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Evaluation of Hypotensive and Antihypertensive Effects of Velvet Bean (*Mucuna pruriens* L.) Hydrolysates

Luis Chel-Guerrero,[1] Saulo Galicia-Martínez,[1] Juan José Acevedo-Fernández,[2] Jesus Santaolalla-Tapia,[2] and David Betancur-Ancona[1]

[1]*Facultad de Ingeniería Química, Universidad Autónoma de Yucatán, Mérida, México.*
[2]*Facultad de Medicina, Universidad Autónoma del Estado de Morelos, Cuernavaca, México.*

ABSTRACT Hypertension could cause significant worldwide health problems that affect 15–20% of all adults; according to National Health and Nutrition Examination Survey, about 29% of the adult population in the United States are hypertensive. Recent research has shown that peptides derived from the hydrolysis of food proteins can decrease blood pressure. This study was carried out to evaluate the hypotensive and antihypertensive potential of *Mucuna pruriens* protein hydrolysates in *in vitro* and *in vivo* models. *M. pruriens* protein concentrate was prepared by wet fractionation and enzymatically hydrolyzed using Alcalase®, Flavourzyme®, and the sequential system Alcalase-Flavourzyme at different times (5–120 min). The biological potential was measured *in vitro* based on the IC_{50} value as well as *in vivo* effect, measuring the systolic (SBP) and diastolic (DBP) blood pressure in normotensive and antihypertensive Wistar-Kyoto rats by the tail-cuff method. Hydrolysis of *M. pruriens* protein concentrates with commercial enzymes generated extensive hydrolysates with angiotensin-converting enzyme (ACE-I) inhibitory activity (IC_{50}: 0.589–0.993 mg/mL) and hypotensive (SBP: 0.6–47.43%, DBP: 1.94–43.47%) and antihypertensive (SBP: 8.84–27.29% DBP: 16.1–29.37%) effect. These results indicate that *Mucuna pruriens* protein hydrolysate (MPPH) could be used as a functional ingredient to prevent blood pressure increase.

KEYWORDS: ***ACE-I*** • ***antihypertensive*** • ***hypotensive*** • **Mucuna pruriens** • ***protein hydrolysates***

INTRODUCTION

HYPERTENSION IS A common health problem in all Western countries. It is a major risk factor for cardiovascular diseases (CVDs), including coronary heart disease, peripheral artery disease, and stroke. Untreated hypertension could cause significant worldwide health problems that affect 15–20% of all adults and carrying significant costs to society.[1] According to the recent National Health and Nutrition Examination Survey,[2] about 29% of the adult population in the United States are hypertensive (>140/90 mmHg) or use antihypertensive drugs. In Mexico, the National Survey of Health and Nutrition found a prevalence of 31.5%.[3] There is a clear and dependent relationship between blood pressure and CVD events.

In the Framingham Heart Study, the risk ratio for coronary heart disease, stroke, peripheral artery disease, and cardiac failure was about 2–3 times higher for hypertensive subjects than for normotensive subjects.[4] Hypertension was also associated with about a four-fold risk of atherothrombotic brain infarction in comparison with normotension.[5] Clinical trials have shown that, in hypertensive subjects, lowering the blood pressure reduces the risk of CVDs.[6] Decreases in diastolic blood pressure (DBP) of 5, 7.5, and 10 mmHg have been associated with at least 34%, 46%, and 56% reductions, respectively, in the incidence of stroke, and with at least 21%, 29%, and 37% lower rates of coronary heart disease.[7] On the other hand, a 20 mmHg increase in systolic blood pressure (SBP) or a 10 mmHg increase in DBP doubles the risk of CVD in the blood pressure range of 115/75 to 185/115 mmHg.[8]

The genus *Mucuna*, belonging to the Fabaceae family, covers perhaps 100–150 species of annual and perennial legumes. This legume is native to Malaysia, South China, China, and India, but nowadays is widely distributed in many tropical regions. Cultivated and wild varieties from America and Africa were originally introduced and propagated by humans along various commercial routes.[9] The main differences among cultivated species are in the character of the pubescence on the pod, the seed color, and number of days to harvest the pod. So far, improved cultivars have only been produced in (a) Australia (White, Mauritius, Black Mauritius, Somerset, Marbilee, and Smith and Jubilack), (b) USA (Georgia, Alabama, Osceola, Yokohama, and Florida), and (c) Zimbabwe (Bengal, White Stigless, SES 30, SES 45, SES 68, SES 74, and SES 108).

Throughout its distribution range, the species has also been given various scientific names, among them *M. deeringianum* Bort, *M. utilis* (Wall) Baker ex Burck, *M. pruriens* (L.) DC,

Address correspondence to: David Betancur-Ancona, PhD, Facultad de Ingeniería Química, Universidad Autónoma de Yucatán, Periférico Nte. Km. 33.5, Tablaje Catastral 13615, Col. Chuburná de Hidalgo Inn, Mérida, Yucatán C.P. 97203, México, E-mail: bancona@correo.uady.mx

M. cochinchinensis (Lour) Burk, *M. nivea* (Roxb.) Kuntze, *M. capitata*, *M. aterrima* Piper & Tracy, *M. hassjo*, *M. diabolica*, *M. cinerum*, *M. haltonii*, and *M. sloanei* are some of which are just synonyms, but others may represent valid names referring to different taxa.[9] The dry weight composition of *Mucuna* seeds is as follows: 10.0% moisture; 19.0–37.5% protein; 4.7–9.0% fat; 51.5% nitrogen-free extract (NFE); 81.7% total digestible nutrients; 5.3–11.5% raw fiber, and 2.9–5.7% ash. Among the amino acids found in seeds, the aspartic and glutamic acids are found to be predominant (8.9–19 and 8.6–14.4%, respectively), whereas the levels of other amino acids are found to be low.[10]

Alkaline extractions are a technological alternative for protein isolation from *M. pruriens*. Recent research has focused on the properties of food protein-derived peptides, their biological activities, and potential health benefits. Peptides extracted from partial enzymatic protein hydrolysates of food proteins as *M. pruriens* can provide specific health benefits such as antihypertensive or antioxidant activities. For the above mentioned, the objective of this study was to evaluate the *in vitro* ACE inhibitory activity and hypotensive and antihypertensive effect of *M. pruriens* protein hydrolysates in experimental animal models.

MATERIALS AND METHODS

Materials

Mucuna pruriens seeds were obtained from the 2007 harvest in the state of Yucatán, México. Reagents were of analytical grade and purchased from J.T. Baker (Phillipsburg, NJ, USA), Sigma Chemical Co. (St. Louis, MO, USA), Merck (Darmstadt, Germany), and Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Alcalase® 2.4 L FG and Flavourzyme® 500 mg enzymes were purchased from Novo Laboratories (Copenhagen, Denmark).

M. pruriens *flour*

Impurities and damaged seeds were removed. Sound seeds were milled in a Mykros impact mill until passing through a 20-mesh screen (0.85 mm), and then in a Cyclotec 1093 (Tecator, Höganäs, Skåne län, Sweden) mill until passing through a 60-mesh screen (0.24 mm).

Nitrogen solubility of M. pruriens *flour*

Nitrogen solubility was determined by the method of Chel-Guerrero *et al.*[11] *M. pruriens* flour (125 mg) was dispersed in 25 mL deionized water and adjusted to pH 3.5, 4.0, 4.4, 4.5, 4.6, and 5.0 with either 0.01 or 1.0 N NaOH or HCl. The dispersions were shaken for 30 min at ambient temperature and centrifuged at 43,503 *g* for 30 min. Nitrogen content in the supernatant was determined by the Bradford method,[12] and the percent soluble protein was calculated as follows:

$$\text{Solubility } (\%) = \frac{\text{Amount of nitrogen in the supernatant}}{\text{Amount of nitrogen in the sample}} \times 100$$

Protein concentrate

A single protein extraction was done with the legume flour. This was processed using the wet fractionation method of Herrera-Chalé *et al.*[13] Briefly, whole flour was suspended in distilled water at a 1:6 (w/v) ratio, pH adjusted to 11 with 1 mol/L NaOH, and the dispersion stirred for 1 h at 400 rpm with a mechanical agitator (Caframo RZ-1, Heidolph Schwabach, Germany). This suspension was wet-milled with a KitchenAid® mill and the fiber solids separated from the starch and protein mix by straining through 80- and 150-mesh sieves, followed by five washings of the residue with distilled water.

The protein–starch suspension was allowed to sediment for 30 min at room temperature to recover the starch and protein fractions. The pH of the separated solubilized protein was adjusted to its isoelectric point (4.6) with 1 mol/L HCl. The suspension was then centrifuged at 1317 *g* for 12 min (Mistral 3000i; Curtin Matheson Scientific, Inc., Morris Plains, NJ, USA), the supernatants discarded, and the precipitates freeze-dried at –47°C and 13×10^{-3} mbar, then stored in polyethylene bags at room temperature until its use.

Proximate composition of M. pruriens *flour and protein concentrate*

Standard AOAC[14] procedures were used to determine nitrogen (method 954.01), fat (method 920.39), ash (method 925.09), crude fiber (method 962.09), and moisture (method 925.09) contents in the *M. pruriens* flour and protein concentrate.[14] Nitrogen content (N) was quantified with the Kjeldahl method using Kjeltec Digestion System (Tecator). Protein content was calculated as $N \times 6.25$. Fat content was obtained from a 1 h hexane extraction. Ash content was calculated from sample weight after burning at 550°C for 2 h. Moisture content was measured based on sample weight loss after oven-drying at 110°C for 2 h. Carbohydrate content was estimated as NFE by difference from the sum of the protein, fat, ash, and crude fiber content. The protein content of the *M. pruriens* hydrolysates was determined by the Lowry *et al.* method.[15]

Enzymatic hydrolysis

Protein extract hydrolysis was performed using a totally randomized design. Treatments were the enzymatic systems: Alcalase 2.4 L FG, Flavourzyme 500 mg (Novo Laboratories), and Alcalase-Flavourzyme sequential system. The response variable was the degree of hydrolysis (DH). The *M. pruriens* protein concentrate (5 g/100 mL) was hydrolyzed for 5, 15, 30, 45, 60, 90, and 120 min with Alcalase, Flavourzyme, and the Alcalase-Flavourzyme sequential system. In this case, half the hydrolysis time was used with each enzymatic system.

Hydrolysis was done under controlled conditions (temperature, pH, and stirring) in a reaction vessel equipped with a stirrer, thermometer, and pH electrode. Hydrolysis temperature was 50°C in all cases and other parameters with Alcalase 2.4 L FG and Flavourzyme 500 mg were as follows: enzyme/substrate ratio, 0.3 AU/g for Alcalase and 50 leucine aminopeptidase units per gram for Flavourzyme;

and pH 8 for Alcalase and pH 7 for Flavourzyme.[16] In all treatments, the reaction was stopped by heating to 80°C for 20 min, followed by centrifuging at 9880 *g* for 20 min to remove the insoluble portion.

Degree of hydrolysis

DH was calculated by determining free amino groups with *o*-phthaldialdehyde following the methodology described by Nielsen *et al.*[17]: $DH = h/h_{tot} \times 100$; where h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds. The h_{tot} factor is dependent on the raw material amino acid composition and was determined by reverse-phase high-performance liquid chromatography (HPLC).[18] Samples (2–4 mg protein) were treated with 4 mL of HCl (6 mol/L), placed in hydrolysis tubes, and gassed with nitrogen at 110°C for 24 h. Then, they were dried in a rotary evaporator and suspended in 1 mol/L sodium borate buffer at pH 9.0.

Amino acid derivatization was performed at 50°C using diethyl ethoxymethylenemalonate. Amino acids were separated using HPLC with a reversed-phase column (300 × 3.9 mm, Nova Pack C18, 4 mm; Waters, Massachusetts, USA) and a binary gradient system with 25 mmol/L sodium acetate containing (A) 0.02 g/L sodium azide at pH 6.0, and (B) acetonitrile as solvent. Flow-rate was 0.9 mL/min, and elution gradient was: time 0.0–3.0 min, linear gradient A:B (91:9) to A–B (86:14); time 3.0–13.0 min, elution with A–B (86–14); time 13.0–30.0 min, linear gradient A–B (86:14) to A–B (69:31); time 30.0–35.0 min, elution with A–B (69:31).

In vitro *ACE inhibitory activity*

The ACE inhibiting activity of protein hydrolysate was analyzed according to Hayakari *et al.*[19] ACE hydrolyzes hippuryl-L-histidyl-L-leucine (HHL) to yield hippuric acid and histidyl-leucine. This method relies on the colorimetric reaction of hippuric acid with 2,4,6-trichloro-*s*-triazine (TT) in a 0.5 mL incubation mixture containing 40 μmol potassium phosphate buffer (pH 8.3), 300 μmol sodium chloride, 40 μmol 3% HHL in potassium phosphate buffer (pH 8.3), and 100 μ/mL ACE. The mixture was incubated at 37°C/45 min and the reaction terminated by the addition of TT (3% v/v) in dioxane and 3 mL 0.2 mol/L potassium phosphate buffer (pH 8.3). After centrifuging the reaction mixture at 10,000 *g* for 10 min, the enzymatic activity in the supernatant was determined by measuring absorbance at 382 nm. All runs were done in triplicate.

The ACE inhibiting activity was quantified by a regression analysis of ACE-inhibiting activity (%) versus peptide concentration, and the IC_{50} value (*i.e.*, the peptide concentration in μg protein/mL required to produce 50% ACE inhibition under the described conditions) defined and calculated as follows:

$$\text{ACE-inhibiting activity } (\%) = (A - B)/(A - C) \times 100$$

where *A* represents absorbance in the presence of ACE and sample, *B* is absorbance of the control, and *C* is absorbance of the reaction blank.

$$IC_{50} = (50 - b)/m$$

where *b* is the intersection and *m* is the slope.

Hypotensive effect of M. pruriens *protein hydrolysates in animal models*

For this experiment, normotensive Wistar-Kyoto rats (8–10 weeks old; 240–320 g initial weight) were housed in individual cages under controlled conditions (12-h light:12-h dark period; 25 ± 1°C; 65 ± 5% relative humidity).[20] All animal experimentations have been conducted in accordance with the guidelines for care and use of experimental animals of the European Communities Council Directive 86/609/EEC.[21] Free access was provided to feed and water. After one week of adaptation period, the rats were randomly divided into groups (five rats per group): each group with three concentrations (5, 10, and 15 mg/kg body weight, diluted in 0.3 mL physiological saline solution [PSS]) of selected hydrolyzed with the best *in vitro* activity, and additionally two groups as positive and negative control. (Captopril® and PSS were used as positive and negative control, respectively).

Before the determination of blood pressure, animals were anesthetized (20 min) with a single dose (30 mg/kg) of sodium barbital by intraperitoneal (IP) injection. The measurements of SBP and DBP were recorded for at least 160 min using an electric sphygmomanometer on the tail. The original method for measuring arterial blood pressure using the tail cuff provides SBP and DBP values detected by a physiographer (CPM Physiograph Narco Bio-System, Inc., Houston, TX, USA). The blood pressure signals were acquired (at 1.0 kHz) on a computer using the analog-digital converter Mini Digi B (Axon Instruments, California, USA) and software AxoScope 10.2. To analyze the blood pressure reading, a 10.2 Clampfit (Axon Instruments) was used.

Blood pressure was recorded for at least 160 min for each rat. The first 20 minutes of this time period were used to measure basal blood pressure and then administered via IP evaluate treatments: negative control (PSS), positive control (Captopril) and hydrolysates. The same person took all measurements to minimize stress-induced variations in blood pressure, in the same peaceful environment. Moreover, to guarantee the reliability of measurements, we established a training period of 2 weeks before the actual trial time, and during this period, the rats were accustomed to the procedure. Determinations were made in triplicate. The percentage of hypotensive effect was calculated from the following formula:

$$\text{Hypotensive effect}\% = 100 \times (\text{PA Exp})/(\text{PA Ctrl})$$

where

PA Exp = SBP and DBP observed after administration of PSS, captopril, and hydrolysates,

PA Ctrl = basal blood pressure observed during the first 20-min registration.

Antihypertensive effect of M. pruriens *protein hydrolysates in animal models*

Adult male Wistar rats (8–10 weeks old) weighing 240–320 g were used in this study. The animals were housed at 25 ± 1°C on a 12-h light:12-h dark cycle and had free access to standard rat chow and drinking water. Fifty rats were used for all treatments and these were divided into seven experimental groups (five rats per group). Hypertension was induced by oral administration of the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 25 mg/kg/day), dissolved in drinking water, for 6 weeks.[22] Moreover, two groups of five animals received normal tap water throughout the experiment (control normotensive groups). The chronic NOS inhibition was evaluated by acute administration of L-NAME (5 and 10 mg/kg) in normotensive and hypertensive rats. The SBP of hypertensive rats was >140 mmHg.

Two hydrolysate concentrations with higher hypotensive effect were evaluated: 5 and 10 mg/kg body weight, diluted in 0.3 mL PSS. Captopril and PSS were used as positive and negative control. Before the determination of blood pressure, animals were anesthetized with a single dose (30 mg/kg) of sodium barbital by IP injection. Measurements of SBP and DBP blood pressure were recorded for at least 160 min using an electric sphygmomanometer on the tail as described before. Also, during treatment, the bodyweight of rats was monitored for 2 weeks, and each week, the chemistry of urine was evaluated to assess their renal function using qualitative analysis with test strips from Axmilab® brand, Uricheck10.

Statistical analysis

A *t*-student test was carried out for comparison of proximate analysis of flour and concentrate, and a one-way analysis of variance was run to evaluate protein extract hydrolysis data, *in vitro* ACE inhibiting activity as well as *in vivo* hypotensive and antihypertensive effect. A Duncan multiple range test was applied to determine differences between treatments. All analyses were done according to Montgomery[23] with the Statgraphics Plus version 5.1 software.

RESULTS AND DISCUSSION

Nitrogen solubility of M. pruriens *flour*

Solubility characteristics of protein are among the most important functional properties since many functional performances of proteins depend upon their capacity to go into solution initially. For that reason, the solubility profile of *M. pruriens* flour was determined (Fig. 1). The nitrogen solubility was pH dependent ($P < .05$) and ranged from 5.5% to 9.0%. The lowest solubility registered in this study was at pH 4.6, which was statistically different to others pH assayed. This result was similar to that reported by Adebowale *et al.*[24] for *M. pruriens* (4–5%) as well as by Chel-Guerrero *et al.*[11] for *Phaseolus lunatus* and *Canavalia ensiformis* (4.5%). The lowest nitrogen solubility value (4.6) was used as reference to separate the solubilized protein during protein concentrate extraction.

Proximate composition of M. pruriens *flour and protein concentrate*

Proximate assay is an important criterion to assess the overall composition and nutritional status of any ingredient intended for food use. In this context, *M. pruriens* flour and protein concentrate were analyzed for different quality attributes such as moisture, crude protein, crude fat, crude fiber, ash, and NFE. Proximate composition showed significant variations among samples. The *M. pruriens* flour and protein concentrate had moisture of 9.29% and 3.18%; ash of 3.56% and 7.12%; crude fiber of 3.71% and 3.45%; fat of 6.85% and 13.11%; protein of 31.28% and 55.12%, and NFE of 45.31% and 18.01%, respectively (Table 1). These findings regarding proximate composition are in conformity with values described in previous literature.

The chemical composition of *M. pruriens* flour and protein concentrate was analyzed by Segura-Campos *et al.*[25] and observed that moisture, ash, fiber, fat, protein, and NFE were 7.61–3.26%, 1.13–0.09%, 1.13–0.13%, 5.59–15.22%, 26.5–57.88%, and 58.05–23.42%, respectively. In array of investigations, variations in proximate composition of different legumes have been observed owing to different environments, genotype, and analytical methods. It was further reported that protein content is sensitive to rainfall, light

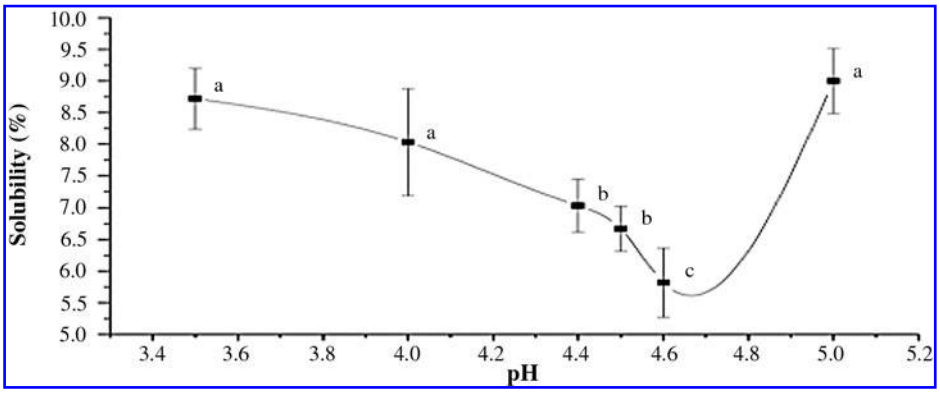

FIG. 1. Nitrogen solubility of *Mucuna pruriens* flour. Data are mean ± standard deviation ($n = 3$). [abc]Different letters indicate statistical difference ($P < .05$).

Table 1. Proximate Composition of the *Mucuna pruriens* Flour and Protein Concentrate

Component	*Flour*	*Protein concentrate*
Moisture	9.29 ± 0.2^{a}	3.18 ± 0.1^{b}
Protein	31.28 ± 0.48^{b}	55.12 ± 1.7^{a}
Fat	6.85 ± 0.15^{b}	13.11 ± 0.34^{a}
Fiber	3.71 ± 0.03^{a}	3.45 ± 0.04^{b}
Ash	3.56 ± 0.06^{b}	7.12 ± 0.15^{a}
NFE	45.31 ± 0.80^{a}	18.01 ± 2.42^{b}

[ab]Different superscript letters in the same row indicate statistical difference ($P < .05$).

NFE, nitrogen-free extract.

intensity, length of growing season, day duration, temperature, and agronomic practices.[26]

The proteins are polymers of amino acids and their relative proportion represents its quality that is dependent on the genetic makeup of legumes. According to Sadiq and Batool,[27] variations in protein contents of different protein isolates or concentrates could possibly by due to the extent of soluble proteins present in raw materials as well as the used extraction method. The protein content of *M. pruriens* protein concentrate (55.12%) makes from this legume a viable alternative to obtain biologically active peptides.

Enzymatic hydrolysis

Protein hydrolysates from *M. pruriens* were obtained by treatment with the endoprotease Alcalase, the endo/exoprotease Flavourzyme, and the sequential system Alcalase-Flavourzyme. Theses proteases are used in the food industry to improve the functional and nutritional properties of protein preparations. According to Pedroche *et al.*,[28] the controlled liberation of biologically active peptides from protein by enzymatic hydrolysis is one of the most promising trends concerning medical applications of protein hydrolysates with DH higher than 10%, while hydrolysates with a low DH (lower than 10%) are used for the improvement of functional properties of flours or protein isolates. Therefore, the results indicate that *M. pruriens* proteins are an appropriate substrate for producing these bioactive peptides when hydrolyzed enzymatically (Fig. 2).

The highest DH for the *M. pruriens* protein concentrate was produced with Flavourzyme at 120 min reaction time (39.39%), while the lowest DH (17.23%) was produced with Alcalase at 5 min reaction time. Variation in DH values among these results was probably the result of protease specificity. The highest DH produced using Flavourzyme to hydrolyze the *M. pruriens* protein concentrate resulted from it being a protease complex produced by *Aspergillus oryzae*, which contains endoproteinases and exoproteinases. This fungal protease complex has a broader specificity, which, when combined with its exopeptidase activity, produces DH values as high as 50% and dipeptides in the hydrolysate.[29]

The highest DH obtained in the *M. pruriens* protein concentrate by hydrolysis with Flavourzyme was lower than that reported by Megías *et al.*[16] for chickpea hydrolysates produced sequentially with Alcalase and Flavourzyme (65% DH) at 150 min. All these hydrolysates had higher DH than mung bean (*Vigna radiata*) protein hydrolysates produced with neutrase (12%) at 10 h, and higher than for soy protein hydrolysates produced with pepsin for 60 min (11% DH) and 180 min (17% DH).[30]

In vitro *ACE inhibitory activity*

The *M. pruriens* protein hydrolysates obtained with Alcalase (90 and 120 min), Flavourzyme (5 and 120 min), and the sequential system Alcalase-Flavourzyme (90 and 120 min) were selected to evaluate the ACE inhibitory activity. These hydrolysates registered IC_{50} values ranging from 0.589 to 0.993 mg protein/mL, similar to that reported for enzymatic hydrolysates from different protein sources ($IC_{50} = 0.2–2.467$ mg protein/mL) with antihypertensive activity in spontaneously hypertensive rats.[31]

After extensive hydrolysis with Alcalase (DH = 24.5% and 24.14%) for 90 and 120 min, an *M. pruriens* concentrate had a 0.809 and 0.589 mg/mL IC_{50}, while with Flavourzyme (DH = 27.31% and 39.39%) the IC_{50} was 0.789 and 0.630 mg/mL at 5 and 120 min. When *M. pruriens* was hydrolyzed for 90 and 120 min with Alcalase-Flavourzyme sequential system (DH = 30.54% and 33.14%), the resulting IC_{50} was 0.940 and 0.993 mg/mL (Fig. 3). This suggests that size of generated peptides is important, since these must fit into slit between ACE domains that contain their active sites. This restricts the access of large polypeptides into active sites. Also the sequence of these is important because the presence of hydrophobic amino acids for ACE inhibitory activity is required.[32] This may occur in the case of Alcalase

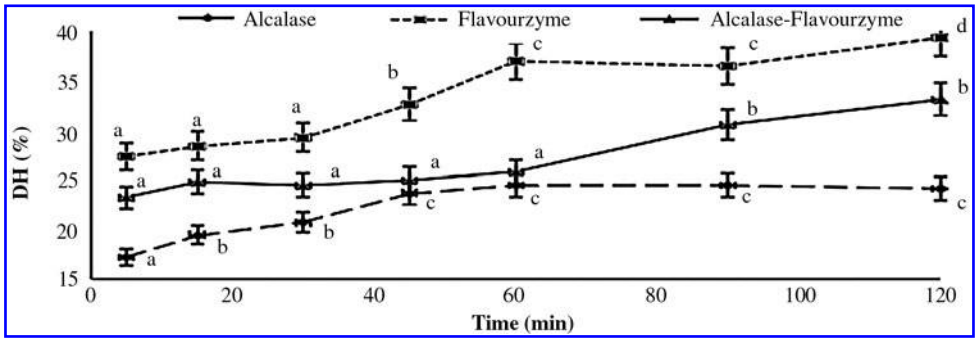

FIG. 2. Degree of hydrolysis of Velvet bean (*M. pruriens*) protein hydrolysates. [abcd]Different letters in the same system indicate statistical difference ($P < .05$).

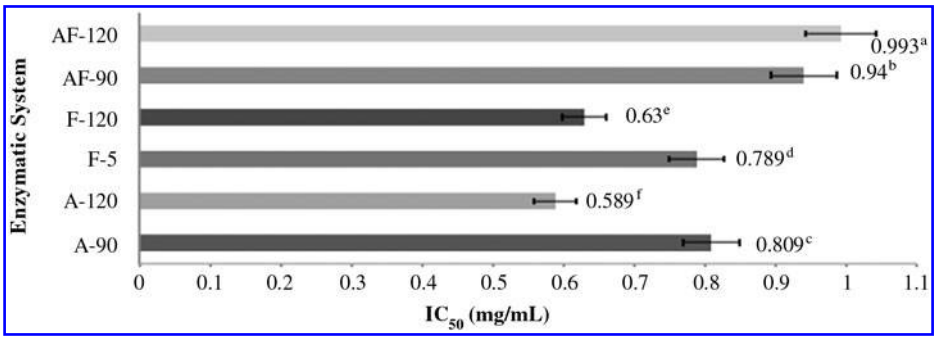

FIG. 3. Angiotensin-converting enzyme (ACE-I) inhibitory activity (IC_{50}) of *M. pruriens* protein hydrolysates. [abcdef]Different letters indicate statistical difference ($P < .05$).

that has demonstrated great ACE inhibitory activity according to Murray and Fitzgerald.[33]

Hypotensive effect of M. pruriens *hydrolysates in experimental animal models*

The *M. pruriens* protein hydrolysates obtained with Alcalase (90 and 120 min), Flavourzyme (5 and 120 min), and the sequential system Alcalase-Flavourzyme (90 and 120 min) were selected to evaluate the hypotensive effect in animal models. In this study, the hypotensive effect of the *M. pruriens* protein hydrolysates was evaluated using a trial where normotensive rats were administered a single dose of hydrolysates (5, 10, and 15 mg/kg) by IP injection and the tail cuff method was used to measure the blood pressure levels. The peptides in the six analyzed *M. pruriens* protein hydrolysates exhibited hypotensive effect in experimental animal model excepting Flavourzyme hydrolysate at 5 min administered at the concentration of 5 mg/kg (Fig. 4).

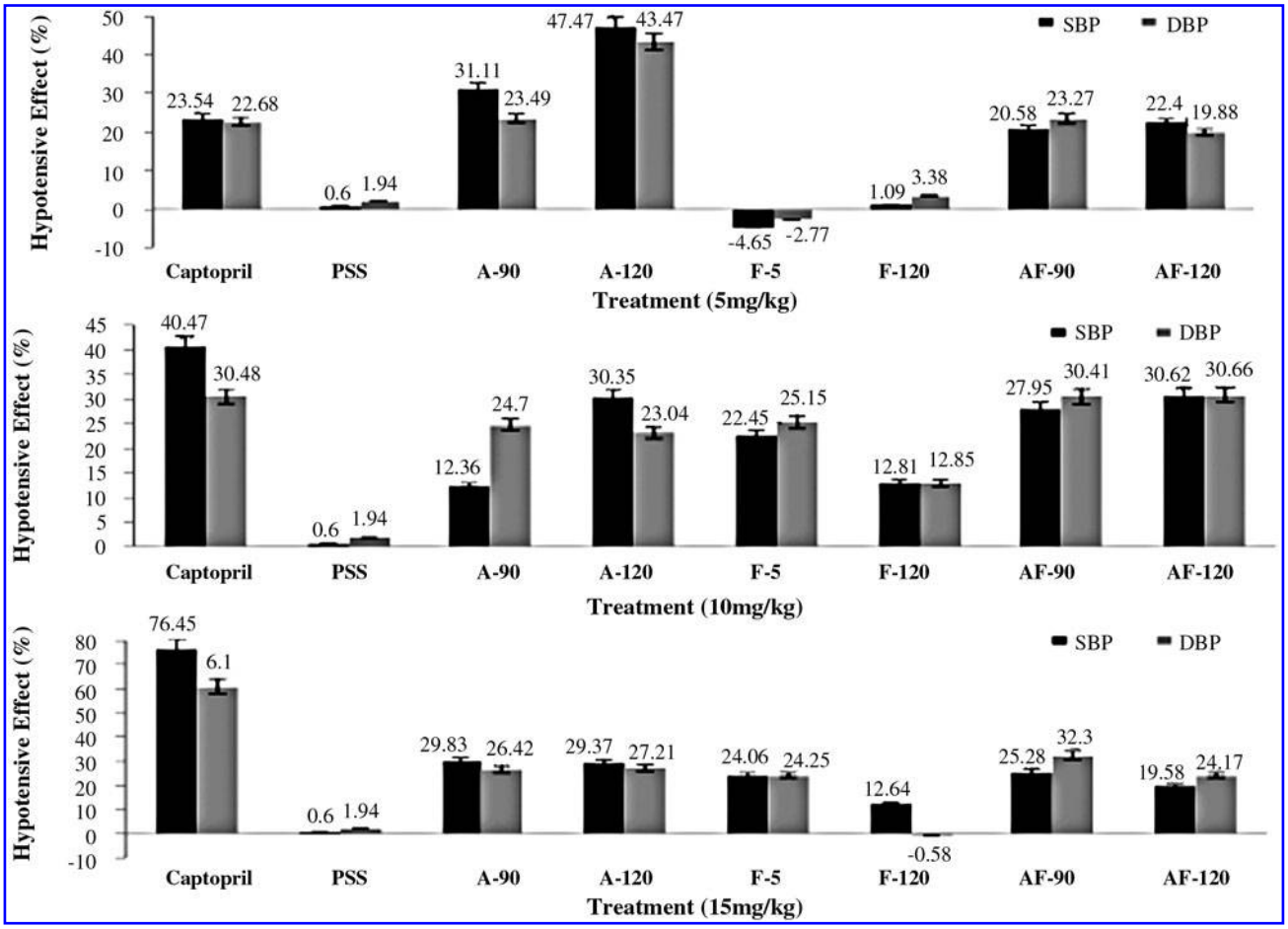

FIG. 4. SBP and DBP reduction of captopril, PSS, and the *M. pruriens* protein hydrolysates administered a single dose (5, 10, and 15 mg/kg) by intraperitoneal injection to normotensive rats. A-90, A-120: Alcalase® at 90 and 120 min; F-5, F-120: Flavourzyme® at 5 and 120 min; AF-90, AF-120: Alcalase-Flavourzyme at 90 and 120 min. DBP, diastolic blood pressure; SBP, systolic blood pressure; PSS, physiological saline solution.

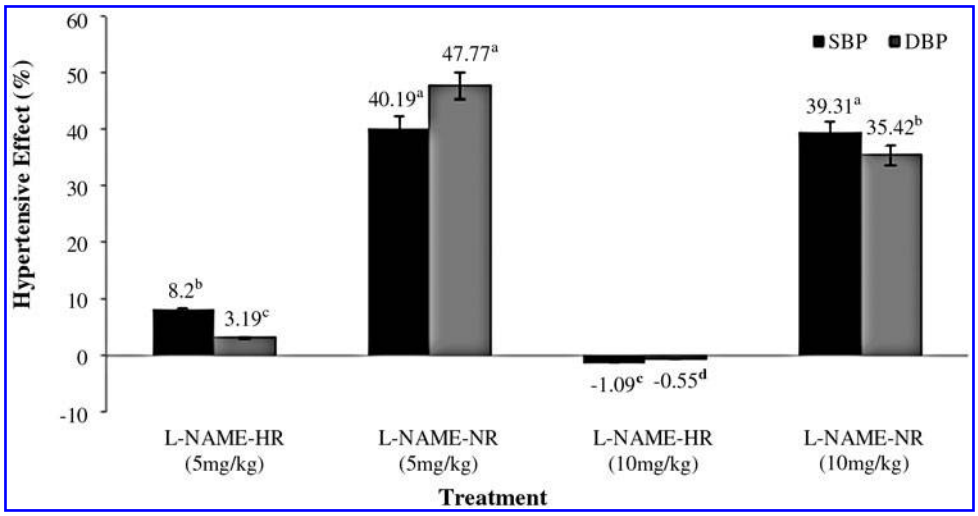

FIG. 5. Hypertensive effect by intraperitoneal administration of the NOS inhibitor L-NAME (25 mg/kg/day) to HR and NR. [abcd]Different letters in the same blood pressure indicate statistical difference ($P < .05$). NOS, nitric oxide synthase; L-NAME; N^{ω}-nitro-L-arginine methyl ester; HR, hypertensive rats; NR, normotensive rats.

The hydrolysate produced with the Alcalase system at 90 min produced reductions in SBP ranging from 12.36% to 31.11% after 160 min, and a maximum reduction of 26.42% in DBP. The hydrolysate produced with the Alcalase system at 120 min produced reductions ranging from 29.37% to 47.43% in SBP and 23.04% to 43.47% in DBP. When Flavourzyme system at 5 min was injected, a maximum reduction of 24.06% and 25.15% in SBP and DBP was observed.

The hydrolysate produced with the Flavourzyme system at 120 min produced reductions in SBP ranging from 1.09% to 12.81% and a maximum reduction of 12.85% in DBP. When Alcalase-Flavourzyme sequential system at 90 and 120 min was injected, a maximum reduction of 27.95% and 30.62% was produced in SBP and 32.30% and 30.66% in DBP. On the other hand, the results show that the negative control (PSS) registered a nonsignificant hypotensive effect (SBP = 0.6%, DBP = 1.191%), while the positive control (Captopril) was dosage dependent with SBP and DBP reductions ranging from 23.54% to 76.45% and 22.68% to 61%, respectively.

The hypotensive effect of all *M. pruriens* protein hydrolysates evaluated at 5, 10, and 15 mg/kg registered statistical equality ($P > .05$), suggesting that the hypotensive effect was in the stable phase of the curve. Therefore, future researches with these hydrolysates should be conducted to lower dosage.

Antihypertensive effect of M. pruriens *hydrolysates in experimental animal models*

The *M. pruriens* protein hydrolysates obtained with Alcalase (90 and 120 min), Flavourzyme (5 min), and the sequential system Alcalase-Flavourzyme (90 and 120 min) were selected to evaluate the antihypertensive effect in animal models. Hypertension was induced by oral administration of the nonselective NOS inhibitor L-NAME (25 mg/kg day) for 6 weeks. According to Doggrell and Brown,[31] nitric oxide (NO, endothelium-derived relaxing factor), a paracrine vasodilator, has been implicated in regulating vascular tone and myocardial contractility, and inhibiting platelet aggregation, and therefore may be critical in the development of hypertension and atherosclerosis. There are at least three forms of the NOS; the cytokine-inducible NOS2 cosegregates with an increased blood pressure in the Dahl salt-sensitive rat, but endothelial cell NOS3 seems uninvolved in human essential hypertension.

Hypertensive effect by IP administration of the NOS inhibitor L-NAME (25 mg/kg/day) to hypertensive and normotensive rats showed that NOS was pharmacologically blocked to not register significant increases in SBP and DBP in the hypertensive rats group in contrast with the normotensive rats

Table 2. Urine Parameters of Wistar-Kyoto Rats Treated and Not Treated (Control) with N^{ω}-Nitro-L-Arginine Methyl Ester

	Weeks				
Parameters	*1*	*2*	*3*	*4*	*5*
Control					
Leukocytes (leu/dL)	—	—	—	—	—
Nitrites (+/–)	—	—	—	—	—
Urobilinogen (mg/dL)	0.2	0.2	0.2	0.2	0.2
Proteins (mg/dL)	40	30	86	58	86
pH	6.4	6	6.5	6.5	6.5
Blood (erythrocytes/μL)	—	—	—	—	—
Specific gravity	1.021	1.025	1.02	1.022	1.023
Ketone bodies (mg/dL)	15	—	13.75	—	—
Bilirubin (mg/dL)	—	—	—	—	—
Glucose (mg/dL)	—	—	—	—	—
Treated with L-NAME					
Leukocytes (leu/dL)	—	—	—	—	—
Nitrites (+/–)	—	—	—	—	—
Urobilinogen (mg/dL)	0.2	0.2	0.2	0.2	0.2
Proteins (mg/dL)	30	44	86	72	30
pH	5.9	6.5	6.3	6.9	8.4
Blood (erythrocytes/μL)	—	—	—	—	—
Specific gravity	1.024	1.023	1.027	1.023	1.027
Ketone bodies (mg/dL)	15	10	5	—	5
Bilirubin (mg/dL)	—	—	—	—	—
Glucose (mg/dL)	—	—	—	—	—

(—) Indicates absence of the measured parameter.

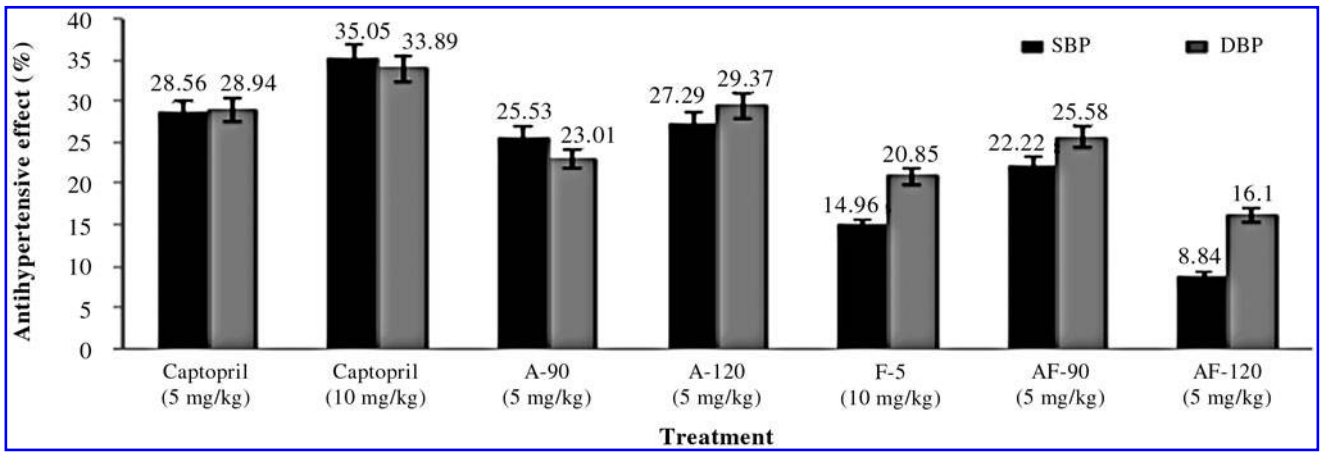

FIG. 6. SBP and DBP reduction of captopril and the *M. pruriens* protein hydrolysates administered a single dose (5 or 10 mg/kg) by intraperitoneal injection to HR. A-90, A-120: Alcalase at 90 and 120 min; F-5: Flavourzyme at 5 min; AF-90, AF-120: Alcalase-Flavourzyme at 90 and 120 min.

group. While other authors demonstrate inhibition of NOS by enzyme assays or by NOS isoforms expression,[18] we demonstrate inhibition of NOS by pharmacological and functional assays, for which the administration of L-NAME has a significantly smaller effect on hypertensive rats versus normotensive rats (Fig. 5).

The experimental models treated and not treated with L-NAME did not record statistical differences ($P < .05$) in corporal weight, as well as the urine-evaluated parameters (Table 2). It is to be noted that the hypertensive status is an important factor for generating kidney damage, which can be determined by protein in urine. If we compare the control group treated with the L-NAME group, we see that protein levels are significantly lower at end of treatment, suggesting that the antihypertensive effect of hydrolysates could also reduce kidney damage and the other parameters, including the higher pH in treatments with L-NAME than control, were in the normal limits according to Laso.[34]

The antihypertensive effect of the *M. pruriens* protein hydrolysates that registered the higher hypotensive effect, at lower doses that were statistically equal ($P > .05$) corresponding to the same enzyme system, were evaluated using a trial where rats with induced arterial hypertension were administered a single dose of hydrolysates by IP injection (Fig. 6). The Alcalase hydrolysate at 90 and 120 min (5 mg/kg) produced maximum reductions of 25.53% and 27.29% in SBP and of 23.01% and 29.37% in DBP, respectively. These values are similar to those obtained with the lowest captopril dose and was observed dose dependent since the increase to 10 mg had statistically better response ($P < .05$). These levels were similar to report by Betancur-Ancona *et al.*[35] for *P. lunatus* and *P. vulgaris* peptide fractions obtained by enzymatic hydrolysis.

The Flavourzyme hydrolysate at 5 min and the Alcalase-Flavourzyme system at 90 and 120 min registered reductions of 14.96%, 22.22%, and 8.84% in SBP and of 20.85%, 25.58%, and 16.10% in DBP, respectively. Antihypertensive peptides from *M. pruriens* are not as potent as synthetic hypertension drugs (Captopril), but hold promise as safe and natural therapeutic agents free of adverse side effects. This result shows the potential of using *M. pruriens* protein hydrolysates as an alternative to prevent/control hypertension and related illness.

Conclusions

The IP injection of *M. pruriens* protein hydrolysates to normotensive and antihypertensive rats produced a hypotensive and antihypertensive effect. By its ACE inhibitory activity *in vitro* and the *in vivo* effect, these hydrolysates could be incorporated as functional ingredients with an antihypertensive activity for humans. However, more studies are necessary to clarify the mechanisms responsible for these antihypertensive properties. Before the routine use of these protein hydrolysates, it would be necessary to carry out clinical studies to demonstrate its long-term antihypertensive efficiency in humans.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Domínguez-Magaña M, Segura-Campos M, Dávila-Ortiz G, Betancur-Ancona D, Chel-Guerrero L: ACE-I inhibitory properties of hydrolysates from germinated and ungerminated *Phaseolus lunatus* proteins. *Food Sci Technol (Campinas)* 2015; 35:167–174.

2. Feibleib M, Rifkind B, Sempos C, *et al.*: Methodological issues in the measurement of CVD risk factors: Within-person variability in selected serum lipid measures-results from the Third National Health and Nutrition Examination Survey (NHANES III). *Can J Cardiol* 1993;9 (suppl D):87D–88D.
3. Gutiérrez JP, Rivera-Dommarco J, Shamah-Levy T, Villalpando-Hernández S, Franco A, Cuevas-Nasu L, Romero-Martínez M, Hernández-Ávila M: *National Survey of Health and Nutrition*, National Institute of Public Health, Cuernavaca, México, 2012.
4. Kannel WB, Wilson PW, Nam BH, D'Agostino RB, Li J: A likely explanation for the J-curve of blood pressure cardiovascular risk. *Am J Cardiol* 2004;94:380–384.
5. Shimizu M, Sawashita N, Morimatsu F, Ichikawa J, Taguchi Y, Ijiri Y, Yamamoto J: Antithrombotic papain-hydrolyzed peptides isolated from pork meat. *Thromb Res* 2009;123:753–757.
6. Khiari Z, Rico D, Barry-Ryan C, Martin-Diana AB: Structure elucidation of ACE-inhibitory and antithrombotic peptides isolated from mackerel skin gelatine hydrolysates. *J Sci Food Agric* 2013;94:1663–1671.
7. Singh BP, Vij S, Hati S: Functional significance of bioactive peptides derived from soybean. *Peptides* 2014;54:171–179.
8. Lewington S, Clarke R, Qizilbash N, Peto R, Collins R: Age-specific relevance of usual blood pressure to vascular mortality: A meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet* 2002;360:1903–1913.
9. Ortiz-Ceballos AI, Aguirre-Rivera JR, Osorio-Arce MM, Peña-Valdicia C: Velvet vean (*Mucuna pruriens* var. *utilis*) cover crop as bioherbicide to preserve the environmental services of soil. In: *Herbicides-Environmental Impact Studies and Management Approaches* (Alvarez-Fernandez R, ed.). InTech, Rijeka, Croatia, 2012, pp. 167–184.
10. Pugalenthi P, Vadivel V, Siddhuraju P: Alternative food/feed perspectives of an underutilized legume *Mucuna pruriens* var. *utilis*—A review. *Plant Foods Hum Nut* 2005;60:201–218.
11. Chel-Guerrero L, Pérez-Flores V, Betancur-Ancona D, Dávila-Ortíz G: Functional properties of flours and protein isolates from *Phaseolus lunatus* and *Canavalia ensiformis* seeds. *J Agric Food Chem* 2002;50:584–591.
12. Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254.
13. Herrera-Chalé F, Ruiz-Ruiz JC, Acevedo-Fernandez JJ, Betancur-Ancona D, Segura-Campos MR: ACE inhibitory, hypotensive and antioxidant peptides fractions from *Mucuna pruriens* proteins. *Process Biochem* 2014;49:1691–1698.
14. AOAC: *Association of Official Analytical Chemists, Official Methods of Analysis*, 20th edn. Washington D.C., USA, 1997.
15. Lowry O, Rousebrough N, Lewis F, Randal R: Protein measurement with the folin phenol agent. *J Biol Chem* 1951;193: 265–275.
16. Megías C, Pedroche J, Yust MM, Alaiz M, Girón-Calle J, Millán F, Vioque J: Sunflower protein hydrolysates reduce colesterol micellar solubility. *Plant Foods Hum Nutr* 2009;64:86–93.
17. Nielsen P, Petersen D, Dammann C: Improved method for determining food protein degree of hydrolysis. *J Food Sci* 2001;66:642–646.
18. Alaiz M, Navarro JL, Girón JE, Vioque E: Amino acid analysis by high performance liquid chromatography after derivatization with diethyl ethoxymethylenemalonate. *J Chromatogr* 1992;591: 181–186.
19. Hayakari M, Kondo Y, Izumi H: A rapid and simple spectrophotometric assay of angiotensin-converting enzyme. *Anal Biochem* 1978;84:361–369.
20. Norma Oficial Mexicana NOM-062-ZOO-1999: Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio, Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación, México, 1999.
21. European Commission: Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Official Journal L* 1986;358:1–28.
22. Kuru O, Kemal-Senturk U, Kocer G, Ozdem S, Baskurt OK, Cetin A, Yesilkaya A, Gunduz F: Effect of exercise training on resistance arteries in rats with chronic NOH inhibition. *J Appl Physiol* 2009;107:896–902.
23. Montgomery DC: *Design and Analysis of Experiments*, 5th edn. John Wiley and Sons, New York, 2004, pp. 21–141.
24. Adebowale Y, Adeyemi I, Oshodi A, Niranjan K: Isolation, fractionation and characterisation of proteins from *Mucuna* bean. *Food Chem* 2007;104:287–299.
25. Segura-Campos MR, Espadas-Alcocer CP, Chel-Guerrero L, Betancur-Ancona D: ACE-I inhibitory peptide fractions from enzymatic hydrolysates of velvet bean (*Mucuna pruriens*). *Agric Sci* 2013;4:767–773.
26. Bampidis VA, Christodoulou V: Chickpea (*Cicer arietinum*) in animal nutrition: A review. *Anim Feed Sci Technol* 2011;168:1–20.
27. Sadiq M, Batool R: Nutritional and functional properties of some promising legumes protein isolates. *Pak J Nut* 2010;9:373–379.
28. Pedroche J, Yust M, Girón-Calle J, Vioque J, Alaiz M, Millán F: Plant protein hydrolysates and tailor-made foods. *Electr J Environ Agric Food Chem* 2003;2:233–235.
29. Udenigwe CC, Aluko RE: Hypolipidemic and hypocholesterolemic food proteins and peptides. In: *Bioactive Food Proteins and Peptides* (Hettiarachchy NS, ed.). CRC Press Taylor and Francis Group, Boca Raton, FL, 2012, pp. 191–218.
30. Hong LG, Wei L, Liu H, Hui SY: Mung-bean protein hydrolysates obtained with alcalase exhibit angiotensin I-converting enzyme inhibitory activity. *Food Sci Technol Int* 2005;11:281–287.
31. Doggrell SA, Brown L: Rat models of hypertension, cardiac hypertrophy and failure, *Cardiovasc Res* 1998;39:89–105.
32. Yust M, Pedroche J, Girón J, Alaiz M, Millan F, Vioque J: Production of ACE inhibitory peptides by digestion of chickpea legumin with alcalase. *Food Chem* 2003;81:363–369.
33. Murray B., Fitzgerald R: Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production. *Curr Pharm Des* 2007;13:773–791.
34. Laso M: Interpretación del análisis de orina. *Arch Argent Pediatr* 2002;100:179–183.
35. Betancur-Ancona D, Dávila-Ortiz G, Chel-Guerrero L, Torruco-Uco JG: ACE-I inhibitory activity from *Phaseolus lunatus* and *Phaseolus vulgaris* peptide fractions obtained by ultrafiltration. *J Med Food* 2015;18:1247–1254.