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DANIELLY CHRISTINE ADRIANI MAIA MOTA

ISOLATION AND CHARACTERIZATION OF PAPAIN-LIKE CYSTEINE
PROTEINASES FROM *MORINDA CITRIFOLIA* SEED WITH ANTI-
TOXOPLASMOSE EFFECT

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Orientadora: Prof^ª Dr^ª Fernanda Maria Santiago

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TOXOPLASMOSE EFFECT

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À todos que fizeram parte dessa etapa tão importante da minha vida

Dedicatória

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Isolation and characterization of papain-like cysteine proteinases from *Morinda citrifolia* seed with anti-toxoplasmosis effect

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Abstract

Morinda citrifolia, known as Noni, is used in folk medicine with several therapeutic applications. The present study investigated the enzymatic and biological characteristics of the papain-like cysteine proteases (PLCP) from *M. citrifolia* seed. Its enzyme was purified in two-step by DEAE-Sephacel and Phenyl-Sepharose CL-4B (10 × 0.6 cm) columns. PLCP enzyme demonstrated an apparent molecular mass of 51 kDa on SDS-PAGE 14%. The proteolytic activity in the presence of fibrinogen with the PLCP of *M. citrifolia* demonstrated total hydrolysis of the fibrinogen chains, occurring in the concentration of 20 µg; with the optimum pH of 7.0; temperatures range from room temperature to 50°C; better activity with calcium ions and the effect of inhibition appeared in the presence of Leupeptin. There was no platelet aggregation or coagulation activity. PLCP enzyme was not cytotoxic for cells such as Human Foreskin Fibroblasts (HFF) and murine embryo fibroblasts strain NIH/Swiss (NIH/3T3) macrophages and showed to be effective when tested directly against *Toxoplasma gondii* infection *in vitro*. In that sense, this study indicates perspectives for pharmacological applications of *M. citrifolia* enzymes.

Keywords: *Morinda citrifolia* - Cysteine proteases - proteolytic activity - *Toxoplasma gondii*

1. Introduction

Morinda citrifolia of the Rubiaceae family, is original from Southeast Asia and grows in almost all tropical areas of the world, known as Noni or Indian mulberry (Wang, et al., 2002). The plant has been widely used by folk medicine to treat inflammatory diseases, such as arthritis, and scientific studies have reported the plant's properties, such as antitumor, antimicrobial, antiseptic, antifungal and antiviral (Wang and Su, 2001; Jayaraman, et al., 2008; Basar, et al., 2010; Assi, et al., 2015; Zhang, et al., 2016; Torres, et al., 2017). Recent studies have shown that daily doses of Noni juice increase the expression of DNA repair genes and selective cell death mediated by apoptosis in cervical cancer cells and, according to these studies, it is the second most frequent type of cancer present in women (Gupta and Singh, 2013; Gupta, et al., 2015).

Proteases, also known as proteolytic enzymes or peptidases, are a large group of enzymes which play a significant role in many biochemical mechanisms to maintain metabolic processes of all organisms. They have been isolated in last years from plants (latex, fruits, and seeds) and animals (bee, snake, scorpion and spiders) mainly for pharmacological purposes, such as blood coagulation and fibrinolysis (Costa Jde, et al., 2010, Siritapetawee, et al., 2012).

Considering the relevance role of plant proteases as potential therapeutic targets, the aim of the present work was to purify and characterize of a cysteine protease present in the seeds of the *Morinda citrifolia* and evaluate its biological and functional effects.

2. Material and Methods

2.1. Plant material

Morinda citrifolia seeds was collected of plants, which were grown under natural conditions in the University Campus localized in Uberlândia, Minas Gerais, Brazil, in April - December 2016. A voucher specimen (HUFU 75261) was identified and deposited at the Herbarium of the Federal University of Uberlândia.

2.2. Human blood

Human blood was obtained by donation from volunteers. The criteria for selection of donors were be in good state of health, have 18 to 65 years old, weighting at least 50 kg, no use of any medication that interferes with hemostasis, no use of illicit drugs and no alcohol consumption for at least 24 h before donation. The experiments were carried out according to the current guidelines for research with humans established by the Committee for Ethics in Human of the Federal University of Uberlandia (CEP/UFU – protocol number 1.627.982/2016).

2.3. Purification of the papain-like cysteine proteinase (PLCP) from *Morinda citrifolia* seed

The crude extract (CE) of *M. citrifolia* (20 mg) was dispersed in 2.0 mL of 0.05 M ammonium bicarbonate buffer (AMBIC, pH 7.8), clarified by centrifugation at $10.000 \times g$ for 10 min and applied on a DEAE Sephacel column ($1.7 \times 15\text{cm}$). Chromatography was carried out at a flow rate of 20 mL/h, with a convex concentration gradient of the same buffer (0.05–1.0 M) of the same buffer. Fractions of 2.0 mL/tube were collected, their absorbances were read at 280 nm and those corresponding to the peak P2 was freeze-dried and then diluted by 0.01M Tris-HCl, pH 8.5 and 4M NaCl buffer prior to the next chromatographic step in a Phenyl-Sepharose CL-4B ($10 \times 0.6\text{ cm}$) column, previously equilibrated with the same buffer. The chromatography was carried out at a flow rate of 50 mL/h with decreasing gradient of NaCl (4 to 0 M), followed by distilled H₂O. Protein concentrations were determined by Bradford method (1976), using bovine serum albumin as standard.

2.4. Electrophoretic analysis

The electrophoretic profile of the of the proteins from *M. citrifolia* before purification was visualized by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (Laemmli, 1970). Molecular size markers (MrS) (BenchMarck™ Protein Ladder) were used in each electrophoretic run. The relative molecular mass of the proteins was estimated by Kodak 1D image analysis software.

2.5 Mass spectrometry

The molar mass and purity of the PLCP were determined using Electrospray ionization mass spectrometry (ES/MS). A Perkin–Elmer SCIEX API300 triple quadrupole mass spectrometer equipped with an electrospray ionization interface was operated in the positive-ion mode. The samples were dissolved in methanol/water/acetic acid (65/35/5) and applied into the mass spectrum by an automatic syringe injection at a flow rate of 20 $\mu\text{L/h}$. The parameters used were: ion spray voltage 5 KV, curtain gas (ultrapure nitrogen) flow 0.6 L/min, ring voltage 280 V and orifice voltage 50 V.

2.6. Proteolytic activity upon fibrinogen

Fibrinogenolytic activity was assayed as previously described by Rodrigues, et al. (2000) with modifications. Samples of 10 μ L of bovine fibrinogen (3 mg/mL saline) were incubated with 10 μ g PLCP from *M. citrifolia* at 37°C for different periods of time (5, 10, 15, 30, 60, 120, 240, 720 and 1440 minutes). The effect of the concentration on the proteolytic activity was verified by varying the incubation concentrations of the enzyme with the fibrinogen in 2.5, 5, 15, 20 and 25 μ g by 60 minutes. The effect of inhibitors on the fibrinogenolytic activity was assayed after preincubation of PLCP from *M. citrifolia* (10 μ g), for 60 minutes with each of following inhibitors: EDTA, 1,10-phenanthroline, benzamidine, leupeptin, and aprotinin. The thermal stability of *M. citrifolia* was tested by preincubating the enzyme (10 μ g), for 60 min at varying temperatures (25 – 90°C). Similarly, 10 μ g of PLCP from *M. citrifolia* was preincubated at different pH 4.0 to 10.0 (0.2 M sodium acetate pH 4.0 and pH 5.0; 0.2 M monobasic sodium phosphate pH 6.0 and pH 7.0; 0.2 M Tris-HCl pH 8.0 and pH 9.0; 0.2 M sodium borate pH 10.0) and in the presence of different ions (KCl); (NaCl); (CaCl₂); (ZnCl₂), for 60 minutes, all reagents at concentration of 5 mM. The treated samples were added to bovine fibrinogen and incubated for 60 minutes at 37°C. The reaction was stopped with 0.0625 mol/L Tris-HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 5% (v/v) β -mercaptoethanol, 2% (w/v) SDS, and 0.001% (w/v) bromophenol blue. Reaction products were analyzed by 14% (w/v) SDS-PAGE.

2.7. Functional characterization

2.7.1. Coagulant activity

The coagulant activity of the PLCP enzyme from *M. citrifolia* sample was evaluated on citrated bovine plasma (Denson, et al., 1972). Thus, the samples of 20 μ g of enzyme were added to aliquots of 200 μ L of bovine plasma and, then, they were incubated at 37°C in triplicate. Bovine plasma samples used were mixed with 3.2% sodium citrate and centrifuged at 3000 rpm for 15 minutes at 4 °C in order to obtain platelet-poor plasma. The evaluation of the activity was observed through the immediate appearance and formation of the fibrin clot.

2.7.2. Platelet aggregation assay

For the aggregation essays, we used the AggRAM™ brand automated 4-channel aggregometer in version 1.1 with platelet-rich plasma (PRP) (de Queiroz, et al., 2014). All experiments were done in triplicate process and the control experiments were performed only

with platelet agonists. For the platelet aggregation essay, the human blood was collected in sodium citrate tubes (3.2%). Within the first four hours after the collection of the blood, the platelet-rich plasma (PRP) was obtained by centrifugation at room temperature for 12 minutes at 1000 rpm, and, then, the platelet-poor plasma (PPP) was centrifuged for 15 minutes at 3000 rpm, in a way that the total aggregation is expressed as a ratio between absorbance and PPP aggregation. Then, the tests were performed using 200 μ l of PRP at 37 °C in siliconized glass cuvettes and held under constant stirring. So, the aggregation agonists were epinephrine (300 μ M), ADP (20 μ M) or collagen (10 μ g / ml), incubated with PLCP from *M. citrifolia* seed with a concentration range of epinephrine and ADP (10, 25 and 50 μ g) and collagen (10 and 25 μ g).

2.7.3. Cell Viability Essay

In order to evaluate if PLCP enzyme of the *M. citrifolia* exhibited cytotoxic activity to Human Foreskin Fibroblasts (HFF) and murine embryo fibroblasts strain NIH/Swiss (NIH/3T3) in MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as previously described with modification (Mosmann, 1983). The cells were cultured separately in 96-well plates (1 x 10⁵ cells/well) in triplicate and treated with PLCP enzyme in different concentrations (1 to 300 μ g/mL) for 18h at 37°C and 5% CO₂. As control the HFF and NIH/3T3 were treated with only complete RPMI medium. This experiment was performed with triplicate wells of each concentration and three different independent observations.

2.7.4. Antiparasitic Activity

The antiparasitic activity of the PLCP enzyme of the *M. citrifolia* was verified on *in vitro* *Toxoplasma gondii* infection following the protocols of de Oliveira, et al. (2009). NIH/3T3 cells were cultured on 13-mm round glass coverslips into 24-well plates (1 x 10⁴ cells/well/200 μ L) for 24 h at 37 °C and 5% CO₂. *T. gondii* tachyzoites (Rh strain) were obtained from previously infected NIH/3T3 cells, washed in RPMI medium and pretreated for 1 h at 37 °C and 5% CO₂ with PLCP enzyme of the *M. citrifolia* in different concentrations (1, 3, 10, 30, 100 and 300 μ g/mL) or with medium alone (control). Then, parasites were washed and incubated with NIH/3T3 cell monolayers on coverslips at 2:1 (parasite: host cell) rate of infection (2 x 10⁴ tachyzoites/well/200 μ L) for 24 h at 37 °C and 5% CO₂. Cells were washed with 0.9% NaCl to remove non-adherent parasites, fixed in 10% buffered formalin for 2 h and stained with 1% toluidine blue (Sigma Chemical Co.) for 5 sec. Coverslips were mounted on glass slides and cells were examined under a light microscope with regards to *T. gondii*

infection index (percentage of infected cells per 100 examined cells) and parasite intracellular replication (mean number of parasites per cell in 100 infected cells).

The results were expressed as percentages of inhibition of infection as well as of parasite intracellular replication for each treatment in relation to controls. The median inhibitory concentration (IC₅₀) of PLCP enzyme was calculated by extrapolation of the corresponding dose-curve response on a log linear plot employing the portions of the curve that transected the 50% response point (Jones-Brando, et al., 2006).

2.8 Statistical Analysis

Statistical analysis was carried out using the GraphPad Prism 5.0 software (Graphpad Software Inc., San Diego, USA). The anticoagulant activity data were analysed by the Student's t-test. Values of $p < 0.05$ were considered statistically significant. The cytotoxic and anti-toxoplasmosis activity data were analysed by one-way ANOVA and Dunnett post-test.

3. Results and Discussion

3.1 Purification of the papain-like cysteine proteinase (PLCP) from *Morinda citrifolia* seed

The *M. citrifolia* crude extract seed samples from several extractions had a mean protein concentration of 5.0 mg/mL. The SDS-PAGE profile of *M. citrifolia* crude extract showed several peptide components, with relative molecular masses (M_r) ranging from 20 to 140 kDa when analysed under nonreducing conditions and intense staining for bands above 28 kDa (**Fig. 1A, lane 1**).

The PLCP enzyme from *M. citrifolia* was isolated by only two steps of purification. *M. citrifolia* crude extract (500 mg) was applied on a DEAE-Sephacel column and produced ten main protein peaks (**Fig. 1B**). The two peaks were further fractionated by Phenyl-Sepharose CL-4B column and the non-adsorbed fraction (peak a) showed a single-band protein with an excellent level of purity (**Fig. 1C**). The electrophoretic analysis indicated that the protein had an apparent molecular mass around 51 kDa (**Fig. 1A, lane 2**). The mass spectrometry revealed the partial sequence of this protein and after it was analysed in comparison with the NCBI databank, we identified as a papain-like cysteine proteinase (**Fig. 2**). The molecular mass of the cysteine proteases may vary according to the organism which it's produced, for example, the cysteine protease from *Zingiber montanum* rhizome, have molecular mass of 48 kDa (Jamir and Seshagirirao, 2018).

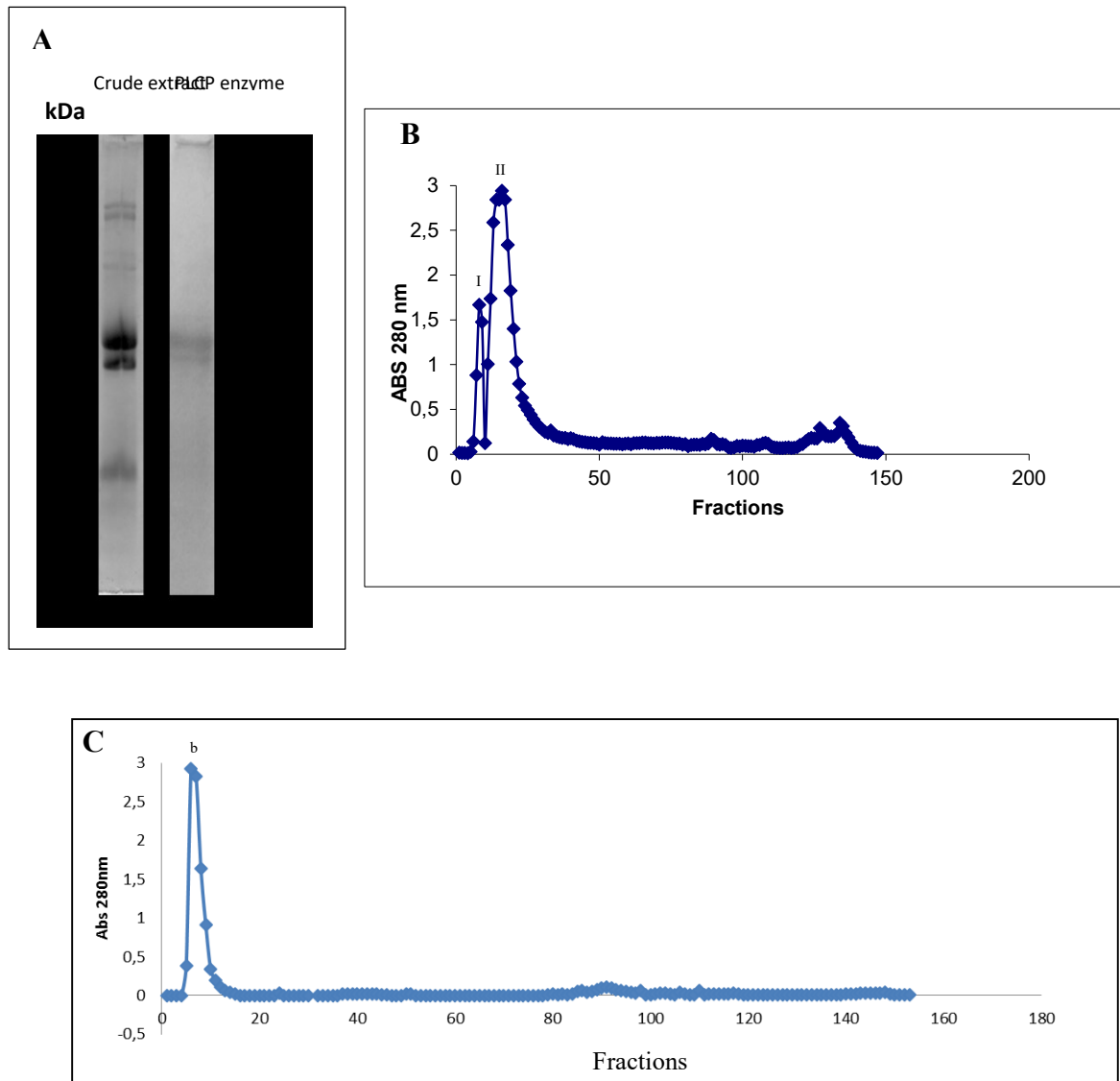


Figure 1: Purification of the crude extract of the seeds of *M. citrifolia*. (A) SDS-PAGE in 12% (w/v) gel. (A) Line 1 - crude extract of the seeds of *M. citrifolia*; Line 2 - Cysteine protease. The gel was stained with Coomassie blue R-250. (B) Separation on DEAE-Sepharose ion exchange chromatography: the crude extract of the *M. citrifolia* seeds was applied to the column and the elution was performed in a flow rate of 20 mL/hr with ammonium bicarbonate gradient buffer (0, 05-1.0M). Fractions of 3.0 mL/tube were collected and their absorbances read at 280 nm. (C) Separation on the chromatography the PII fraction of DEAE was applied to a Phenyl Sepharose hydrophobic interaction resin and eluted in a gradient decreasing of NaCl (4-0M), followed by distilled water. Fractions of 3.0 mL/tube were collected at a flow rate of 20 mL/hr.

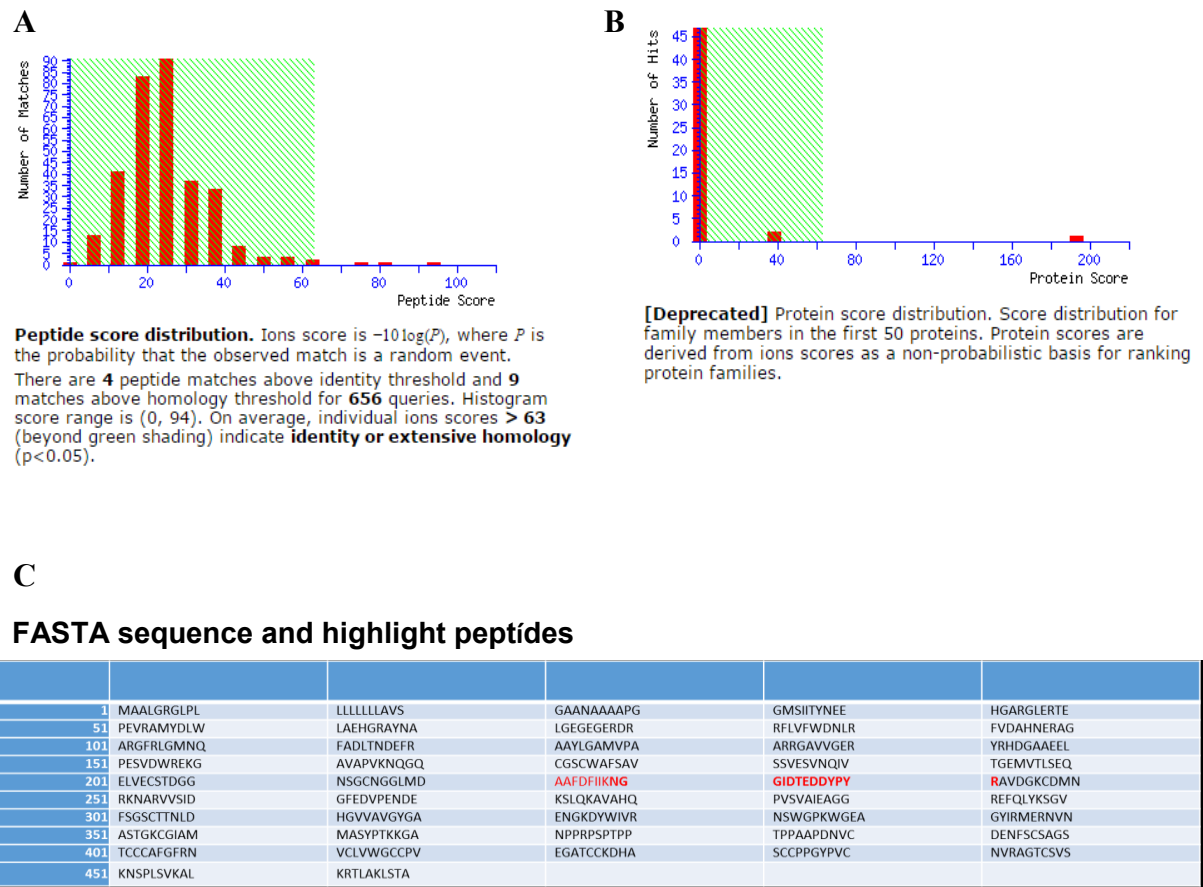


Figure 2. Construction of the polypeptide sequence of papain-like cysteine proteinase (PLCP) from *Morinda citrifolia*. (A) Peptide score distribution; (B) Protein score distribution; (C) Cluster assembled from of peptides obtained by MS/MS.

3.2 Fibrinolytic activity

The effect of the proteolytic activity of the PLCP enzyme of the *M. citrifolia* was evaluated in the fibrinogen substrate. The enzyme demonstrated time-dependent fibrinolytic activity with only 5 minutes of incubation (**Fig. 3A, line 2**) and it occurred more markedly from the 20µg concentration (**Fig. 3B, line 5 and Fig. 3C, line 6**). Our results show that there was total degradation of the fibrinogen chains. Recent studies show that the serine protease from *Curcuma aromatica* and cysteine protease from *Ervatamia heyneana* latex also show the complete hydrolysis of all three fibrinogen subunits (Shivalingu, et al., 2016, Uday, et al., 2017).

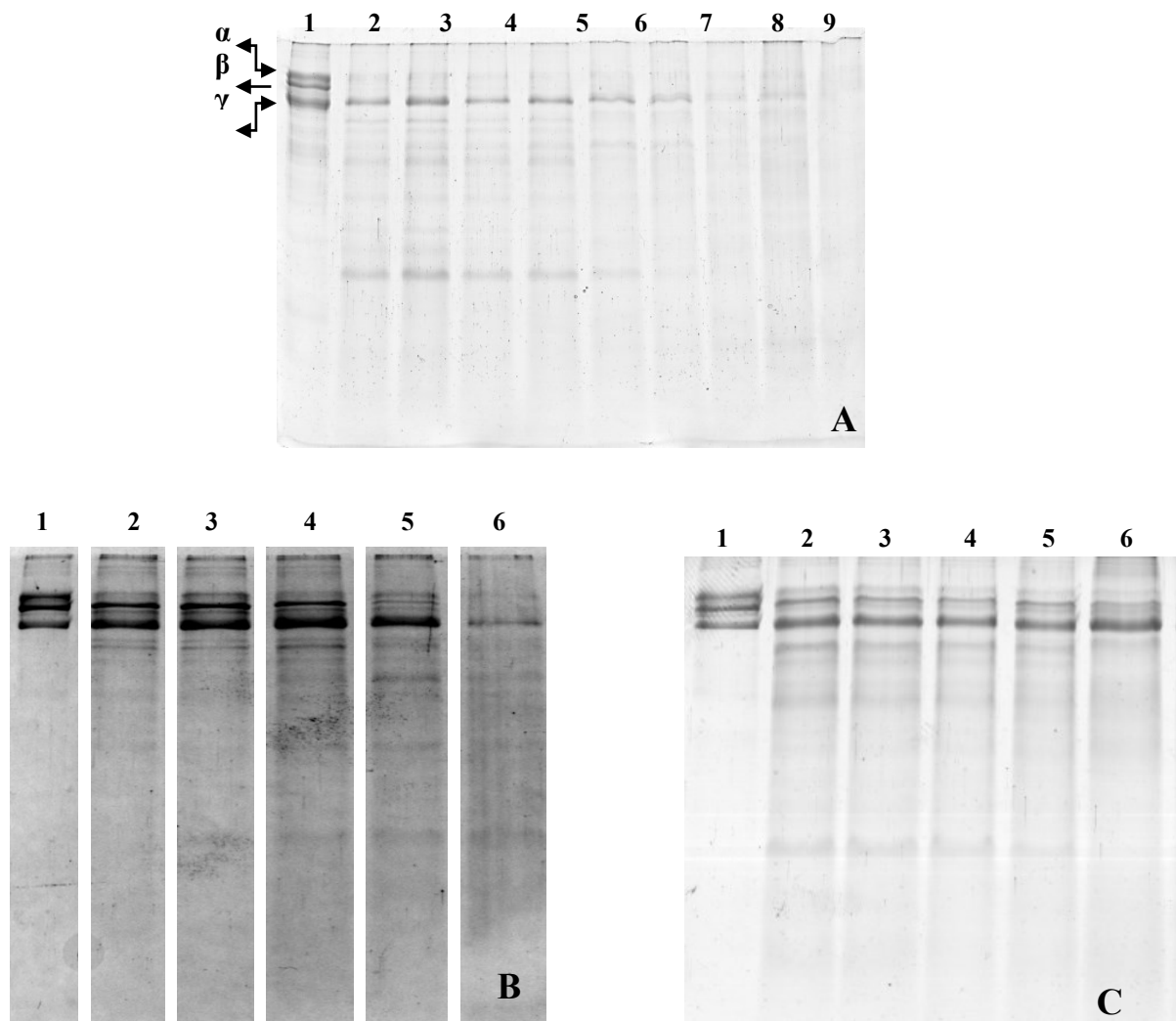


Figure 3: Polyacrylamide gel electrophoresis in 14% of the results of the fibrinogen digestion made by the PLPC for *Morinda citrifolia* seed. Line 1: negative control-fibrinogen incubated with enzyme for 0 minutes. **(A)** Time-dependent. Lanes 2-10: fibrinogen incubated with enzyme for 5; 10; 15; 30; 60; 120; 240; 720 and 1440 minutes, respectively. **(B)** Concentration-dependent, Lanes 2-6: fibrinogen incubated with the enzyme at 2,5; 5; 15; 20 and 25 μg. **(C)** Period in function of the 20 μg concentration. Lanes 2-6: Incubated fibrinogen with the enzyme for 5; 10; 15; 30 and 60 minutes.

The evaluation of the effect of temperature on the fibrinogen proteolysis activity of the PLCP enzyme showed high activities between 25°C to 50°C (**Fig. 4, lines 2 to 4**), optimal activity at between 25°C and 37°C and impaired activity above 60°C. Thus, the following experiments were performed with 20 µg of the enzyme at 37°C for one hour. Studies have demonstrated that proteases present fibrinogenolytic activity, as observed with cysteine protease from *Curcuma longa* rhizomes (saffron) that exhibited optimal activity between 37°C and 60°C and the serine protease from *Diopatra sugokai* with high activity at 50°C to 70°C. (Nagarathnam, et al., 2010; Kim, et al., 2017).

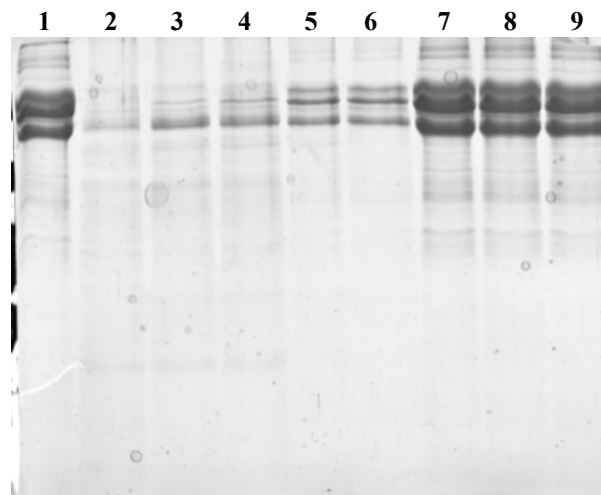


Figure 4: Polyacrylamide gel electrophoresis in 14% of the results of the fibrinogen digestion made by the PLCP for *Morinda citrifolia* seed. Dependent-temperature Line 1: negative control-fibrinogen incubated with enzyme for 0 minutes, lanes 2-9: fibrinogen with the pre-incubated enzyme at 25; 37; 50; 60; 70; 80; 90 and 100°C, respectively.

The fibrinogenolytic activity of the PLCP enzyme was evaluated over a wide pH range varying between 4.0 and 10.0. The results showed that the enzyme of *M. citrifolia* exhibits an optimal activity at pH 7.0 (**Fig. 5, line 5**) and its activity drastically reduced in acid pH (4.0, 5.0 and 6.0) (**Fig. 5, lines 2 and 3**) and alkaline pH (10.0) (**Fig. 5, line 8**). The extreme pHs can modify the three-dimensional structure of the enzymes, influencing the interaction of the enzyme with the substrate and causing the loss of the enzymatic activity (Robinson, 2015, Smeller, 2015). Previous studies with the cysteine protease from *Echinochloa crus-galli*, also had the maximum effect for its activity at pH 7.0 and the serine protease from *Curcuma aromatica* demonstrated to have high activity in pH 7.5 (Hirase and Molin, 2001; Shivalingu, et al., 2016).

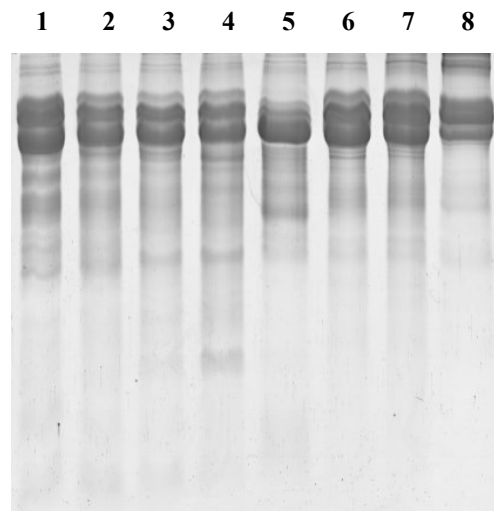


Figure 5: Polyacrylamide gel electrophoresis in 14% of the results of the fibrinogen digestion made by the PLCP for *Morinda citrifolia* seed. pH-dependent Line 1: negative control-fibrinogen incubated with enzyme for 0 minutes. Lanes 2-8: fibrinogen incubated with enzyme for 60 minutes after preincubation of enzyme in pH= 4,0; 5,0; 6,0; 7,0; 8,0; 9,0 and 10,0, respectively

The enzymatic activity of PLCP enzyme in the presence of different protease inhibitors showed reduced proteolytic activity after pre-incubation particularly with leupeptin (**Fig. 6A, line 3**). According to the literature, leupeptin has inhibitory activity against cysteine proteases (Solomon, et al., 1999).

The enzymatic activity of PLCP enzyme of the *M. citrifolia* in the presence of different ions demonstrated there was no proteolytic activity for monovalent ions such as K^+ and Na^+ . Zn^{2+} also did not reach proteolytic activity. The proteolytic activity was achieved with bivalent Ca^{2+} ion (**Fig. 6B, line 5**). Previous studies demonstrated that calcium ion could increase the proteolytic activity by maintaining the enzyme structure to the active form when subjected to high temperatures as observed with enzymes purified from *Bacillus* sp SMIA-2 strain and *Euphorbia cf. lactea* (Nascimento and Martins, 2004; Siritapetawee, et al., 2015). Thus, our results suggest that the ion calcium was able to stabilize the PLCP enzyme and increased its proteolytic activity.

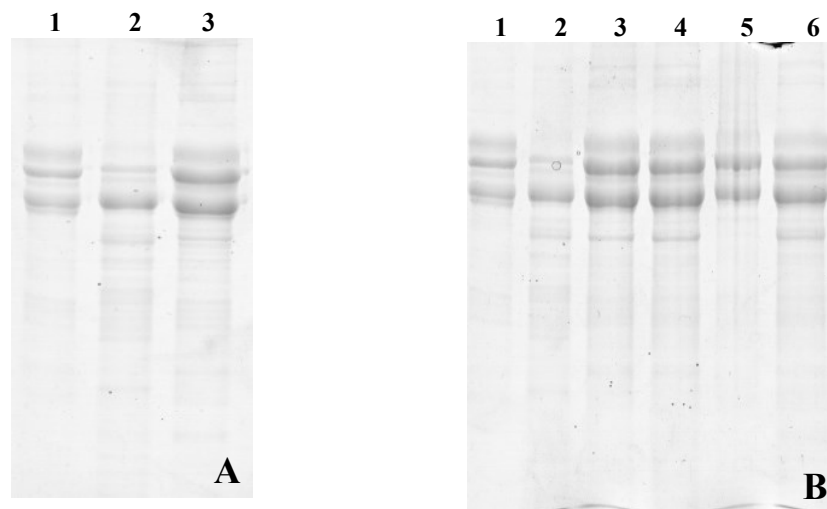


Figure 6: Polyacrylamide gel electrophoresis in 14% of the results of the fibrinogen digestion made by the PLPC for *Morinda citrifolia* seed. Line 1: negative control-fibrinogen incubated with enzyme for 0 minutes. **(A)** Inhibitor-dependent. Lane 2: fibrinogen incubated with enzyme (considering that the enzyme is at 20 g) for 30 minutes; Lane 3: fibrinogen incubated with enzyme for 60 minutes after preincubation of enzyme with Leupeptin. **(B)** Ion-dependent. Lanes 2-6: fibrinogen incubated with enzyme for 60 minutes after preincubation of enzyme with Potassium Chloride (KCl); Sodium Chloride (NaCl); Calcium Chloride ($CaCl_2$); Zinc Chloride ($ZnCl_2$).

3.3 Coagulant activity and Platelet aggregation assay

The enzyme presented high fibrinogen degradation activity, however, did not present any coagulant or platelet aggregation activity (results not shown).

3.4 Cell viability assay

Cytotoxic effects from different concentrations of PLCP enzyme were tested on Human Foreskin Fibroblasts (HFF) and murine embryo fibroblasts strain NIH/Swiss (NIH/3T3) macrophages. After incubating 18 hours, the PLCP enzyme of the *M. citrifolia* did not change the viability of these cells even at higher concentrations (**Fig. 7**). Similar results were observed with *Porphyra tenera* crude extract that did not show cytotoxicity in macrophages cells (Song, et al., 2017).

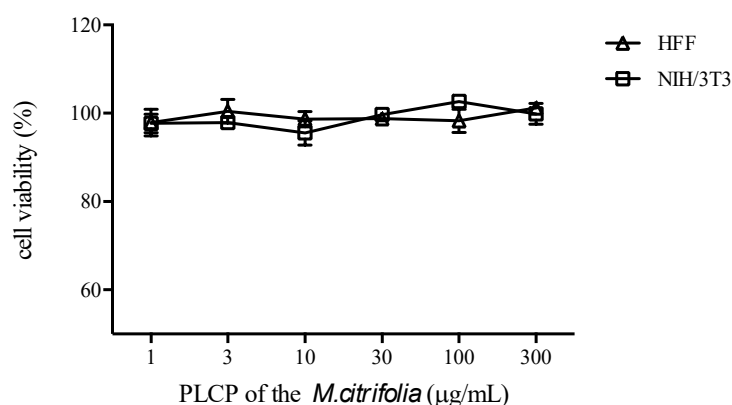


Figure 7: Cytotoxic effects of PLCP enzyme of the *M. citrifolia* against cells lineages. Percentage of cell viability in HFF, and NIH/3T3 demonstrating the non-cytotoxic effects of PLCP enzyme even at higher concentrations.

3.5 Antiparasitic activity

The pretreatment of *T. gondii* tachyzoites with PLCP from *M. Citrifolia* before infection of NIH/3T3 cells showed a dose-response inhibitory curve that reached up to 52.5% of inhibition and showed an IC_{50} of 570.98 µg/mL for the infection index (**Fig. 8A**). Concerning the inhibition of intracellular parasite replication, the pretreatment of *T. gondii* tachyzoites with PLCP from *M. Citrifolia* before infection of NIH/3T3 cells showed a dose-dependent inhibition, reaching rates of 59% and IC_{50} of 507.02 µg/mL (**Fig. 8B**). These findings indicate that can be effective when tested directly against the parasite in both infection parameters. Similar results have been demonstrated by others herbs extract. For instance, it was reported in study done in Western Africa, that aqueous extracts from *Vernonia colorata* were found to be inhibit

Toxoplasma gondii activity (Benoit-Vical, et al., 2000). In addition, de Oliveira, et al. (2009) demonstrated that *Artemisia annua* L. was able to control *T. gondii* infection.

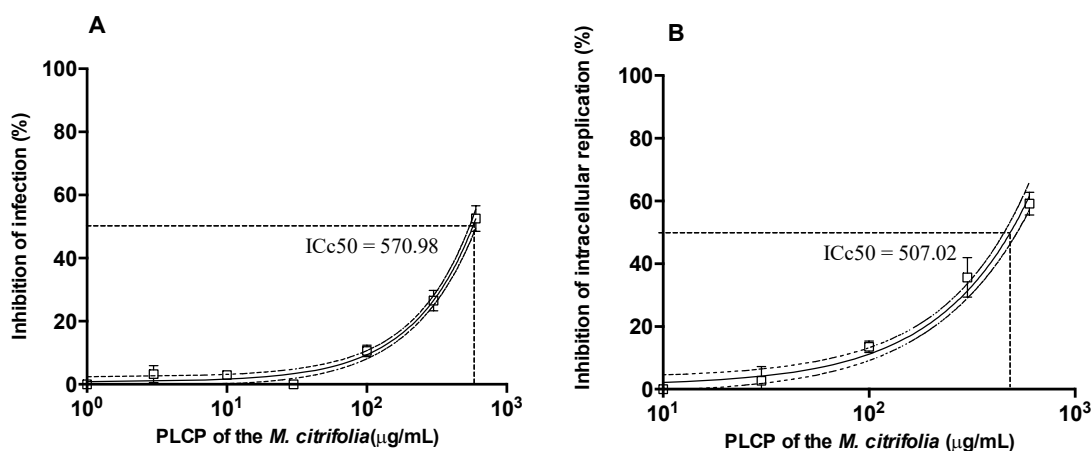


Figure 8. Effect of pretreatment of *T. gondii* tachyzoites with PLCP from *M. Citrifolia* in different concentrations (1, 3, 10, 30, 100, 300 and 600 $\mu\text{g/mL}$) or with medium alone (control) (A) on *T. gondii* infection and (B) intracellular replication in NIH/3T3 cells. Results are expressed as mean and SD of the percentages of inhibition of infection and intracellular replication related to controls. Dotted lines show the inhibitory concentration of 50% (IC_{50}).

4. Conclusion

In summary, the present study demonstrated the enzymatic and biological characteristics of the PLCP enzyme purified from *M. citrifolia* seed. This enzyme demonstrated apparent molecular weight of 51 KDa and hydrolysis of the fibrinogen chains, with the optimum pH of 7.0; temperatures range from room temperature to 50°C ; better activity with calcium ions and the effect of inhibition appeared in the presence of Leupeptin. PLCP enzyme was not cytotoxic for HFF and NIH/3T3 cells and demonstrated antiparasitic activity by *T. gondii*. Then, the present work demonstrated that the medicinal properties of the enzyme presented in *M. Citrifolia* Seed should be explored in more detail.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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