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MICROBIOLOGY

Antileishmanial effects of *Crotalaria spectabilis* Roth aqueous extracts on *Leishmania amazonensis*

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Abstract: Fifteen polar extracts from leaf, seed, pod, stem, flower and root of *Crotalaria spectabilis* were prepared using aqueous systems, based on the principles of green chemistry, and showed different protease inhibitor (PI) activities on trypsin, papain, pepsin and the extracellular *L. amazonensis* serine protease (LSPIII). The most pronounced inhibitory effect on LSPIII was observed in leaf (CS-P), root, stem, flower (CS-FPVPP) and pod (CS-VA) extracts. *Crotalaria* extracts exhibited low cytotoxicity on macrophages; however, they decreased the viability of *L. amazonensis* promastigotes and amastigotes, as observed in leaf (CS-AE, CS-P, CS-T and CS-PVPP), seed (CS-ST), flower and root (CS-RA) extracts. CS-P was chosen to study PI and secondary metabolites and a 10-12 kDa protein, analyzed by mass spectrometry, was identified as a serine PI homologous with papaya latex serine PI. Glycosylated flavonoids, such as quercetins, vitexin and tricin were the major secondary metabolites of CS-P. The presence of PIs in *C. spectabilis* is a new finding, especially in other organs than seeds since PIs have been reported only in seed legumes. Besides, this is the first report of antileishmanial activity of *C. spectabilis* extracts and the identification of serine polypeptide PI and glycosylated flavonoids from leaf.

Key words: *Crotalaria spectabilis*, aqueous extracts, protease inhibitors, *Leishmania* proteases, *Leishmania* viability, flavonoids.

INTRODUCTION

Leishmania sp. are a group of protozoan parasites responsible for a spectrum of chronic diseases, ranging from self-healing cutaneous lesions to mucocutaneous skin ulcers and a long-lasting diffuse cutaneous leishmaniasis in cell-mediated immune response-deficient hosts and to a lethal visceral form (i.e., visceral leishmaniasis or kala-azar) and post-kala-azar dermal leishmaniasis (Gradoni 2018). Leishmaniasis is one of the most significant neglected diseases and is endemic in the tropical and subtropical regions of America, Asia, Africa and Europe. Its incidence is increasing, no effective human vaccine is available, and programs of vector control and drug therapy are the main strategies used to deal with the disease (WHO 2022). Currently, chemotherapy is the only treatment available for these diseases and pentavalent antimony compounds have been the mainstays of antileishmanial therapy since the 1940s. Although these drugs are usually effective, they produce serious side effects and are difficult to administer, the treatment is costly, the parasite persists in the scars of clinically cured patients, and drug resistance has been observed (Garza-Tovar et al. 2020). Second-line

drugs have been used in areas with high rates of unresponsiveness to antimonial treatment. However, they are even more toxic and expensive than antimonials and have a lower therapeutic index (Blum et al. 2018, Palić et al. 2022). New therapies for leishmaniasis are currently being investigated and strategic chemotherapeutic targets identified via rational drug development (Novais et al. 2021).

While *Leishmania* is in the host, many changes are induced in parasite metabolism and enzymes are expressed and secreted, including proteases that catalyze the hydrolysis of peptide bonds, which irreversibly modify the structure and function of proteins and peptides (Silva-López 2012). They comprise seven protease families known as the asparagine, aspartic, cysteine, glutamic, metallo, serine and threonine proteases (Rawlings & Bateman 2021). Proteases are important virulence factors and they have been identified in *Leishmania*, playing crucial roles in the life cycle, host-parasite interactions, and pathogenesis of leishmaniasis (Marshall et al. 2018). In this context, our group isolated and characterized three distinct serine proteases from intracellular and extracellular fractions of *Leishmania amazonensis* promastigotes (Silva-López & De Simone 2004a, b, Silva-López et al. 2005). Subsequently, synthetic serine protease inhibitors (PIs) and a natural polypeptide serine PI were assayed against promastigotes. Only the best serine PIs of LSPI, LSPII and LSPII were able to induce *Leishmania* death and parasite morphological changes, on the other hand, poor inhibitors of these serine proteases did not affect the viability and the morphology of promastigotes (Silva-López et al. 2007). Besides, the morphological changes induced by these PIs were observed in the cell site where proteases were localized (Silva-López et al. 2005). Therefore, the inhibition of *L. amazonensis* serine proteases LSPI, II and III by PIs induced the parasite death, and they are promising molecules with antileishmanial effect (Silva-López et al. 2007). The antileishmanial potential of PIs targeted to parasite serine proteases have been recognized by other authors (Paik et al. 2020, Gomes et al. 2022).

Plants produce antimicrobial substances that provide the first line of defense; among these, the most remarkable are PIs (Cohen et al. 2019, Karray et al. 2020). These polypeptides are broadly found in nature, mainly in plants, and form very stable and specific stoichiometric complexes with proteases (Silva-López 2009). PIs have been classified by the type of protease inhibited and are primarily defined as serine, cysteine, aspartic, or metallo PIs (Kellici et al. 2021). They are abundant in storage organs such as seeds and tubers, especially in the seeds of *Fabaceae* or *Leguminosae*, *Brassicaceae*, and *Poaceae*, as well as in tubers of *Solanaceae* (Hellinger & Gruber 2019). Serine PIs are the most studied and have been isolated from various *Leguminosae* seeds and mainly classified as Kunitz-type, Bowman-Birk-type, squash and serpin (Ferreira et al. 2019).

Many species of *Leguminosae* have been investigated to assess their inhibitory action with pathogen proteases (Rodríguez-Sifuentes et al. 2020). Species of the *Crotalaria* genus belong to the Fabaceae family and are found throughout the world, most of them being well adapted to tropical climates. They are erect annual herbs or woody shrubs, originated from India, and have been used for many purposes such as animal feed, control of soil nematodes, trap plants in endemic regions of dengue, nurse species during early reforestation, protection against soil erosion, and covers for control of weeds (Pacheco & Silva-López 2010). In addition, there are reports of *C. spectabilis* and *C. juncea* being used in small communities of South America and India to treat infectious diseases

(Maregesi et al. 2007). *Crotalaria* produces a diverse array of compounds which have various ecological, adaptive and defensive functions (Pacheco & Silva-López 2010, Scupinari et al. 2020). The activity of PIs in *Crotalaria* was first reported in *C. cratalia* seeds as an antitrypsin activity (Norioka et al. 1988). However, the first inhibitor was obtained from *C. paulina* seeds and was a 20-kDa single polypeptide chain characterized as a Kunitz-type trypsin inhibitor (Pando et al. 1999). Moreover, another 32-kDa trypsin inhibitor with two polypeptide chains was purified from seeds of *C. pallida*, which reduced the activities of digestive enzymes from the insect gut and killed lepidopterans and coleopterans, considered to be agricultural pests, in the larval stage (Gomes et al. 2005). These results indicated that *Crotalaria* PIs have potential activity against proteases of invading organisms. Thus, the aims of this work were: investigate the PI activity of aqueous extracts from *C. spectabilis* on reference proteases and *L. amazonensis* extracellular serine protease; evaluate the cytotoxicity effects of these extracts on macrophages and, *L. amazonensis* promastigotes and intracellular amastigotes; study the composition, of the extract with desirable activities, in terms of polypeptide serine PIs and flavonoids, the major class of secondary metabolites in *C. spectabilis* aqueous extracts.

Abbreviations

BSA, bovine serum albumin; CC_{50}, half maximal inhibitory concentration CpaTI, inhibitor of *C. pallida*; CS, *Crotalaria spectabilis*; CS-CA, aqueous stem extract from CS, CS-CPVPP, polyvinylpolypyrrolidone stem extract from CS; CS-EA, aqueous leaf extract from CS; CS-ED detergent leaf extract from CS; CS-FA, aqueous flower extract from CS; CS-FPVPP, polyvinylpolypyrrolidone flower extract from CS; CS-P, phosphate leaf extract from CS; CS-PVPP, polyvinylpolypyrrolidone leaf extract from CS; CS-RA, aqueous root extract from CS; CS-RPVPP, polyvinylpolypyrrolidone root extract from CS; CS-SP, phosphate seed extract from CS; CS-ST, Tris seed extract from CS; CS-T, Tris leaf extract from CS; CS-VA, aqueous pod extract from CS; CS-VPVPP, polyvinyl- polypyrrolidone pod extract from CS; EC, enzyme classification; IC_{50}, half maximal inhibitory concentration; PIs, protease inhibitors; SDS-PAGE, polyacrylamide gel electrophoresis containing sodium dodecyl sulfate; $_{L}$-TAME, *N*-r-tosyl-$_{L}$-arginine methyl ester; High-performance liquid chromatography-diode array detection/electrospray ionization mass spectrometry from HPLC-DAD-ESI/MS.

MATERIALS AND METHODS

Plants

Crotalaria spectabilis Roth organs were collected in the morning on sunny days from Campus Mata Atlântica of Fundação Oswaldo Cruz (FIOCRUZ) in the state of Rio de Janeiro, Rio de Janeiro city, Brazil (S:22°56´24.10"/WO:43°24´09.22"). The plant specimen was deposited in the Rio de Janeiro Botanical Garden, Rio de Janeiro, Brazil, under the number RB-488.839.

Parasites

L. amazonensis (IFLA/BR/67/PH8) were cultivated to obtain fractions III and serine protease. *L. amazonensis* (MHOM/BR/77/LTB/0016) was grown to perform the viability assays. All parasites were obtained from the Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

Preparation of *Crotalaria spectabilis* extracts

All extracts were prepared from fresh organs of *C. spectabilis* as previously described (Pacheco & Silva-López 2012). Briefly, leaves and flowers were powdered using N_2 and proteins were

extracted using different extraction systems for 2 h at 24 °C, with gentle stirring and supernatants were collected (10,000 x *g* for 30 min at 4 °C) and lyophilized. The extracts were obtained from leaves: an aqueous extract (CS-EA) using distilled water; a detergent extract (CS-ED) using 1% Triton X-100; a phosphate extract (CS-P) using 50 mM sodium phosphate (pH 6.5); a CS-PVPP extract using 50 mM sodium phosphate (pH 6.5) and 5% polyvinylpyrrolidone (PVPP); a Tris extract (CS-T) using 50 mM Tris-HCl (pH 7.5). From flowers, only aqueous (CS-FA) and PVPP (CS-FPVPP) extracts were obtained. Fresh seeds, pods, stems, and roots were homogenized in different extraction systems using a blender, and the supernatants obtained after centrifugation were lyophilized. From seeds CS-ST and CS-SP extracts were obtained using Tris-HCl and phosphate buffers, respectively, and from pods, stems and roots the aqueous extracts CS-VA, CS-CA and CS-RA, and the PVPP extracts CS-VPVPP, CS-CPVPP and CS-RPVPP, respectively, were obtained as described above. Protein content of plant extracts was determined by the Bradford method (1976), using bovine serum albumin (BSA) as a standard.

Leishmania culture conditions

Promastigotes of *L. amazonensis* (IFLA/BR/67/PH8) was maintained at 28 °C, in brain heart infusion medium [(BHI) Difco, USA] supplemented with 10% (v/v) heat-inactivated fetal-calf serum (SFB), 0.5 mg of hemin and 0.5 mg/L of folic acid for serine protease production. *L. amazonensis* (MHOM/BR/77/LTB 0016) was obtained from BALB/c mouse lesions and maintained at 26 °C, in Schneider´s insect medium (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated SFB, 100 µg/ml of streptomycin and 100 U/mL of penicillin. All experiments were performed with parasite cultures in logarithmic phase of growth (on day 4 of cultivation). Cell growth was estimated by counting the parasites in a Neubauer chamber (Silva-López & De Simone 2004a, b).

Preparation of Leishmania fractions and protease purification

The cultures (1 x 10^9 cells) were harvested by centrifugation (3,000 x *g* for 15 min at 4 °C), and the parasites were washed three times in cold PBS, pH 7.2 (3,000 x *g* for 15 min at 4 °C) and stored for further analysis. Solid $(NH_4)_2SO_4$ was added to the culture supernatants to 45% saturation. After gently stirring at 4 °C overnight, the suspension was centrifuged (12,000 x *g* for 60 min at 4 °C). The pellet was collected and ressuspended in 10 mM Tris-HCl (pH 7.5) and dialyzed overnight against 200 volumes of the same buffer at 4 °C. After removal of the insoluble material (10,000 x *g* for 60 min at 4 °C), the clear supernatant (fraction III) was obtained. This fraction III was loaded on to an aprotinin-agarose affinity column (2.5 mL, Sigma-Aldrich) previously equilibrated using 10 mM Tris-HCl, pH 7.5 containing 5 mM $CaCl_2$. After exhaustive washing (20 resin bed volumes) the active material was eluted with the same buffer with 1.5 M NaCl, without calcium. Fractions of 1 mL were collected on ice, and the Abs_{280nm} was monitored. The enzymatic activity of these fractions was assayed with *N*-r-tosyl-$_L$-arginine methyl ester ($_L$-TAME) as substrate, and serine proteases were obtained from *L. amazonensis* named LSPIII (Silva-López et al. 2005). Protein concentrations were estimated by the Bradford method as described before.

Inhibitory effect of *Crotalaria spectabilis* extracts on protease activity

Inhibitory assays were performed by pre-incubating the references proteases trypsin, papain and pepsin (1 mg/mL, Sigma-Aldrich) with 10 µg of protein from *C. spectabilis* extracts

for 30 min at 24 °C. The reaction commenced upon addition of the substrate casein (0.5 mg/mL) at 24 °C for 30 min with slightly agitation. The reactions were stopped with thrichloroacetic acid, and the Abs $_{280nm}$ was monitored. Trypsin, papain and pepsin are representative proteases of serine, cystein and aspartic classes, respectively. The buffers used in the inhibition assays were Tris-HCl pH 8.2, sodium phosphate pH 5.5 and sodium acetate pH 4.5 for trypsin, papain and pepsin, respectively.

The inhibitory activity of *C. spectabilis* extracts was assessed with LSPII and fractions III, both obtained from culture supernatant of *L. amazonensis*. Ten µg of protein from fraction III was incubated for 30 min with 10 µg of protein from *C. spectabilis* extracts at 24 °C in Tris-HCl pH 7.5. Then, 125 µM $_{L}$-TAME was added and the reaction proceeded for 15 min and the absorbance was measured at 247 nm. In all experiments controls were carried out in parallel using the same enzyme solutions free of extracts. Inhibition was expressed as a percentage of the control activity (100%). All buffers were used at 50 mM. The data represent the average and standard error of the mean of three independent experiments performed in quadruplicates.

Quantification of macrophage cytotoxicity

Resident macrophages were harvested from the peritoneal cavities of normal BALB/c mice in ice-cold RPMI supplemented with 1% glutamine and pyruvate. This study was performed in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (COBEA) and had the approval of the Animal Ethics Committe of Fundação Oswaldo Cruz (license number LW07/2010).

The cells were plated at 1.0 × 10^6/mL (0.4 mL/well) in 96-well plates at 37 °C under an atmosphere of 5% CO_2 for 1 h. Nonadherent cells were removed by washing with pre-warmed complete medium. Adherent macrophages were incubated with *C. spectabilis* extracts at concentrations ranging from 0 to 200 µg/mL at 37 °C. After 72 h of incubation, the culture supernatant was removed, and 200 µL of sterile PBS and 22 µL of 500 µM resazurin were added, and the *C. spectabilis* extracts (25, 50, 100 and 200 µg/mL) were incubated for an additional 3 h. The fluorescence was measured using a SpectraMax GEMINI XS spectrofluorometer (Molecular Devices, Silicon Valley, USA) at excitation and emission wavelengths of 560 nm and 590 nm, respectively. Cell viability (%) was calculated relative to the controls (0 and 100% of viability). The half maximal inhibitory concentration (CC_{50}) was determined by nonlinear regression analysis in the software GraphPad Prism 7.0 software. Differences were considered significant when $P < 0.01$. All data represent the average and standard error of the mean of three independent experiments performed in quadruplicates. The data were analysed by two-way ANOVA with the Bonferroni post-test (Andrade-Neto et al. 2021). All data represent the average and standard error of the mean of three independent experiments performed in quadruplicates.

Promastigote viability measurements by resazurin assay

The anti-*Leishmania* activity of extracts was evaluated using *L. amazonensis* promastigotes maintained at 26 °C in Schneider's medium supplemented with 10% fetal bovine serum. Tests were performed in 96-well plates at 26 °C with an initial inoculum of 4.0 x 10^5 parasites/mL and different concentrations of *C. spectabilis* extracts (0, 25, 50, 100 and 200 µg/mL). Cells in culture medium plus DMSO (0.5%) were used as control of viability. Parasite viability was assessed

by a fluorescent assay employing resazurin (Sigma-Aldrich). After 72 h of incubation, the culture supernatant was removed, and 200 µL of sterile PBS and 22 µL of 500 µM resazurin were added, and the samples were incubated for an additional 3 h. The fluorescence was measured as described before. The viable cells were estimated by counting in a Neubauer chamber after 72 h of incubation, and parasite viability (%) was calculated relative to the controls (0 and 100% of viability). The 50% inhibitory concentration (IC_{50}) was determined by nonlinear regression analysis in the software GraphPad Prism 7.0. Differences were considered significant when $P < 0.01$. Pentamidine isethionate (1.25-10 µM) was used as reference, which is a second-line drug for the management of antimonial-resistant visceral cases of leishmaniasis, and it was also used in the treatment of cutaneous leishmaniasis (Sereno et al. 2019). All data represent the average and standard error of the mean of three independent experiments performed in quadruplicates.

Quantification of *L. amazonensis* intracellular growth in macrophages

Resident macrophages were harvested from the peritoneal cavities of normal BALB/c mice in ice-cold RPMI supplemented with 1% glutamine and pyruvate. The cells were plated at 1.0×10^6/mL (0.4 mL/well) in Lab-Tek 8-chamber slides (Nunc) and incubated at 37 °C under an atmosphere of 5% CO_2 for 1 h. Nonadherent cells were removed by washing with pre-warmed complete medium. Stationary phase *L. amazonensis* promastigotes were added at a 3:1 parasite/macrophage ratio, and the cultures were incubated for a further 3 h. The cell monolayers were washed three times with pre-warmed complete medium to remove free parasites, and 0.4 mL of *C. spectabilis* extracts in complete RPMI at different protein concentrations (0, 12.5, 25, 50 and 100 µg/mL) was added for 72 h. Coverslips were stained with a quick Romanowsky-type stain (Panótico, Rápido, Laborclin, Pinhais, Brazil) according to the manufacturer's instructions. The number of intracellular amastigotes was determined by counting at least 200 macrophages per sample, and the results were expressed as percent inhibition relative to controls without *C. spectabilis* extracts. Peritoneal macrophage viability (%) was calculated as a percentage of the controls (0 and 100% of viability). The results were expressed as selective index (SI) (% infected cells × number of amastigotes/total number of macrophages) using pentamidine isethionate (1.25-10 µM) as a control. The 50% inhibitory concentration (IC_{50}) was determined by nonlinear regression analysis in the software GraphPad Prism 7.0. Differences were considered significant when $P < 0.01$ (Andrade-Neto et al. 2021). All data represent the average and standard error of the mean of three independent experiments performed in quadruplicates.

Protease inhibitor isolation by gel filtration

Sample of *C. spectabilis* extracts were subjected to column chromatography on Sephadex G-75 gel filtration column (23 x 2.4 cm, I.D., GE Healthcare) equilibrated with 100 mM sodium phosphate buffer (pH 7.6) containing NaCl 100 mM and calibrated with conalbumin (Mr 75000), ovalbumin (Mr 43000), ribonuclease A (Mr 13700) and aprotinin (Mr 6500). Fractions of 1.0 mL were collected (flow rate of 0.5 mL/min) and assayed for determination of PI activity against LSPIII and protein content (A_{280} nm) (Pando et al. 1999).

Protease inhibitor identification by mass spectrometry analysis

Active fractions from gel filtration showing higher than 50% of LSPIII inhibition were pooled and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

12 % according to the method of Laemmli (1970), using a Mini Protean Tetra Cell Systems (Bio-Rad) and the gels were silver stained using a Bio-Rad kit. For molecular mass characterization, molecular mass standards (250-10 kDa) from Bio-Rad were used.

Protein bands from the gels were sliced and digested with trypsin (Shevchenko et al. 2006) and analyzed by liquid chromatography coupled to mass spectrometry (LC-MS/MS). The peptide mixture was injected into a NanoACQUITY HPLC chromatographic system coupled to the LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source. Peptides were initially loaded onto a trap column 2.0 cm long (100 µm internal diameter) packed in-house with C18 resin (5 µm, 100 Å pore, Magic C18 AQ, Bruker-Michrom, Auburn, CA) and fractionated on a RP-HPLC column 30 cm long (75 µm internal diameter) using a linear gradient composed of 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in acetonitrile for 38 min. The eluted peptides were directly introduced to an LTQ Orbitrap XL MS for analysis. Precursor scans were performed in Orbitrap mass detector at resolution of 60,000 in a mass range from 300 m/z to 1700 m/z, while MS/MS scans were acquired in a linear trap analyzer. With exclusion of singly charged ions, up to ten of the most intense precursor ions were subjected to product ion scans using collision-induced dissociation with a normalized collision energy of 35.0. Moreover, MS/MS scans were only triggered for precursor ions having a minimum signal threshold of 10,000 counts. Precursors selected for MS/MS scans were dynamically excluded for 30 s from a repeated product ion scan within a ± 10 ppm mass error (Gonçalves et al. 2021).

The plant genome database was downloaded from NCBInr databank. Protein searches of the tandem mass spectra acquired on LTQ Orbitrap XL mass spectrometer were performed using Peaks Studio 8.5 software. A fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 20 ppm were used. All searches were performed using the following post translation modification: carbamidomethylation of cysteine, oxidation of methionine and deamidation of asparagine. Only peptides presenting score above 19 were considered as positive identification. False discovery rates were estimated through the PEAKS decoy fusion approach. A peptide-spectrum match FDR of 1% and protein identifications with at least 2 unique peptide were the criteria used to establish FDR values at peptide and protein levels ≤1% (Shevchenko et al. 2006).

Flavonoid identification by mass spectrometry analysis

The analysis were conducted using a high-performance liquid chromatography-diode array detection/electrospray ionization mass spectrometry (HPLC-DAD-ESI/MS). The system was equipped with an SLC10A controller, high-pressure binary pump LC-30AD, SIL-10AF auto sampler, SPDM10A PDA detector and column oven CTO-30A. A BEH C-18 column (100 x 2.1 mm, 1.7 mm) was used. The mobile phase consisted of formic acid (A) and acetonitrile (B) at a flow rate of 0.4 mL min-1 using the following gradients: 0.01–8.30 min, 5–25% of solvent B in A; 8.30–13.80 min, 25-80% of solvent B in A; 13.80–16.60 min, 80-5% of solvent B in A, and 16.60–20 min, 5% solvent B and 95% solvent A. The detection was done on a DAD detector was programmed in the range 190 - 600 nm with resolution at the level of 4 nm. The mobile phase was prepared, filtered through a 0.45 mm membrane filter (Millipore), and sonicated before use (Tostes et al. 2019). The MS analyses were performed with a photodiode array detector (model SPD-M20A) and coupled to a quadrupole mass spectrometer

MS ZQ (Micromass-Waters) equipped with an ESI source and an ion trap mass analyzer. The following parameters were set in the positive ion mode (by the decrease of a hydrogen) and operated according to defined conditions: capillary voltage 4.5 KV, cone voltage 40.0 V, extractor voltage 1.0 V, RF lens 1.0 V, source block temperature 220˚C, desolvation temperature 350˚C, N2 flow 10 L/min, and nebulizing pressure 5 bar. Mass acquisition was set in the range m/z 100 - 1000 (resolution 0.4).

RESULTS

Effect of *C. spectabilis* extracts on reference and Leishmania proteases

The reference proteases trypsin, papain and pepsin were employed for PI activity identification in plant extracts (Spelbrink et al. 2011). Trypsin activity was inhibited by extracts of all organs of *C. spectabilis*, especially the leaf (CS-P), seed (CS-ST and SP), root (CS-RA), flower (CS-FA and FPVPP) and pod extracts (CS-VA and CS-VPVPP), except for stem extracts (Table I). There is no reports about trypsin inhibitor in stems. However, bromelain cysteine protease inhibitors, are very well studied in stems of many species of pineapples, Ananas genus (Hatano et al. 2018).

Papain activity was completely abolished by seed, root, stem, flower and pod extracts; however, no interference with protease activity was observed for leaf extracts. The activity of pepsin inhibitors can be found in all organs of *C. spectabilis,* especially in the CS-RPVPP root extract, which completely inhibited pepsin activity (Table I).

Extracts from all organs of *C. spectabilis*, except flowers, inhibited the protease activity of *L. amazonensis* fraction III, and the most pronounced effect was observed for the aqueous stem extract (CS-CA). *C. spectabilis* extracts exhibited distinctive patterns of inhibition of LSPIII. CS-P, both root extracts, the stem extract CS-CPVPP, the flower extract CS-FPVPP and the aqueous pod extract CS-VA completely inhibited LSPIII. CS-CA reduced LSPIII activity by approximately 85%. The aqueous leaf extract, CS-AE, did not affect LSPIII, but reduced fraction III activity by approximately 20% (Table I).

Effect of *C. spectabilis* extracts on macrophage cytotoxicity

The CC_{50} was estimated to study the macrophage cytotoxicity of *C. spectabilis* extracts. The leaf detergent extract CS-DE was the most cytotoxic and at 12.5 µg/mL protein it killed 50% of the macrophages (Figure 1). Due to its high toxicity, it was removed from the study. Other leaf extracts showed minimal toxicity at the highest concentration assayed of 200 µg/mL. Macrophage viability was reduced in a concentration-dependent manner by both seed extracts and, at 200 µg/mL, the number of cultured cells was approximately 50%. All *C. spectabilis* extracts were less toxic to macrophages than pentamidine, a second-line drug for leishmaniais treatment (Sereno et al. 2019) that was used as control in our studies (Table II).

Although all *C. spectabilis* extracts were used at concentrations up to 200 µg/mL, it was not possible to calculate CC_{50} values for root, flower, stem, pod and leaf extracts because most of them did not reduce viability by approximately 50%, except for CS-DE, which was toxic at all concentration assayed. This was the maximal assayable extract amount (200 µg/mL) because it is dark brown and the higher extract concentrations interfere with the cytotoxicity assays which are colorimetric.

Table I. Effect of *C. spectabilis* extracts on references and *Leishmania amazonensis* proteases activities.

Crotalaria spectabilis extracts[a]	Inhibition (%)[b]				
	Trypsin	Papain	Pepsin	*L. amazonensis* Fraction III	*L. amazonensis* LSP III
CS-AE	0.00 ± 0.8	0.00 ± 1.2	38.47 ± 2.5	19.84 ± 1.7	0.00 ± 0.0
CS-DE	0.00 ± 0.0	0.00 ± 5.5	76.93 ± 2.8	19.73 ± 2.1	19.00 ± 0.6
CS-PVPP	0.00 ± 0.0	0.00 ± 0.2	0.00 ± 10.0	30.73 ± 0.8	56.31 ± 2.6
CS-T	0.00 ± 0.1	0.00 ± 1.3	9.62 ± 0.5	0.00 ± 0.0	60.2 ± 5.5
CS-P	56.60 ± 1.1	0.00 ± 0.2	0.00 ± 0.1	0.00 ± 0.6	100.00 ± 1.1
CS-ST	23.92 ± 2.4	100.00 ± 0.0	21.43 ± 3.1	17.32 ± 4.8	47.81 ± 8.5
CS-SP	19.57 ± 0.8	100.00 ± 0.0	17.86 ± 1.2	6.52 ± 1.5	38.00 ± 2.6
CS-RA	39.13 ± 2.9	100.00 ± 0.0	0.00 ± 0.5	16.49 ± 3.2	85.00 ± 7.5
CS-RPVPP	0.00 ± 0.0	100.00 ± 0.1	100.00 ± 0.0	0.00 ± 0.0	100.00 ± 5.8
CS-CA	0.00 ± 0.0	100.00 ± 0.0	42.86 ± 3.4	80.00 ± 9.8	84.62 ± 13.5
CS-CPVPP	0.00 ± 0.0	100.00 ± 0.0	67.86 ± 0.9	0.00 ±0.0	100.00 ± 9.0
CS-FA	21.74 ± 1.7	100.00 ± 0.0	64.29 ± 3.6	0.00 ± 0.5	29.42 ± 2.7
CS-FPVPP	50.00 ± 0.9	100.00 ± 0.0	0.00 ± 4.5	0.00 ± 0.0	100.00 ± 7.6
CS-VA	51.20 ± 5.3	63.90 ± 7.8	19.24 ± 2.8	0.00 ± 0.0	81.00 ± 8.7
CS-VPVPP	70.00 ± 6.5	41.67 ± 6.3	9.24 ± 1.2	0.00 ± 0.0	0.00 ± 0.5

[a]Ten µg of protein from extracts were pre-incubated with each protease for 30 min at 24°C. [b]Inhibition was assayed by incubation with substrates (casein or $_L$-TAME). Values are inhibition of protease as a percentage of the activity on substrate without *C. spectabilis* extracts, as described in Materials and methods section and represent the average of 3 separate experiments carried out in quadruplicates.

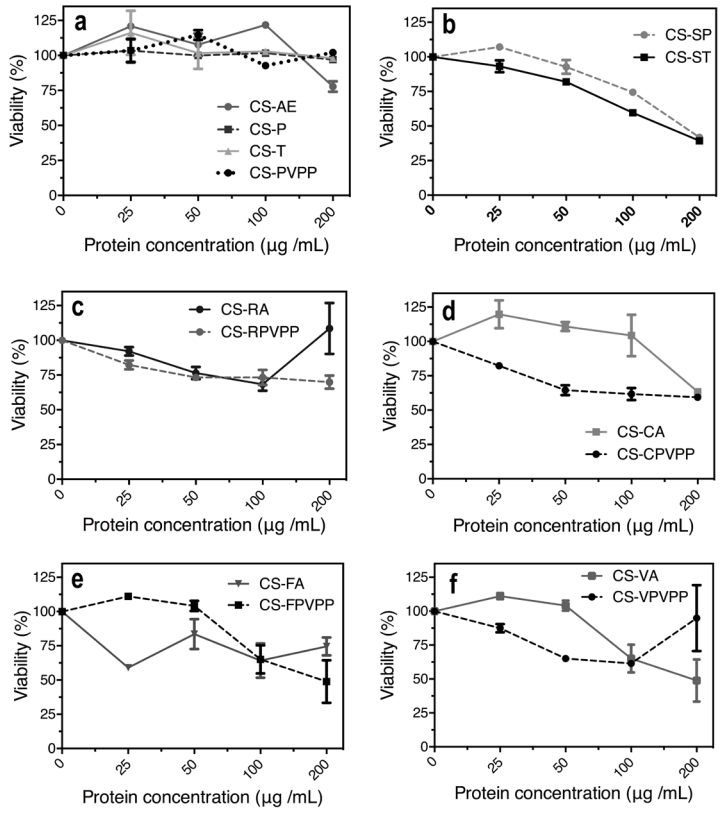

Figure 1. Effects of *Crotalaria spectabilis* extracts protein concentration on macrophage cytotoxicity. (a) Leaf extracts; (b) seed extracts; (c) root extracts; (d) stem extracts; (e) flower extracts; (f) pod extracts.

Table II. Antileishmanial activity, cytotoxicity, and selectivity index for *Crotalaria spectabilis* extracts.

Crotalaria spectabilis extracts	CC_{50} (µg/mL) M_{ϕ} peritoneal murine	IC_{50} (µg/mL) *Leishmania amazonensis* promastigotes	IC_{50} (µg/mL) *Leishmania amazonensis* amastigotes	Selectivity Index (SI)
CS-AE	>200.00	16.30 ±12.0	22.17 ± 1.3	ND
CS-DE	12.50 ± 0.5	ND	ND	ND
CS-T	>200.00	33.03 ± 2.8	13.91 ± 17.9	ND
CS-PVPP	>200.00	27.79 ± 3.9	71.61 ± 8.7	ND
CS-P	>200.00	24.35 ± 2.6	31.74 ± 3.0	ND
CS-ST	150.00 ± 2.2	31.50 ± 0.9	81.65 ± 5.8	1.84
CS-SP	175.00 ± 1.5	>200.00	ND	ND
CS-RA	>200.00	33.90 ± 8.2	>100.00	ND
CS-RPVPP	>200.00	61.00 ± 3.3	ND	ND
CS-CA	>200.00	>100.00	ND	ND
CS-CPVPP	>200.00	48.69 ± 2.5	52.11± 28.3	ND
CS-FA	>200.00	36.12 ± 7.8	91.98 ± 5.0	ND
CS-FPVPP	>200.00	32.36 ± 3.5	92.00 ± 31.0	ND
CS-VA	>200.00	>200.00	ND	ND
CS-VPVPP	>200.00	>200.00	ND	ND
Pentamidine	9.8 ± 1.0	4.2 ± 0.3	2.2 ± 0.1	4.5

ND Not determined.
(SI) = CC_{50} in macrophages/IC_{50} in intracellular amastigotes.

Effect of *C. spectabilis* extracts on Leishmania viability

All extracts showed different levels of promastigotes cytotoxicity, and their effect was dose-dependent (Figure 2). Leaf extracts were the most effective and CS-AE was the best. Although CS-T, CS-PVPP and CS-P were not as effective as CS-AE, they were important inhibitors of LSPIII, particularly CS-P, which completely abolished the activity of this protease (Table I). CS-RA from root, CS-CPVPP from stem and flower extracts also expressively reduced the viability of *Leishmania* promastigotes. CS-SP from seed, CS-CA from stem and both pod extracts did not reduce substantially promastigote viability. The *C. spectabilis* extracts induced changes in promastigotes morphology. Extracts that inhibited LSPIII activity induced a significant change in parasite shape from elongated to rounded, in addition to the formation of cytoplasmic vacuoles. These morphological changes were concentration dependent, as demonstrated by CS-P (Figure 3). All *C. spectabilis* extracts were less toxic to *L. amazonensis* promastigotes than pentamidine, employed as control (Table II).

Mouse peritoneal macrophages infected with *L. amazonensis* were incubated for 72 h with different concentrations of *C. spectabilis* extracts (Figure 4). The leaf and flower extracts and root and stem PVPP extracts were selected for the anti-amastigote assays because of their strong inhibition of LSPIII activity and/or anti-promastigote effect. CS-ST was also chosen because *Leguminosae* seeds are known sources of PIs (Ferreira et al. 2019). All extracts decreased the number of amastigotes inside the

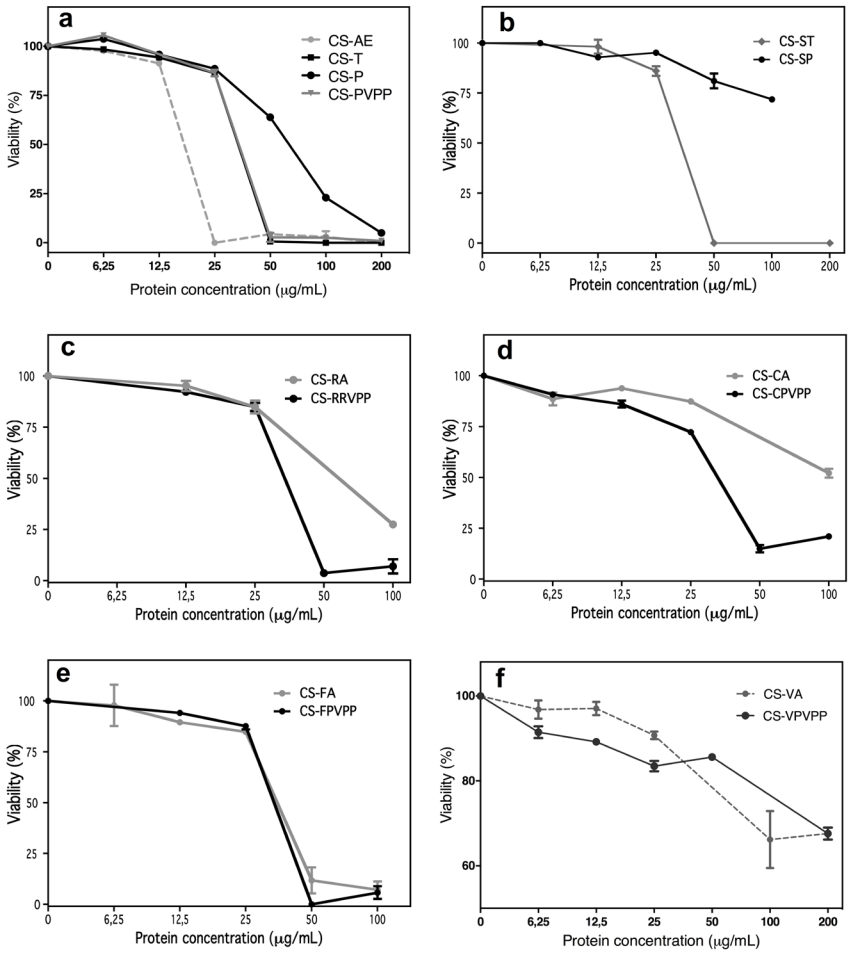

Figure 2. Effects of *Crotalaria spectabilis* extracts protein concentration on *L. amazonensis* promastigotes viability. (a) Leaf extracts; (b) seed extracts; (c) root extracts; (d) stem extracts; (e) flower extracts; (f) pod extracts.

macrophages in a dose-dependent manner, and the most effective were CS-T (IC_{50} = 13.91 mg/mL), CS-AE (IC_{50} = 22.17 mg/mL) and CS-P (IC_{50} = 31.79 mg/mL) (Table II). The effect of *C. spectabilis* extracts on amastigote growth was also monitored by optical microscopy, revealing that the parasite number inside the phagolysosomes was reduced by increasing extract protein concentrations (Figure 5). Parasite-free macrophages were commonly observed at the highest extract doses, especially for CS-P, demonstrating the expressive leishmanicidal effect of *C. spectabilis* extracts. Vacuoles inside the cytoplasm were also found in macrophages treated only with leaf extracts. However, these morphological changes did not affect the viability of phagocytes because the extracts used in the anti-amastigote assays and their concentrations were not cytotoxic for these cells.

Serine protease inhibitor isolation and identification by mass spectrometry

CS-P was selected to investigate serine PIs because it completely abolished LSPIII activity, with low macrophage cytotoxicity and important *L. amazonensis* cytotoxicity. Thus, it was submitted to gel filtration chromatography and 105 fractions (1 mL) were collected and pooled into thirty new fractions (I to XXX) (Figure 6). The inhibitory activity against LSPIII was evaluated in fractions I to XXV (Table III). Fractions XVI, XVII, and XVIII, which showed higher than 50% LSPIII inhibition, were analyzed by MS to identify serine PIs. They were first fractionated by 12% SDS-PAGE

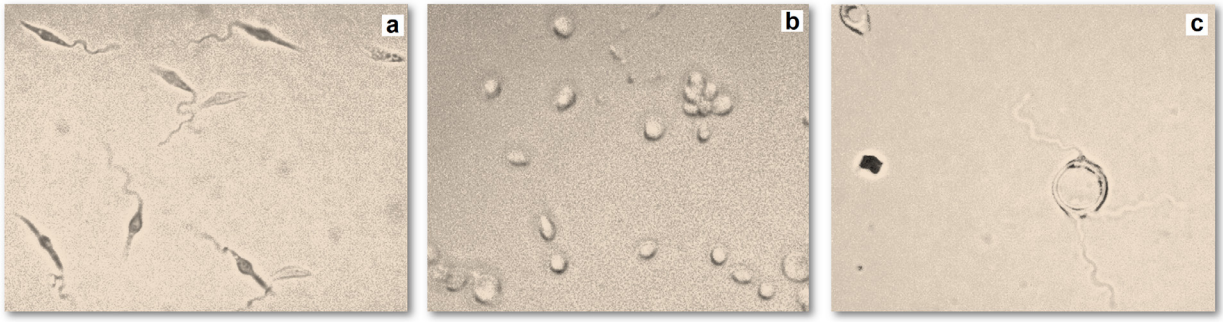

Figure 3. Effects of CS-P on *L. amazonensis* promastigotes by optic microscopy (100 x). (a) Control (untreated); (b) 50 µg of CS-P protein/mL; (c) 100 µg of CS-P protein/mL.

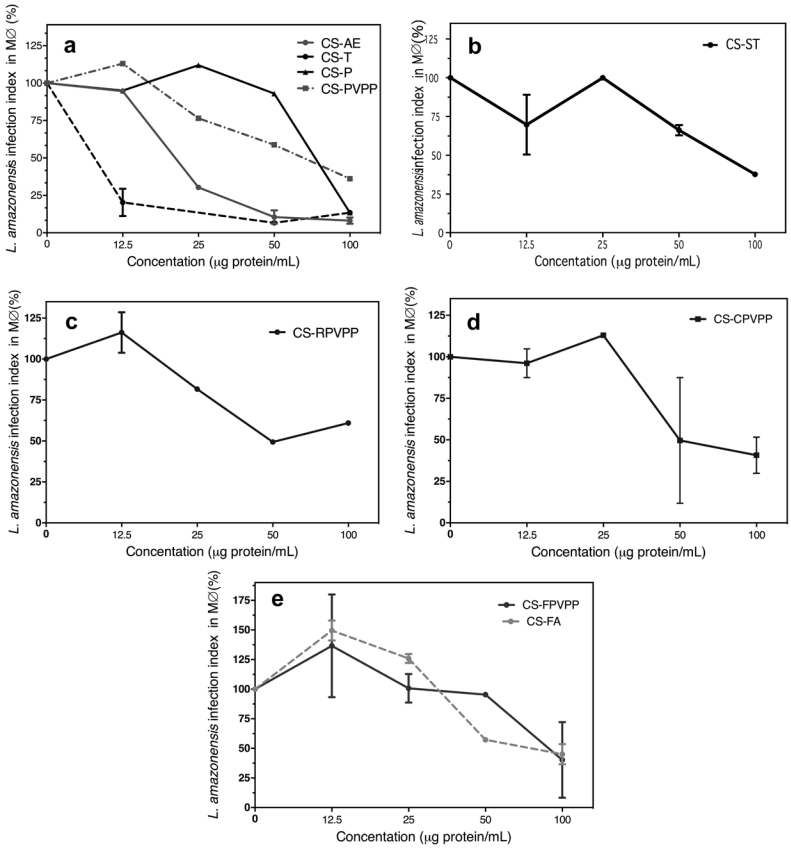

Figure 4. Effects of *Crotalaria spectabilis* extracts protein concentration on intracellular *L. amazonensis* amastigotes viability. (a) Leaf extracts; (b) seed extract; (c) root extracts (d) stem extract; (e) flower extracts.

under reducing conditions and silver stained, and bands were sliced and digested with trypsin and analyzed by LC-MS/MS as described before. Only fraction XVIII exhibited protein bands in SDS-PAGE. The 10-12 kDa protein band resulted in five peptides whose sequences identified the orthologous protease inhibitor named Papaya latex serine protease inhibitor (PPI) from *Carica papaya* (in red, Figure 7), which belongs to the Kunitz type serine protease inhibitor family and has 20.6 kDa (Azarkan et al. 2011).

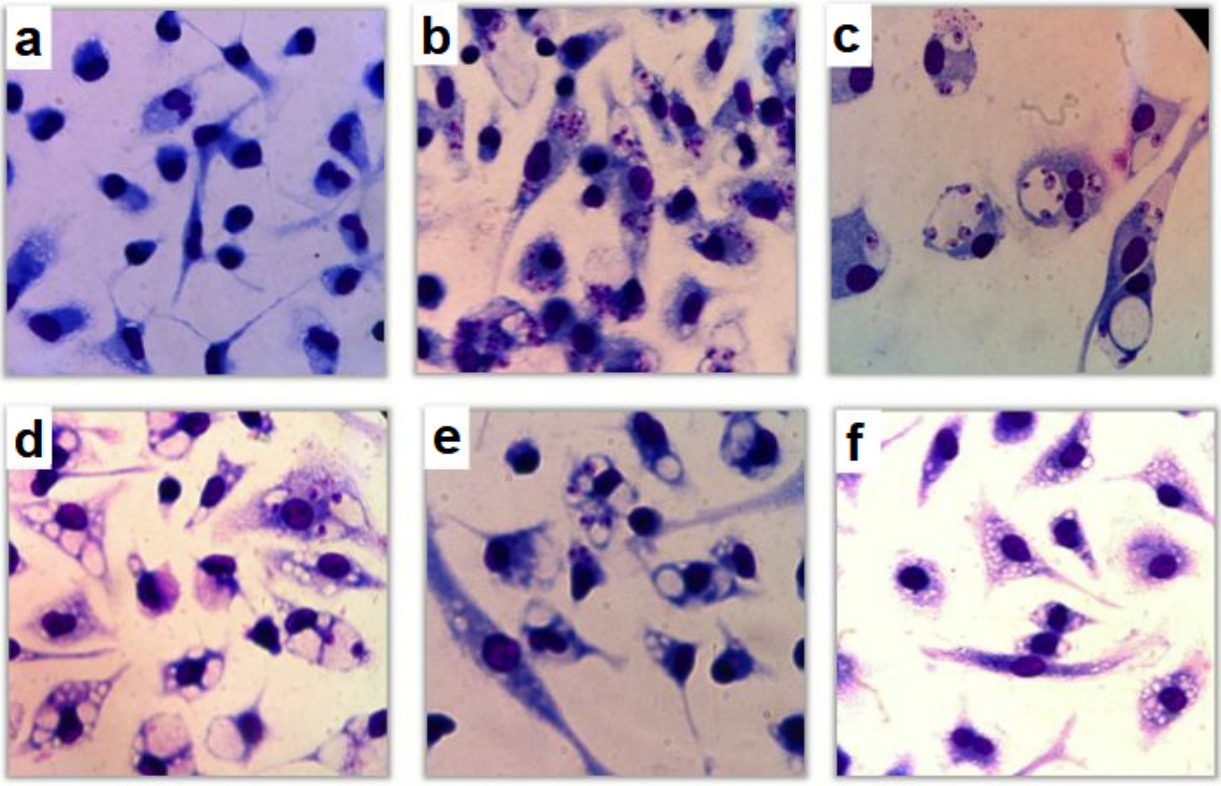

Figure 5. Effects of CS-P on peritoneal macrophages infected with *L. amazonensis* amastigotes by optic microscopy (100 x). (a) negative control with non-infected macrophages; (b) positive control with macrophages infected with *L. amazonensis* amastigotes; (c) macrophages infected with 12,5 µg; (d) 25 µg; (e) 50 µg and (f) 100 µg of CS-P protein/mL.

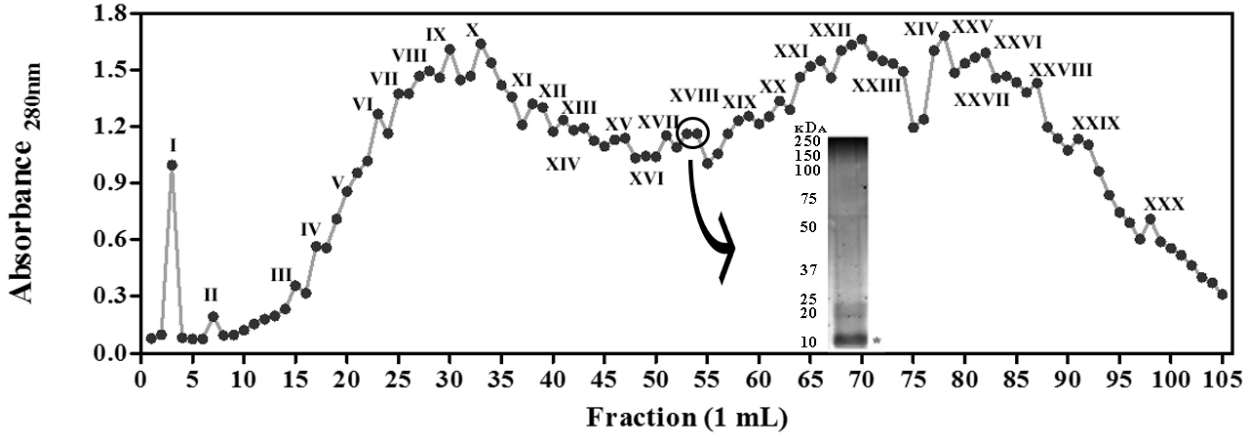

Figure 6. CS-P gel filtration separation profile and 12% SDS–PAGE under reducing conditions of fraction XVIII SDS–PAGE analysis. The values of standard molecular mass proteins (kDa) are located to the left side of the gels.

Table III. Inhibition of CS-P fractions on LSPIII activity.

CS–P fractions	Molecular mass (Mr)	LSPIII inhibition (%)	CS–P fractions	Molecular mass (Mr)	LSPIII inhibition (%)
I	890	47.84	XIV	60-57	28.90
II	520-480	20.75	XV	50	0.00
III	325-300	1.69	XVI	44-38	51.79
IV	220-208	10.56	XVII	28-20	52.40
V	190-180	0.00	XVIII	15-12	54.80
VI	165	10.22	XIX	12	16.19
VII	158-150	0.00	XX	11	36.09
VIII	135-130	25.68	XXI	10	56.67
IX	120	9.45	XXII	< 10	35.35
X	105-98	9.55	XXIII	< 10	41.15
XI	87-84	30.33	XXIV	< 10	3.04
XII	79-76	32.38	XXV	< 10	36.59
XIII	71-68	9.88			

gi|355333050 Mass: 20642 Score: 73 Matches: 7(2) Sequences: 7(2) emPAI: 0.35
Chain A, Crystal Structure Of A Papaya Latex Serine Protease Inhibitor (Ppi) At 2.6a Resolution

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
289	391.7270	781.4394	781.4374	2.48	0	12	28	8	U	K.EFVFIK.T
649	441.7410	881.4674	881.4647	3.12	0	19	15	1	U	K.AGLPFSYK.F
686	452.2425	902.4705	902.4709	-0.45	0	27	3.8	1	U	R.ESVDLNVK.F
2980	386.6717	1542.6577	1542.6562	0.97	0	19	10	1	U	K.GYHGFESTHSMFK.I
3011	780.9288	1559.8431	1559.8348	5.32	0	56	0.0054	1	U	R.FPGVIGWTVTLGGEK.G
3077	798.4036	1594.7926	1594.7701	14.1	0	12	1.3e+002	6	U	R.LIPCNNVDIFFDK.Y
4410	816.4194	2446.2363	2446.2090	11.1	0	52	0.019	1	U	K.CPLSVVQDPFDNGEPIIFSAIK.N

Figure 7. LC-MS/MS analysis of peptides obtained by CS-P gel filtration fraction XVIII trypsin digestion using NCBI protein database (Mascot). Search parameters: fixed modification of carboxyamido methylation at Cys and variable modification of oxidation at Met and deamination of Asn to Asp. Asparagine residue (N) in N-glycosylation site (N-X-S/T) is underlined and peptides residue number with N-glycosylation site is underlined.

Evaluation of secondary metabolites in the phosphate leaf extract

A previous report from our group identified flavonoids as the major secondary metabolites of CS-P (Silva-López et al. 2018). LC-MS analysis provided a good separation profile and detected intense peaks at retention times of 2.7 (compound 6), 3.1 (compound 7), 4.4 (compound 12), 5.6 (compound 17), 7.1 (compound 22), 10.3 (compound 25), and 11.0 min (compound 26) (Figure 8). The analysis identified the flavonoids, considering the elements C, O and H (Table IV). The signals from the 12th minute correspond to the solvent that was used to dilute the sample (methanol) and were not considered. They were also observed in the blank analysis, without the presence of the CS-P (Supplementary Material - Figure S1).

The major signal (7) had a molecular ion mass of 757.2188, which was identified by MassBank as the quercetin-3-neohesperidoside-7-rhamnoside with a molecular mass of 756.7 Da ($C_{33}H_{40}O_{20}$). This molecular mass was confirmed by the masses suggested by the LC-MS program. Two fragments of 610.1 that confirmed the presence

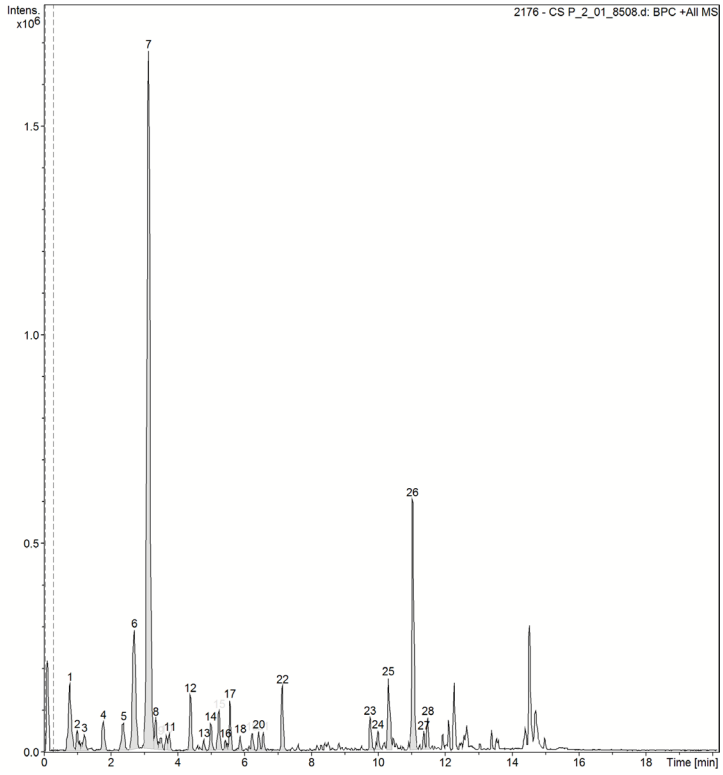

Figure 8. LC-MS chromatogram obtained from the analysis of the leaf *Crotalaria spectabilis* phosphate extract.

of this molecule were: 3-Glu-7-rha quercetin and 3-O-neohesperidoside quercetin (Figure 9). In addition to the molecular formula, the ultraviolet (UV) spectrum was also used to support the identification of substances. The compound 6 was proposed as 5-[3,5-Bis(hexopyranosyloxy)-7-hydroxy-2-chromeniumyl]-2-hydroxyphenyl hexopyranoside. The molecular formulas $C_{34}H_{42}O_{20}$ and $C_{33}H_{40}O_{20}$ were proposed for signals 12 (RT = 3.1 min; m/z 771.3556) and 17 (RT = 4.4 min; m/z 993.2669), respectively. After analysis using the Massbank database and the UV-Vis spectra maximum absorbance, 7-methylquercetin-3-galactoside-6-rhamnoside-3-rhamnoside and quercetin-3-neohesperidoside-7- rhamnoside were suggested for signal 12 and 17, respectively. The fragmentation corroborated thin layer chromatography, indicating the presence of the flavonoid quercetin (Figure S2). Compound 22 was characterized as isovitexin-2-rhamnoside with 579.1715 Da. The residual fragment observed at 433.1134 m/z was identified as glycosilated isovitexin by MassBank and UV spectrum. Tricin 7-diglucuronoside (compound 25) was identified as O-methylated flavone with a molecular mass of 773.2132 Da and a formula of $C_{29}H_{30}O_{19}$, and the fragmentation of 506.3533 showed a tricin 7-o-glucuronide that confirmed this suggested compound. The second most intense compound (26) was proposed to be a flavone, a chemical derivative of luteolin, ie., chrysoeriol-O-glucosylglucoside malonylated ($C_{31}H_{34}O_{19}$), with 711.3786 Da of molecular mass (Table IV).

DISCUSSION

Many plant extracts and compounds have been assayed against *Leishmania* and have demonstrated leishmanicidal effects, such as peptides, tannins, flavonoids, coumarins, quinones, alkaloids, sesquiterpenes, triterpenes, steroids, and saponins (Bekhit et al. 2018). However, the poor water solubility and high cytotoxicity of the great majority of these plant

Table IV. Chemical constituents identified in *Crotalaria spectabilis* aqueous extract with corresponding retention times, molecular ions in positive mode, and key fragments.

Compound	RT (min)	$[M+H]^+$ (m/z)	MS fragmentation $[M+H]^+$ (m/z)	Suggested compound
6	2.7	773.2132	773.2132; 627.1536; 506.3533	5-[3,5-Bis(hexopyranosyloxy)-7-hydroxy-2-chromeniumyl]-2-hydroxyphenyl hexopyranoside
7	3.1	757.2188	757.2188; 611.1604	Quercetin-3-neohesperidoside - 7 -rhamnoside
12	4.4	386.1816	770.3556; 386.1816	7-Methylquercetin-3-Galactoside-6''-Rhamnoside-3'''-Rhamnoside
17	5.6	933.2669	757.2168; 667.2624	Quercetin-3-neohesperidoside - 7 -rhamnoside
22	7.1	579.1715	579.1715; 433.1134	Vitexin-2-rhamnoside
25	10.3	507.3533	682.53998; 653.4072; 507.3533	Tricin 7-diglucuronoside
26	11.0	711.3786	711.3786; 679.21600	Chrysoeriol O-glucosylglucoside malonylated

compounds and their extracts are limiting factors for their use as anti-*Leishmania* agents (de Paula et al. 2019). Plant natural products were specific for different targets in the parasite and affected distinctive biochemical pathways, and many enzymes, such as proteases, have been investigated for their capacity to regulate the *Leishmania* life cycle, the host-parasite interaction, the pathogenesis of leishmaniasis, and infection progression (Silva-López & De Simone 2004a, b, Silva-López 2012, Islamuddin et al. 2014, Koko et al. 2022). The importance of these proteases has been confirmed by the finding that specific inhibitors killed *Leishmania* parasites (Silva-López et al. 2007, Machado et al. 2019, Gomes et al. 2022).

The characterization of *C. spectabilis* extracts reported in an earlier publication was useful to determine the experimental conditions for PI analysis and serine proteases were the most important activities observed (Pacheco & Silva-López 2012). Furthermore, all *C. spectabilis* extracts were prepared using aqueous systems and only high polarity compounds such as, peptides, proteins, carbohydrates, and flavonoids were extracted. On the other hand, CS-DE was prepared with detergent and some compounds with moderate polarity were obtained, such as alkaloids and phenols. Thus, the inhibition of protease activities observed in the present study was due to the action of peptides since because natural plant PIs are generally polypeptides (Gomes et al. 2022).

The reference proteases were employed for PI activity identification in plant extracts (Spelbrink et al. 2011). Serine peptidases are among the most studied enzymes and participate in a vast number of biological processes so that their inhibitors belong to the largest and most widely distributed superfamily of PIs (Rawlings et al. 2018). Trypsin is the representative serine peptidase and trypsin-like serine proteases are found in all living organisms (Gurumallesh et al. 2019). The inhibition of trypsin activity was observed in extracts of all organs of *C. spectabilis*, especially leaf, flower and pod extracts. Surprisingly, the most pronounced trypsin inhibition was not observed in seed extracts. However, plant PIs are almost exclusively studied in seeds because they accumulate these inhibitors to protect their genetic material (Avilés-Gaxiola et al. 2018). *Leguminosae* species store large amounts of proteins in seeds and *C. spectabilis* seed extracts

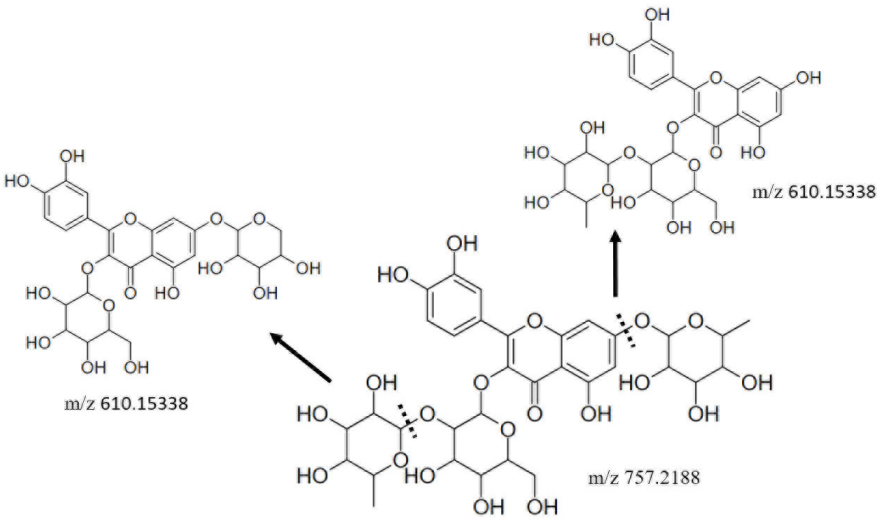

Figure 9. Chemical structures of flavonoid derivatives and the proposed MS fragmentation in positive ion mode. Left: Quercetin-3-glucoside-7-rhamnoside; Right - Quercetin-3-O-Neohesperidoside; C - Quercetin-3-neohesperidoside-7-rhamnoside.

also showed the highest protein content of all extracts (Pacheco & Silva-López 2012). Moreover, antitrypsin activity has already been described in seeds from the *Crotalaria* genus (Norioka et al. 1988, Pando et al. 1999, Gomes et al. 2005).

Papain activity was completely abolished by seed, root, stem, flower and pod extracts. As stated before, Kunitz-type inhibitors also inhibit papain activity, but plant cystatins are the most specific inhibitors and react reversibly and competitively with papain and papain-like cysteine proteases (Hellinger & Gruber 2019). The activity of pepsin inhibitors can be found in all organs of *C. spectabilis,* especially in the PVPP root extract, which completely inhibited this activity. An aspartic protease is expressed in plant roots and contributes to root development, which involves proteolysis of root proteins (Soares et al. 2019); thus, PIs that control this activity are necessary. *C. spectabilis* root extract (CS-RPVPP) showed this aspartic acid protease activity (Pacheco & Silva-López 2012). Taken together, these results indicate the presence of this type of PI in *C. spectabilis* roots. This finding is notable because peptide aspartic PIs are rare and unevenly distributed among classes of organisms, in contrast to serine and cysteine proteases inhibitors (Silva-López 2009).

L. amazonensis extracellular fraction III had many released and secreted proteins directly involved in pivotal processes related to host–parasite interactions, such as serine protease (Silva-López et al. 2005). *C. spectabilis* extracts reduced LSPIII activity differently and, as expected, fraction III inhibition was lower than that of the purified secreted serine protease of *L. amazonensis* because the amount of LSPIII in the fraction was lower. Furthermore, LSPIII inhibition has also been studied using the purified Kunitz type inhibitor from sea anemone *Stichodactyla helianthus* (ShPI-I), which abolished LSPIII activity at 10^{-5} M (Silva-López et al. 2007). Despite the unknown molar concentration of *C. spectabilis* inhibitors in the extracts, we can infer that *C. spectabilis* extracts are important sources of PIs that could affect the course of cutaneous leishmaniasis infection because the inhibition of *Leishmania* serine proteases induced parasite death in culture (Silva-López et al. 2007).

Screening of plant natural products is a valuable approach to the discovery of new compounds with anti-*Leishmania* activity. However, plant compounds and their extracts also show toxicity in mammalian cells *in vitro*, particularly when they are extracted

using organic solvents and have limited water solubility (Saleem et al. 2021). Thus, it was necessary to analyze the toxic effects of *C. spectabilis* extracts on a mammalian host cell of *Leishmania*. In general, these extracts were nontoxic to macrophages, with the exception of the detergent leaf extract that was obtained using Triton X-100. This detergent may possibly extract pyrrolizidine alkaloids, which are highly toxic to mammalian cells (Huang et al. 2017). At high protein concentrations, both seed extracts showed macrophage toxicity that could be explained by the fact that *C. spectabilis* accumulates many toxic peptides in its seeds that work as unspecific defense mechanisms (Gomes et al. 2005).

Promastigotes are employed in the initial investigation of leishmanicidal activity of natural products (Bekhit et al. 2018, de Paula et al. 2019). All *C. spectabilis* extracts were cytotoxic for *L. amazonensis*, and the leaf extracts were the most effective, especially CS-AE. Although this extract did not inhibit LSPIII, the parasite death could be caused by the inhibition of other protease types such as aspartic proteases because the extract inhibited pepsin activity and reduced the protease activity of fraction III, which contains all secreted proteases (Marshall et al. 2018). Although CS-P was not as effective at reducing promastigote viability as CS-AE (Table II), it was the best inhibitor of LSPIII among the leaf extract. LSPII is an important drug target in *L. amazonensis*. The correlation between *Leishmania* serine protease inhibition and parasite death was first reported when specific PIs reduced the viability of *L. amazonensis* promastigotes in a dose-dependent manner. These PIs induced morphological changes, intracellular vesicular bodies, autophagic vacuoles, and alterations in parasite shape (Silva-López et al. 2007). It is important to note that *C. spectabilis* also changed the morphology of normal promastigotes, probably because they affected same targets in *Leishmania* such as serine proteases.

C. spectabilis leaf extracts demonstrated the most important dose-dependent effect in anti-amastigote activity assays. In others words, these extracts passed through the macrophage membranes with no toxic effects on the host cells, and killed amastigotes inside the phagolysosomes. Due to the very low cytotoxicity of *C. spectabilis* extracts, it was not possible to calculate the selective index (SI). However, if the CC_{50} was 200 µg/mL, the leaf extracts CS-AE, CS-T and CS-P would have a higher SI than the pentamidine control, also used in the treatment of leishmaniasis (Sereno et al. 2019). The correlation between *Leishmania* protease inhibition and anti-*Leishmania* activity was also investigated in leaf apolar extracts of *Arrabidaea chica*, *Coccinia grandis* and *Aloe vera* using hexane, ethyl acetate and ethanol extracts, respectively, a potato (*Solanum tuberosum*) tuber etanolic extract, and a *Garcinia brasiliensis* ethanolic pericarp extract. The correlation between PI and antileishmanial activity was only found in polar extracts containing polypeptides (de Paula et al. 2019, Figueredo et al. 2022). A serine protease polypeptide inhibitor (PTF3) from potato tubers also demonstrated strong antileishmanial activity against intracellular amastigotes of *L. donovani* and curative efficacy in infected hamsters (Paik et al. 2020). The great interest in polypeptide PIs for leishmaniasis chemotherapy is due to their higher specificity and lower toxicity compared to conventional drugs employed for the treatment of these diseases. Furthermore, the study of innovative plant-derived components with antileishmanial activity is necessary for the development of new affordable drugs.

CS-P was chosen to study PIs because of its complete inhibition of LSPIII, low macrophage

cytotoxicity and expressive anti-*Leishmania* activity. The extract was fractionated and the 10-12 kDa polypeptide, homologue to the Papaya latex serine protease inhibitor (PPI) from *Carica papaya*, which is a Kunitz type protease inhibitor (Azarkan et al. 2011), was identified. It is important to note that *C. spectabilis* belongs to the Leguminosae family, which comprises about 19,320 species. However, there are only 14 sequenced leguminous plant genomes with predicted genes which are: *Ammopiptanthus nanus, Amphicarpaea edgeworthii, Arachis duranensis*, *Arachis ipanensis, Cajanus cajan, Cicer arientum, Dalbergia odorifera, Faidherbia albida, Glycine max, Lablab purpureus, Lotus japonicus*, *Medicago trucatula*, *Phaseolus vulgaris* and *Vigna subterranea* (Chen et al. 2018, Liu et al. 2020). Consequently, the proteins identified in the present study were based on orthologs from other plant species.

Many investigations have shown that *C. spectabilis* is a source of flavonoids and alkaloids (Scupinari et al. 2020). However, CS-P was prepared using an aqueous phosphate buffer, which made it difficult to compare it with previous works, all of which employed organic solvents to extract *C. spectabilis* secondary metabolites. Phosphate buffer is highly suitable for the extraction of plant proteins and other polar molecules, but not for the extraction of apolar substances such as alkaloids, widely found in *C. spectabilis* (Pacheco & Silva-López 2012). LC-MS and MassBank analysis identified glycosilated flavonoids and their derivatives quercetins, vitexin, tricin and chrysoeriol as major compounds in CS-P. 5-[3,5-Bis(hexopyranosyloxy)-7-hydroxy-2-chromeniumyl]-2-hydroxyphenyl hexopyranoside, quercetin-3-neohesperidoside-7-rhamnoside (major signal), 7-methylquercetin-3-galactoside-6-rhamnoside-3-rhamnoside and quercetin-3-neohesperidoside-7-rhamnoside were found in many legumes species (Wojakowska et al. 2013, Hostetler et al. 2017, Wen et al. 2020), and are widely distributed in the *Crotalaria* genus (Ibrahim et al. 2017), but they were found for the first time in *C. spectabilis*. Vitexin-2-rhamnoside is particularly abundant in *Crotalaria sessiliflora* (Tang et al. 2017). The flavone tricin 7-diglucuronoside, also identified for the first time in CS-P, is a predominant flavonoid amongst monocots and unrelated dicot lineages such as some legumes species (Lui et al. 2020). The second major signal was chrysoeriol-O-glucosylglucoside malonylated, a flavone chemically derived from luteolin, that was identified in leaves of different plants species, but was not identified in legumes (Wojakowska et al. 2013, Hostetler et al. 2017). Flavonoids are particularly abundant in legumes; they are the legume chemical markers, are polar substances and have a great diversity of pharmacological and biological activities, including antioxidant, anti-inflammatory, and protective effects against many infectious agents (Hostetler et al. 2017, Ullah et al. 2020). The low mammalian host cell toxicity of *C. spectabilis* extracts could also be explained by their glycosilated flavonoid content. Besides, they can also have a synergic antileishmanial effect since some plant extracts rich in flavonoids reduced *Leishmania* viability in *in vitro* assays (Imperatori et al. 2019, Fadel et al. 2019). Thus, a patent application concerning pharmaceutical formulations containing these aqueous *C. spectabilis* extracts for the treatment of cutaneous leishmaniasis was filed in Brazil in 2018 under register number BR 10 2108 011550 2 (Silva-López et al. 2018).

In summary, aqueous *C. spectabilis* extracts contain serine, cysteine and aspartic PIs. Some of them inhibited the activity of *L. amazonensis* serine protease, exhibited low macrophage cytotoxicity and reduced the viability of promastigotes and intracellular

amastigotes. Furthermore, the 10-12 kDa serine PI, homologous with papaya latex serine PI, and polar flavonoids such as quercetins, vitexin, tricin and a derivative of luteolin was isolated from CS-P. Finally, the presence of serine-type PIs in *C. spectabilis* leaf extracts is a new finding since legume PIs have always been previously reported only in seeds. This is the first report on the effect of PIs and their leishmanicidal activity from *C. spectabilis* extracts.

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Figures S1, S2.

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