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Antiparasitic effect of a fraction enriched in tight-binding protease inhibitors isolated from the Caribbean coral *Plexaura homomalla*

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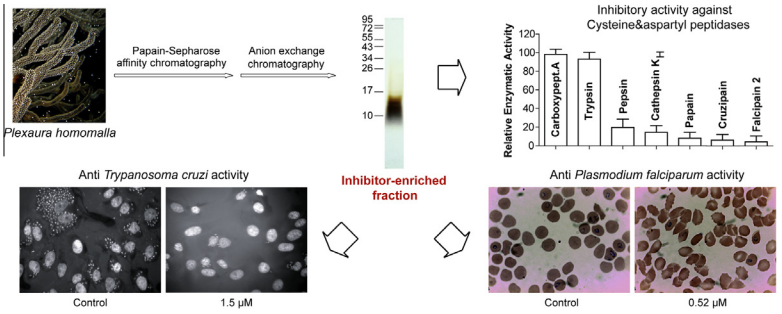
HIGHLIGHTS

- *Plexaura homomalla* extract inhibits Cruzipain and Falcipain 2 in the nanomolar range.
- An inhibitor-enriched fraction is active against cysteine(CP) and aspartyl proteases.
- CP inhibitors represent >90% of total inhibitory activity in enriched fraction.
- Inhibitor-enriched fraction reduces infection and replication of *Trypanosoma cruzi*.
- Inhibitor-enriched fraction also inhibits the replication of *Plasmodium falciparum*.

GRAPHICAL ABSTRACT



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ABSTRACT

Malaria and American Trypanosomiasis constitute major global health problems. The continued emergence and spreading of resistant strains and the limited efficacy and/or safety of currently available therapeutic agents require a constant search for new sources of antiparasitic compounds. In the present study, a fraction enriched in tight-binding protease inhibitors was isolated from the Caribbean coral *Plexaura homomalla* (Esper, 1792), functionally characterized and tested for their antiparasitic activity against *Trypanosoma cruzi* and *Plasmodium falciparum*. The resultant fraction was chromatographically enriched in tight-binding inhibitors active against Papain-like cysteine peptidases (92%) and Pepsin-like aspartyl peptidases (8%). Globally, the inhibitors present in the enriched fraction showed no competition with substrates and apparent K_i values of 1.99 and 4.81 nM for Falcipain 2 and Cruzipain, the major cysteine peptidases from

Abbreviations: AAFP, N-(4-metoxiphenylazoformyl)-L-phenylalanine; ACN, acetonitrile; AMC, 7-amino-4-methyl coumarin; AU, absorbance units; BAPA, benzoyl-arginyl-p-nitro-anilide-HCl; CMD, carboxymethyl dextran; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; IF, intensity fading; NaAc, sodium acetate buffer; RP, reverse-phase; RT, room temperature; TFA, trifluoracetic acid; Z-FR-AMC, benzyloxycarbonyl-Phenyl-Arginyl-7-amino-4-methyl coumarin.

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Cysteine peptidases
Tight-binding inhibitor
Antiparasitic agents

P. falciparum and *T. cruzi*, respectively. The inhibitor-enriched fraction showed promising antiparasitic activity in cultures. It reduced the growth of the chloroquine-resistant *P. falciparum* strain Dd2 (IC_{50} = 0.46 - μM) and promoted the apparent accumulation of trophozoites, both consistent with a blockade in the hemoglobin degradation pathway. At sub-micromolar concentrations, the inhibitor-enriched fraction reduced the infection of VERO cells by *T. cruzi* (CL Brener clone) trypomastigotes and interfered with intracellular differentiation and/or replication of the parasites. This study provides new scientific evidence that confirms *P. homomalla* as an excellent source of tight-biding protease inhibitors for different proteases with biomedical relevance, and suggests that either the individual inhibitors or the enriched fraction itself could be valuable as antiparasitic compounds.

1. Introduction

Malaria and American Trypanosomiasis are prevalent neglected tropical protozoal diseases, constituting serious global health problems (Mathers et al., 2007). Malaria, caused in humans by five species of the genus Plasmodium, generates annually about 660,000 deaths, more than 219 million new infections and 3.2 billion persons are in risk of infection (http://www.dndi.org/diseases-projects/diseases/malaria/global-view.html, date of last access: July 23, 2013). Meanwhile, American Trypanosomiasis or Chagas disease (caused by Trypanosoma cruzi) is endemic in Latin America and is a leading cause of death by heart failure in the region. About 12,000 people die annually from this disease, 8 millions are currently infected and another 100 millions at continuous risk of infection (http://www.dndi.org/diseases-projects/diseases/chagas/global-view.html.

Over the last 10 years, the study of peptidases from protozoan parasites has acquired considerable importance. Some of these enzymes have been proposed to play central roles in diverse processes such as cell invasion, differentiation, cell cycle progression, catabolism of host proteins, parasite feeding and evasion of the host immune response (Alvarez et al., 2012; Klemba and Goldberg, 2002; Wegscheid-Gerlach et al., 2010); raising as attractive targets for therapeutic intervention. Falcipain 2 is the major papain-like cysteine protease of *Plasmodium falciparum* (Sijwali et al., 2001), the most deadly human malaria parasite. It occurs in the food vacuole as a 27 kDa mature enzyme during trophozoite stage, although it is synthesized as an inactive zymogen that undergoes proteolytic auto-activation. This enzyme is considered a promising therapeutic target (Rosenthal et al., 2002; Teixeira et al., 2011). It is actively involved in (i) hemoglobin hydrolysis (Goldberg, 2005), (ii) proteolytic activation of pro-plasmepsins (a family of food-vacuole aspartic hemoglobinases) (Drew et al., 2008) and (iii) release of parasites from red blood cells (Dhawan et al., 2003). Finally, Falcipain 2 inhibitors effectively blocked hemoglobin degradation, leading to parasites with swollen food vacuoles and abnormal morphology (Rosenthal, 2011), the same phenotype observed in Falcipain 2-knockout parasites (Sijwali and Rosenthal, 2004). Falcipain 2 inhibitors arrested *P. falciparum* development in the mature trophozoite form *in vitro* (Naughton et al., 2010; Shenai et al., 2002), reduced parasitaemia levels *in vivo* (Micale et al., 2006; Olson et al., 1999) and showed potent synergistic effect when combined with Plasmepsin inhibitors (Semenov et al., 1998).

Cruzipain, the main cysteine protease of *T. cruzi*, is an important virulence factor. It is a member of a large family of polymorphic genes encoding highly similar isoforms (approximately 88–98% amino acid identity) which are globally responsible for the majority of the proteolytic activity present in epimastigotes lysates (Duschak and Couto, 2009; Kosec et al., 2006). Cruzipain is expressed in the four main stages of *T. cruzi* life cycle, and is present in lysosome-related organelles (Alvarez et al., 2012). The highest concentration is found in epimastigote-specific pre-lysosomal organelles called reservosomes (Sant'Anna et al., 2008; Souto-Padron et al., 1990), although it could also be associated to plasma membrane in amastigotes (Parussini et al., 1998) and secreted to extracellular medium by trypomastigotes (Aparicio et al., 2004). Cruzipain is involved in parasite nutrition, invasion of mammalian cells and evasion of the host immune response (Cazzulo et al., 2001; McKerrow et al., 2006). In addition, Cruzipain participates in differentiation steps of the parasite's life cycle, which are blocked by permeant irreversible inhibitors of the enzyme (Franke de Cazzulo et al., 1994) and enhanced by its over-expression (Tomas et al., 1997). The use of specific inhibitors of this enzyme has effectively inhibited parasite invasion and replication in mammalian cells (Franke de Cazzulo et al., 1994; Harth et al., 1993; Meirelles et al., 1992), as well as promote direct parasite killing in culture (Ashall et al., 1990). The treatment of *T. cruzi*-infected mice with the potent Cruzipain inhibitor N-methyl-piperazine-Phe-homoPhe-vinyl sulphone phenyl (K777) resulted in their effective rescue form lethal infection (Doyle et al., 2007; Engel et al., 1998), confirming the validity of this enzyme as a chemotherapeutic target for Chagas disease.

Natural products constitute a very attractive source of chemical diversity for drug discovery, showing a relatively high rate of success in comparison to synthetic compounds obtained by combinatorial chemistry (Koehn and Carter, 2005; Li and Vederas, 2009). Although only a very small fraction of known organisms has been included in some type of screening for bioactive compounds, about 80% of the drugs in use until 1990 came directly from natural products or had derived from them, with plants, bacteria and marine organisms as the most studied (Li and Vederas, 2009; Molinski et al., 2009; Harvey, 2007, 2008). Among marine fauna, invertebrates from tropical seas constitute a particularly attractive and little-explored source of bioactive compounds. They comprise an enormous biological diversity, from where a large group of bioactive molecules, including antimalarial compounds (Watts et al., 2010; Gademann 2009) and protease inhibitors for all mechanistic classes (Alonso-del-Rivero et al., 2012; Covaleda et al., 2012; Delfin et al., 1996; Fusetani et al., 1999; Gonzalez et al., 2007; Lenarcic et al., 1997; Pascual et al., 2004) have been identified.

The Caribbean gorgonian *Plexaura homomalla* (Esper, 1792) (*Metazoa*, *Cnidaria*, *Anthozoa*, *Alcyonaria* (*Octocorallia*), *Alcyonacea*, *Holaxonia*, *Plexauridae*, *Plexaura*) has especially proved to be a prolific source of bioactive compounds with pharmacological interest. This sea whip coral contains the highest concentrations of different kinds of prostaglandins and prostaglandin esters found so far in nature, accounting for 2–3% of its dry weight (Weinheimer and Spraggins, 1969). In addition, a glutathione transferase inhibitory activity has been found in organic extracts of *P. homomalla* (Whalen et al., 2010), associated with the defensive and antipredatory roles of prostaglandins (Gerhart, 1984). More recently, a reversible and tight-binding Plasmepsin II inhibitor was purified from the aqueous extract of *P. homomalla* (Ramirez et al., 2009), suggesting the potential of this organism as a source for protease inhibitors active against different mechanistic classes. Using a combination of different screening methodologies, we further confirmed the existence of potent Papain inhibitory activity in the same extract (unpublished results), although the purification and

characterization of their active components were not addressed until now. In the present paper, we re-evaluate the inhibitory activity of *P. homomalla* aqueous extract against Papain and extend the analysis to Falcipain 2 and Cruzipain. The molecules responsible for cysteine protease inhibition were partially purified by chromatographic procedures and the resultant fraction was functionally characterized in terms of global inhibitor concentration, enzymatic specificity and binding kinetics and affinity to the target enzymes. In addition, the antiparasitic effect of the partially-purified fraction was evaluated against *T. cruzi* and *P. falciparum* cultured parasites. Finally, the diversity and identification of the inhibitory components were explored by intensity fading MALDI-TOF MS.

2. Materials and methods

2.1. Chemicals

Proteolytic enzymes Papain from *Carica papaya* (EC 3.4.22.2), Carboxypeptidase A from bovine pancreas (EC 3.4.17.1), Trypsin from bovine pancreas type I (EC 3.4.21.4) and porcine Pepsin (EC.3.4.23.1) and inhibitors [E-64 and pepstatin A] were obtained from Sigma–Aldrich (St. Louis, MO, USA). The fluorogenic substrate for cysteine proteases benzyloxycarbonyl-phenyl-arginyl-7-amino-4-methyl coumarin (Z-FR-AMC), and the chromogenic substrates benzoyl-arginyl-p-nitro-anilide-HCl (BAPA), N-(4-metoxiphenylazoformyl)-L-phenylalanine (AAFP) and leucine-serine-nitrophenilalanine-norleucine-alanine-leucine-methyl ester (Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe) were obtained from Bachem Bioscience (Bubendorf, Switzerland).

2.2. Collection of P. homomalla. Preparation and clarification of aqueous extract

Specimens of *P. homomalla* (Esper, 1792) were identified "*in situ*" and collected by snorkelling and scuba diving in the main marine habitats (sea grass beds, mangroves, coral reefs, sand bottoms and rocky coasts), from low tide to a depth of 10 m, in various locations along the northwest Cuban coast from Havana to Puerto Esperanza (Pínar del Río).

Crude aqueous extracts were prepared by homogenizing the external structures (polyps and coenenchyme) of the wet specimen in distilled water (1:2 w/v) followed by centrifugation (10,000*g*, 30 min, 4 °C) and subsequent filtration by glass wool. For clarification, the extract was heated (60 °C, 30 min) and centrifuged (15,000*g*, 30 min, 4 °C) to eliminate insoluble components. The clarified extract was kept at −20 °C until use.

2.3. Selective concentration of the protease inhibitors present in the clarified extract of P. homomalla

A papain–Sepharose support was synthesized via a Glyoxyl–Sepharose derivative (Otero et al., 1988) at an immobilization grade of 2 mg mL^{-1} as it facilitates further recovery of tight-binding inhibitors under milder elution conditions. The papain–Sepharose resin was equilibrated with 100 mM NaAc, 150 mM NaCl, pH 5.5 and then incubated overnight (with gentle stirring at 4 °C) with the clarified extract. The resin was packed into a column and washed with equilibrium buffer until $DO_{280nm} \sim 0$. The retained components were eluted from the column with 100 mM Na_2HPO_4, 2 M NaCl, pH 9.5 and immediately neutralized with 1 M NaAc, pH 5.5. Fractions carrying papain inhibitory activity were pooled, concentrated with a 3 kDa Cut off ultra-filtration device (Millipore SAS, Molsheim, France) and dialyzed overnight against 4 L of 50 mM Na_2HPO_4, pH 7.5. The dialyzed fraction was applied to a 5 mL HiTrap Q HP column (GE Healthcare, Little Chalfont, UK) equilibrated with 50 mM Na_2HPO_4, pH 7.5 using an AKTA Purifier HPLC/FPLC system (GE Healthcare). After washing the column to remove unadsorbed substances, the bound components were eluted with a linear NaCl (0–2 M in 50 mM Na_2HPO_4, pH 7.5) gradient (Total volume: 40 mL). Fractions containing papain inhibitory activity were pooled, dialyzed and concentrated (inhibitor-enriched fraction) as above.

2.4. Expression and purification of enzymes

Cruzipain was purified to homogeneity from epimastigotes of the Tul 2 strain, as described previously (Labriola et al., 1993; Parussini et al., 2003). Falcipain 2 was expressed as inclusion bodies in BL21(DE3) *Escherichia coli* strain; purified under denaturing conditions and refolded to active enzyme as previously described (Sarduy et al., 2012). A *Pichia pastoris* strain expressing human Cathepsin K was kindly donated by Dr. Dieter Bromme (University of British Columbia, Vancouver, Canada). The enzyme was expressed, activated and purified as previously described (Linnevers et al., 1997).

2.5. Enzymatic assays

Cruzipain activity was assayed fluorimetrically with 12.5 μM Z-FR-AMC as substrate in 100 Tris–HCl, and 10 mM DTT, pH 7.6. The release of 7-amino-4-methyl coumarin was monitored (excitation 355; emission 460 nm) over 5 min at RT with an Aminco Bowman Series 2 spectrofluorometer (Thermo Spectronic, Madison, WI, USA). The activity of human Cathepsin K and Falcipain 2 was assayed using the same substrate Z-FR-AMC (12.5 μM) in 100 NaAc, and 10 mM DTT, pH 5.5 (Linnevers et al., 1997; Sarduy et al., 2012) The evaluation of porcine Pepsin activity was performed in 100 mM NaAc, pH 4.4 using the substrate Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe (215 μM) (Ramirez et al., 2009). Trypsin (20 Tris–HCl, 150 NaCl, and 20 mM $CaCl_2$, pH 8.0) and Carboxypeptidase A (20 Tris–HCl, and 500 mM NaCl, pH 7.5) activity were assayed spectrophotometrically using the substrates BAPA (Erlanger et al., 1961) and AAFP (Mock et al., 1996), respectively.

All the inhibition assays included a 10 min pre-incubation of the sample with the enzymes on the activity buffer at RT before the substrate was added. Inhibition was detected through the decrease in residual activity, which is the ratio of v_i/v_0, where v_i and v_0 are the initial rates of the reaction in the presence and absence of the inhibitor, respectively. The IC_{50} values (concentration of the inhibitor required to produce half-maximal degree of inhibition) were estimated by fitting the equation $\frac{v_i}{v_0} = \frac{1}{1+\frac{[I]}{IC_{50}}}$ (Copeland, 2000) to experimental data from dose–response curves using the GraphPad Prism program (version 5.03). To determine the inhibition type exerted by the inhibitor-enriched fraction against cysteine proteases, the effect of substrate concentration on the IC_{50} value against Falcipain 2 was determined as described previously (Copeland, 2000).

2.6. Estimation of the apparent concentration of active inhibitors by titration with enzymes

The enzymatic assays described above were used to estimate the active concentration of class-specific inhibitors present in the *P. homomalla* inhibitor-enriched fraction, using the prototypical enzymes Papain (26.3 nM, titration with E-64) and Pepsin (35.0 nM, titration with Pepstatin A). Titration assays were conducted under experimental conditions ($[E_0]/K_i \geqslant 10$–100) ensuring titrant behavior (Williams and Morrison, 1979). Residual enzymatic activity was evaluated after the addition of increasing volumes of the inhibitor-enriched fraction. The titration volume was estimated by extrapolating the linear portion of dose–response curves to the volume (X) axis (Copeland, 2000). The active concentration of the inhibitor was calculated assuming a

stoichiometric ratio 1:1. As this value characterizes the concentration of multiple inhibitors (specific either for Papain or Pepsin) it was considered as apparent.

2.7. Experiments with IAsys optical biosensor

Kinetic analyses of interaction between active components from inhibitor-enriched fraction with Cruzipain and Falcipain 2 was performed using an IAsys Biosensor instrument (ThermoLabSystems, Cambridge, UK). Purified Cruzipain and Falcipain 2 were immobilized in 10 mM NaAc pH 4.0 on a carboxymethylated dextran layer (CMD cuvettes) using EDC/NHS chemistry following manufacturer instructions (8–15 ng of active enzymes were immobilized on a typical experiment). For kinetic constant determination, dilutions of the inhibitor-enriched fraction in 50 NaAc, and 150 mM NaCl, pH 5.5 were added to the cuvette. Binding curves (25 °C) were recorded for at least six different concentrations. Apparent kinetic association (k_{ass}) and dissociation (k_{diss}) rate constants were calculated by fitting the equations of monophasic association and dissociation models to experimental data using the FASTfit software (ThermoLabSystems). The obtained k_{diss} value was used to calculate the time for 50% of ligate dissociation to occur ($t_{1/2}$) by using a first-order dissociation kinetic. Equilibrium constants (K_D) were determined by direct analysis of binding curves using equilibrium response data.

2.8. Effect of the inhibitor-enriched fraction on intracellular Cruzipain

T. cruzi epimastigotes (Cl Brener clone) (Zingales et al., 1997) at 5×10^7 mL^{-1} were incubated with the inhibitor-enriched fraction at apparent concentration of 1 μM for 6 h at 28 °C. Fractions (5×10^7 parasites) were taken at different time points (1, 3 and 6 h) and washed 10 times in PBS buffer, without fetal calf serum, in order to remove all unbound inhibitor. Parasites extracts were then obtained (Cazzulo et al., 1989) and subjected to evaluation of Cruzipain activity. A similar non-treated parasite culture was used as control.

2.9. Effect of the inhibitor-enriched fraction on the growth and differentiation of T cruzi

T. cruzi epimastigotes (Cl Brener clone) were inoculated to 5×10^6 mL^{-1} and cultured at 28 °C in brain–heart–tryptose (BHT) medium containing 10% inactive fetal calf serum (Franke de Cazzulo et al., 1994); 1×10^6 mL^{-1} trypomastigotes, obtained as in (Franke de Cazzulo et al., 1994), were incubated in MEM medium with 4% fetal calf serum at 37 °C, 5% CO_2, both in the absence (control) or presence of the inhibitor-enriched fraction at apparent concentration of 1 μM. The motile parasites were counted in a Neubauer chamber after 48 and 24 h, respectively.

For infection experiments, Vero cells were seeded (1×10^4 cells/well) in 24-well cell culture cluster flat bottom (Corning Inc., Corning, NY, USA) containing glass coverslips with 500 μL of MEM containing 10% (v/v) fetal calf serum. Cells were allowed to attach for 24 h in a humidified 5% CO_2/95% air atmosphere at 37 °C. The culture was inoculated with trypomastigotes (Cl Brener clone) previously "matured" for 3 h at 37 °C. After infection for 24 h, performed either in the presence (at apparent concentrations 0.01–1.5 μM) or absence of the inhibitor-enriched fraction and using 1×10^6 mL^{-1} parasites, the medium containing the extracellular parasites was removed. Fresh medium, containing 4% fetal calf serum and without inhibitors was added, and the coverslips were incubated at 37 °C, 5% CO_2 for 48 h. The cells were then fixed and stained either with May Grunwald-Giemsa or DAPI. For each well (control, control with PBS buffer and with inhibitor), the number of intracellular parasites was estimated by observing approximately 500 cells in 10–20 random fields. The results (means ± SD) are expressed as percent of cells infected, average number of intracellular parasites per infected cell, and endocytic index (calculated by multiplying the first two values).

2.10. Plasmodium falciparum cultures

P. falciparum strains Dd2 (clone MRA-150) were used for this study. Erythrocytes were obtained from type A+ human healthy local donors and collected in Vacuette® tubes with citrate–phosphate–dextrose anticoagulant (Greiner Bio-One, Frickenhausen, Germany). The culture media consisted of standard RPMI 1640 (Sigma–Aldrich) supplemented with 0.5% Albumax I (Gibco, Carlsbad, CA, USA), 100 μM hypoxanthine, 25 mM HEPES, 12.5 μg mL^{-1} gentamicin (Sigma–Aldrich) and 25 mM $NaHCO_3$ (Moneriz et al., 2011). Each culture was started by mixing uninfected and infected erythrocytes to achieve a 1% hematocrit and incubated in 5% CO_2 at 37 °C in tissue culture flasks (Iwaki Glass, Funabashi, Chiba, Japan). The progress of growth in the culture was determined by microscopy in thin blood smears stained with Wright's eosin methylene blue solution (Merck, Darmstadt, Germany), using the freely available Plasmoscore software (Proudfoot et al., 2008) to monitor the parasitaemia. The detailed description of the culture and synchronization methods used has been reported previously (Radfar et al., 2009).

2.11. Effect of the inhibitor-enriched fraction on the growth and differentiation of P. falciparum

A PicoGreen® microfluorimetric DNA-based assay was used to monitor parasite growth inhibition (Moneriz et al., 2009). PicoGreen® (Invitrogen, Carlsbad, CA, USA) was diluted as indicated by the manufacturer in TE buffer (10 Tris–HCl, and 1 mM EDTA, pH 7.5). Synchronized rings from stock cultures were used to test different apparent concentrations (0.027–5.27 μM) of the inhibitor-enriched fraction in 96-well culture microplates. Thus, 150 μL of parasites at 2% hematocrit and 1% parasitaemia were allowed to grow for 48 h in 5% CO_2 at 37 °C. The parasites were then centrifuged at 600*g* for 10 min and re-suspended in saponin (0.15%, w/v in PBS) to lyse the erythrocytes and release the malaria parasites. To eliminate all hemoglobin traces, the pellet was washed (twice) by the addition of 200 μL of PBS followed by centrifugation at 600*g*. Finally, pellets were re-suspended in 100 μL of PBS and 100 μL of PicoGreen® diluted in TE were added to each well. Plates were incubated for 30–60 min in the dark and the fluorescence intensity was measured at 485 excitation and 528 nm emission. Growth inhibition was calculated as previously described (Moneriz et al., 2009). Parasite morphology was then evaluated from replicate experiments by microscopic analysis of Wright's -stained thin blood smears.

2.12. Identification of active components from inhibitor-enriched fraction by intensity fading MALDI-TOF MS

The identification of cysteine and aspartic-specific protease inhibitors in the enriched fraction was carried out using the IF MALDI-TOF MS approach, as previously reported (Villanueva et al., 2003; Yanes et al., 2007) using prototypical enzymes. Papain and Pepsin immobilized on agarose microbeads were kindly donated by Dr. Giovanny Covaleda (Universitat Autònoma de Barcelona, Barcelona, Spain). In the experiment, 100 μL of the inhibitor-enriched fraction was mixed with 100 μL of interaction buffer 2× and 100 μL of enzyme-agarose suspension. After 10 min incubation at RT, the resin was collected by centrifugation and washed with 100 μL of interaction buffer (four times). Finally, the interacting molecules were eluted by changing the pH. Every fraction (sample, flow-through, every wash and eluate) were ana-

lyzed by MALDI-TOF MS. On the Papain IF assay, 100 Na_2HPO_4, 100 KCl, 0.1 EDTA, and 3 mM DTT, 0.05% Brij 35, pH 6.5 was used as interaction buffer and TFA 0.1% for the elution step, whereas on the Pepsin assay the interaction buffer was 50 mM NaAc, pH 4.0, and the elution of the bound molecules was carried out in successive steps using 100 μL of 0.1% TFA, 100 μL of 1% NH_4OH and 100 μL of H_2O:ACN:TFA (65.9:33:1), respectively.

All samples were mixed with a matrix solution (1:1, v/v) of 10 mg mL^{-1} sinapinic acid (Bruker Daltonics, Bremen, Germany) dissolved in H_2O: ACN: TFA (65.9:33:1); 0.5 μL of the mixture was spotted onto the MALDI-TOF groundsteel plate by using the dried-droplet method. Mass spectra were acquired in an UltrafleXtrem mass spectrometer (Bruker Daltonics) equipped with a smartbeam™ II laser in linear-mode geometry under 20 kV and 1000 laser shots. A mixture of proteins from Bruker Daltonics (protein calibration standard I; mass range 3000–25,000 Da) was used as a standard.

3. Results

3.1. The aqueous extract of Plexaura homomalla inhibits the cysteine proteases Falcipain 2 and Cruzipain

Given that a potent Papain inhibitory activity had been observed by us in aqueous extract of *P. homomalla*, we decided to re-evaluate this activity (for batch-to-batch reproducibility) and further extend the analysis to Falcipain 2 and Cruzipain. To increase the confidence in the detection of inhibitors, we performed the evaluations using two orthogonal methodologies. First, inhibitory activity of clarified extract was evaluated against both enzymes by using a fluorogenic (continuous) enzymatic assay. The heat-clarified extract of *P. homomalla* inhibited the activity of both Cruzipain and Falcipain 2 in a dose–response manner (Fig. 1A and 1B). The IC_{50} ranged from 0.1125 ± 0.0125 μg mL^{-1} for Cruzipain to 0.093 ± 0.0179 μg mL^{-1} for Falcipain 2, and clearly showed a biphasic behavior, suggesting the presence of tight-binding cysteine protease inhibitor(s).

Then, an IAsys Biosensor instrument was used to confirm the occurrence of Falcipain 2 and Cruzipain interacting components (potential inhibitors) in the clarified extract. Both enzymes were immobilized to CMD cuvettes via amine-coupling and used to test the specific binding of the extract components to the activated surface (immobilized lysozyme was used as a reference surface). The resultant sensograms clearly indicated the presence of molecules able to interact specifically with the immobilized proteases, showing a dose–response behavior (Fig. 1C and D).

3.2. Selective concentration of cysteine protease inhibitory activity present in the extract of Plexaura homomalla

Aiming to isolate and selectively concentrate the active component(s) responsible for the inhibition of cysteine proteases in the extract of *P. homomalla*, we performed an affinity chromatography step using a preparative Papain–Sepharose column (Fig. 2A). The chromatographic profile showed a maximum of absorbance at 280 nm that coincided with the maximum in Papain inhibitory activity. Eluted fractions were pooled, desalted and further fractionated by anion exchange chromatography (Fig. 2B). The fractions containing inhibitory activity were pooled and finally concentrated. The SDS–PAGE analysis of the resultant fraction displayed a complex pattern of bands, ranging from approximately 6 to 16 kDa after silver staining (Fig. 2C). A summary of the purification procedure is presented in Table 1.

3.3. The inhibitor-enriched fraction showed functional characteristics similar to a reversible, tight-binding and non-competitive cysteine protease inhibitor

Given that tight-binding inhibitors ($K_i \leqslant 10^{-7}$ M) have the greatest interest for biomedical and biotechnological applications,

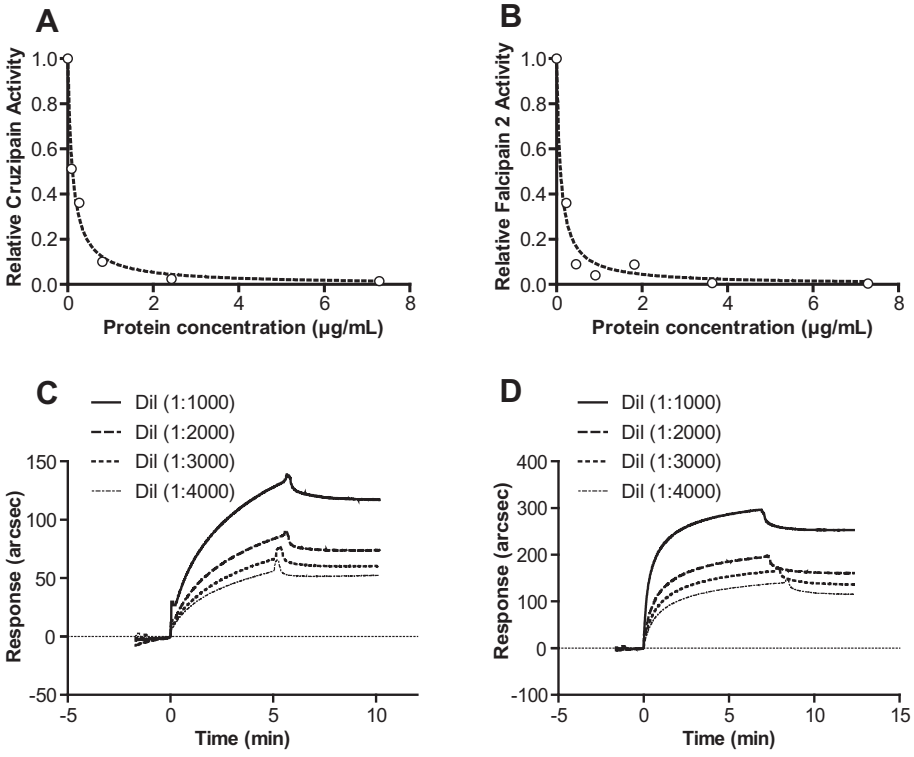

Fig. 1. Inhibition of Cruzipain and Falcipain 2 by the aqueous extract of *P. homomalla*. Biphasic dose–response curves showing the inhibition of Cruzipain (A) and Falcipain 2 (B) by the heat-clarified extract of *P. homomalla*. IAsys biosensor sensograms confirming the dose-dependent binding of extract components to Cruzipain- (C) and Falcipain 2-CMD cuvettes (D).

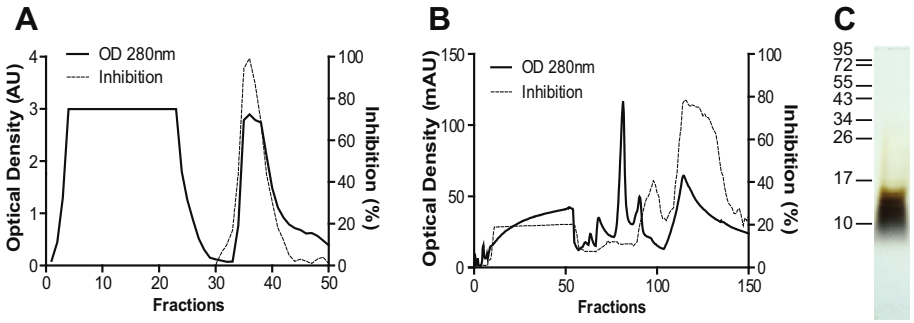

Fig. 2. Partial purification of the protease inhibitors present in the clarified extracts of *P. homomalla*. (A) Affinity chromatography of clarified extract in a Papain–Sepharose support. (B) Ionic exchange chromatography of the inhibitory fractions resultant from affinity chromatography. (C) Tris-tricine SDS–PAGE (16.5% T; 6% C) analysis of the inhibitor-enriched fraction (silver staining). Position of molecular protein markers (kDa) is indicated. Optical density at 280 nm was represented by a continuous (▬) line and Papain inhibitory activity by dotted (---) lines.

Table 1
Summary of the partial purification of cysteine protease inhibitors present in the aqueous extract of *P. homomalla*.

Purification step	Total protein (mg)	Total inhibitor (U)	Specific inhibitory activity (U/mg)	Yield (%)	Purification (times)
Crude extract	111.8	480208.9	4295.3	100	1
Clarification	64.1	421735.6	6579.3	87.8	1.5
Affinity chromatography	9.6	294368.1	30625.5	61.3	7.1
Anion exchange chromatography	3.4	166152.3	48966.4	34.6	11.4

Values are from a representative purification experiment from 500 mL of crude extract.

Table 2
Apparent kinetic constants for the interaction of Cruzipain and Falcipain 2 with inhibitors present in the enriched fraction.

Enzyme	k_{ass} (M^{-1} s^{-1})	k_{diss} (s^{-1})	$t½$(s)	k_{diss}/k_{ass} (M)
Cruzipain	$(1.45 \pm 0.43) \times 10^{7}$	$(1.7 \pm 0.66) \times 10^{-2}$	40.8	$(1.17 \pm 0.9) \times 10^{-9}$
Falcipain 2	$(1.98 \pm 0.32) \times 10^{6}$	$(2.67 \pm 0.33) \times 10^{-3}$	259.6	$(1.35 \pm 0.4) \times 10^{-9}$

Values (mean ± SD) for k_{ass} and k_{diss} represent typical behavior observed during this investigation and were obtained from three independent experiments. The values for $t½$ and k_{diss}/k_{ass} were mathematically determined from primary data.

we estimated the apparent K_i values for the inhibitor-enriched fraction against both target enzymes. The global concentration of active inhibitor in the enriched fraction was estimated as 9.67 μM after titration with E-64-titrated Papain. Using this global inhibitor estimation, kinetic assays were performed in the IAsys biosensor to determine apparent kinetic constants k_{ass}, k_{diss} and k_{diss}/k_{ass} for the interaction of the inhibitor(s) with immobilized Cruzipain and Falcipain 2 (Table 2). Apparent thermodynamic dissociation constant K_D determined from binding curves (Fig. 3A and B) were (4.81 ± 1.8) and $(1.99 \pm 0.43) \times 10^{-9}$ M for Cruzipain and Falcipain 2, respectively, confirming the occurrence of at least one tight-binding inhibitor for these enzymes in the enriched fraction.

Furthermore, we assessed the functional specificity of the inhibitors present in the enriched fraction against prototypical proteases of the four major mechanistic classes. As expected, the enriched fraction was able to inhibit Papain (Cysteine) and human Cathepsin K (Cysteine), but not bovine Trypsin (Serine) or Carboxypeptidase A (Metallo) (Fig. 3C). The apparent K_i value estimated for human Cathepsin *K* was $(4.41 \pm 0.29) \times 10^{-7}$ M (Fig. 3D), indicating a marginal tight-binding inhibition. Surprisingly, the enriched fraction showed significant inhibitory activity against porcine Pepsin (Aspartic). The dose–response curve showed a linear shape, suggesting tight-binding inhibition and titrant behavior. After titration of active enzyme with pepstatin A, the global concentration of aspartic-specific inhibitors in the enriched fraction was estimated as 0.89 μM, about 10 times smaller than the specific for cysteine proteases. However, the apparent K_i value for the inhibition of porcine Pepsin by enriched fraction components was estimated as $(2.1 \pm 0.7) \times 10^{-9}$ M (Fig. 3E), suggesting possible relevance in Plasmepsins inhibition in living parasites. At least for Cysteine proteases, a 10-fold increment in substrate concentration was not able to increase the value of IC_{50}, suggesting no competition between substrate and inhibitor(s) (Fig. 3F).

3.4. Inhibitor-enriched fraction of Plexaura homomalla is active against Trypanosoma cruzi and Plasmodium falciparum in cultures

Given that Cruzipain and Falcipain 2 play central roles in different critical processes for parasite development, we further explored the effect of the inhibitor-enriched fraction in cultures of *T. cruzi* and *P. falciparum*. At the apparent cysteine-specific inhibitor concentration of 1 μM, no differences in growth and morphology were observed in *T. cruzi* epimastigotes when compared with non-treated controls after 24 or 48 h of incubation. In order to explore whether the inhibitors were able to penetrate and inhibit their target enzyme(s) inside the living parasites, we evaluated the activity of Cruzipain in cell-free lysates of treated *T. cruzi* epimastigotes. Treated epimastigotes for 1 or 3 h with inhibitor at 1 μM resulted in ~30% reduction in Cruzipain activity in the cell-free lysate (Supplementary data). Interestingly, the observed decrease in Cruzipain activity was transient, as no differences were found after 6 h of incubation, explaining the lack of physiological effects at longer times. At the same concentration, a 24 h incubation of the inhibitor-enriched fraction with *T. cruzi* trypomastigotes did not cause any significant change in viability and

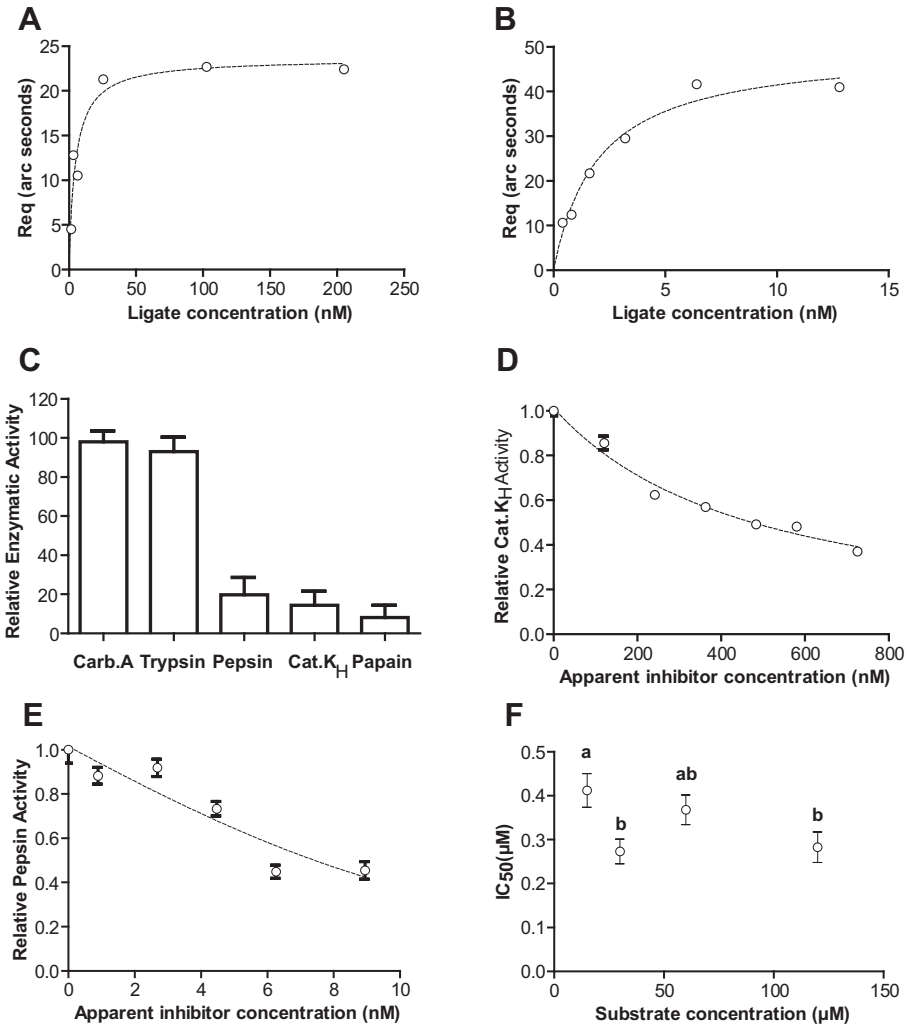

Fig. 3. Functional characterization of the inhibitor-enriched fraction. Binding curves analysis of the interaction of inhibitors in the enriched fraction with Cruzipain- (A) and Falcipain 2-CMD cuvettes (B). (C) Enzymatic specificity of the inhibitor-enriched fraction. Concave dose–response curve for the inhibition of human Cathepsin K (D) and porcine Pepsin (E) with the inhibitor-enriched fraction. Each point (mean ± SD) was evaluated in triplicate in two independent experiments. (F) Effect of substrate concentration on the IC_{50} value obtained for the inhibition of Falcipain 2 by the inhibitor-enriched fraction. Different letters are assigned to significantly different results (post-ANOVA Tukey tests, $p < 0.05$).

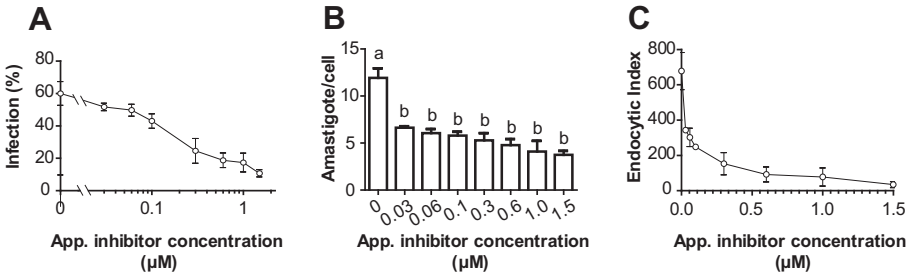

Fig. 4. Antiparasitic effect of inhibitor-enriched fraction against *T. cruzi*. (A) Dose-dependent reduction in Vero cell infection by *T. cruzi* trypomastigotes. (B) Reduction in the number of intracellular amastigotes present in infected Vero cells promoted by the inhibitor-enriched fraction. Different letters are assigned to significantly different results (post-ANOVA Tukey tests, $p < 0.05$). (C) Dose-dependent reduction in endocytic index promoted by the inhibitor-enriched fraction. Results are the mean ± SD of three independent experiments.

morphology of treated parasites. However, the presence of the inhibitor-enriched fraction during the infection of Vero cells by trypomastigotes, the subsequent differentiation of the parasites to amastigotes and the intracellular multiplication of this stage, resulted in a dose–response decrease in the number of infected Vero cells (Fig. 4A). A statistically significant reduction in the average number of intracellular amastigotes per infected cell was concomitantly observed (Fig. 4B), although it doesn't showed a clear dose-dependent behavior. The endocytic index, used as a convenient measure of parasite load, also displayed a dose-dependent and statistically significant reduction in the range of concentration tested (Fig. 4C). These results suggest that the inhibitors present at the enriched fraction interfere with trypomastigote-to-amastigote differentiation and/or intracellular

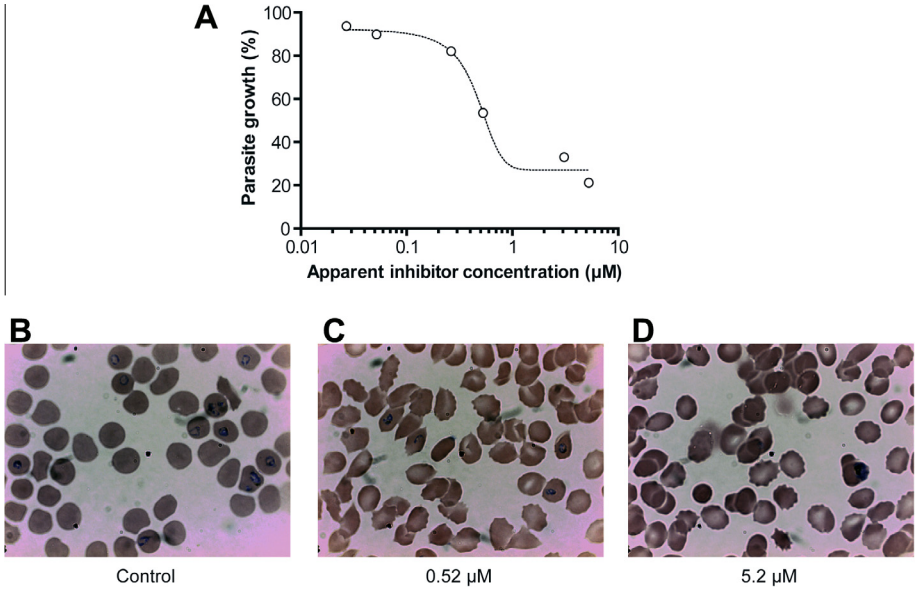

Fig. 5. Antiparasitic effect of inhibitor-enriched fraction against *P. falciparum*. (A) Dose–response curve of inhibitor-enriched fraction on *P. falciparum* (Dd2 strain) infected erythrocytes. *P. falciparum* was grown in erythrocyte cultures at 1% initial parasitaemia and incubated with different apparent concentrations of inhibitor-enriched fractions. The DNA content was determined at 48 h by microfluorimetry. Results are the mean of three independent cultures. Representative microscopic images of *P. falciparum* cultures, incubated or not (B) with apparent concentrations of inhibitor-enriched fraction corresponding to IC_{50} (C) and 10 × IC_{50} (D). The total red blood cell numbers and the parasitized cells number in B, C and D were 49 and 9; 64 and 4 and 67 and 1, respectively, corresponding to 18.4%, 6.3% and 1.5% parasitaemia, respectively.

amastigote replication process at apparent sub-micromolar concentrations.

The effect of the inhibitor-enriched fraction on the growth of the chloroquine-resistant Dd2 strain of *P. falciparum* in human erythrocyte cultures was determined by analyzing the dose–response curve in the sub-micromolar range by using a microfluorometric assay on DNA-stained cultures. The culture showed a dose-dependent inhibitory effect on the parasite growth (IC_{50} = 0.46 ± 0.07 μM) (Fig. 5A). Synchronized Dd2 infected erythrocytes at mature ring stage (1% parasitaemia) were incubated with the inhibitor-enriched fraction at different apparent concentrations during one infective cycle (48 h), sampled and examined by thin blood smears. Typically, the untreated culture reached >10% parasitaemia and contained predominantly trophozoites at sampling time (Fig. 5B), indicating that a complete invasive cycle had taken place, producing new trophozoites. Cultures treated with the inhibitor-enriched fraction at apparent concentration of 0.52 μM (IC_{50} range) accumulated trophozoites at the same time, concomitant with a 50% reduction in parasitaemia as compared to the un-treated culture (Fig. 5C). The increase in parasitaemia suggests that the parasite was able to invade new erythrocytes, although at lower rates, and the observed accumulation of trophozoite stage may account for both first and delayed second generation parasites. Apparent concentrations of 5.2 μM (10 × IC_{50}) led to a complete arrest of parasite growth (1.4% of parasitaemia). At this concentration, parasite population consisted mainly in trophozoites (Fig. 5D), likely from the previous cycle as suggest the absence of further increase in parasitaemia.

3.5. Identification of a bifunctional protease inhibitor specie by Intensity fading MALDI-TOF MS

Given that protease inhibitors must bind their target enzymes (formation of the EI complex) in order to exert enzymatic inhibition, we performed an intensity fading MALDI-TOF MS analysis of the inhibitor-enriched fraction to identify and characterize class-specific protease inhibitors responsible for the observed antiparasitic effects. This technique is based on the analysis of the relative intensities derived from the MALDI ions (m/z) to study the formation of complexes between biomolecules. Complexes are detected through the decrease (fading) of the relative intensities of the m/z signal corresponding to a ligand or mixture of potential ligands after the addition of the target molecule immobilized to an appropriate support. To confirm the binding, the formed complexes are then dissociated to regenerate the faded ion signals corresponding to the interactors.

As shown in Fig. 6A, the spectra corresponding to the inhibitor-enriched fraction exhibited two heterogeneous groups of ion signals around m/z^+ of 6000 and 14000–15000, respectively. The intensity of these ion signals were greatly diminished or completely disappeared in the mass spectra after the addition of Papain–Sepharose support, suggesting the presence of putative interactors. After four washing steps (not bound material was practically undetected) the bound molecules were eluted from the resin by addition of TFA. Five major ion signals were restored, corresponding to m/z^+ of 5973.8, 6074.2, 7357.7, 14711.0 and 14736.9; showing relative intensities considerably higher than in reference (not-bound) MS spectra. Given that the control (without sample) and the blank (using Sepharose) run spectra showed no ion signals in these ranges (data not shown), the few ion signals perturbed (affected by the addition of the enzyme and subsequently restored by elution) were attributed to molecules that specifically bind to the immobilized cysteine protease.

We followed a similar approach to identify the aspartic-specific inhibitors present in the enriched fraction by using this time a Pepsin–Sepharose support. The addition of the immobilized enzyme greatly reduced the intensity of several of the ion signals observed in the initial MS spectra of the inhibitor-enriched fraction, that were later restored by elution under acidic conditions (Fig. 6B). In contrast with the previous experiment, the control run using Pepsin–Sepharose exhibited several ion signals of moderate intensities (~300 AU) in the m/z^+ range 13,000–13,400 and some minor but detectable signals around m/z^+ 6000 (⩽150 AU), consistent with auto-processing of the enzyme (data not shown). However, an intense (1400–2000 AU) signal at m/z^+ 5973.7; not present in

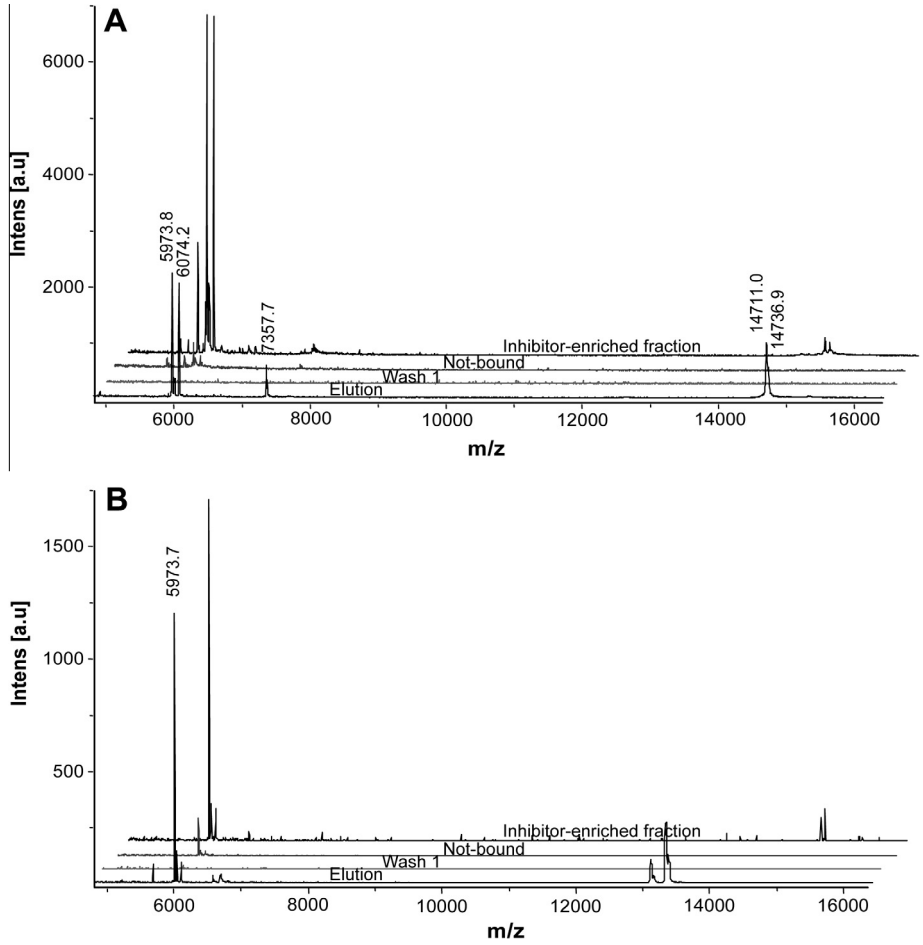

Fig. 6. Identification of protease inhibitors present in the inhibitor-enriched fraction of *P. homomalla*. (A) Intensity fading MALDI-TOF MS analysis of the cysteine protease-specific inhibitors using a Papain–Sepharose support. (B) Intensity fading MALDI-TOF MS analysis of the aspartic protease-specific inhibitors using a Pepsin–Sepharose support.

the control run, was clearly identified and assigned to a Pepsin binding specie. This signal was also identified as a Papain interactor, suggesting that it may be due to a bi-functional molecule capable to interact both with aspartic and cysteine proteases.

4. Discussion

In addition to the broad range of biological activities previously reported for *P. homomalla*, in this paper we describe for the first time the antiparasitic effect of the aqueous extract of *P. homomalla* against both *P. falciparum* and *T. cruzi*. This effect is elicited in part by the cysteine protease inhibitors present in the extract, which are able to inhibit in the nanomolar range the major Papain-like cysteine proteases Cruzipain and Falcipain 2 present in these human parasites. In addition, these inhibitors are able to penetrate and reach their target(s) enzyme(s) at least in *T. cruzi* epimastigotes, as demonstrated by the transient reduction of Cruzipain activity in cell-free lysates after 1–3 h incubation with the inhibitor mixture. More important, the inhibitor-enriched fraction caused enzyme inhibition at the same concentration range they elicit antiparasitic activity ($\sim$1 μM) in amastigote/trypomastigote intracellular stages in culture.

Initially, we intended the purification to homogeneity of individual inhibitors present in the enriched fraction by using a combination of size exclusion chromatography and C18 RP-HPLC. This strategy failed to provide pure inhibitors, mainly due to the heterogeneity of the inhibitor-enriched fraction, the low recovery of individual components and/or the significant loss of their biological activity after acetonitrile elution. Considering the limited number of individuals of *P. homomalla* that can actually be removed without causing damage to marine ecosystems and the low concentration of these inhibitors in the raw extract, we decided to treat the enriched fraction as a natural "cocktail" of inhibitors active against cysteine and aspartic proteases. For those inhibitors active against the same class of enzymes (i.e. cysteine or aspartic proteases), they were functionally considered (in both cases) as a single inhibitor with global features (such as active concentration, specificity, etc.) characterizing the behavior of the whole population. The term "apparent" was used for all the functional parameters estimated under these circumstances.

The inhibitor-enriched fraction showed activity primarily against Papain-like cysteine proteases belonging to Clan CA, family C1, although with clear bias to Cruzipain/Falcipain 2 over human Cathepsin K. The analysis of *P. falciparum* genome indicated the presence of 16 of these enzymes, comprising Falcipain 1, food-vacuole Falcipains (2, 2′ and 3), three dipeptidyl-peptidases and nine serine-repeated antigens (SERA 1-9) (Rosenthal, 2011, 2004). However, the protease activity of many of them has not been biochemically confirmed, or they have been proven to be non-essential for parasite development at erythrocytic stages (Rosenthal, 2011). Only food-vacuole Falcipains and dipeptidyl dipeptidase 1 seem to be involved in hemoglobin degradation, a well-known essential pathway in *P. falciparum*. Similarly, SERAs 4, 5 and 6 have been identified in the parasitophorous vacuole and implicated in

erythrocyte rupture, a critical process to complete parasite cycle that is blocked by cysteine protease inhibitors (Rosenthal, 2004). The accumulation of *P. falciparum* trophozoites after treatment of synchronized parasite cultures with the inhibitor-enriched fraction provides useful evidence to discriminate between these two processes, given that they occur at different time-points in the parasite life cycle. The observed impairment of treated parasites to progress through the life cycle after trophozoite stage is compatible with a blockade in hemoglobin degradation caused by protease inhibition. In addition, it is in good agreement with the timing of expression for these enzymes and the reported anti-parasitic effects of cysteine and aspartic protease inhibitors (Naughton et al., 2010).

Given that no pepsin-like aspartic proteases have been identified in the genome of *T. cruzi* (Alvarez et al., 2012), the anti-parasitic effects observed can be attributed to the inhibition of cysteine proteases belonging to Clan CA, family C1. In addition to Cruzipain isoforms, three other proteases of the same family have been identified in the parasite genome: a 30 Cathepsin B, and a 30 kDa Cathepsin S and a putative bromelain-like protease (Kosec et al., 2006). The relevance of Cruzipain for *T. cruzi* life cycle is well documented (Cazzulo et al., 2001; McKerrow et al., 2006). However, little is known about the functions and essentiality of the other enzymes, although the reservosome location for Cathepsin B and S may suggest house-keeping functions. The impaired ability to invade new cells and the limited capacity for intracellular differentiation and multiplication observed in this work for parasites treated with the inhibitor-enriched fraction; point out to Cruzipain as the most obvious target, although additive or cooperative effects on the other enzymes cannot be dismissed.

The existence of several molecules able to target the same or, even better, different enzyme specificities would be advantageous in two different ways for the potential application of this inhibitor-enriched fraction. First, their combination could elicit additive or synergistic effects on the parasite physiology, targeting simultaneously different processes and metabolic pathways. Secondly, this multi-target inhibition could greatly reduce the possibility of parasite escape, diminishing the chance of generation of drug resistance (Capela et al., 2009; Muregi and Ishih, 2010). In this regard, the existence of several cysteine protease inhibitors (probably belonging to different evolutionary families, with different molecular and functional features) are expected to be important for the global antiparasitic effect of the extract, given the high number of Cruzipain isoforms existent in *T. cruzi* (Campetella et al., 1992) and the presence of several cysteine hemoglobinases in *P. falciparum* (Rosenthal, 2011). In addition, the inhibitory activity against Pepsin-like aspartic proteases found in the enriched extract is also attractive as an additive to the main anti-cysteine protease activity, given the synergistic effect demonstrated for Plasmepsin and Falcipain inhibitors (Semenov et al., 1998).

Although it is not expected that all of the interactors identified by intensity fading MALDI-TOF MS would be, indeed, inhibitors of the target enzymes, it is highly probable that true inhibitors appear as intense signals given the previous concentration of these inhibitory components during partial purification process. The ion signal at m/z^+ 5973.7 detected after specific binding analysis to Papain and Pepsin suggests the presence of a bi-functional (and potentially inhibitory) component in the extract, which would explains, at least partially, the existence of aspartic-specific inhibitory activity after Papain–Sepharose affinity chromatography. In addition, bifunctional inhibitors capable to target simultaneously different kinds of proteases are relatively abundant among marine invertebrates (Alonso-del-Rivero et al., 2012; Gonzalez et al., 2004; Lenarcic et al., 1997). Interestingly, a recent Intensity Fading MALDI-TOF MS study (using Pepsin-Sepharose as binding partner) of the Plasmepsin II inhibitor purified from *P. homomalla* (Ramirez, et al., 2009) showed a predominant signal at m/z^+ 5974.4 (unpublished results). This finding, in addition to the coincidence in the low-nanomolar range apparent K_i values exhibited by both preparations against aspartic proteases, could indicate that the pepsin-specific inhibitory activity observed by us in the inhibitor-enriched fraction would correspond to the previously reported Plasmepsin II inhibitor (Ramirez et al., 2009). This molecule can be already considered as a lead compound against malaria, given the potent inhibition displayed against Plasmepsin II, and the potential for tight-binding inhibition of other Plasmepsin isoforms relevant for parasite physiology (Meyers and Goldberg, 2012). However, the biomedical or biotechnological attractiveness of this molecule could be greatly enhanced if its postulated bi-functionality could be experimentally confirmed.

All together, these findings highlight the general importance of natural products as an abundant and unexplored source for the identification of bioactive molecules with potential biomedical applications. In particular, the aqueous extract of the Caribbean gorgonia *P. homomalla* stands as a prominent source of tight-binding inhibitors for different proteases with biomedical relevance for parasitic infections. New efforts are currently underway in order to isolate and characterize the individual inhibitors present in the inhibitor-enriched fraction.

Authors' contributions

ESS, MACP and JJC designed the experiments and wrote the manuscript. ESS and ACM performed purification and characterization of inhibitors, participated in data analysis and interpretation of results. ACauerhff and ESS designed, performed and interpreted IAsys experiments. SAT performed MALDI-TOF MS experiments, analyzed experimental data and performed the interpretation of Intensity Fading results. AChidichimo and YG performed the evaluation of antiparasitic effects on *T. cruzi* and *P. falciparum*, respectively; analyzed experimental data and performed the interpretation of results. All authors participated in editing the manuscript and all have read and approved the final version.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.exppara.2013.09.013.

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