# A Novel Trypsin and α-Chymotrypsin Inhibitor from *Maclura pomifera* Seeds

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**Abstract:** A new peptidic protease inhibitor (MpI) has been isolated from *Maclura pomifera* seeds, being the first trypsin and chymotrypsin inhibitor from a species belonging to the family Moraceae. MpI was purified by acetone precipitation, gel filtration and ion exchange chromatography, successively, with purification factors of 112 and 109 for the aforementioned enzymes, which are infrequent high values for inhibitors isolated from seeds. MpI showed a unique band in SDS-Tricine PAGE (Mr 11 kDa) and isoelectric focusing (pI = 5.2), inhibited the serine proteases trypsin and α-chymotrypsin (IC50 0.17 and 0.7 μg/ml, respectively), but not cathepsin B (cysteine protease), cathepsin D (aspartic protease) nor carboxypeptidase A (metallo protease). The N-terminal sequence was determined (AREPKFSTHCEEEESR) but no homology was detected with other peptide inhibitors isolated from seeds. Preliminary assays related to blood clotting reactions showed that the isolated inhibitor significatively increased the activated partial thromboplastin time (APTT), suggesting its potential use in the treatment of blood coagulation disorders.

**Keywords:** *Maclura pomifera* seeds, Peptide trypsin inhibitor, Peptide α-chymotrypsin inhibitor, Moraceae.

## INTRODUCTION

Protease inhibitors (PI) have been found in multiple forms in many animals and plant tissues, as well as in microorganisms [1,2]. Their physiological function, in general, is related to the prevention of unwanted proteolysis or to the control/ termination of specific protein degradation processes. Proteolytic activity is one of the most important biological function in living organisms, because it is involved in practically all life cycle of a protein, that is, its biosynthesis, control of destiny, activity and degradation (metabolic turnover), tissue and organ development and reshaping. In addition, proteases have been shown to be essential for a variety of viruses and other pathogenic microorganisms. Thus, protease inhibitors (PIs) are also involved in many aspects of normal and altered physiological processes [3-7].

The major incentive in inhibitor research is that control of limited proteolysis represents a valuable therapeutical tool. PIs have proven to be succesful in influencing pathogenesis in many experimental models, which demonstrate their great potentialities as drugs. Among those more successful are the synthetic inhibitors of ACE and renin (two proteolytic enzymes, the former is a metalloprotease and the latter is an aspartic one), which gave rise to efficient drugs for the regulation of blood pressure. Also very useful in therapy have been the synthetic inhibitors of HIV-PR (the aspartic protease of HIV) for AIDS, and of thrombin for blood coagulation disorders. In the latter case, the natural inhibitor hirudin, is noteworthy the natural inhibitor hirudin, isolated from the medicinal leech *Hirudo medicinalis*, which is the most potent anticoagulant and antithrombotic agent knew up to now and it is the best example of the superior protein tailoring made by nature which surpasses most of the man made inhibitors [8].

Plants are particularly good sources of protease inhibitors [2], as these compounds protect against diseases, pests, and consumption by herbivores. It is common to find several PIs present in the same tissue and species, presumably acting synergistically as an integrated defense system. Diversity and abundance of PIs in plants make them excellent sources for discovering novel protease inhibitors with specific pharmacological effects [9]. For example, a natural plant protease inhibitor with human activity has been extracted from *Leucaena leucocephala* seeds; this peptide (L1TI) inhibits human plasmin and exhibits anticoagulant properties *in vivo* [10]. BBI, a well-characterized soybean peptide, has exhibited broad anticancer activities in clinical trials and cell-based assays. Soybean extract enriched with BBI has been shown *in vitro* to inhibit tumor cell proliferation, invasion and survival in several models of prostate cancer without adversely affecting normal cells [11]. The chemopreventive properties of BBI have been attributed to proteasome inhibition, presumably *via* an antichymotrypsin mechanism [12]. Additionally, protease inhibitors from plants potently inhibited the growth of a variety of pathogenic bacterial and fungal strains and are therefore excellent candidates for use as the lead compounds for the development of novel antimicrobial agents [13].

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The present paper deals on the isolation, purification and partial characterization of a new trypsin-chymotrypsin inhibitor from *Maclura pomifera* seeds. The species belongs to the family Moraceae, comprising about 40 genera and over 1000 species, most of them widespread in tropical and subtropical regions [14].

## EXPERIMENTAL

### Plant Material

Fruits of *Maclura pomifera* (Raf.) Schneid*. (Moraceae)* were collected in May 2007 in La Plata, Buenos Aires province. Individual fruits were carefully cleaned with distilled water, chopped and seeds manually harvested and stored at -20 ºC.

A voucher specimen was deposited by Dr. Ana María Arambarri in the Herbarium of Área de Botánica, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Argentina, under the accession code LPAG 5445.

### Chemicals

Bovine serum albumin, N-benzoyl-D,L-tyrosine p-nitroanilide (BTPNA), Carboxypeptidase A (CPA, C9268) Cathepsin B (C6286), Cathepsin D (C3138), α-Chymotrypsin (C4129), Hemoglobin (H2625), Trypsin (T8802) and Trypsin inhibitor from *Glycine max* (soybean) (T9128), was purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). Benzyloxycarbonyl-L-arginine-L-arginine-4-methyl-7-coumarylamide (Z-Arg-Arg-Mec), N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and N-(4-methoxyphenylazoformyl)-Phe-OH (Aaf-Phe-OH) were purchased from Bachem AG (Torrance, CA, U.S.A.). Coomassie brillant blue R-250, acrylamide, and bisacrylamide were obtained from Bio-Rad (Hercules, CA, U.S.A.). Molecular weight standard was purchased to GE Healthcare-Bioscience AB (Uppsala, Sweden). Other reagents used were of the highest grade available.

### Crude Extract Preparation

Seeds (50 g) were extensively washed with distilled water and crushed in an Omni Mixer (Sorval) with 250 ml of 50 mM Tris-HCl buffer (pH 7.2) containing 0.15 M NaCl, by applying six pulses of 10 s separated by 30 s intervals. Homogenates were centrifuged at 16,000 *g* for 30 min at 4 ºC; the resulting supernatant was filtered to obtain the crude extract (CE).

### Partial Purification

As crude extracts contained phenolic compounds, which could oxidize and irreversibly react with proteins and peptides, a previous treatment was carried out with organic solvent (acetone). One volume of crude extract was treated with four volumes of cold (−20 °C) acetone with gentle agitation, the suspension was settled for 24 h at −20 °C and then centrifuged at 16,000 *g* for 30 min at 4 ºC. The precipitate was redissolved in 125 ml of 50 mM Tris-HCl buffer (pH 7.2); this preparation, named "redissolved acetone precipitate" (RAP), was freeze-dried and stored.

### Trypsin Inhibition Assay

The antitrypsin activity of bovine trypsin was assayed using the chromogenic synthetic substrate BAPNA in 0.05 M Tris-HCl buffer (pH 8.0), containing 0,04 M $CaCl_2$ at 37 ºC according to Erlanger *et al*. [15] with minor modifications. Briefly, 100 µl of trypsin (13 µM) solution and 100 µl of sample or buffer (as blank) were incubated for 1 min at 37 ºC, then 750 µl of buffer was added and the assay was initiated by the addition of 50 µl of 20 mM BAPNA in DMSO. The reaction rate was determined by measuring the absorbance at 410 nm for 3 min. One unit of trypsin activity was defined as the increase of 0.1 absorbance units at 410 nm per min and one unit of inhibitory activity (TIU) was defined as the decrease of 0.1 absorbance units at 410 nm per min, in the assay conditions.

### Chymotrypsin Inhibition Assay

The antichymotrypsin activity of bovine α-chymotrypsin was assayed using the chromogenic synthetic substrate BTPNA in 0.05 M Tris-HCl buffer (pH 7.8), containing 0.04 M $CaCl_2$ at 37 ºC according to Tasneem *et al*. [16] with minor modifications. Briefly, 100 µl of chymotrypsin solution (20 µM) and 100 µl of sample or buffer (as blank) were incubated for 1 min at 37 ºC, then 700 µl of buffer was added and the assay was initiated by the addition of 100 µl of 1 mM BTPNA in dimethylformamide. The reaction rate was determined by measuring the absorbance at 410 nm for 3 min. One unit of trypsin activity was defined as the increase of 0.1 absorbance units at 410 nm and one unit of inhibitory activity (ChIU) was defined as the decrease of 0.1 absorbance units at 410 nm, in the assay conditions.

### Cathepsin B Inhibition Assay

The cathepsin activity was determined using the spectrofluorometric method of Sarath *et al*. [17]. The specific cathepsin B fluorimetric substrate Z-Arg-Arg-Mec was employed and its hydrolysis was monitored using the excitation wavelength 380 nm and the emission wavelength 460 nm, the reaction was recorded for 3 min. For the inhibition experiments, the cathepsin activity was determinated after pre-incubation with the sample. Briefly, 100 µl of cathepsin B solution (85 nM) and 150 µl of sample or buffer (as blank) were incubated for 1 min at room temperature, then 2.5 ml of phosphate buffer pH 6.5, containing 4 mM EDTA and 1 mM DTT was added and the assay was initiated by the addition of 50 µl of 0.2 mM Z-Arg-Arg-Mec in DMSO.

### Cathepsin D Inhibition Assay

The Cathepsin D activity assay is based on the hydrolysis of acid denatured hemoglobin [18]. The reaction mixture contained 730 µl of 100 mM formic acid buffer (pH 3.3), 50 µl of substrate solution (2.5% hemoglobin) and 20 µl of cathepsin D (5 units/ml). After 10 min of incubation at 37 ºC the reaction was interrupted by adding 10% trichloroacetic acid, then each test tube was centrifuged at 4,000 g for 20 min and the absorbance of the supernatant was read at 280 nm. For the inhibition experiments, the cathepsin activity was determinated after pre-incubation with the sample.

### Carboxipeptidase A Inhibition Activity

The CPA activity assay is based on the hydrolysis of the substrate N-(4-methoxyphenylazoformyl)-Phe-OH (Aaf-Phe-OH) [19]. For the inhibition experiments, the CPA activity was determinated after pre-incubation with the sample. Briefly, 50 µl of CPA solution (12 µM), 100 µl of sample and 1.8 ml of buffer Tris-HCl 0,05 M, pH 7.5 containing 0.1 M sodium chloride and 50 µl of 10 mM Aaf-Phe-OH were incubated for 3 min at 37 ºC; the reaction progression is reflected by a decrease in absorption at 350 nm.

### Protein Estimation

Protein concentration was measured by the Bradford´s method [20] using bovine serum albumin (BSA) as standard. In chromatographic eluates, protein content was determined by measuring the absorbance of eluates at 280 nm.

### Denaturing Electrophoresis (SDS-PAGE)

Samples were subjected to denaturing electrophoresis in tricine gels composed of a stacking gel (4%T, 3%C), a spacer gel (10%T, 3%C) and a separating gel (16.5%T, 6%C), which is especially suitable to resolve peptides mixture [21]. The electrophoresis was performed in a Mini-Protean III dual slab cell (Bio-Rad, Hercules, CA 94547, USA). Electrophoresis was performed at room temperature using a voltage stepped procedure: voltage was kept constant (30 V) until the samples completely left the stacking gel and then voltage was increased 15 V per min for 4 times. Voltage was maintained constant (90-100 V) until the tracking dye reached the bottom of the gel. Gels were fixed for 30 min with a fixative solution containing methanol, acetic acid and water (5:1:4) and then stained with a Coomassie Brilliant Blue G-250 solution.

### Isoelectric Focusing

Isoelectric focusing (IEF) was developed on 5% polyacrylamide gels containing broad pH range ampholytes (Biolyte 3-10, Bio-Rad) in a Mini IEF Cell (Model 111, Bio-Rad). Samples were precipitated with 3 volumes of cold (-20ºC) acetone, centrifuged, the protein sediments redissolved and precipitated once again with acetone and finally redissolved in half a volume of deionized water. About 1-10 µg of protein was loaded in each case. Focusing was carried out under constant voltage conditions in a stepped procedure: 100 V for 30 min, 200 V for 15 min and 450 V for 60 min. Gels were fixed and then stained by Coomassie Brilliant Blue R-250.

### Chromatographic Purification

Size exclusion chromatography of the RAP, the first step of purification, was carried out onto a K15/30 column filled with Sephadex G50 Fine (GE Healthcare-Bioscience AB, Uppsala, Sweden). The proteins resolved into three main fractions. Fractions which exhibited inhibitory activity higher than 50% were pooled and submitted to anion exchange chromatography using an Äkta Purifier and a 5 ml HiTrap Q HP column (GE Healthcare-Bioscience AB), equilibrated with 50 mM Tris–HCI buffer, pH 7.2. After washing with the same buffer, the proteins were eluted with a 0–0.3 M NaCl linear gradient at a flow rate of 60 ml/h. Proteins were detected by monitoring the absorbance at 280 nm.

### N-Terminal Sequence

The N-terminal sequence was determined by Edman's automated degradation using an Applied Biosystems (Procise 492) peptide sequencer. Protein homology searches were performed using the BLAST network service [22].

### Coagulation Tests

Prothrombin time (PT), thrombin time (TT) and activated partial thromboplastin time (APTT) were measured by standard procedures [23, 24]. All incubations were performed in the presence of MpI to study the inhibitory activity on clotting time, in the control experiments 50 mM Tris–HCI buffer was used.

## RESULTS AND DISCUSSION

As far as we know, no information exists on protease peptide inhibitors isolated from seeds of species belonging to the family Moraceae, except a gene sequence from *Ficus carica* consigned in the MEROPS peptidase database [2]. A crude inhibitor preparation (CE) was obtained by saline extraction of ground seeds of *Maclura pomifera* (13.6 mg of protein/g of seed) showing inhibitory activity on trypsin and chymotrypsin (serine proteases), but not on cathepsin B (cysteine protease), cathepsin D (aspartic protease), nor on CPA (metalloprotease).

The CE was purified by acetone precipitation, gel filtration and ion exchange chromatography, successively (Table

**Table 1. Purification Scheme**

| Step | Total Protein[a] (mg) | Specific Activity[b] | | % Recovery (Protein) | Purification Factor | |
|---|---|---|---|---|---|---|
| | | T Inhibition (TIU/mg) | Ch Inhibition (ChiU/mg) | | T | Ch |
| CE | 135.85 | 67 | 13 | 100.00 | 1.00 | 1.00 |
| RAP | 72.15 | 82 | 24 | 53.11 | 1.44 | 1.90 |
| SEC | 11.4 | 750 | 361 | 8.39 | 11.20 | 28.30 |
| MpI | 0.9 | 7500 | 1396 | 0.67 | 112.00 | 109.30 |

[a]based on extraction of 10 g of *Maclura pomifera* seeds; [b]one unit of trypsin inhibitory activity (TIU) or chymotrypsin inhibitory activity (ChIU) was defined as the decrease of 0.1 absorbance units at 410 nm per min. CE: Crude extract: RAP: redissolved acetone precipitate; SEC: Size exclusion chromatography; MpI: purified inhibitor.

**1**). Acetone precipitation allowed to obtain a partially purified preparation devoided of pigments and soluble carbohydrates (RAP), containing about 53% of initial proteins. Fig. (**1**) shows the results of size exclusion chromatography of RAP, which afforded three fractions, only one presenting inhibitory activity against trypsin (fraction II). This fraction was submitted to anion exchange chromatography for further purification (Fig. **2**): a unique active fraction was obtained when a sodium chloride gradient was applied, exhibiting inhibitory activity against trypsin and α-chymotrypsin, and was denomined MpI, whereas the unretained fraction did not show inhibitory activity.

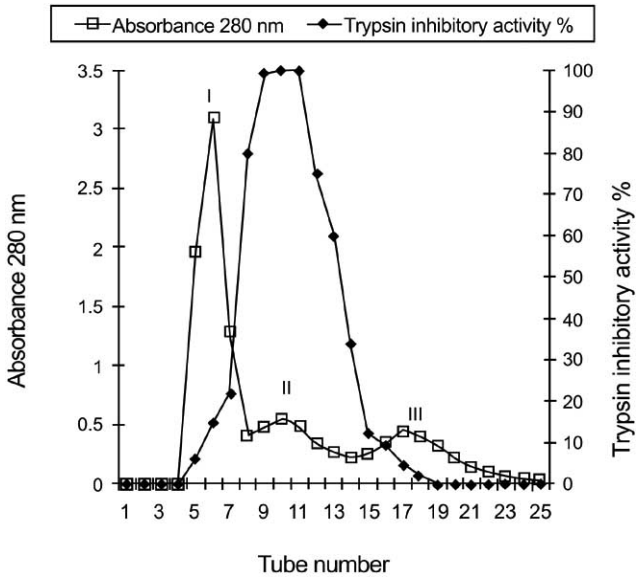


**Fig. (1).** Size exclusion chromatography (Sephadex G50 Fine) of RAP. Column diameter: 1.5 cm; column height: 30 cm; buffer: 50 mM Tris–HCI buffer, pH 7.2; flow rate, 1.0 ml/min. Fractions of 2 ml were collected.

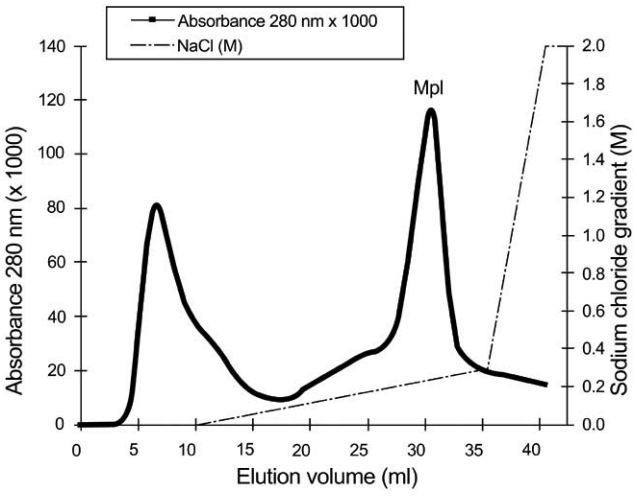


**Fig. (2).** Anion exchange chromatography (5 ml HiTrap Q-HP, column). Starting buffer: 50 mM Tris–HCI buffer, pH 7.2; elution saline gradient: 0.0 - 0.3 M of sodium chloride in the starting buffer. Flow rate, 1.0 ml/min. Fractions of 2 ml were collected.

The electrophoretic profile of each purification step is shown in Fig. (**3**). A similar pattern can be seen in CE and RAP, whereas SEC allowed the exclusion of high molecular weight proteins and IEC (MpI) produced a unique band of 11 kDa. Inhibitors of serine proteases with similar molecular weights has been isolated from seeds of dicotyledonous plants (like those belonging to the Bowman Birk inhibitors familiy), as well as the cereal trypsin/α-amylase inhibitors and those found in the family Cruciferae, as the mustard trypsin inhibitors [25].

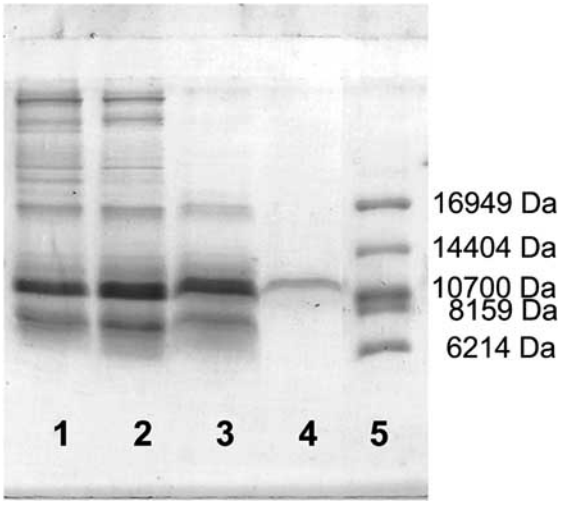


**Fig. (3).** SDS-Tricine-PAGE. Lane 1, CE; lane 2, RAP; lane 3 fraction II (size exclusion chromatography); lane 4, MpI; lane 5, Peptide Molecular Weight Markers (GE Healthcare, horse myoglobin peptides).

The purification process was also verified by means of the 50% inhibitory concentration (IC50), that is, the protein quantity necessary to reduce the enzyme activity to 50%. As can be seen in Fig. (**4**), a considerable reduction of IC50 for

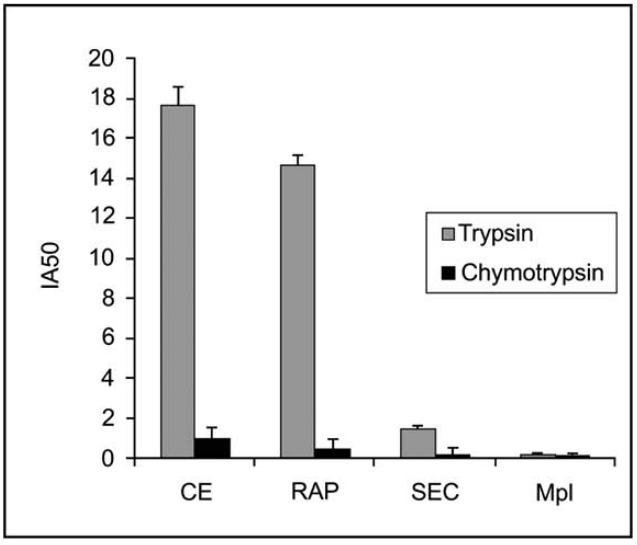


**Fig. (4).** IC50 of purification steps.

IC50: protein quantity of inhibitor (μg/ml) necessary to reduce the enzyme activity to 50%. Data points represent the mean value of four determinations and each experiment was repeated twice.

chymotrypsin and trypsin was determined from CE to IEC steps (from 84.4 to 0.7 and from 17.62 to 0.17 μg/ml, respectively), results coincident with those shown in the purification scheme (Table **1**), with purification factors of 109.3 and 112 for the aforementioned enzymes, that are unfrequent high values for inhibitors isolated from seeds [26]. For comparative purposes the IC50 was also determined for commercial Trypsin inhibitor from *Glycine max* (soybean), and the value obtained was 7.68 μg/ml.

Analyisis by IEF (Fig. **5**) confirmed the homogeneity of the isolated inhibitor, as well as its pI value (5.2). The acidic nature of the isoelectric point of MpI is a feature generally found in serine protease inhibitors isolated from seeds, such as the inhibitors from *Bauhinia variegata* [27], *Archidendron ellipticum* [28], *Dolichos biflorus* [29], *Derris trifoliata* [30], *Vigna unguiculata* [31], *Acacia victoriae* [32] and *Acacia plumosa* [33], belonging either to Bowman-Birk or Kunitz type.

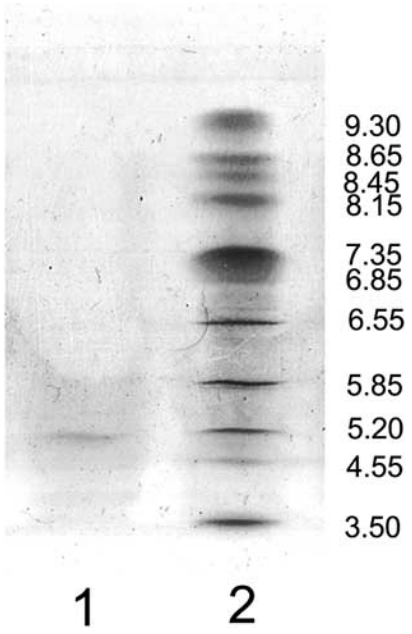


**Fig. (5).** IEF. Lane 1, MpI; lane 2, pI markers: amyloglucosidase (pI 3.50), trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), carbonic anhydrase II (pI 5.85), carbonic anhydrase I (pI 6.55), myoglobin (pI 6.85 and 7.35), lectins from *Lens culinaris* (pI 8.15, 8.45 and 8.65), and trypsinogen (pI 9.30).

The N-terminal sequence (AREPKFSTHCEEEESR) was compared by means of the SIB BLAST Network Service (http://www.expasy.ch/tools/blast/) with those of other protease inhibitors, but no homology was detected, probably because MpI is the first peptide inhibitor isolated from seeds of a species belonging to the family Moraceae, which is not phylogenetically close to Fabaceae, source of most seed inhibitors up to date studied.

Preliminary assays related to blood clotting reactions (Table **2**) showed that the novel inhibitor ("MpI") significatively increased the activated partial thromboplastin time (APTT) but not the thrombin time (TT) nor the prothrombin time (PT), suggesting its potential use in the treatment of blood coagulation disorders. The action of MpI on enzymes that participate in the blood clotting extrinsic pathway is confirmed by the prolongation of activated partial thromboplastin time, a similar behavior to that reported for the protease inhibitor (L1TI) extracted from *Leucaena leucocephala* seeds [10]. Complementary assays are being carried out to stablish which coagulation factors are specifically inhibited by MpI.

**Table 2. Coagulation Tests**

| Sample | PT | APTT | TT |
|---|---|---|---|
| Human plasma | 14.0±0.9 | 33.7±1.2 | 19.3±0.6 |
| MpI | 13.5±0.8 | 66.7±3.1 | 20.0±2.0 |
| Control | 13.7±0.7 | 34.3±1.5 | 18.7±0.6 |

The clotting times (s) for human plasma were determined in the presence and absence of inhibitor. In control experiments MpI was replaced by 50 mM Tris–HCl buffer. Data represent the mean value of three determinations. PT: Prothrombin Time; APTT: Activated Partial Thromboplastin Time; TT: Thrombin Time.

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