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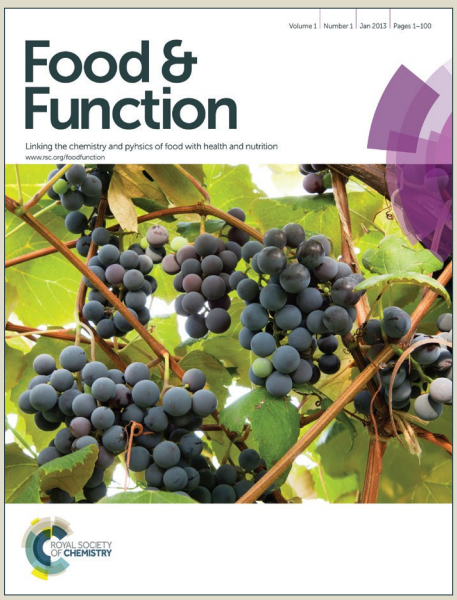

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Hypolipidemic Effect and Antithrombotic Activity of *Mucuna pruriens* Protein Hydrolysates

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ABSTRACT

Hydrolysates and peptide fractions (PF) obtained from *M. pruriens* protein concentrate with commercial and digestive enzymatic systems were studied for their hypolipidemic and antithrombotic activities. Hydrolysate obtained with Pepsin-Pancreatin (PP) and its peptide fractions inhibited cholesterol micellar solubility with a maximum value of 1.83% in PP. Wistar rats were used to evaluate the hypolipidemic effect of hydrolysates and PF. The higher reductions of cholesterol and triglycerides levels were exhibited by PP and both peptide fractions < 1 kDa obtained from PP and Alcalase®-Flavourzyme® hydrolysate (AF) at a dose of 15 mg/kg of animal weight. PF > 10 kDa from both hydrolysates showed the maximum antithrombotic activity with values of 33.33% for PF >10 kDa from AF and 31.72% for PF >10 kDa from PP. The results suggest that *M. pruriens* bioactive peptides with hypolipidemic effect and antithrombotic activity might be utilized as nutraceuticals.

Keywords: *Mucuna pruriens*, protein hydrolysis, peptide fractions, hypolipidemic effect, antithrombotic activity.

INTRODUCTION

Elevated concentrations of some lipids in the plasma have been associated with increased risk for heart diseases in humans.[1] These increases are elicited by changes in the lipid metabolism pathways induced by environmental and genetic factors leading to hyperlipidemia and hypercholesterolemia, which are not diseases but health conditions characterized by elevated serum lipids and cholesterol, respectively.[2,3] Raised cholesterol is estimated to cause 2.6 million deaths (4.5% of total) and 29.7 million disability adjusted life years. Although several drugs have been developed to manage

severe cases of hypercholesterolemia in humans, dietary and lifestyle modification approaches have been recommended for maintaining low blood level of proatherogenic cholesterol.[1] In addition, various research investigations have focused on the use of less toxic functional foods and nutraceuticals to treat and manage mild cases of hypercholesterolemia.[4-8]

Thrombosis is a serious pathological issue that causes high rates of morbidity and mortality. In the U.S. alone, 100,000 to 180,000 deaths occur annually because of venous thromboembolism. Thrombosis occurs as the result of excessive adhesion and aggregation of platelets due to the disruption, rupture and erosion of the atherosclerotic plaque. The suppression or the inhibition of platelet aggregation is believed to decrease the incidence of the disease.[9] Antiplatelet agents act as metabolic inhibitors of platelet activation, drugs such as aspirin and ticlopidine have some ability to prevent arterial thrombi during chronic use but do not have a predictable effect when given during acute arterial thrombosis.[10] Food derived peptides that inhibit the blood platelet aggregation can be useful natural ingredients for the prevention of thrombosis.[11] For instance, papain-hydrolyzed pork meat was found to inhibit platelet reactivity. In addition, a tetrapeptide, asp-gly-glu-ala (DGEA), derived from type I collagen was found to inhibit collagen-induced platelet aggregation.[12]

Ingestion of a fatty meal appears to cause venous endothelial dysfunction in healthy adults, while postprandial lipemia has been associated with transient changes in factor VII coagulant activity in humans, suggesting a temporary and reversible hypercoagulable state.[13] Dyslipidaemia may exert influence on the risk of venous thromboembolism, as determined by a recent meta-analysis in which patients with

venous thromboembolism had high triglyceride and low HDL cholesterol levels, while no effect of total cholesterolaemia on venous thromboembolism was seen.[14]

Mucuna pruriens is a legume grown in Africa, South America and South Asia.[15] It is rich in protein (23-35%) and has a nutritional quality comparable to that of other pulses like lima bean and has good potential as a cheap and alternate source of proteins.[16] The aim of the present study was to evaluate the hypolipidemic effect and the inhibition of platelet aggregation of protein hydrolysates and ultrafiltered peptide fractions from *Mucuna pruriens* protein concentrates hydrolyzed with digestive and commercial sequential enzymatic systems.

MATERIALS AND METHODS

Seeds and chemicals

Pods of *Mucuna pruriens* were collected in Yucatan, Mexico. After drying pods were thrashed to remove seeds. Matured and dried seeds were stored in airtight plastic jars at room temperature. All chemicals were reagent grade or better and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Protein concentrate

Selected grains were ground in a disk mill (model 4-E Quaker, Mill Straub Co., Philadelphia, PA, USA) and then sifted through 4.76 and 2.38 mm screens in order to remove the smallest particles. Hulls were removed with a fluidizing air bed and the material resulted was milled in a Cyclotec mill (Tecator, Höganas, Sweden) until passing through a 0.841 mm screen. The protein concentrate of *M. pruriens* bean was obtained by wet fractionation.[17]

Enzymatic hydrolysis

Hydrolysis of the protein isolates was done using a totally randomized design. Treatments were the sequential enzymatic system applied: Alcalase® 2.4L FG and Flavourzyme® 500MG (Novo Nordisk, Bagsvaerd, Denmark); or pepsin from porcine gastric mucosa (Sigma, P7000-100G) and pancreatin from porcine pancreas (Sigma, P3292-100G). The response variable was degree of hydrolysis (DH). The hydrolysis with the AF system was done according to Pedroche et al.[18]. The hydrolysis with the sequential PP system was done according to Megías et al.[19]. Degree of hydrolysis was calculated by determining free amino groups with o-phthaldialdehyde[20] : DH = h/htot * 100; where htot is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds.

Hydrolysate fractionation

The Alcalase®-Flavourzyme® (AFH) and pepsin-pancreatin (PPH) hydrolysates were fractionated by ultrafiltration[21] with a high performance ultrafiltration cell (Model 2000, Millipore). The supernatants of AFH and PPH were collected. Part of them was studied as whole hydrolysates, and the rest was fractionated with a high performance ultrafiltration cell (Model 2000, Millipore). Five fractions were prepared using four molecular weight cut-off (MWCO) membranes: 1 kDa, 3 kDa, 5 kDa and 10 kDa. Protein content in ultrafiltered peptide fractions was quantified using the method of Lowry et al.[22] Ultrafiltration-membrane-based separations of enzymatic hydrolysates can achieve the removal of peptides from non-hydrolyzed proteins and proteolytic enzymes. In addition, ultrafiltration can also be used to perform peptide separation according to their molecular mass and also to their charge.

Protein and peptide pattern

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Schägger et al.[23] using 15% (w/v) resolving polyacrylamide gel. Protein concentrate (i.e., control sample), hydrolysates and ultrafiltered peptide fractions were dissolved at 2 mg/mL in distilled water then diluted to a final concentration of 1 mg/mL with sample buffer containing β-mercaptoethanol and heated to 85 °C for 10 min. 10 μL of each sample and molecular weight markers, containing low molecular weight peptides and high molecular weight proteins, were loaded onto the gel. The analysis was run at a constant current of 25 mA. The gel was silver stained and de-stained according to Sorensen et al.[24].

***In vitro* cholesterol micellar solubility inhibition**

The *in vitro* micellar solubility of cholesterol with hydrolysates and peptide fractions was measured according to the method described Zhong et al.[6]. This assay consist in a micellar solution (7.0 mL) containing 10.0 mM sodium taurocholate, 2.0 mM cholesterol, 5.0 mM oleic acid, 132.0 mM NaCl, 15.0 mM sodium phosphate (pH 7.4), 1.0 mL of hydrolysate or peptide fractions was prepared by sonication. The mixture was incubated at 37 °C for 24 h and ultracentrifuged at 100,000 x *g* for 60 min at 37 °C. The supernatant was collected for the determination of total cholesterol content using a COBAS C 111 analyzer (Roche Diagnostics, Indianapolis, IN, U.S.A.).

Hypocholesterolemic effect in rats

The experimental procedures were approved by the Care and Use Committee of Laboratory Animals (Faculty of Medicine, Autonomous University of Morelos) and were performed according to the Official Mexican Norm (NOM-062-ZOO-1999)

"Technical specifications for the care and use of laboratory animals" as well as all Federal and Institutional regulations. Female Wistar alloxan-induced diabetic rats of 8-10-week-old and weighing 280 ± 40 g were used to evaluate the hypocholesterolemic effect of the following products: AFH, PPH, and peptide fractions < 1 kDa (5, 10 and 15 mg/kg). All the above-mentioned products were administered to the rats by intraperitoneal injection (IP), between 9 and 10 a.m. Five animals were used per treatment. Animals were housed in an air conditioned room (22-24 °C with a 12 h light cycle) and consumed tap water and a standard diet for rats (nu3lab, Research Global Solution. Brulington, Ontario, Canada) *ad libitum* during the experiments. To induce diabetes in rats a solution of alloxan at 2% diluted in Physiological saline solution (PSS) (Sigma, USA H4034) at 0.9% was administered to the animals in a single dose corresponding to 40 mg of alloxan per kg of animal weight injected intraperitoneal. PSS served to dilute hydrolysates and as positive control, and pravastatin sodium (0.5 mg/kg of animal weight), a known lipid-lowering compound which reduce cholesterol biosynthesis as negative control. The content of cholesterol was measured weekly for a period of four weeks.

In vitro inhibition of human platelet aggregation

The *in vitro* inhibition of human platelet aggregation was analyzed following Miyashita et al.[25]. Blood from human volunteers, diluted with 130 mM trisodium citrate (4.5mL), was centrifuged at 127 x *g* for 15 min at room temperature in a Solbat J-600 centrifuge to give platelet-rich plasma (PRP) as the supernatant. After removing most of PRP, the residual phase was centrifuged at 1918 x *g* for 15 min at room temperature to obtain platelet-poor plasma (PPP). Immediately, a platelet adjustment was done to the PRP (200 X 10^3/μL) using a hematic cytometer (Sysmex KX-21). PRP (450 μL) was

incubated with each test compound, which was dissolved in saline (154mM NaCl at pH 6.4) for 1 min at 37 °C. Platelet aggregation was induced by adding 10 μL of an aqueous solution of 10 μm ADP. Aggregation was measured by an aggregometer (Chrono-Log 400) as the increase in the light transmission through PRP. PPP, which was used as the control, gave 100% transmission. The platelet aggregation inhibition percentage was determined to subtract the platelet aggregation percentage from the PRP with protein hydrolyzed to the platelet aggregation percentage from the PRP in basal conditions.

Amino acid composition

Amino acid composition was determined for the ultrafiltered peptide fractions by high performance liquid chromatography.[26] Samples (4 mg of protein) were treated with 4 mL of HCl 6.0 N, placed in hydrolysis tubes and gassed with nitrogen at 110 °C for 24 h. They were then dried in a rotavapor (Büchi, Rotavapor R-215, Flawil, Switzerland) and suspended in sodium borate buffer (1.0 M, pH 9.0). Derivatization was performed at 50 °C using diethyl ethoxymethylenemalonate. Amino acids were separated using HPLC with a reversed-phase column (300×3.9mm, Nova Pack C18, 4mm; Waters), and a binary gradient system with sodium acetate containing 25 mM (A) 0.02 g/L sodium azide at pH 6.0, and (B) acetonitrile as solvent.

Statistical analysis

All results were analyzed using central tendency and dispersion measures. One-way ANOVAs were run to evaluate protein isolate hydrolysis. A LSD multiple range test was used to determine differences between treatments and biological activities in

hydrolysates and ultrafiltered peptide fractions. All analyses were done according to Montgomery [27] and processed with the Statgraphics Plus version 5.1 software.

RESULTS AND DISCUSSIÓN

Hydrolysate fractionation

Protein content was determined in filtrates and retentates of both sequential enzymatic systems (Figure 1). Some differences between the protein content and molecular weight distribution profiles of both hydrolysates were observed.

Figure 1

In Alcalase®-Flavourzyme® system the content of protein did not differ between F > 10 kDa and F 3-5 kDa, and between F 5-10 kDa and F 1-3 kDa, the lowest content was observed in F < 1 kDa. The hydrolytic activity of this enzymatic system generated peptides with a wide range of molecular weights.[28,29,30] Protein hydrolysis with endoproteinases and exopeptidases like Alcalase® and Flavourzyme® is known to yield a large proportion of small peptides with molecular weight < 2 kDa.[31,32,33] For Pepsin-Pancreatin system the highest content of protein was observed in the F > 10 kDa and the lowest in the F < 1 kDa. Although the hydrolytic activity of the Pepsin-Pancreatin enzymatic system was higher (40.15%) this produced primarily high molecular weight peptides > 10 kDa and a little amount of low molecular weight peptides.

Protein and peptide patterns

224 The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) pattern
225 of *Mucuna bean* protein concentrate and hydrolysates are depicted in Figure 2. The gel
226 indicates the presence of five polypeptide protein sub-units of apparent molecular
227 weights 200, 116, 82, 63, and 59 kDa. Similar ranges of polypeptide molecular weight
228 distribution have also been reported for several other legume proteins.[34]
229 Figure 2
230
231 At the end of both hydrolysis, the majority of high molecular weight proteins (200-30
232 kDa) were hydrolyzed into peptides with lower molecular size as shown in lanes 3 and 4
233 (Figure 3). The electrophoresis pattern reveals higher hydrolysis efficiency for Pepsin-
234 Pancreatin. These results agree with those observed in the degree of hydrolysis. Gel
235 electrophoresis (SDS-PAGE) pattern of ultrafiltered peptide fractions are depicted in
236 Figure 3:
237
238 Figure 3.
239
240 The SDS-PAGE patterns of Alcalase®-Flavourzyme® ultrafiltered peptide fractions
241 from F > 10 kDa to F < 1 kDa, respectively, showed that intensity of some bands
242 decreased after ultrafiltration steps. The same behavior was observed by Pepsin-
243 Pancreatin ultrafiltration. The average molecular weight of protein hydrolysates is one
244 of the most important factors, which determines their functional properties.[35] An
245 ultrafiltration membrane system could be a useful method for obtaining peptide
246 fractions with a desired molecular size and enhanced biological activity. This system
247 has been successfully applied in the fractionation and functional characterization of
248 hydrolysates;[36] and also as a first step in the isolation and further purification of

bioactive peptides from similar sources.[37,38] Under conditions studied in the present study the ultrafiltration process with different membranes could be used to partially concentrate peptides with a defined molecular weight range.

***In vitro* inhibition of cholesterol micellar solubility**

Hydrolysate obtained with the sequential enzymatic system Alcalase®-Flavourzyme® and its peptide fractions did not exhibit hypocholesterolemic activity. Inhibition of *in vitro* cholesterol micelle solubility of hydrolysate obtained with the sequential enzymatic system Pepsin-Pancreatin and its ultrafiltered fractions is shown in Table 1.

Table 1

The *in vitro* cholesterol micellar solubility inhibition of Pepsin-Pancreatin hydrolysate and ultrafiltered peptide fraction ranged between 1.83% (Hydrolysate) and 0.53% (F < 1 kDa). This values not agree with those obtained by others researchers. Zhong et al.[6] reported the cholesterol micellar solubility inhibition of soy Alcalase protein hydrolyzed and found that cholesterol was reduced by 48.6%. Also casein tryptic hydrolysate reduced the cholesterol micellar solubility by 20%.[39] The *in vitro* inhibition of cholesterol micellar solubility is a strategy that has been used toward the search for potent hypolipidemic and hypocholesterolemic food proteins and peptides.[6] However this method depends only on the reduction of cholesterol in the micellar solubility solution. Generally, proteins and peptides could exhibited hypocholesterolemic and hypolipidemic *in vivo* effect by means of increased cholesterol catabolism, reduced cholesterol synthesis or increased expression of LDL reception genes.[28] Thus the *in*

vitro evaluation of the hypocholesterolemic activity could not be directly correlated with the *in vivo* effect. Considering the above both hydrolysates and their ultrafiltered peptide fractions obtained in the present study could exhibit hypocholesterolemic effect *in vivo*.

Hypocholesterolemic and hypolipidemic effect in rats

Significant hyperlipidemia that characterized the diabetic state may therefore be regarded as a consequence of uninhibited actions of lipolytic hormones on the fat depots. The *in vivo* hypocholesterolemic activity of both hydrolysates and their ultrafiltered peptide fractions (F < 1 kDa) was studied with female Wistar alloxan-induced diabetic rats. As shown in Table 2, after 1 week of being administration of pravastatin sodium, hydrolysates and ultrafiltered peptide fractions, the controls and the samples exhibited hypocholesterolemic effect in rats. During the study, the hypocholesterolemic effect generated by the administration of hydrolysates and ultrafiltered peptide fractions was higher than the effect of the drug pravastatin sodium. The hypocholesterolemic effect of hydrolysates and peptide fractions depended on the dose delivered and was greater for peptide fractions.

Table 2

The hypocholesterolemic effect of hydrolysates and peptide fractions at a dose of 5 mg/kg of animal weight is showed in Figure 4a. In general, serum cholesterol content remained stable throughout the study period in rats IP administered with SPS. For pravastatin sodium the hypocholesterolemic effect decay from second week at the dose evaluated. Hydrolysates and peptide fractions exhibited higher hypocholesterolemic

effect than pravastatin sodium. The highest reductions in serum cholesterol content were observed at the end of the third week by the IP administration of both hydrolysates, at the end of the four week the serum cholesterol content of animals administered with hydrolysate increased to reach a value close to that of first week. Only PP < 1 kDa showed a tendency to reduce serum cholesterol content for fourth week.

Figure 4

For the dose of 10 mg/kg of animal weight serum cholesterol content remained stable throughout the study period in rats IP administered with SPS. For pravastatin sodium the hypocholesterolemic effect decay from second week at the dose evaluated (Figure 4b). AF hydrolysate reduced the serum cholesterol content during the study period with the highest reduction at the end of the fourth week. On the contrary, PP hydrolysate showed high serum cholesterol contents during the study period with a similar content than pravastatin sodium at the end of the fourth week. From both peptide fractions only PP < 1 kDa showed a tendency to reduce serum cholesterol content for fourth week. At a dose of 15 mg/kg of animal weight serum cholesterol content remained stable throughout the study period in rats IP administered with SPS. For pravastatin sodium the hypocholesterolemic effect decay from second week at the dose evaluated (Figure 4c). AF hydrolysate increased the serum cholesterol content after the second week of the study period with the highest content at the end of the fourth week. On the contrary PP hydrolysate and both peptide fractions reduced the serum cholesterol content after the second week of the study period with the highest reduction at the end of the fourth week. In general, the higher reductions in serum cholesterol contents were observed

after the second week of the study period at the three doses (5, 10 and 15 mg/kg animal weight). Only at the high dose PP hydrolysate and peptide fractions increased the hypocholesterolemic effect after the second week to the end of the study period.

The hypolipidemic effect of hydrolysates and peptide fractions at a dose of 5 mg/kg of animal weight is showed in Figure 5a. In general, serum triglycerides content remained stable throughout the study period in rats IP administered with SPS. For pravastatin sodium the hypocholesterolemic effect decays from second week and remains stable at the end of the study. The highest reductions in serum triglycerides content were observed at the beginning of the third week by the IP administration of both hydrolysates. At the end of the study the hypolipidemic effects of both hydrolysates decay. The ultrafiltered peptide fractions exhibited hypolipidemic effect only at de beginning of the second week after that their effects decay until reach similar values than SPS at the end of the study.

Figure 5

For the dose of 10 mg/kg of animal weight serum triglycerides content remained stable throughout the study period in rats IP administered with SPS. For pravastatin sodium the hypocholesterolemic effect decays from second week and remains stable at the end of the study (Figure 5b). PP hydrolysate only exhibited a slight hypolipidemic effect at the beginning of the second week. On the contrary, AF hydrolysate showed a reduction in triglycerides content at the beginning of the second week with successive reductions in its hypolipidemic effect at weeks three and four. Both peptide fractions exhibited higher reduction of serum triglycerides content than pravastatin sodium at the end of the

study. At a dose of 15 mg/kg of animal weight serum triglycerides content remained stable throughout the study period in rats IP administered with SPS. For pravastatin sodium the hypocholesterolemic effect decays from second week and remains stable at the end of the study (Figure 5c). During three weeks AF hypolipidemic effect remains stable after that the effect was lost until reach the higher value of triglycerides of all the study. PP hydrolysate reduced the serum triglyceride content after the second week and maintained a reducing trend throughout the study. Both peptide fractions reduced the serum triglyceride content after the second week of the study period with the highest reduction at the end of the fourth week. In general, the higher reductions in serum triglycerides contents were observed after the second week of the study period at the three doses (5, 10 and 15 mg/kg animal weight). At a dose of 10 mg/kg animal both peptide fractions exhibited the highest hypolipidemic effect.

The present study showed the hypocholesterolemic and hypolipidemic effects of *Mucuna pruriens* hydrolysates and ultrafiltered peptide fractions. Other studies that examined the effect of plant proteins and their hydrolysates also showed the hypolipidemic effect.[40,41] Suggested mechanisms for this hypolipidemic effect are the excretion of fat by the hydrophobicity of hydrolysate itself or peptide made during the ingestion process and the changes of hepatic enzymes related to lipid metabolism. Because a high concentration of serum cholesterol and triglycerides is a key factor for cardiovascular risk, the results suggested that the hydrolysates and the ultrafiltered peptide fractions were an effective hypocholesterolemic and hypolipidemic peptide mixture, and the serum cholesterol and triglycerides lowering effect of hydrolysate and ultrafiltered peptide fractions was dosage and time dependent.

Inhibition of human platelet aggregation

The peptide fractions obtained with the AF and PP sequential system showed significant inhibition of ADP-induced aggregation of human platelets in platelet-rich plasma (Table 1). The maximum inhibitory effect of protein hydrolysates obtained with the AF and PP systems was 33.33 and 31.72 % in F >10 kDa at a concentration of 0.9269 and 1.8130 mg/mL, respectively that corresponding to a moderate antithrombotic activity which is similar activity to the synthetic antithrombotic compounds (aspirin and indomethacin). Probably the most active ultrafiltered peptide fraction is F >10 kDa from hydrolysate AF because needs less protein concentration than F >10 kDa from hydrolysate PP to cause the same inhibition. Previous studies have identified three distinct fibrinogen-derived peptides that are inhibitors of platelet aggregation. The amino acid sequences of these peptides form RGD, which recurs twice in the fibrinogen α chain; HHLGGAKQAGDV, the carboxyl terminus of the fibrinogen γ-chain; and GPRP. RGD-containing peptides and γ-chain-related peptides appear to inhibit platelet aggregation by binding to the platelet receptor complex GPIIb/IIIa and blocking adhesive glycoprotein attachment. GPRP binds directly to the fibrinogen D domain and inhibits the ability of fibrinogen to bind to GPI1b/IIIa. GPRP is also able to inhibit fibrin gel formation via binding to the D domain of fibrinogen. Agents that interfere with fibrinogen and von Willebrand factor binding to GPIIb/IIIa or with von Willebrand factor binding to GPIb have already been proven effective antithrombotics in *in vivo* studies. Using models in which platelet-dependent thrombus formation occurred after coronary artery constriction, various investigators have demonstrated inhibition of thrombosis after infusion of a monoclonal antibody that binds to GPIIb/IIIa or by peptides that contained the RGD sequence. An antibody that bound to von Willebrand factor and prevented its attachment to activated platelets also inhibited arterial thrombus

formation. Similarly, the agent aurintricarboxylic acid, which binds to von Willebrand factor and prevents its attachment to GPIb, has been demonstrated *in vivo* to be a potent inhibitor of platelet-dependent thrombus formation within constricted arteries. Inhibition of platelet aggregation by direct blockade of platelet-adhesive glycoprotein interactions is a potentially effective means by which to prevent or arrest acute arterial thrombus formation.[11]

Amino acid composition

During hydrolysis, asparagine and glutamine partially converted to aspartic acid and glutamic acid, respectively; the data for asparagine and/or aspartic acid were therefore reported as Asx while those for glutamine and/or glutamic acid were reported as Glx. The results of this study suggest that *Mucuna pruriens* protein hydrolysates and ultrafiltered peptide fractions could contain peptides that inhibit platelet aggregation in plasma and exhibit both *in vitro* and *in vivo* hypolipidemic effect (Table 3).

Table 3

The ability of a peptide to inhibit the platelet aggregation can be linked to its amino acid composition and its primary sequence. Laudano and Doolittle[42] demonstrated that peptides comprising gly-pro-arg (G-P-R) at the beginning of their sequences have the ability to bind fibrinogen and prevent fibrin monomer polymerization. Different studies related the inhibition of platelet aggregation by non-food derived peptides (i.e., snake venom) to the presence of tripeptide arg-gly-asp (R-G-D) which blocks the binding of fibrinogen to the activated platelets receptors.[43] Milk, egg and rapeseed derived peptides have been reported to possess antithrombotic properties.[12] Shimizu et al.[9] suggested that

424 the antithrombotic activity is probably associated with the higher contents of Ile, Leu
425 and Phe. Ultrafiltered peptide fractions F > 10 kDa from both hydrolysates that
426 exhibited the highest values of inhibition of human platelet aggregation, presented in
427 their amino acid composition high amounts of amino acids reported in sequences of
428 peptides with antithrombotic activity.
429
430 Hydrophobicity plays a major role in the hypocholesterolemic activity of peptides
431 especially in binding bile acids. The hydrophobic amino acids of the
432 hypocholesterolemic peptides and proteins are thought to interact with bile acids by
433 hydrophobic interactions leading to the formation of insoluble complexes for fecal
434 removal.[28] Kongo-Dia-Moukala et al.[44] suggest that hydrophobic amino acids contain in
435 hydrolysates favors its immersion in the lipid micelles. Other factors such as amino acid
436 sequences of peptides may also contribute to cholesterol-lowering activity. Kritchevsky
437 et al.[45] considered that a high arginine-lysine ratio in food proteins might contribute to
438 increased cholesterol-lowering ability. This hypothesis was supported by the
439 hypocholesterolemic activity reported for fish protein hydrolysate, which had a high
440 arginine–lysine ratio when compared to casein.[31] Kwon et al.[46] isolated a tetrapeptide
441 with the amino acid sequence (Leu-Pro-Tyr-Pro) from soy glycinin. They reported that
442 the hypocholesterolemic effect of this peptide was related to a Leu residue at the N
443 terminus. In this study hydrolysates and their peptide fractions content high amounts of
444 hydrophobic amino acids but not showed a significant *in vitro* inhibition of cholesterol
445 micellar solubility. However hydrolysates and peptide fractions exhibited
446 hypocholesterolemic and hypolipidemic effects in alloxan induced diabetes. In this
447 sense the amino acid composition could be more related to the biological activity than
448 hydrophobicity.

449

450

451

452 **Conclusions**

453 The *in vitro* inhibition of human platelet aggregation and cholesterol micellar solubility
454 of the ultrafiltered peptide fractions was significantly higher than those of the non-
455 fractionated hydrolysates. Intraperitoneal administration of PP and the ultrafiltered
456 peptide fractions AF < 1 kDa and PP < 1 kDa at a dose of 15 mg/kg of animal weigh
457 decrease serum cholesterol content in alloxan-induced diabetic rats during a period of
458 four weeks. For triglycerides intraperitoneal administration of ultrafiltered peptide
459 fractions AF < 1 kDa and PP < 1 kDa at a dose of 10 mg/kg of animal weigh decrease
460 serum triglycerides content in alloxan-induced diabetic rats during a period of four
461 weeks. The findings from the present study suggest that hydrolysis of *Mucuna pruriens*
462 proteins with sequential enzymatic systems combined to ultrafiltration fractionation of
463 hydrolysates could provide new opportunities for the development of health-promoting
464 ingredients. Although it is necessary to evaluate the antithrombotic *in vivo* effect of the
465 hydrolysates and their ultrafiltered peptide fractions and determine the sequences of the
466 peptides presents in the ultrafiltered fractions, the results of this study may provide a
467 model by which to develop new antithrombotic, hypocholesterolemic and
468 hypolipidemic strategies such as the development of physiologically functional foods or
469 therapeutic agents aimed at preventing and/or treatment thrombosis and dyslipidemias.

470

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473 desarrollo de derivados proteínicos de *Mucuna pruriens* con potencial actividad

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Table 1. *In vitro* inhibition of human platelet aggregation and cholesterol micellar solubility of hydrolysates and ultrafiltered peptide fractions.

In vitro biological activity	Inhibition of human platelet aggregation (%)		Inhibition of cholesterol micellar solubility (%)
Enzymatic system	AF	PP	PP
Hydrolysate	22.58^{d}	5.38^{d}	1.83^{e}
F > 10 kDa	33.33^{f}	31.72^{e}	1.25^{d}
F 5 - 10 kDa	2.15^{a}	0.54^{a}	1.12^{c}
F 3 - 5 kDa	3.76^{b}	1.08^{b}	1.05^{b}
F 1 - 3 kDa	4.84^{c}	5.38^{d}	1.12^{c}
F < 1 kDa	3.22^{b}	2.15^{c}	0.53^{a}

The data are expressed as means ± SEM (n = 3). Within a column, data with different superscript letters are significantly different.

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Table 2. Serum cholesterol (C) and triglycerides (TG) levels in alloxan-induced diabetic rats during four weeks at doses of 5, 10 and 15 mg/kg of animal weigh.

Time	After 1 week		After 2 weeks		After 3 weeks		After 4 weeks	
mg/dL	C	TG	C	TG	C	TG	C	TG
5 mg/kg of animal weight								
SPS	159.50^{a}	476.00^{f}	162.25^{c}	245.50^{f}	162.75^{b}	336.00^{e}	153.75^{a}	130.25^{a}
Pravastatin sodium	160.60^{a}	191.00^{c}	157.40^{a}	113.40^{b}	164.75^{c}	138.50^{b}	179.75^{d}	157.50^{b}
AF	170.00^{b}	262.60^{e}	160.20^{b}	145.00^{d}	154.00^{a}	134.2^{b}	164.00^{c}	343.80^{f}
PP	168.60^{b}	122.60^{a}	157.00^{a}	120.20^{c}	153.75^{a}	82.00^{a}	163.50^{c}	297.25^{e}
AF F < 1 kDa	159.40^{a}	208.40^{d}	160.00^{b}	154.60^{e}	155.60^{a}	192.00^{d}	158.60^{b}	239.20^{d}
PP F < 1 kDa	165.80^{b}	134.00^{b}	162.80^{c}	97.80^{a}	169.00^{d}	178.80^{c}	155.00^{a}	170.40^{c}
10 mg/kg of animal weight								
SPS	159.50^{a}	476.00^{f}	162.25^{c}	245.50^{c}	162.75^{b}	336.00^{d}	153.75^{a}	130.25^{b}
Pravastatin sodium	160.60^{b}	191.00^{a}	157.40^{b}	113.40^{a}	164.75^{b}	138.50^{a}	179.75^{d}	157.50^{c}
AF	169.50^{c}	342.25^{d}	174.25^{d}	255.25^{d}	176.00^{c}	342.25^{e}	160.00^{b}	417.50^{d}
PP	165.25^{c}	254.25^{b}	177.25^{d}	301.50^{f}	176.75^{c}	412.00^{f}	175.50^{c}	506.00^{e}
AF F < 1 kDa	159.75^{a}	363.00^{e}	158.00^{b}	199.50^{b}	150.25^{a}	291.25^{c}	155.25^{a}	153.00^{c}
PP F < 1 kDa	157.00^{a}	318.00^{c}	154.00^{a}	268.75^{e}	162.50^{b}	146.00^{b}	152.50^{a}	123.00^{a}
15 mg/kg of animal weight								
SPS	159.50^{a}	476.00^{e}	162.25^{c}	245.50^{f}	162.75^{b}	336.00^{e}	153.75^{b}	130.25^{c}
Pravastatin sodium	160.60^{a}	191.00^{c}	157.40^{b}	113.40^{a}	164.75^{b}	138.50^{b}	179.75^{d}	157.50^{d}
AF	166.00^{b}	237.25^{d}	155.74^{a}	183.75^{d}	169.75^{c}	190.50^{d}	164.00^{c}	600.00^{e}
PP	159.75^{a}	146.75^{a}	162.50^{c}	210.50^{e}	155.75^{a}	146.25^{c}	150.00^{a}	131.00^{c}
AF F < 1 kDa	157.75^{a}	155.00^{b}	159.75^{b}	129.50^{b}	154.25^{a}	78.75^{a}	150.00^{a}	119.00^{b}
PP F < 1 kDa	159.25^{a}	143.00^{a}	161.75^{c}	163.00^{c}	154.75^{a}	149.75^{c}	150.75^{a}	109.00^{a}

The data are expressed as means (n = 4). Within a line, data with different superscript letters are significantly different.

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Table 3. Amino acid composition of hydrolysates and ultrafiltered peptide fractions.

Amino acid	Alcalase®-Flavourzyme®			Pepsin-Pancreatin		
	Hydrolysate	F > 10 kDa	F < 1 kDa	Hydrolysate	F > 10 kDa	F < 1 kDa
Asp	19.33	16.56	20.03	17.71	15.12	14.35
Glu	10.89	13.50	12.51	12.87	13.32	13.77
Ser	8.66	17.47	14.80	14.33	2.38	7.89
His	10.87	4.85	5.32	5.22	9.09	3.47
Gly	7.13	7.85	5.02	4.98	7.98	4.52
Thr	8.20	7.72	7.89	8.60	13.28	5.79
Arg	4.27	4.54	4.75	5.28	6.15	7.67
Ala	2.89	3.10	3.67	4.09	4.23	4.89
Pro	1.01	0.59	0.81	0.66	0.65	0.22
Tyr	5.55	4.27	4.24	3.84	3.88	2.40
Val	5.26	0.00	3.07	2.94	2.42	1.57
Met	0.00	0.00	0.00	0.00	0.00	0.00
Cys	3.90	5.11	4.50	4.30	3.91	6.91
Ile	2.93	4.69	4.26	4.27	3.68	8.29
Trp	0.67	0.41	0.54	0.51	0.29	0.26
Leu	2.21	2.22	1.32	2.43	2.30	2.39
Phe	1.69	1.02	0.89	0.80	2.31	8.06
Lys	4.53	6.10	6.38	7.17	9.00	7.55
Amino acid distribution (%)						
Hydrophobic	16.66^{d}	12.03^{a}	14.56^{b}	15.70^{c}	15.89^{c}	25.68^{e}
Hydrophilic	52.49^{e}	47.31^{c}	47.63^{c}	46.06^{b}	51.66^{d}	43.78^{a}
Neutral	30.85^{a}	40.67^{d}	37.81^{c}	38.24^{c}	32.45^{b}	30.54^{a}

The data are expressed as means (n = 3). Within a line, data with different superscript letters are significantly different.

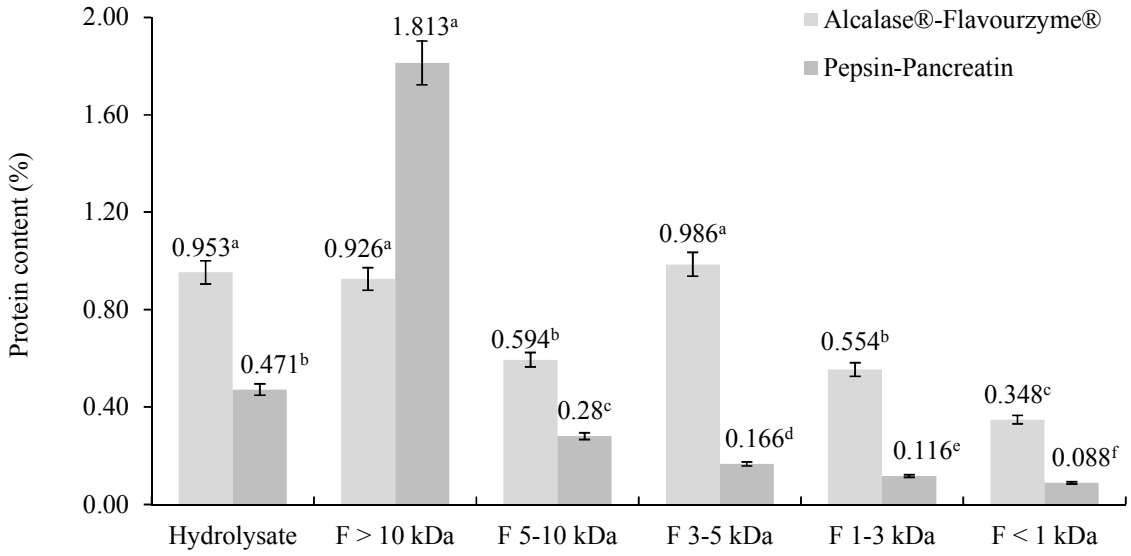

Figure 1. Peptide content (mg/mL) in total hydrolysates and ultrafiltered peptide fractions obtained from *M. pruriens* protein concentrate hydrolyzed with Alcalase®-Flavourzyme® and Pepsin-Pancreatin. [a-f]Different letters in the same system indicate statistical difference ($p<0.05$).

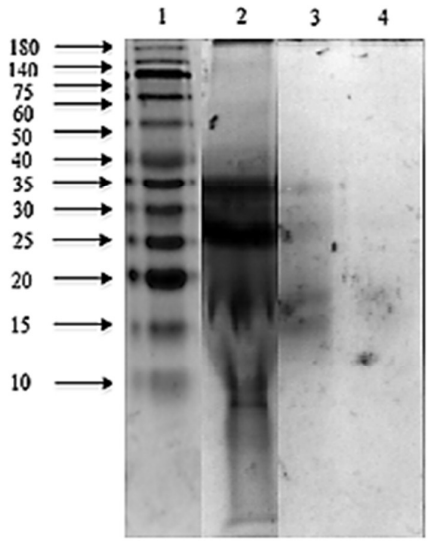

Figure 2. SDS-PAGE patterns of *Mucuna pruriens* protein concentrate and hydrolysates obtained with Alcalase®-Flavourzyme® and Pepsin-Pancreatin enzymatic systems. Lane 1: molecular weight markers (10 - 180 kDa); lane 2: *M. pruriens* protein concentrate; lane 3: Alcalase®-Flavourzyme® hydrolisate; lane 4: Pepsin-Pancreatin hydrolysate.

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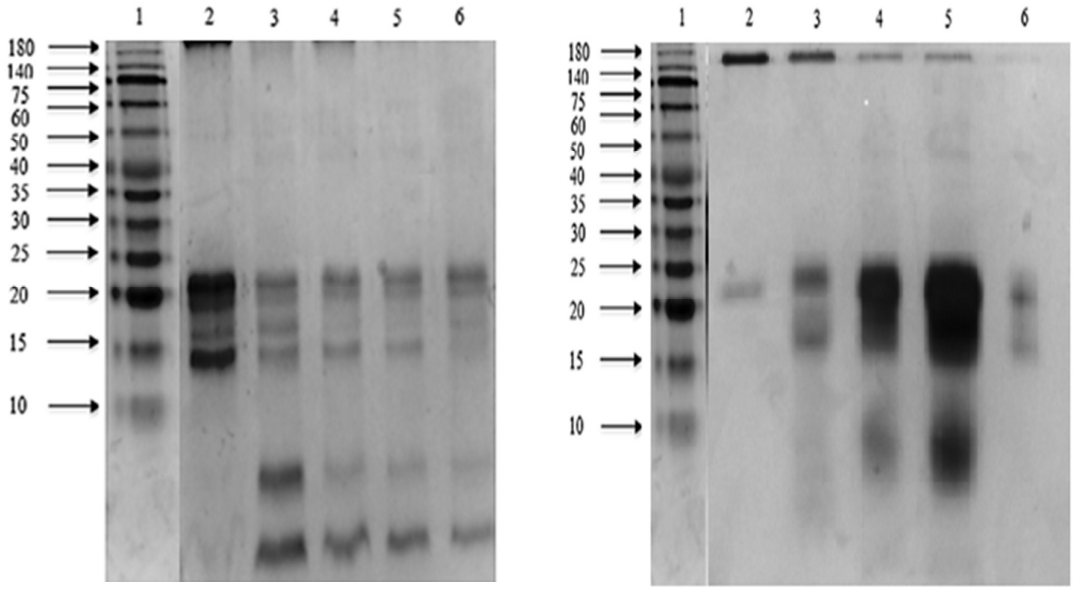

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680 a) b)

681 **Figure 3.** SDS-PAGE patterns of *Mucuna pruriens* ultrafiltered peptide fractions. a)
682 Ultrafiltered peptide fractions from Alcalase®-Flavourzyme® system. Lane 1: molecular
683 weight markers (10 - 180 kDa); lane 2: F > 10 kDa; lane 3: F 5-10 kDa; lane 4: F 3-5
684 kDa; lane 5: F 1-3 kDa; lane 6: F < 1 kDa. b) Ultrafiltered peptide fractions from
685 Pepsin-Pancreatin system. Lane 1: molecular weight markers (10 - 180 kDa); lane 2: F
686 > 10 kDa; lane 3: F 5-10 kDa; lane 4: F 3-5 kDa; lane 5: F 1-3 kDa; lane 6: F < 1 kDa.

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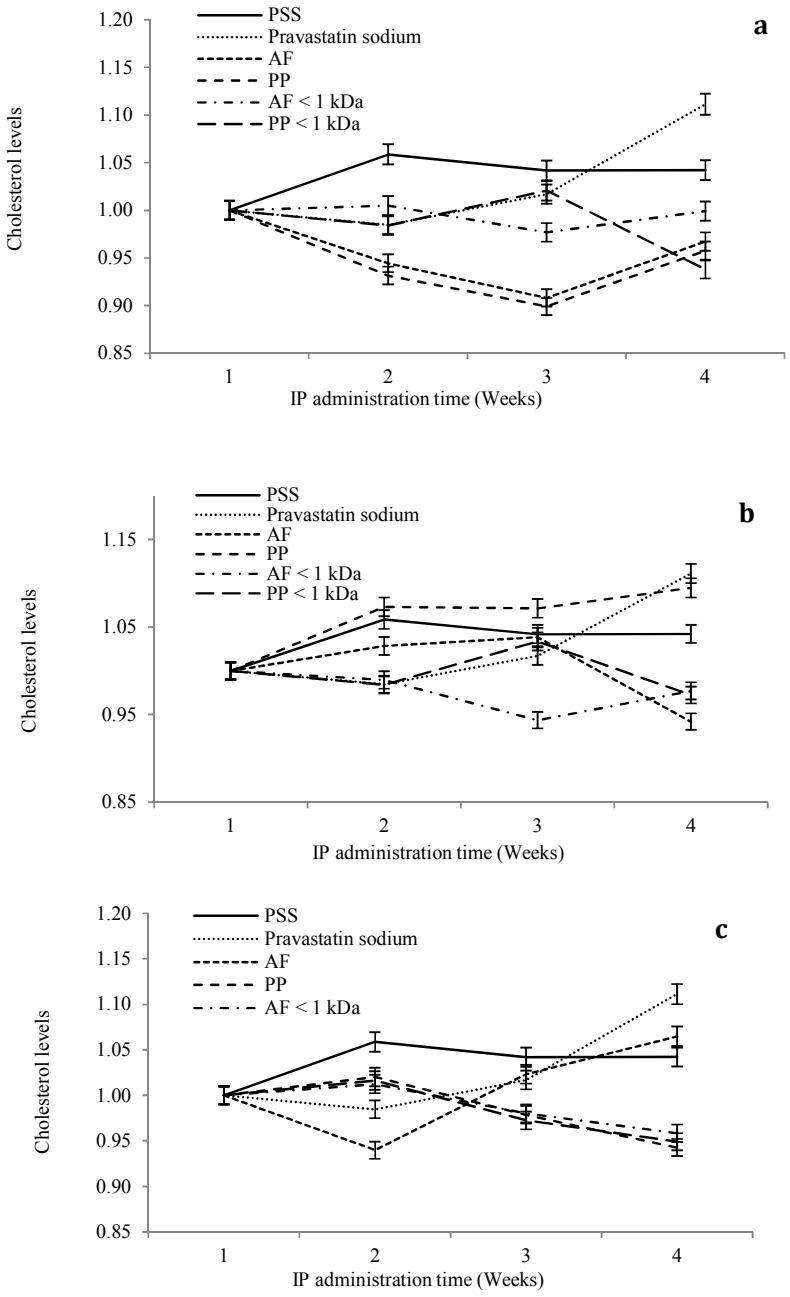

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698 **Figure 4.** Serum cholesterol levels in alloxan-induced diabetic rats for four weeks of IP

699 administration of hydrolysates and peptide fractions a) 5 mg/kg of animal weight; b) 10

700 mg/kg of animal weight; c) 15 mg/kg of animal weight. Saline physiological solution

(SPS); Alcalase®-Flavourzyme® hydrolysate (AF); Pepsin-Pancreatin hydrolysate (PP), Alcalase®-Flavourzyme® peptide fraction < 1 kDa (AF < 1 kDa); Pepsin-Pancreatin peptide fraction < 1 kDa (PP < 1 kDa).

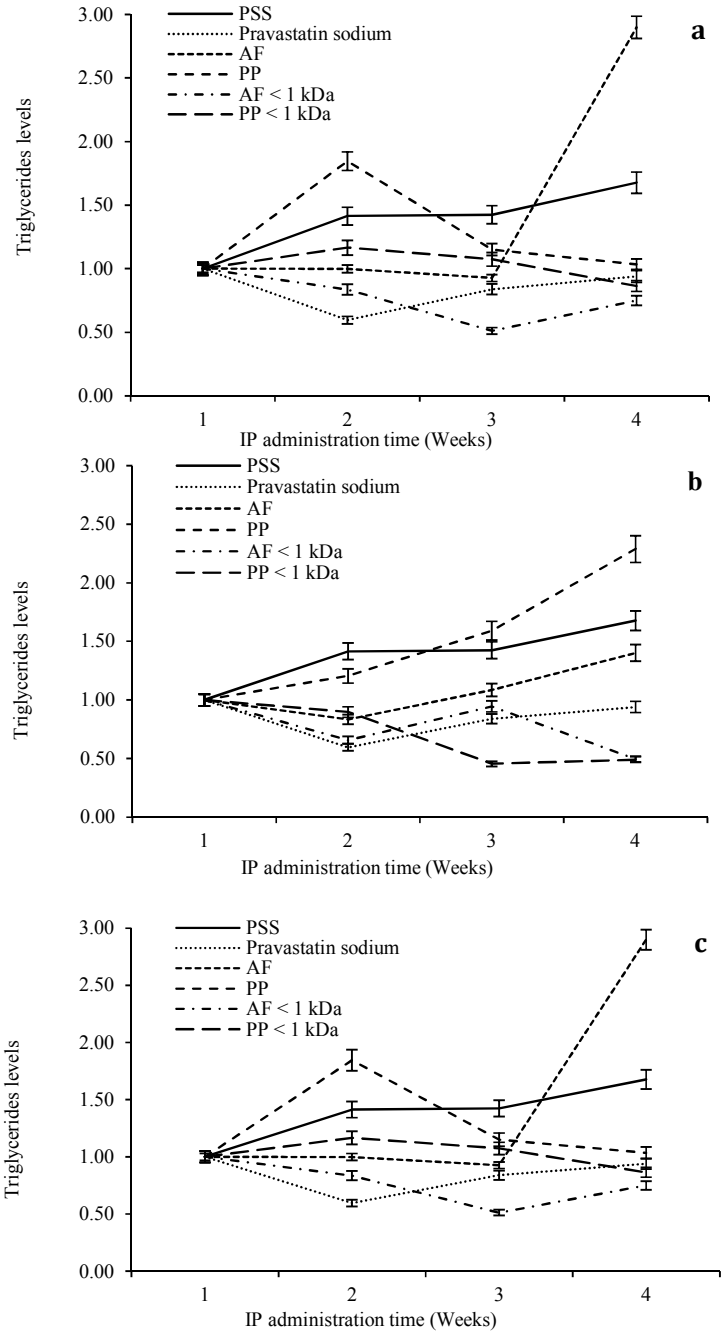

Figure 5. Serum triglycerides levels in alloxan-induced diabetic rats for four weeks of IP administration of hydrolysates and peptide fractions a) 5 mg/kg of animal weight; b) 10 mg/kg of animal weight; c) 15 mg/kg of animal weight. Saline physiological solution

710 (SPS); Alcalase®-Flavourzyme® hydrolysate (AF); Pepsin-Pancreatin hydrolysate (PP),
711 Alcalase®-Flavourzyme® peptide fraction < 1 kDa (AF < 1 kDa); Pepsin-Pancreatin
712 peptide fraction < 1 kDa (PP < 1 kDa).