtong, O., Karnchanatat, A., 2019. Free radical scavenging and anti-inflammatory potential of a protein hydrolysate derived from salmon bones on RAW 264.7 macrophage cells. J. Sci. Food Agric. 99, 5112–5121. https://doi.org/10.1002/jsfa.9755.
Schlegel, K., Sontheimer, K., Hickisch, A., Wani, A.A., Eisner, P., Schweiggert-Weisz, U., 2019. Enzymatic hydrolysis of lupin protein isolates—Changes in the molecular weight distribution, technofunctional characteristics, and sensory attributes. Food Sci. Nutr. 7, 2747–2759. https://doi.org/10.1002/fsn3.1139.
Sharma, J.N., Al-Omran, A., Parvathy, S.S., 2007. Role of nitric oxide in inflammatory diseases. Inflammopharmacology 15, 252–259. https://doi.org/10.1007/s10787-007-0013-x.
Shu, G., Huang, J., Bao, C., Meng, J., Chen, H., Cao, J., 2018. Effect of different proteases on the degree of hydrolysis and angiotensin I-converting enzyme-inhibitory activity in goat and cow milk. Biomolecules 8, 101. https://doi.org/10.3390/biom8040101.
Silva, A.C.S., Silveira, J.N., 2013. Correlation between the degree of hydrolysis and the peptide profile of whey protein concentrate hydrolysates: effect of the enzyme type and reaction time. Am. J. Food Technol. 8, 1–16. https://doi.org/10.3923/ajft.2013.1.16.
Silvestre, M. P. C., Morais, H. A., Silva, V. D. M., Silva, M. R., 2013. Degree of hydrolysis and peptide profile of whey proteins using pancreatin. Revista da Sociedade Brasileira de Alimentação e Nutrição, 38, 278-290. http://dx.doi.org/10.4322/nutrire.2013.026
Su, B., Chen, X., 2020. Current status and potential of *Moringa oleifera* leaf as an alternative protein source for animal feeds. Front. Veterinary Sci. 7. http://doi.org/10.3389/fvets.2020.00053.
Sultana, S., 2020. Nutritional and functional properties of *Moringa oleifera*. Metabolism Open 8, 100061. https://doi.org/10.1016/j.metop.2020.100061.
Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., Byrne, D.H., 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. J. Food Compos. Anal. 19, 669–675. https://doi.org/10.1016/j.jfca.2006.01.003.
Tshabalala, T., Ncube, B., Madala, N.E., Nyakudya, T.T., Moyo, H.P., Sibanda, M., Ndhlala, A.R., 2019. Scribbling the Cat: A Case of the "Miracle" Plant, *Moringa oleifera*. Plants 8, 510. https://doi.org/10.3390/plants8110510.
Tsopmo, A., Cooper, A., Jodayree, S., 2010. Enzymatic hydrolysis of oat flour protein isolates to enhance antioxidative properties. Adv. J. Food Sci. Technol. 2, 206–212.
Vidal, A.R., Cechin, C.d.F., Cansian, R.L., Mello, R.d.O., Schmidt, M.M., Demiate, I.M., Dornelles, R.C.P., 2018. Enzymatic hydrolysis (pepsin) assisted by ultrasound in the functional properties of hydrolyzates from different collagens. Ciência Rural 48, 1–9. https://doi.org/10.1590/0103-8478cr20170649.
Víteček, J., Lojek, A., Valacchi, G., Kubala, L., 2012. Arginine-based inhibitors of nitric oxide synthase: therapeutic potential and challenges. Mediators Inflamm., 318087. https://doi.org/10.1155/2012/318087.
Xu, S., Shen, Y., Li, Y., 2019. Antioxidant activities of sorghum kafirin alcalase hydrolysates and membrane/gel filtrated fractions. Antioxidants 8, 131. https://doi.org/10.3390/antiox8050131.
Yang, G., Lee, K., Lee, M., Ham, I., Choi, H.Y., 2012. Inhibition of lipopolysaccharide-induced nitric oxide and prostaglandin E 2 production by chloroform fraction of *Cudrania tricuspidata* in RAW 264.7 macrophages. BMC Complement. Alternat. Med. 12, 1–7. https://doi.org/10.1186/1472-6882-12-250.
Yun, Y.R., Oh, S.J., Lee, M.J., Choi, Y.J., Park, S.J., Lee, M.A., Park, S.H., 2020. Antioxidant activity and calcium bioaccessibility of *Moringa oleifera* leaf hydrolysate as a potential calcium supplement in food. Food Sci. Biotechnol. 29, 1563–1571. https://doi.org/10.1007/s10068-020-00820-9.
Zheng, L., Su, G., Ren, J., Gu, L., You, L., Zhao, M., 2012. Isolation and characterization of an oxygen radical absorbance activity peptide from defatted peanut meal hydrolysate and its antioxidant properties. J. Agric. Food Chem. 60, 5431–5437. https://doi.org/10.1021/jf3017173.
Zou, T.B., He, T.P., Li, H.B., Tang, H.W., Xia, E.Q., 2016. The structure-activity relationship of the antioxidant peptides from natural proteins. Molecules 21, 72. https://doi.org/10.3390/molecules21010072.