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RESEARCH

Methanolic extracts of paratoid gland secretions from Cuban *Peltophryne* toads contain inhibitory activities against peptidases with biomedical relevance

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ABSTRACT

Peptidases regulate most biochemical and physiological processes and are essential for propagation of infectious agents. Many became established targets for new therapeutic drugs. Amphibian's skin secretions have evolved as potent defense mechanism against predators and microbials. Although numerous biomedical applications have been documented for this natural source, few examples of peptidase inhibitors have been described. In this contribution we evaluated the inhibitory effect of the methanolic extracts obtained from paratoid gland secretions of four Cuban endemic species of toads (*Peltophryne fustiger*, *P. florentinoi*, *P. peltocephala* and *P. taladai*) against nine peptidase enzymes from different mechanistic classes. The qualitative chemical analysis of each methanolic extract indicated the presence of triterpenoids and steroids, reducing sugars, phenolic compounds, aminoacids, cardiac glycosides and alkaloids. In all cases, the inhibitory activities vs pAPN, pAPA, and pDPP-IV were dose-dependent; inhibition was reduced at the higher substrate concentration. In addition, *P. fustiger* secretion dose-dependently inhibited papain, but inhibition was not affected by substrate concentration. We did not detect a dose-dependent inhibition of bovine trypsin and pepsin, and of mPPII, pAPB and pLAP. Additionally, Bufalin inhibited pAPN with an IC_{50} value of 6.23 ± 0.11 μM and a kinetic behavior indicative of a competitive or non-competitive $\alpha > 1$ mode of inhibition. Thus, extracts from paratoid gland secretions from Cuban toads contain inhibitory activities directed against metallo, serine and cysteine peptidases, whose chemical characterization may generate new lead inhibitors with biomedical relevance. Bufalin emerges as a new classical inhibitor of pAPN, with potential applications in biomedical studies targeting APN.

Keywords: enzyme inhibition, paratoid gland secretion, methanolic extracts, peptidase mechanistic classes, porcine aminopeptidase N, Bufalin

RESUMEN

Extractos metanólicos de secreciones de glándulas paratoideas de sapos cubanos del género *Peltophryne* contienen actividades inhibitorias de peptidasas de relevancia biomédica. Las peptidasas regulan la mayoría de los procesos bioquímicos y fisiológicos de los organismos vivos y facilitan la propagación de agentes infecciosos. Muchas son blancos para el diseño de nuevos agentes terapéuticos. En este contexto, las secreciones de la piel de los anfibios han evolucionado con potentes mecanismos de defensa contra depredadores y microorganismos, aunque se ha informado sobre pocos inhibidores de peptidasas en ellas. En este trabajo se evaluó el efecto inhibitorio de extractos metanólicos obtenidos de las secreciones de las glándulas paratoides de cuatro especies de sapos endémicos cubanos (*Peltophryne fustiger*, *P. florentinoi*, *P. peltocephala* y *P. taladai*) contra nueve peptidasas de diferentes clases mecanísticas. El análisis químico cualitativo de los extractos indicó la presencia de triterpenoides y esteroides, azúcares reductores, compuestos fenólicos, aminoácidos, glucósidos cardíacos y alcaloides. Las actividades inhibitorias frente a pAPN, pAPA y pDPP-IV fueron dependientes de la dosis; la inhibición disminuyó al incrementar la concentración de sustrato. La secreción de *P. fustiger* inhibió a la papaína de forma dosis-dependiente, aunque la inhibición no dependió de la concentración del sustrato. Tampoco se detectó inhibición dosis-dependiente para tripsina, pepsina bovina, mPPII, pAPB ni pLAP. Además, Bufalina inhibió a pAPN (IC_{50} de 6.23 ± 0.11 μM) y el comportamiento cinético sugirió un modo de inhibición no competitivo ($\alpha > 1$) o competitivo. Los extractos metanólicos estudiados contienen actividades inhibidoras dirigidas contra peptidasas de tipo metalo, serino y cisteíno, cuya caracterización química podría generar nuevos inhibidores con relevancia biomédica. Además, Bufalina emerge como un nuevo inhibidor clásico de pAPN.

Palabras clave: inhibición enzimática, secreciones de glándulas paratoideas, extractos metanólicos, clases mecanisticas de peptidasas, aminopeptidase N porcina, Bufalina

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Introduction

Proteolytic enzymes are critical in all living organisms [1]. They can act as exo- and/or endo-peptidases and are segregated in mechanistic classes [1]. Proteases control the activation, synthesis and turnover of other proteins, and regulate most biochemical and physiological processes [1]. Consequently, they are major

regulators of homeostasis, ageing, diseases and death. Proteases are also essential for propagation of infectious agents, being major contributors of pathogenesis in several infectious diseases [1, 2].

The relevance of proteases has driven the search and identification of potent and selective inhibitors [2, 3], a major tool to control proteolysis. Many protease inhibitors are characterized by high chemical diversity, biochemical specificity, molecular flexibility and other molecular properties that make them more favorable than synthetic inhibitors as lead structures for drug discovery [4, 5]. Protease inhibitors have been isolated from natural sources; however, few have been identified in paratoid secretions of toads [9], making them a potential and unexplored source.

Anuran species have a permeable skin with mucous and granular glands, generally associated to maintenance of humidity and cutaneous respiration, and with chemical defense against predators and/or microbial infection, respectively [6, 7]. Bufonids produce potent toxins in parotoid macroglands located in the head in the postorbital region [8, 9]. The biochemistry of parotoid glands toads' secretion has been relatively well studied. The isolated active compounds have been classified into four main categories: biogenic amines, bufadienolides, alkaloids, peptides and proteins [9]. The diversity of chemical compounds in the paratoid glands of toads makes them important sources, from which new therapeutic agents can be developed.

Within the Bufonidae family, the genus *Peltophryne* Fitzinger, 1843, represents a monophyletic radiation of Caribbean toads. The Cuban archipelago hosts the bulk of species comprised within *Peltophryne*, with eight endemic species [10]. The chemistry of the paratoid gland secretions from Cuban toads remained understudied until recently, when an inhibitory effect on Na+/K+ ATPase was reported for *Peltophryne fustiger* [11].

Therefore, in this work, exploratory qualitative chemical analysis of methanolic extracts of the paratoid gland secretions from four Cuban *Peltophryne* species was conducted. Methanolic extracts were evaluated for their inhibitory effect against nine peptidases of biomedical relevance belonging to the major mechanistic classes of proteases [1]. These included porcine trypsin and dipeptidyl peptidase IV (pDPP-IV) (serine endo- and amino-peptidases, respectively), porcine neutral aminopeptidase (pAPN), glutamyl aminopeptidase (pAPA), arginyl aminopeptidase (pAPB) and mouse pyroglutamyl peptidase II (mPPII) (metallo aminopeptidases from the M1 family), porcine leucine aminopeptidase (pLAP, M17 family), papain (cysteine endopeptidase) and porcine pepsin (aspartic endopeptidase). Most of them are current targets for development of therapeutics drugs [2, 3, 12-15]. Finally, we explored the inhibitory activity of Bufalin, one of the major constituents of *Peltophryne* venom.

Materials and methods

Materials and reagents

Porcine kidneys were kindly donated by the Porcine Research Institute, Cuba. Porcine kidney cortex APN, APA and DPP-IV were prepared in form of microsomes [16]. Porcine kidney cortex aminopeptidase B (APB) and leucyl aminopeptidase (LAP) were partially purified from the soluble fraction of a microsome preparation, as described for human LAP [17].

Bovine pepsin and pancreatic trypsin were purchased from Sigma-Aldrich, USA, while papain from *Carica papaya* was acquired from Roche (USA). Mouse brain pyroglutamyl peptidase II (PPII) was prepared as described by Vargas *et al*. [18]. Bestatin, an inhibitor of metallo-aminopeptidases, (1S,2S)-2-(((S)-1-((4-Guanidinobutyl)amino)-4-methyl-1-oxopentan-2-yl)carbamoyl) cyclopropanecarboxylic acid (E64) and phenylmethanesulfonyl fluoride (PMSF), inhibitors of papain and trypsin respectively, were purchased from Sigma-Aldrich, USA. l-threo-Ile-thiazolidide (P32/98) were acquired from Enzo Life Sciences, and thyrotropin-releasing hormone (TRH)-aminomethylcoumarin (AMC), L-Leu-pnitroanilide (pNA), L-Arg-pNA, L-Glu-pNA, L-Gly-Pro-pNA, Bz-Arg-pNA, Z-Phe-Arg-pNA and Casein from Bachem (Switzerland). Bufalin was purified from *P. fustiger* as described [11]. Analytical grade laboratory chemical reagents were purchased from Sigma-Aldrich, USA.

Collection of paratoid gland secretion and preparation of methanolic extracts

Paratoid gland secretions were obtained by mechanical compression of both paratoid glands from 2-5 adult specimens of four species of the genus *Peltophryne* from Western and Central Cuba (Figure 1). Secretions for each species were individually collected on a surface of sterile lab watch glasses and then animals were released. Only one specimen from each species was preserved as a voucher and deposited in the Herpetological Collection of the Museum of Natural History "Felipe Poey", Faculty of Biology, University of Havana, Cuba. The species, collection locality, geographic coordinates and voucher numbers are as follows: *P. fustiger* (Schwartz, 1960) from Santo Tomas

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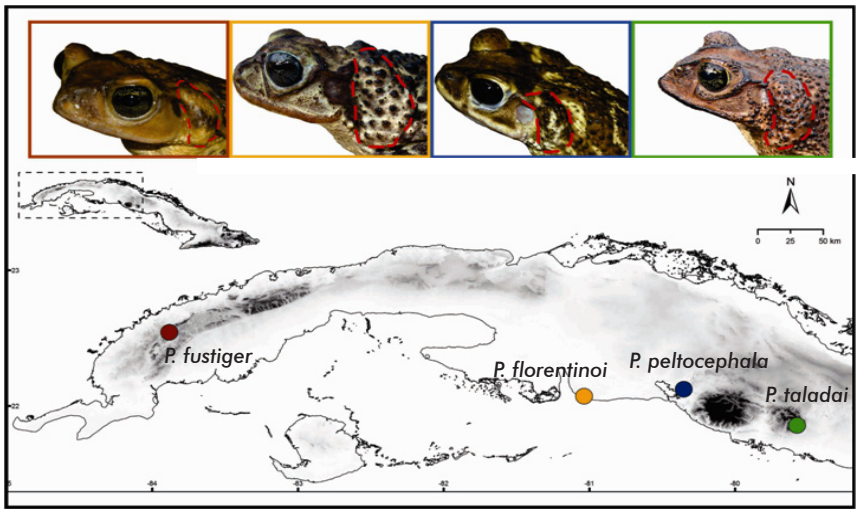

Figure 1. Localities sampled in Western and Central Cuba for the collection of paratoid gland secretions of four species of Cuban toads. From left to right: *Peltophryne fustiger* (red circle) from Santo Tomas stream, El Moncada, Viñales municipality, Pinar del Río province; *Peltophryne florentinoi* (yellow circle) from the surroundings of Playa Girón, Zapata Swamp, Matanzas province; *Peltophryne peltocephala* (blue circle) from Soledad Botanical Garden, Cienfuegos province; *Peltophryne taladai* (green circle) from Jarico river, Banao, Sancti Spíritus province. Details of the heads of these four species are shown; paratoid glands are indicated by dotted lines.

stream, El Moncada, Viñales municipality, Pinar del Río province (22.543 N–83.882444 W), MFP 11599; *P. florentinoi* (Moreno and Rivalta, 2007), from the surroundings of Playa Girón, Zapata Swamp, Matanzas province (22.072222 N–81.036111 W), MFP 11595; *P. peltocephala* (Tschudi, 1838), from Soledad Botanical Garden, Cienfuegos province (22.123528 N -80.352389 W), MFP 11596; and *P. taladai* (Schwartz, 1960) from Jarico river, Banao, Sancti Spíritus province (21.857222 N–79.574722 W), MFP 11597 (Figure 1). The species were identified by Roberto Alonso Bosch.

Fresh secretions (0.35 g from *P. peltocephala*, 0.13 g from *P. florentinoi*, 0.14 g from *P. fustiger* and 0.26 g from *P. taladai*) were extracted three times with MeOH (50 mL per g of secretion), by shaking at room temperature (25 ºC) for 24 h. Extracts were pooled, filtered through 0.22 µm sterile filter and evaporated under reduced pressure; yield was: from *P. peltocephala* (73 mg), *P. florentinoi* (35 mg), *P. fustiger* (84 mg) and *P. taladai* (78 mg).

Qualitative chemical analysis of methanolic extracts

The qualitative chemical quality of each methanolic extract was analyzed according to standard methods of phytochemical analysis [19]. For triterpenoids and steroids, we used the Liebermann-Burchard's test. 1 mg of each extract was dissolved in 1 mL of chloroform and treated with 1 mL of acetic anhydride, boiled and cooled. Two-three drops of concentrated sulfuric acid were added to a side of the test tube, which showed a brownish ring at the junction of the two layers. The formation of a deep reddish color indicated the presence of triterpenoids and/or steroids.

The Fehling assay was used to detect reducing sugars; 2 mL of Fehling reagent (Sol 1: 3.5 % cupric sulphate in water; Sol 2: 15 % sodium and potassium tartrate with 4 % sodium hydroxide in water; Sol 1:Sol 2 1:1 v:v) were added to 1 mg of each extract dissolved in 1 mL of water. The mixture was warmed in hot water for 5-10 min. A reddish color precipitate indicated a positive result.

The ferric chloride test was used to detect phenolic compounds. 1 mg of extract dissolved in ethanol was treated with 3 drops of ferric chloride at 5 % in saline solution. Appearance of a bluish black precipitate indicated the presence of phenolic compounds.

Amino acids were detected by adding a few drops of 2 % ninhydrin solution in distilled water to 1 mg of methanolic extract. Appearance of bluish color indicated the presence of amino acids.

For cardiac glycosides detection, 1 mg of extract in ethanol was treated with 1 mL of Kedde reagent (Sol 1: 2 % 3,5 dinitrobenzoic acid in methanol; Sol 2: 5.7 % potassium hydroxide in water; Sol 1:Sol 2 1:1 v:v) and let stand for 5-10 min. The assay was considered positive when a violet color appeared and persisted for 1-2 h.

Alkaloids were detected by dissolving 1 mg of extract in 1 mL chloroform, followed by evaporation. The residue was acidified with 1 % hydrochloric acid and few drops of Dragendorff's reagent was added (potassium bismuth iodide). Appearance of an orange reddish precipitate indicated the presence of alkaloids.

The Borntrager test was used for quinones; 1 mg of extract was dissolved in 1 mL chloroform and 1 mL of 5 % ammonium hydroxide solution in water was added. A pink or red color above the aqueous phase indicated a positive reaction. This assay was used as a negative control, since the extracts were of animal source.

Monitoring of aminopeptidase activities

Porcine aminopeptidase activities were determined with procedures as described by Tieku and Hooper [20] for porcine kidney cortex APN and APA, Pascual *et al*. [17] for APB and LAP, and Pascual *et al*. [22] for porcine kidney DPP-IV.

Porcine kidney cortex APN activity was determined in microsomes (0.05 mg/mL) using 0.3 mM L-Leu-pNA substrate in the assay, in 50 mM Tris-HCl, pH 8 (buffer A) and 1 mL of assay final volume. Porcine APA activity in microsomes (0.1 mg/mL) was measured using buffer A, supplemented with 1 mM $CaCl_2$, in the presence of 0.5 mM L-Glu-pNA substrate in the assay, and 300 µL of assay final volume. Porcine APB activity (0.32 mg/mL) was measured in buffer A, using the substrate L-Arg-pNA (0.2 mM in the assay), in a final assay volume of 300 µL [17]. A similar procedure was followed for determining the porcine LAP activity (0.16 mg/mL), which was measured in buffer A with 1 mM $CoCl_2$, using the 0.3 mM L-Leu-pNA substrate in the assay, in a final assay volume of 300 µL [17]. Porcine kidney DPP-IV activity in microsomes (0.05 mg/mL) was measured using the substrate L-Gly-Pro-pNA (0.15 mM in the assay) in buffer A and 1 mL of assay final volume. The kinetic assays were run at 37 ºC, and the release of p-nitroaniline was measured every 15 s during 5 min at 405 nm, in a multiplate reader Multiskan FC (Thermo Fisher Scientific, USA), except for porcine kidney DPP-IV, determined using a Genesys 10 UV kinetic spectrometer (Thermo Fisher Scientific, USA).

Membrane mouse brain PPII activity was determined with the substrate TRH-AMC in a coupled assay in the presence of excess DPP-IV according to Friedman and Wilk [21], with few modifications. The incubation mixture contained 1.6 µg/mL membrane protein, DPP-IV (4 nM Gly-Pro-ßNA hydrolyzed per min), bacitracin and N-ethylmaleimide (200 µM each) in 50 mM phosphate buffer, pH 7.5 (buffer B; 100 µL total volume). The mixture was pre-incubated at 37 ºC for 30 min, followed by adding 4 µL of TRH-AMC (400 µM final concentration) in buffer B. Reaction was run at 37 ºC and 8-µL aliquots were taken at 15, 30, 45 and 60 min. The reaction was stopped by adding 8 µL of methanol. AMC was detected fluorometrically using a NanoDrop 3300 system (Thermo Fisher Scientific, USA). Wavelengths for excitation and emission were set at 335 nm and 410 nm, with slit widths of 15 and 20 nm, respectively.

Monitoring of serine, cysteine and aspartic endopeptidases activities

Bovine pancreatic trypsin activity (0.1 µM) was measured using the substrate Bz-Arg-pNA (1 mM in the assay) in Tris-HCl 20 mM, NaCl 150 mM, pH 8.0 and 1 mL of final volume assay [23]. Papain from *Carica papaya* activity (92.5 nmol/L) was measured using the substrate z-Phe-Arg-pNA (0.15 mM in the assay) in

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Porcine pepsin activity was determined by the Anson method, using 1 % casein as substrate [25]. For an assay volume of 1 mL, 300 µL of substrate and 600 µL of sodium acetate 50 mM, pH 4.0, were added. The mixture was preincubated at 37 ºC for 30 min, the reaction stopped by 100 µL of TCA 100 %, and then, the mixture was centrifuged at 10 000 *g* for 10 min and the amount of peptide quantified by measuring the absorbance at 280 nm using a Genesys 10 UV kinetic spectrometer (Thermo Fisher Scientific, USA).

In vitro evaluation of inhibitory activities

Inhibitory activity and dose-response studies

The inhibitory activity of the methanolic extracts was assessed by quantifying the decrease in each enzyme activity (pAPN, pAPA, pAPB, pLAP, pDPP-IV, mPPII, trypsin, papain, pepsin) pre-incubated with the extract. Stock solutions of the methanolic extracts for inhibition assays were prepared at 20 mg/mL in DMSO. In a first study, the effect of the methanolic extract (600 µg/mL) on residual activity was determined using a pre-incubation time of enzyme and extract of 30 min at 37 ºC prior to adding the specific substrate. Dose-response studies (0.05-0.8 mg/mL) were performed when inhibition was at least 50 %. For each inhibitory sample concentration, residual activity was calculated as the ratio of the activity in presence of the inhibitory sample over that of the control assay (v_i/v_o). Then, the half maximal inhibitory concentration (IC_{50}) values were estimated by non-linear regression fitting of the dose-response curves (plotting the residual activity vs. concentration of each treated extract) to the IC_{50} equation included in the Graphpad Prim software version 5.0. IC_{50} was defined as the concentration of the extract needed to inhibit 50 % the control enzyme activity [26].

Dose-response studies in the presence of bestatin (0.10-200 µM), P32/98 (0.020-10 µM), PMSF (0.025-0.2 mM), and E64 (8-120 nM) were run in parallel, as a positive control of inhibition for some of the target peptidases. Inhibition assays were done at 1 K_M substrate concentration value (see below). The dose-response inhibition with Bufalin (0.1-50 µM) isolated from *P. fustiger* [11] was studied for the peptidases targeted by the methanolic extracts, as described above. In these cases, IC_{50} values were established by non-linear regression fitting of the dose-response curves to the IC_{50} equation included in the GRAFIT software 6.0.

Effect of substrate concentration on inhibitory activity

The capacity of the substrates to induce dissociation of the EI complex was analyzed through the effect of substrate concentration on the inhibitory activity of each extract against a positive target enzyme. In this experiment, two substrate concentrations close to 1 and 5 K_M were tested, and a fixed amount of each methanolic extract (Table 1), including pAPN inhibition by Bufalin (5 µM). Assays were performed in quadruplicate.

*R*esults and discussion

Qualitative chemical analysis of the methanolic extracts

Methanolic extracts from the Cuban toad species *P. fustiger*, *P. florentinoi*, *P. peltocephala* and *P. taladai* (Figure 1) were qualitatively screened for specific chemical compound classes. Triterpenoids/steroids, alkaloids, phenolic compounds and amino acids were detected in all toad species. *P. fustiger* was the only extract that was negative for the presence of reducing sugars. Cardiac glycosides were only detected in *P. peltocephala* and *P. taladai* (Table 2). Similar compositions (steroids, indole alkaloids and cardiac glycosides) had been previously reported for closely related species of the genus *Rhaebo* and *Rhinella* from South America, using RP-HPLC, mass spectrometry and NMR as analytical techniques [9, 28-30]. Alkaloids may include lipophilic and guanidine alkaloids, which were described from skin preparations of bufonids [9].

Phenolic compounds, amino acids and reducing sugars were previously described in bufonids skin preparations, but not from paratoid gland secretions [9]. As far as we know, this is the first report about the qualitative analysis of these compounds in *P. peltocephala*, *P. florentinoi* and *P. taladai*. In a previous study, we were able to identified nine major bufadienolides (steroids) and azelayl arginine from the parotoid gland secretion of *P. fustiger* [11]. Moreover, our results also indicate the presence of alkaloids, reducing sugars, phenolic compounds and cardiac glycosides in *P. fustiger* paratoid gland secretion. Based on conserved morphology, distribution ranges, ecological affinities and closed phylogenetic relationships among these species [10, 31], it can be speculated that bufadienolides and azelayl arginine, previously reported for *P. fustiger*, might be present in glandular secretions from the three additional Cuban species, something requiring further insight.

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Table 1. Summary of the assay conditions to determine substrate concentration effect on the inhibitory activity of methanolic extracts for each target enzyme

Inhibitors	K_M (mM)*	[Substrate] (mM)	[*P. peltocephala*] (µg/mL)	[*P. florentinoi*] (µg/mL)	[*P. fustiger*] (µg/mL)	[*P. taladai*] (µg/mL)
pAPN	0.3	0.3, 1.5	80	60	60	120
pAPA	0.49	0.5, 2.5	60	80	160	80
pDPP-IV	0.138	0.15, 0.75	120	100	140	140
Papain	0.14	0.15, 0.75	–	–	200	–

*The K_M value for pAPA was determined in the present contribution (not shown). Values for pAPN, pDPP-IV and Papain were taken from references [17], [22] and [27], respectively. Methanolic extracts were prepared from paratoid gland secretions of Cuban *Peltophryne* toads.

Table 2. Qualitative chemical analysis of methanolic extracts of paratoid gland secretions from four Cuban *Peltophryne* species (Amphibia: Anura: Bufonidae)

Assay	[*P. peltocephala*] (µg/mL)	[*P. florentinoi*] (µg/mL)	[*P. fustiger*] (µg/mL)	[*P. taladai*] (µg/mL)
Liebermann-Burchard (triterpenoids and steroids)	+++	+++	+++	+++
Fehling (reducing sugars)	++	+*	–	+*
Ferric Chloride (phenolic compounds)	++	+	+	+
Ninhydrin (amino acids and peptides)	+	+	++	++
Kedde (cardiac glycosides)	+*	–	–	+*
Dragendorff (alkaloids)	+++	+++	+++	+++
Bortrager (quinones)	–	–	–	–

*Light color.

Effects of methanolic extracts on peptidase activities

An initial evaluation of the inhibitory activity of the extracts was performed at a fixed extract concentration (600 µg/mL). All samples reduced pDPP-IV, pAPN and pAPA activities 50 % or more. A similar decrease was observed for *P. taladai* extracts against trypsin, and for *P. peltocephala* and *P. fustiger* extracts against papain (Table 3). pAPN was the enzyme most susceptible to the inhibitory activity of extracts, and, remarkably, the *P. fustiger* extract was the most potent inhibitor against papain. The other enzymes showed residual activities higher than 0.5 (with 1.0 as the control activity for each target enzyme; Table 3).

Dose-response studies were performed with extracts decreasing residual activity below 0.5. Dose-dependent relationships were detected for pDPP-IV, pAPN and pAPA inhibition by the four methanolic extracts and for papain vs. *P. fustiger* extract. In each case, inhibition was characterized by a concave curve (Figure 2 A-D), indicating the reversible nature of inhibition and corroborating the lack of artifacts interfering with the enzyme assay [26]. IC_{50} values for pDPP-IV were in the range 99-236 µg/mL, 84-369 µg/mL for pAPA, 19-133 µg/mL for pAPN. Extracts from *P. florentinoi*, *P. fustiger* and *P. peltocephala* displayed IC_{50} values lower than 100 µg/mL vs. pAPN, indicating the high potency of these extracts for isolating pAPN inhibitors [32]. Selectivity for pAPN vs. pAPA (IC_{50} APN/IC_{50} pAPA) was detected for *P. fustiger*, *P. florentinoi* and *P. peltocephala*. IC_{50} values for bestatin (vs. pAPN, pAPA, pAPB and pLAP), P32/98 (vs. pDPP-IV), PMSF (vs. trypsin) and E64 (vs. papain) obtained in parallel assays were consistent with known IC_{50} values (Table 4, Figure 3 A-F).

Regarding substrate concentration, its increment for the four methanolic extracts, (from 1 to 5 K_M values) significantly increased the residual activity of pDPP-IV, pAPN and pAPA (Figure 4 A-C). This indicated that extracts contain predominantly inhibitors with competitive and/or non-competitive ($\alpha > 1$) performance, which are characterized by a total or partial effect on the K_M value of the target enzyme [26]. By contrast, the *P. fustiger* extract vs. papain, did not significantly change the residual activity despite increased substrate concentrations. Hence, this indicated the global prevalence of inhibitors with a non-competitive performance ($\alpha = 1$), not affecting K_M value but decreasing the V_{max} value (Figure 4D) [26].

Effects of Bufalin on pAPN, pAPA, pDPP-IV and papain activities

It has been previously reported that APN and APA accommodate preferentially aromatic (rather than aliphatic) compounds in the S1 and S1' pockets, which can act as competitive inhibitors [33]. Additionally, heteroatoms at voluminous lateral chains in the P1 position favor selectivity for APN over other metallo-aminopeptidases. It is thus possible that alkaloids and steroids like bufadienolides may contribute to the inhibition of pAPN and pAPA by the assayed methanolic extracts, although it cannot be ruled out the simultaneous contribution of different types of compounds. To test this hypothesis, the inhibitory effect of purified Bufalin on pAPN and pAPA activities was tested, this inhibitor as one of the major components of *P. fustiger* paratoid gland secretion [11]. A dose-response concave curve was obtained for pAPN (Figure 5A), indicating a reversible inhibition, with an IC_{50} of 6.23 ± 0.11 µM. The effect of Bufalin on pAPN activity was attenuated at a higher substrate concentration (Figure 5B), depicting a competitive or non-competitive performance ($\alpha > 1$), as for the effect of methanolic extracts on pAPN. Thus, Bufalin was regarded as a new classical natural inhibitor of pAPN.

Bufalin did not inhibit pAPA up to 100 µM. Residues of the S1 pocket of APN favor the accommodation of bulky hydrophobic side chains [34]. Conversely, the S1 pocket of APA is well suited to

Table 3. Residual activity of peptidases of different mechanistic classes incubated with methanolic extracts from paratoid gland secretions from Cuban *Peltophryne* species (Amphibia: Anura: Bufonidae)*

Inhibitors	[*P. peltocephala*] (µg/mL)	[*P. florentinoi*] (µg/mL)	[*P. fustiger*] (µg/mL)	[*P. taladai*] (µg/mL)
DPP-IV (Serine)	**0.370 ± 0.010**	**0.513 ± 0.001**	**0.378 ± 0.017**	**0.439 ± 0.021**
Trypsin (Serine)	0.690 ± 0.020	0.690 ± 0.093	0.660 ± 0.030	**0.580 ± 0.040**
APN (Metallo)	**0.370 ± 0.009**	**0.088 ± 0.004**	**0.060 ± 0.003**	**0.161 ± 0.016**
APA (Metallo)	**0.425 ± 0.003**	**0.344 ± 0.016**	**0.447 ± 0.005**	**0.380 ± 0.002**
APB (Metallo)	0.860 ± 0.010	0.840 ± 0.010	0.820 ± 0.010	0.800 ± 0.040
PPII (Metallo)	1.110 ± 0.050	0.850 ± 0.090	0.830 ± 0.050	1.000 ± 0.070
LAP (Metallo)	0.940 ± 0.080	0.820 ± 0.020	0.700 ± 0.010	0.790 ± 0.020
Papain (Cystein)	**0.580 ± 0.070**	0.880 ± 0.010	**0.160 ± 0.007**	0.820 ± 0.090
Pepsin (Aspartic)	1.120 ± 0.006	1.140 ± 0.003	1.070 ± 0.014	1.130 ± 0.009

*Assays were performed with 600 µg/mL of each methanolic extract; data presented as mean ± SD (n = 3). Results in blue stand for residual activities lower than or equal to 0.6, indicating a moderate-to-potent inhibitory effect.

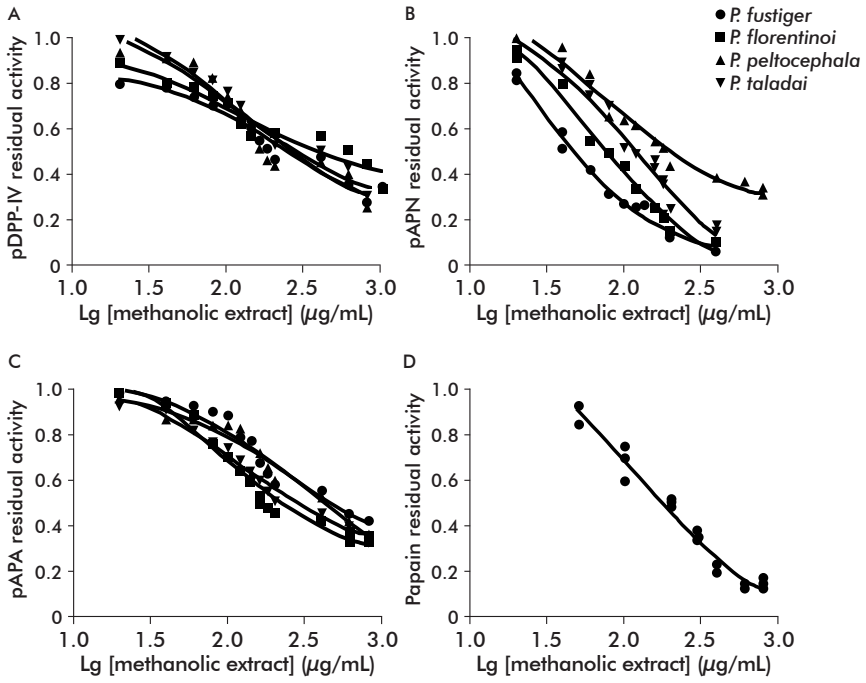

Figure 2. Typical dose-response curves for the inhibition of peptidases of different mechanistic classes by methanolic extracts of Cuban *Peltophryne* species: *P. fustiger*, *P. florentinoi*, *P. peltocephala* and *P. peltocephala*. A) pDPP-IV (0.05 mg/mL; 50 mM Tris HCl, pH 8; 0.15 mM L-Gly-Pro-pNA). B) pAPN (0.05 mg/mL; 50 mM Tris HCl, pH 8; 0.30 mM L-Leu-pNA). C) pAPA (0.10 mg/mL; 50 mM Tris HCl, pH 8; 1 mM $CaCl_2$; 0.5 mM L-Glu-pNA), D) Papain (92.5 nM; 100 mM acetate buffer; 10 mM DTT, pH 5.5; 0.15 mM z-Phe-Arg-pNA). Data are presented as means of three determinations.

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Table 4. IC_{50} values for methanolic extracts from paratoid gland secretions from Cuban species of *Peltophryne* (Amphibia: Anura: Bufonidae) against peptidases of different mechanistic classes*

Peptidase (mechanistic class)	Positive control[a]		*P. peltocephala*		*P. florentinoi*		*P. fustiger*		*P. taladai*	
	IC_{50} (range) (μg/mL)	r^2	IC_{50} (range) (μg/mL)	r^2	IC_{50} (range) (μg/mL)	r^2	IC_{50} (range) (μg/mL)	r^2	IC_{50} (range) (μg/mL)	r^2
DPP-IV (Serine)	0.048 (0.034-0.058)	0.9867	**113.40** (65.70-195.80)	0.9213	**122.40** (66.38-225.60)	0.9234	236.30 (138.10-404.30)	0.9355	**99.33** (62.53-157.80)	0.9423
Trypsin (Serine)	1.23 (0.073-20.65)	0.9552	> 600	–	> 600	–	> 600	–	> 600	–
APN (Metallo)	1.50 (1.17-19.31)	0.9900	**64.47** (43.55-95.44)	0.9664	**57.78** (40.42-82.59)	0.9830	**19.99** (12.25-32.62)	0.9833	**132.50** (79.67-220.20)	0.9638
APA (Metallo)	5.81 (3.25-10.40)	0.9625	368.80 (230.20-590.70)	0.9611	**84.76** (56.17-127.90)	0.9546	222.55 (130.60-378.90)	0.9348	**128.40** (96.28-171.30)	0.9769
APB (Metallo)	6.40 (2.72-15.08)	0.9233	> 200	–	> 200	–	> 200	–	> 200	–
PPII (Metallo)	ND	–	> 200	–	> 200	–	> 200	–	> 200	–
LAP (Metallo)	4.65 (3.26-6.64)	0.9851	> 200	–	> 200	–	> 200	–	> 200	–
Papain (Cystein)	0.012 (0.006-0.026)	0.9659	>600	–	>600	–	**144.30** (87.30-238.60)	0.9732	> 600	–
Pepsin (Aspartic)	ND	–	> 600	–	> 600	–	> 600	–	> 600	–

* Data are IC_{50}, 95 % confidence interval, and r^2 of the fitting to the IC_{50} equation.
[a]: A positive control of inhibition assay was included for pAPN, pAPA, pAPB, pLAP vs. bestatin; for pDPP-IV vs. P32/98; trypsin vs. PMSF and for papain vs. E64. ND: not determined. Results in blue correspond to IC_{50} values lower than 150 μg/mL

accommodate the side chains of acidic residues [35]. Moreover, the methanolic extracts that inhibit pAPN and pAPA exhibit an apparent competitive performance and Bufalin inhibits pAPN but not pAPA. Therefore, it is likely that the inhibitory activities of pAPN and pAPA detected in the methanolic extracts are caused by different compounds. Most of the potent inhibitors for these M1 family enzymes came from chemical synthesis and are highly hydrophobic [3]. Natural APN inhibitors are scarce and isolated mainly from microorganisms, like bestatin which is an analog of the Phe-Leu peptide [3]. There are two examples of APN inhibitors isolated from marine invertebrates and betulinic acid from plants [36-38]. Recently, inhibitory activities of porcine and human APN were also identified in marine invertebrates from the Cuban coastline [17]. Since pAPN was inhibited by *Peltophryne* species venoms and Bufalin, it is possible that some of the anticancer effects reported up to now for toads' venoms and Bufalin could involve the inhibition of membrane APN [5, 39]. Our data suggest that further search for M1 peptidase inhibitors from Bufonids, and amphibians in general, is justified and that Bufalin emerges as a new inhibitor of APN with potential application in biomedical studies.

Natural DPP-IV inhibitors have been described mainly from plants, identified as steroids, phenolic compounds and peptides, coincidently the type of components identified in the present work [13]. In fact, DPP-IV inhibitors have not been described for amphibian skin secretions. Nevertheless, Neerati [40] reported an antidiabetic activity for methanolic extract from paratoid gland secretion of the Indian toad *Duttaphrynus melanostictus* [40], which alone or in combination with glimepiride had a positive effect on glycemic control in diabetic rats. Our data suggest that part of the antidiabetic activity could be due to DPP-IV inhibition, a validated target for type 2 diabetes treatment [13]. We did not detect DPP-IV inhibition by Bufalin up to 100 μM; therefore, the DPP-IV inhibitory activity of the Cuban toads methanolic extracts could

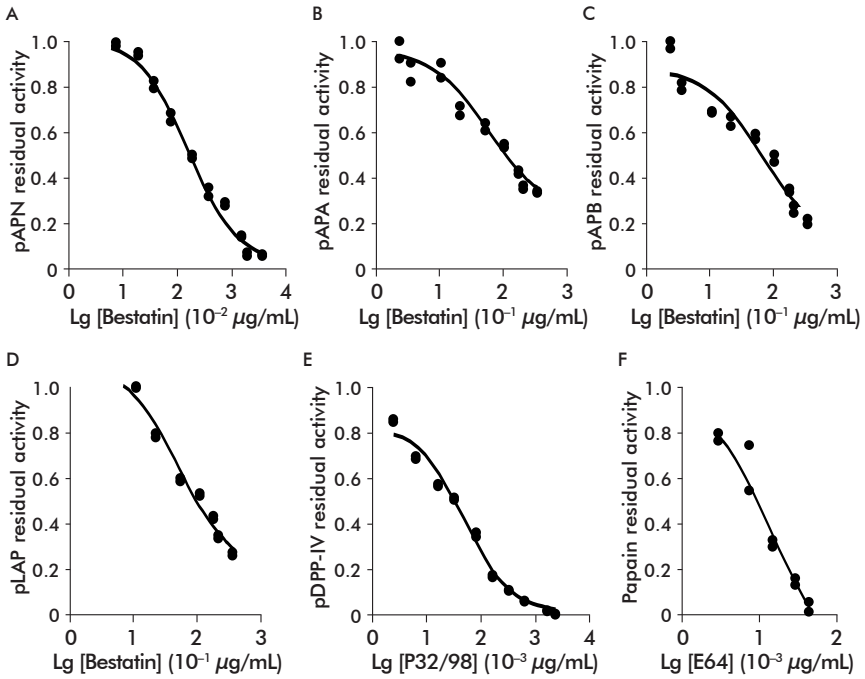

Figure 3. Typical dose-response curves for positive controls of inhibition for some of the target peptidases. A) pAPN vs. bestatin. B) pAPA vs. bestatin. C) pAPB vs. bestatin. D) pLAP vs. bestatin. E) pDPP-IV vs. P32/98. F) Papain vs. E64.

be associated with other extract components (either steroids different from Bufalin and/or phenolic compounds, peptides, among others).

Another type of inhibitors, cysteine protease inhibitors, have been detected in diverse natural sources including amphibians [41]. The major cysteine protease inhibitors isolated from frogs come from skin secretions or plasma, as cystatins or other peptide inhibitors [41]. Otherwise, cysteine protease inhibitors from paratoid gland venoms had never been reported. Our data suggest that the *P. fustiger* extract may contain

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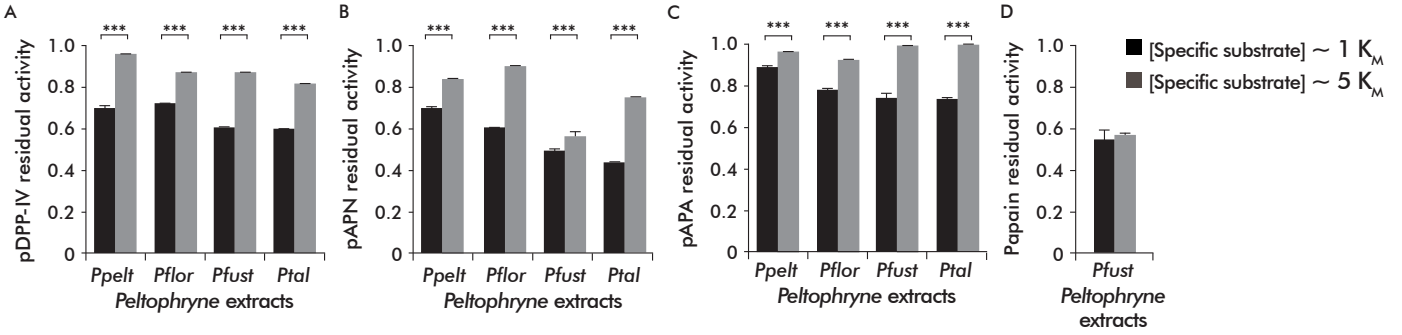

Figure 4. Effect of substrate concentration on inhibition of peptidases by methanolic extracts of paratoid glands' secretion of Cuban *Peltophryne* species (*Pfust*: *P. fustiger*; *Pflor*: *P. florentinoi*; *Ppelt*: *P. peltocephala*; *Ptal*: *P. taladai*). A) pDPP-IV. B) pAPN. C) pAPA. D) Papain. Inhibition assays were done at two substrate concentrations (1 and 5 K_M). Data are presented as means ± SD (n = 4). *** Highly statistically significant differences ($p < 0.001$; Student's t test as assessed by using the GraphPad Instat.

papain inhibitor(s). Bufalin up to 100 μM did not inhibit papain. Therefore, alkaloids and triterpenoids from the *P. fustiger* extract are candidate papain inhibitors, although we cannot exclude the action of other compounds, such as peptides, contributing to this inhibition.

Conclusions

Triterpenoids/steroids, reducing sugars, phenolic compounds, amino acids, cardiac glycosides and alkaloids were identified in methanolic extracts of paratoid gland secretions from Cuban *Peltophryne* species contain. Some of those compounds could be characterized as promising inhibitors for peptidases pAPN, pAPA, pDPP-IV and papain (a model for cysteine peptidases) of biomedical relevance. Moreover, Bufalin was identified as a classical inhibitor of pAPN. It neither inhibited the closely related pAPA, nor pDPP-IV or papain. Since these peptidases are relevant targets in many human pathologies, our encouraging results fully justify further identification and characterization of peptidase inhibitors from Bufonids paratoid gland`s secretions, as well as amphibians. Studies involving bio-guided fractionation of each extract will be required to chemically identify additional inhibitors, and the mechanism of pAPN inhibition by Bufalin and its biomedical impact should be established.

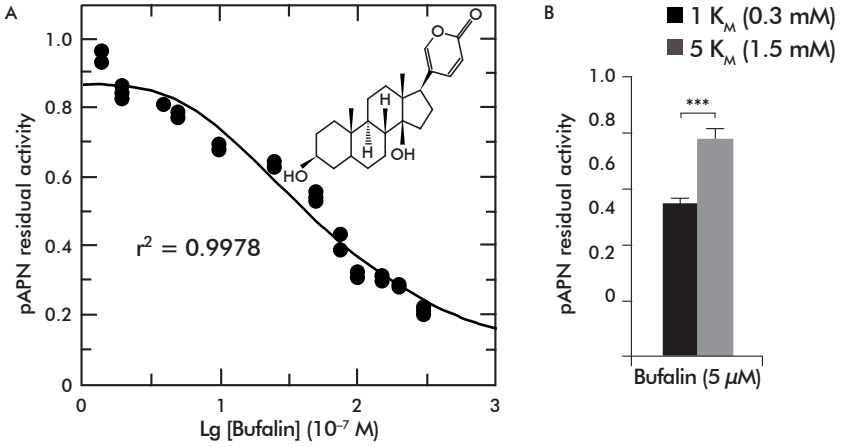

Figure 5. Inhibition of pAPN by bufalin. A) Typical dose-response curve for the inhibition of pAPN (0.05 mg/mL, 50 mM Tris HCl, pH 8; 0.30 mM L-Leu-pNA) by bufalin (plotted individual data; n = 3). B) Effect of substrate concentration on inhibition of pAPN activity by bufalin. Inhibition assays were done at two substrate concentrations (1 and 5 K_M). Data are presented as means ± SD (n = 4). *** Highly statistically significant differences ($p < 0.0001$, Student's t-test, GraphPad Instat).

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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