

Screening of protease inhibitory activity in extracts of five Ascidian species from Cuban coasts

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ABSTRACT

One of the most promising strategies to counteract malaria is by controlling the infectivity of *Plasmodium* parasites with drugs aimed against their main targets. The participation of proteases (belonging to different classes) has been demonstrated in its infection mechanism, hence these proteins have become promising targets in treating this illness. This study involved the screening of crude and clarified extracts of five Cuban ascidian species for inhibitory activity of proteases belonging to different mechanistic classes. Ascidiarians are invertebrates that present many bioactive molecules, but, as yet, only a few protease inhibitors had been isolated from them. In this research we reported for the first time the presence of inhibitory activity of bovine pancreatic carboxypeptidase A and B, aminopeptidase N from porcine kidney and subtilisin A from *Bacillus licheniformis* in some ascidian extracts tested. The most promising extracts were selected in terms of percentage of inhibition and they were characterized in terms of the behaviour of the extract concentration against residual enzymatic activity; the determination of IC₅₀ values and Specific Inhibitory Activity (SIA), and the evaluation of their proteolytic activity through zymographic assays.

Keywords: ascidiarians, proteolytic activity, inhibitors, malaria, proteases

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RESUMEN

Búsqueda de actividad inhibidora de proteasas en extractos de cinco especies de ascidias de las costas cubanas. Una de las estrategias más promisorias para confestrar la malaria es controlar la infectividad del parásito *Plasmodium*, con fármacos dirigidos hacia sus blancos principales. Se ha demostrado que proteasas (pertenecientes a diferentes clases mecanísticas) participan en el mecanismo de infección, por lo que se han convertido en blancos promisorios para el tratamiento de la enfermedad. En este trabajo se investigaron extractos crudos y clarificados de cinco especies de ascidiarias cubanas en busca de actividad inhibidora de proteasas pertenecientes a diferentes clases mecanísticas. Las ascidiarias son animales invertebrados que presentan numerosas moléculas bioactivas, aunque solo unos pocos inhibidores de proteasas han sido aislados a partir de ellas. En esta investigación se informa por primera vez la presencia de actividad inhibidora de carboxipeptidasa A y B pancreática bovina, aminopeptidasa N de riñón porcino y subtilisina A de *Bacillus licheniformis* en algunos de los extractos de ascidiarias evaluados. Los extractos más promisorios se caracterizaron con relación al comportamiento de la concentración de extracto en función de la actividad enzimática residual, la determinación de los valores de IC₅₀ y actividad inhibidora específica (SIA), y la evaluación de su actividad proteolítica a través de ensayos zimográficos. Investigaciones actuales se encuentran dirigidas a la purificación y caracterización de estas moléculas.

Palabras clave: ascidiarias, actividad proteolítica, inhibidores, malaria, proteasas

Introduction

Malaria is a parasitic disease present in many regions of the world and produces approximately 1.5 - 2.7 million deaths each year [1]. The resistance to current anti-malarial drugs is spreading rapidly and the development of new vaccines is very difficult. The World Health Organization (WHO) has therefore argued the urgent need to develop new anti-malarial drugs aimed at new therapeutic targets. In this sense, the isolation of several proteases that play important roles during *Plasmodium falciparum* infection, the parasite that causes the most lethal form of Malaria, has been described. In its intra-erythrocyte state, *P. falciparum* degrades almost all of the haemoglobin for its growth and maturation. This process is carried out by proteases belonging to different mechanistic classes such as: aspartic proteases (plasmepsins) [2], cysteine proteases (falcipains) [3] and metallo-amino-peptidases

[4]. In addition, serine proteases (subtilases) [5] and genes encoding for metallo-carboxy-peptidases [6] have been found in the *P. falciparum* genome, and they have been related to its infection mechanisms. Certain inhibitors from these enzymes have been tested in *in vitro* experiments blocking *P. falciparum* growth and its expansion to other erythrocytes [7-10]. Therefore, the search for inhibitors from these proteases has become a new strategy for developing new antimalarial drugs.

On the other hand, marine organisms are very important sources of lead compounds in current drug development. Among them, the ascidiarians (Phylum Chordata, Subphylum Urochordata) are animals found in every ocean, which are fixed onto rocks or shells [11]. Several bioactive molecules have been isolated from ascidiarians, but only a very small fraction corresponds

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to protease inhibitors [12-14] and only two of them have been isolated and characterized until now. Taking into account, the importance of ascidians as a source of bioactive compounds, as well as the need to find active inhibitors against *Plasmodium* proteases, we carried out this study where we screened extracts of five Cuban ascidian species for protease inhibitors, against model proteases belonging to the same classes as those found in the parasite, such as: bovine pancreatic carboxypeptidase A (CPA) and B (CPB) (metallo-carboxy-peptidases), pancreatic trypsin and *Bacillus licheniformis* subtilisin A (serine proteases), porcine pepsin (aspartic protease), *Carica papaya* papain (cysteine protease) and porcine kidney amino-peptidase N (APN) (metallo-amino-peptidase). This is a preliminary characterization of the inhibitory activity shown by these ascidian extracts that are useful for the future selection and purification of the most promissory extracts.

Materials and methods

Materials

Enzymes: CPA (EC 3.4.17.1), CPB (EC 3.4.17.2), trypsin from bovine pancreas type I (EC 3.4.21.4), porcine pepsin (EC 3.4.23.1) and subtilisin A from *B. licheniformis* (EC 3.4.21.62) were purchased from Sigma Chemical Company (EUA). Papain from *C. papaya* (EC 3.4.22.2) was purchased from Calbiochem-Novabiochem (EUA). APN was partially purified from porcine kidney following the procedure described by Golich and colleagues [15]. A partially purified mixture of aminopeptidases, containing APN, aminopeptidase A (APA) and aminopeptidase B (APB) was obtained, which was termed APN. Synthetic substrates: N-(4-Metoxiphenylazofomaryl)-L-phenylalanine (AAFP), Metoxyphenylazofomaryl-L-arginine (AAFR)-L-arginine (AAFR), Benzoyl-arginine-p-nitro-aniline-HCl (BAPA), t-butyloxycarbonyl-glycyl-glycyl-leucil-p-nitroaniline (Boc-Gly-Gly-Leu-pNA), Leucine-p-nitroaniline (Leu-pNA) and leucine-serine-nitrophenylalanine-norleucine-leucine-methyl ester (Leu-Ser-Phe(NO₂)-Nle-Ala-Leu-OMe) were purchased from the Bachem Company (Germany).

Preparation and clarification of ascidian crude extracts

Five ascidian species were studied: *Ascidia nigra*, *A. sydneiensis*, *Ecteinascidia turbinata*, *Diplosoma liseterianum* and *Potoclinum constellatum*. The specimens were collected from the Northwestern coast of Havana City (Cuba). To obtain the crude extract, whole animals were homogenized in distilled water at a 1:2 proportion (w/v) at 4 °C using a blender (10 sec for 5 repeated cycles). Then, the extracts were centrifuged at 10 000 x g for 30 min at 4 °C and the supernatant was filtered on fiberglass [16].

Clarified extracts were obtained by heating or with the acid treatment of crude extracts. Heating was done for 20 min at 60 °C followed by extract centrifugation at 12 100 x g for 30 min at 4 °C. Alternatively, extracts were treated with 2.5% trichloroacetic acid (TCA) and incubated for 1 h at room temperature. Then, they were centrifuged at 17 400 x g for 30 min at 4 °C. The supernatant was adjusted to pH 7.0 with

1M NaOH and extensively dialyzed against distilled water (1:100, supernatant/water) using membranes with 500 Da of the cutoff (Spectra/Por®, USA).

Determination of protein concentration

Protein concentration of all extracts was determined by absorbance at 280 nm (Ultrospec 4000 spectrophotometer) assuming a $\xi_{280\text{nm}}^{0.1\%} = 1$ [17] and enzyme concentration was calculated using their extinction coefficient [18]. In the case of APN, total protein concentration was determined using the Bradford method [19].

Inhibitory activity of the extracts

The enzymatic activity for CPA (9.0 x 10⁻⁷ M) and CPB (9.0 x 10⁻⁷ M) was assayed using a specific substrate for each enzyme: AAFP (0.1 mM, 1 K_M (Michaelis-Menten constant)) [20] and AAFR (0.1 mM, 1 K_M) [21], respectively. Trypsin (3.1 x 10⁻⁷ M) and papain (3.0 x 10⁻⁸ M) were tested using BAPA (1 mM, 1 K_M) as the substrate [22-23]. The enzymatic activity of subtilisin A (3.0 x 10⁻⁷ M) was determined using Boc-Gly-Gly-Leu-pNA (1 mM, 1 K_M) [24] and for pepsin (7.1 x 10⁻⁹ M) it was monitored using Leu-Ser-Phe(NO₂)-Nle-Ala-Leu-OMe (0.22 mM, 11.3 K_M) as the substrate [25]. APN (0.2 mg/mL) was evaluated against Leu-pNA (0.3 mM), a specific substrate for this enzyme and not for APA and APB [26]. In all cases the enzyme and substrate concentrations are referred to the assay.

The enzymatic assays were carried out in an Ultrospec 4000 spectrophotometer. The inhibition assays included a 10 min pre-incubation of the sample with the enzymes on the activity buffer at 25 °C or 37 °C before the substrate was added (temperature and buffer used depended on the enzyme). Inhibition was detected through the decrease in residual activity, which is the ratio of v_i/v_o, where v_i and v_o are the initial rates of the reaction in the presence and absence of the inhibitor, respectively. The behavior of residual activity for each extract was evaluated through the increase in extract concentration. To calculate the IC₅₀ values (concentration of the inhibitor producing 50% inhibition), the experimental data were adjusted to the IC₅₀ equation using the GRAFIT program (version 3.01).

Additionally, in all cases, the percentage of inhibition (%I) and the initial inhibitor ([I_o]) and enzyme ([E_o]) concentration ratio ([I_o]/[E_o]) were calculated in the assay. For these determinations we used the extract concentration producing the highest inhibition (lower residual activity values) and we assumed total initial protein concentration as [I_o]. We considered the extracts that could produce more than 65% inhibition with [I_o]/[E_o] values lower than 50 as the positive selection criteria for a more thorough characterization.

Also, the proteolytic activity of the most promissory extracts was evaluated in order to avoid false positive inhibition due to proteolytic enzymes that could hydrolyze the enzyme used in the inhibitory assay. Enzymatic activity against the different chromogenic substrates (specific for each type of enzyme) was measured under the same conditions described above for each enzyme, but without the enzyme used as the control in the inhibitory assay.

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Zymography

Proteolytic activity of the extracts was also evaluated through zymographic assays using gelatine (16%) as the substrate included in 12.5% polyacrylamide gel [27]. The crude and clarified extracts were run through the gel. Gels were washed with Triton X100 (twice) and incubated overnight with 20 mM Tris-HCl, 0.5 M NaCl buffer, pH 8.0. Subsequently, they were stained and de-stained according to the Lantz and Ciborowski procedure [27].

Results

Screening of protease inhibitory activity

The inhibitory activity was evaluated in the crude and clarified extracts. The crude extracts did not show inhibitory activity against proteases tested, with the exception of the *D. listerianum* extract, which was active against APN, reaching 80.3% of inhibition (result not shown). Hence, we only show the results obtained with clarified extracts because most of them were able to inhibit some of the enzymes tested in this screening.

Regarding the trypsin and papain enzymes, only the heated extract of *D. listerianum* inhibited trypsin and the TCA treated extract of *E. turbinata* inhibited papain with the inhibitory activity percentage above 65%. However the $[I_o]/[E_o]$ ratio was very high (taking into account our selection criteria; table 1). No extract showed inhibitory activity for pepsin (Table 1). For this reason, we did not continue working with these three enzymes and focused only on the results related to the inhibition of the enzymes CPA, CPB, APN and subtilisin by certain clarified extracts.

In the case of *A. sydneiensis* and *P. constellatum*, only TCA treated extracts showed inhibition, in particular against CPA (Table 1). The Specific Inhibitory Activity (SIA) calculated for them was 1499 U/mg and 725 U/mg, respectively (with $[I_o]/[E_o]$ ratio of 2.5 and 1.9) and the former had the largest SIA value of all in the screening. For these extracts, the effect of their concentration against CPA residual activity presented a concave behaviour, which is characteristic of reversible inhibition, with IC_{50} values of 13 μ g/mL and 38 μ g/mL, respectively (Figure 1).

On the other hand, clarified extracts of *D. listerianum* showed a broad specificity against metallo and

serine proteases (Table 1). They were able to reach a high inhibition of CPA, CPB, APN and subtilisin showing a concave behaviour for these enzymes (Figure 2). The TCA treated extract inhibited CPA and APN with IC_{50} values of 24 μ g/mL and 340 μ g/mL, whereas the heated extract inhibited APN and CPB, showing IC_{50} values of 480 and 426 μ g/mL, respectively. The heated extract also inhibited subtilisin, but produced total inhibition with an IC_{50} value of 40 μ g/mL. The SIA of the heated extract against APN, CPB and subtilisin was 81 U/mg, 41 U/mg and 75 U/mg, respectively; while the SIA obtained for the TCA treated extract against APN and CPA were 118 U/mg and 435 U/mg, being the latter the largest value obtained for this ascidian specie.

On the other hand, interesting results related to the metallo-carboxy-peptidases tested in this screening were obtained with clarified *A. nigra* extracts. The TCA treated extract only showed CPA inhibitory activity with a concave behaviour when plotting extract concentration against residual activity, an IC_{50} value of 168 μ g/mL and a SIA value of 197 U/mg (Table 1; Figure 3A). Nevertheless, the heated extract was not able to inhibit CPA; in contrast, it has CPA-like enzymatic activity, because it is able to hydrolyze AAFP, a specific substrate for CPA. Moreover, the heated extract showed CPB inhibitory activity and was able to reach 100% inhibition but with a $[I_o]/[E_o] = 225$ (Table 1).

Proteolytic activity and zymography of clarified extracts

In order to evaluate the presence of false positive inhibitors in the extract mentioned above due to proteases (which could degrade the enzyme used as the control), we evaluated the proteolytic activity of all crude and clarified extracts through colorimetric assays and zymography described in the experimental section. Only the specie *A. nigra* (both crude and heated extracts) showed enzymatic activity against chromogenic substrates, in particular against AAFP, a typical substrate for CPA. However, this activity was not found when the extract was treated with TCA. Figure 3B shows the linear relationship between the initial rate of the enzymatic reaction and extract concentration obtained for the heated extract. This reac-

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Table 1. Screening the inhibitory activity of proteases present in clarified ascidian extracts*

Extract/Treatment	CPA ^a [I _o]/[E _o]	% [I _o]	CPB ^b [I _o]/[E _o]	% [I _o]	APN ^c [I _o]/[E _o]	% [I _o]	Subtilisin [I _o]/[E _o]	% [I _o]	Trypsin [I _o]/[E _o]	% [I _o]	Papain [I _o]/[E _o]	% [I _o]	Pepsin [I _o]/[E _o]	% [I _o]
<i>A. nigra</i> /TCA	27.5	78	36	27	5.5	40	137.5	5	157	2	3914	40	6850	0
<i>A. nigra</i> /heat	ned ^d	PA ^e	225	100	15	41	375	32	429	12	2686	5	18 750	8
<i>A. sydneiensis</i> /TCA	2.5	84	3.6	0	0.27	3	13.8	1	16	2	757	14	650	2
<i>A. sydneiensis</i> /heat	8.1	3	ne	PA	1.9	36	47.5	24	54	0	2714	17	2350	0
<i>E. turbinata</i> /TCA	1.9	54	2	0	0.3	15	7.5	8	9	0	400	67	350	0
<i>E. turbinata</i> /heat	18.8	19	17	0	2.6	39	63.8	5	73	0	3600	54	3150	0
<i>D. listerianum</i> /TCA	3.8	67	13.6	13	4.1	71	28.5	39	59	1	2214	9	2600	29
<i>D. listerianum</i> /heat	20	3	25.6	60	3.8	76	10.8	100	380	100	10 642	55	9300	0
<i>P. constellatum</i> /TCA	1.9	70	4.6	13	0.7	10	17.5	6	20	0	1014	44	900	0
<i>P. constellatum</i> /heat	15.6	0	30.7	0	4.6	33	115	6	131	1	5304	0	5700	8

* The total protein concentration in the assay assumed as inhibitory concentration [I_o]/ assayed enzyme concentration [E_o] ratio ([I_o]/[E_o]) and %I values shown, correspond to the point of maximum inhibition achieved by the clarified extracts. [E_o] were 9.0 \times 10⁻⁷ M for CPA and CPB, 0.2 mg/mL APN, 3.0 \times 10⁻⁸ M subtilisin, 3.1 \times 10⁻⁸ M trypsin, 3.0 \times 10⁻⁸ M papain and 7.1 \times 10⁻⁹ M pepsin.

^aCPA: carboxypeptidase A.

^bCPB: carboxypeptidase B.

^cAPN: aminopeptidase N.

^dne: No enzyme added to the assay.

^ePA: extract with proteolytic activity.

tion was inhibited by the potato carboxypeptidase inhibitor, an inhibitor of a metallo-carboxy-peptidase belonging to the M14A subfamily (data not shown).

On the other hand, as a result of zymographic assays we found that crude and heated extracts of *P. constellatum*, *A. sydneiensis* and *A. nigra* displayed clear bands in the gels, which represent gelatine degradation by the presence of proteolytic enzymes in the extracts. In contrast, when the extract was treated with TCA the proteolytic activity disappeared and no clear bands were observed (Figure 4A-C). In the case of the *D. listerianum* crude extract, two clear bands were observed (with different molecular weights), and only one band disappeared with each treatment.

Discussion

One of the most important strategies to counteract malaria is to design a drug composed of natural inhibitors against the main protease parasite targets. In this paper we used model proteases to screen ascidian extracts for new natural inhibitors that in the future could be purified and characterized for this purpose.

Ascidians constitute a very important group of organisms for the isolation of bioactive molecules, being the most promising of them the drug Yondelis, isolated from *E. turbinata* and used against different types of cancer [28]. However, only a few protease inhibitors have been isolated from these organisms. The first one was an inhibitor of trypsin (enzyme belonging to PA clan, S1 family) isolated from the *Halicynthia roretzi* [14]. The second were two inhibitors active against trypsin and elastase, respectively, isolated from *Polyandrocarpa misakiensis* [13]. The third is an inhibitor of matrix metalloprotease 2 (MA clan, M10 family), isolated from a tunicate of the *Polyclinidae* family [12].

The ability of marine invertebrates to inhibit enzymes belonging to the M1 family of the MA clan was described by our group in the case of HcPI, an inhibitor isolated from *Hermodice carunculata* that is able to inhibit pyroglutamyl aminopeptidase II [29]. Nevertheless, in this study we report for the first time the inhibition of APN (MA Clan, M1 family) by extracts of *D. listerianum*. It is important to emphasize that the molecules responsible for APN inhibition in this species are stable under both clarification conditions used in this work.

In *P. falciparum* a metallo-amino-peptidase (PfA M1) belonging to the same family of APN was found, which had broad substrate specificity and was involved in haemoglobin degradation [30]. This fact suggests that PfAM1 constitutes a crucial target for the inhibition of parasite growth and development [31]. It is possible that APN inhibitors also could be able to inhibit PfAM1, which could be very promising in obtaining new antimalarial drugs [31].

Also for *D. listerianum* we reported for the first time the inhibitory activity of CPA, CPB (MC clan, M14 family) and subtilisin (SB clan, S8 family). Recently it was reported that the *P. falciparum* genome contains a gene of a metallo-carboxy-peptidase like enzyme belonging to the Nna1 family [6]. Its recombinant *Caenorhabditis elegans* homologous presented broad substrate specificity and is inhibited by inhibitors of metallo enzymes [6]. These authors suggest that these

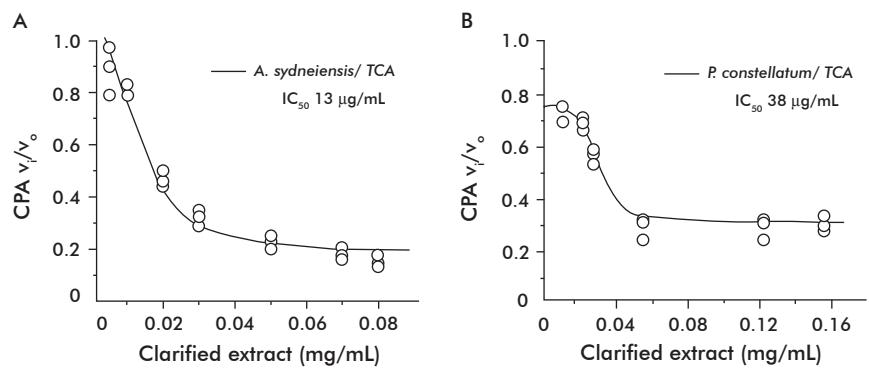


Figure 1. CPA inhibitory activity of *A. sydneiensis* (A) and *P. constellatum* (B) clarified extracts. v/v_0 represents the residual activity of the enzyme, where v_i and v_o are the initial rate in the presence and absence of the inhibitor. Data were fitted to an IC_{50} equation supported by the GRAFIT program.

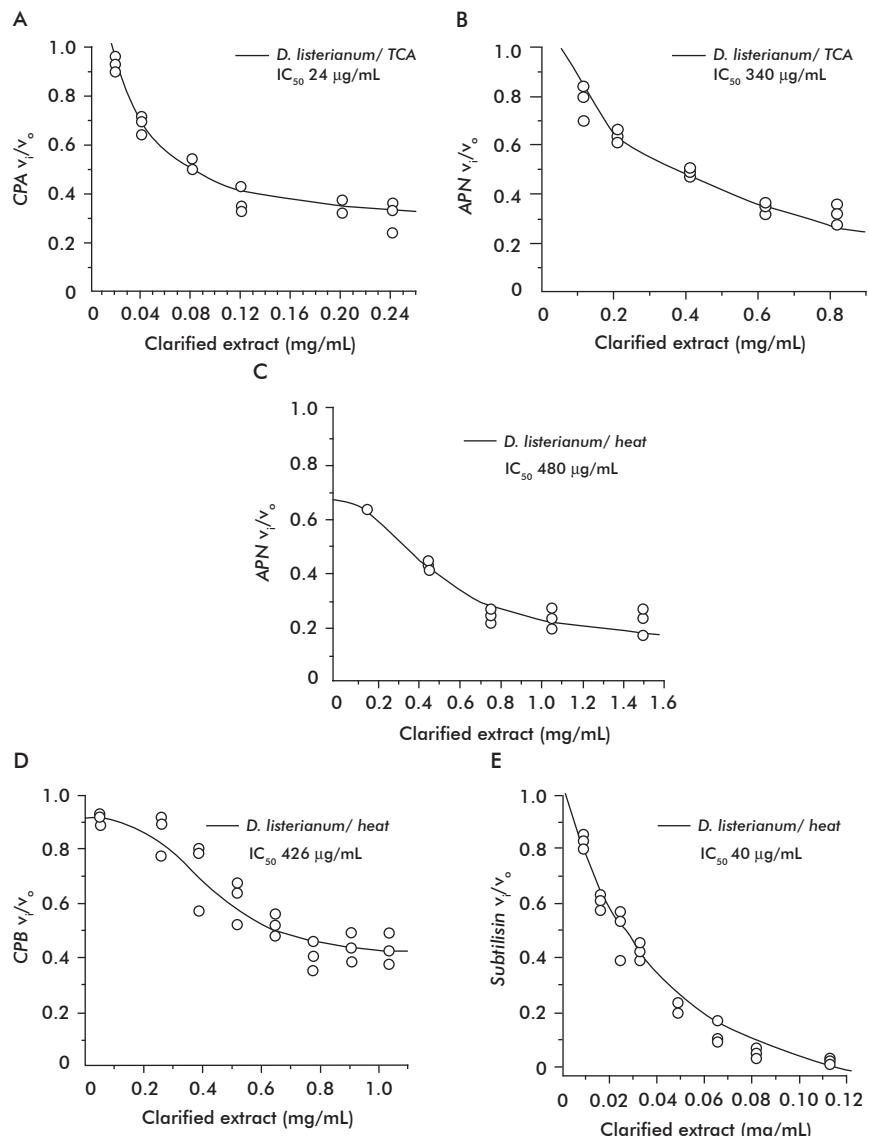


Figure 2. Inhibitory activity of *D. listerianum* clarified extracts. A, B-C, D and E represent the inhibitory activity of: CPA, APN, CPB and subtilisin, respectively. v/v_0 represents the residual activity of the enzyme, where v_i and v_o are the initial rate in the presence and absence of the inhibitor. Data were fitted to an IC_{50} equation supported by the GRAFIT program.

types of enzymes could have tubulin carboxypeptidase activity and could therefore be involved in the infection mechanism of the parasite [6]. In addition, the expression of two midgut carboxypeptidase B gene (*cpbAg1* and *cpbAg2*) in *Anopheles gambiae* has been found to be up-regulated by *P. falciparum* infection and the addition of antibodies against CPB_{Ag1} to a *P. falciparum*-containing blood meal, inhibited CPB activity and blocked parasite development [32]. Therefore, the inhibition of both CPA and CPB like enzymes could result in a promissory strategy for developing drugs and transmission-blocking vaccines against the parasite [6, 32].

TCA treated extracts of *A. nigra*, *A. sydneiensis*, *P. constellatum* and *D. listerianum* inhibit CPA in a reversible way, taking into account the behaviour of the extract concentration against CPA residual activity. For CPA, the analysis of IC_{50} and SIA values suggests that *A. sydneiensis* represents the most efficient extract in CPA inhibition. The biological activity (inhibitory or proteolytic) of *A. nigra* and *A. sydneiensis* extracts found in this work could be related to the recently reported inhibition of *P. falciparum* growth by these extracts [33].

On the other hand, the presence of CPA like proteolytic activity in the heated extract of *A. nigra* (observed by colorimetric assay), general proteolytic activity (observed by zymography), as well as CPA inhibitory activity in TCA clarified extract, constitute an interesting result obtained in this study. This fact could be explained in two ways. Perhaps the crude extract has both types of activities (enzymatic and inhibitory), but proteolytic activity is predominant and hindered the detection of inhibition. This possibility was eliminated when the extract was treated with TCA. On the other hand, it is known that TCA is able to dissociate the non covalent complex, such as protease-inhibitors, allowing inhibitor detection [34]. This behaviour was previously observed with the dipeptidyl peptidase IV inhibitory activity of *Xetospongia muta* extracts [35] and ShPI I, a proteinase inhibitor from *Stichodactyla helianthus* [36]. Nevertheless, heat treatment does not eliminate CPA proteolytic activity as observed in *A. nigra* crude extract, suggesting the presence in this species of a CPA like enzyme that is stable at least at this incubation temperature and time. In addition, in zymographic experiments we observed that the heated extract maintains proteolytic activity, suggesting the presence of endoproteinases in this extract, which were eliminated with TCA treatment. Hence, it is possible to suggest that the CPB inhibitory activity observed in *A. nigra* heated extract (results not shown considering the selection criteria) could be explained due to the fact that the proteases found in this extract could degrade the pancreatic CPB used in the assay causing apparent inhibition. The presence of proteolytic enzymes that are resistant to heat was also observed for the enzyme isolated from the mollusc *Aplysia dactylomela* [37].

On the other hand, it is known that the inhibition of the subtilisin-family serine protease PfSU B1 and the cysteine protease dipeptidyl peptidase 3 (DPAP3) of *P. falciparum* impairs its infection mechanism [38]. We used subtilisin from *B. licheniformis* as a model enzymes and obtained total inhibition by the heated

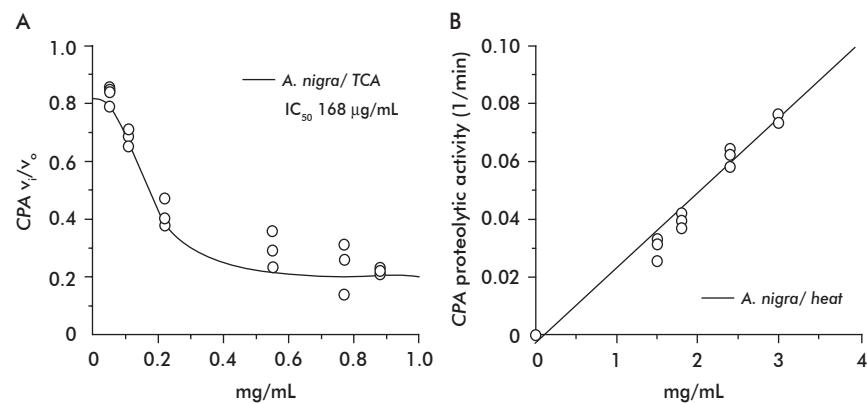


Figure 3. Inhibitory and proteolytic activity of *P. nigra* clarified extracts. A) CPA inhibitory activity of *P. nigra* clarified extract. v/v_0 represents the residual activity of the enzyme, where v_i and v_o are the initial rate in the presence and absence of the inhibitor. Data were fitted to an IC_{50} equation supported by the GRAFIT program. B) CPA like proteolytic activity of the heated extract measured as the initial rate of the enzymatic reaction.

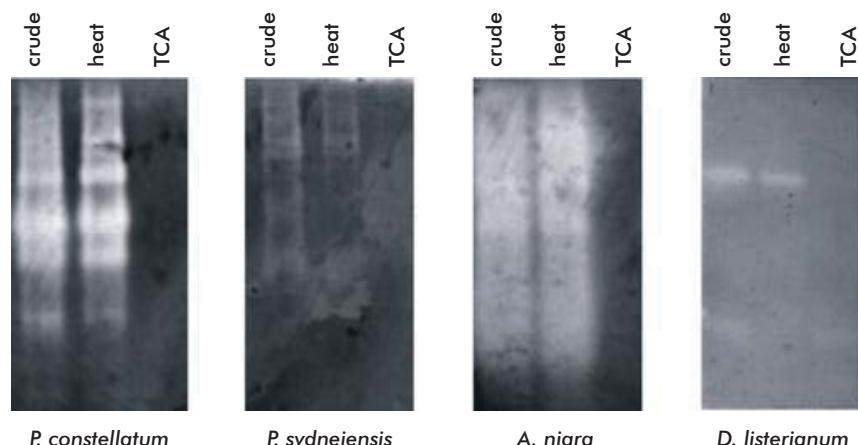


Figure 4. Zymographic assays of the most promising extracts. A 12.5% polyacrylamide gel containing 16% of gelatine was used. The incubation buffer was 20 mM Tris-HCl, 0.5M NaCl, pH 8.0. A staining and de-staining procedure was carried out according to Lantz and Ciborowski (27). In all cases, lanes 1, 2 and 3 correspond to the crude, heated and TCA-treated extracts, respectively.

extract of *D. listerianum* suggesting the efficiency of this inhibitor even when it is not purified. Nevertheless, the only clear band observed in the zymography of the *D. listerianum* heated extract, could mean that there is a proteolytic enzyme present, which is responsible for subtilisin degradation and hence apparent inhibition. Further experiments are being carried out to elucidate this.

Here, for the first time we carried out a screening for protease inhibitors in a group of ascidians using model proteases belonging to the same mechanistic classes as those found in *P. falciparum* parasite. We described the inhibitory activity of CPA, CPB, APN and subtilisin in extracts of *A. nigra*, *A. sydneiensis*, *P. constellatum* and *D. listerianum* with promising results; being this the first report of biological activity described for *P. constellatum* and *D. listerianum* extracts. In addition, this study could be the first step in the line of developing new anti-malarial compounds from inhibitors of the main target of *P. falciparum*. Current efforts are directed to the further purification and characterization of these molecules.

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