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LAUDICÉIA ALVES DE OLIVEIRA

Prospecção das funções biotecnológicas da crotapotina e suas subunidades em protozoários, bactérias e células de mamíferos

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutora em Doenças Tropicais.

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Palavras-chave: Crotalus durissus terrificus; Crotapotina; Doenças negligenciadas; Peptídeos biologicamente ativos; Toxinas.

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Aos meus pais,

o remanso da minha vida

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...

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[João Guimarães Rosa]

RESUMO

O potencial biotecnológico dos venenos de serpentes sempre foi estudado e reconhecido. Esses venenos já foram utilizados desde a produção do medicamente anti-hipertensivo mais vendido no mundo até a produção de uma cola de fibrina capaz de cicatrizar úlceras venenosas.Além disso, os venenos são excelentes para a prospecção de efeitos biológicos em diversos modelos causadores de doenças, incluindo doenças negligenciadas, como por exemplo, a leishmaniose e a malária. Estas possuem tratamentos altamente tóxicos, além de problemas de resistência dos parasitas aos medicamentos existentes. Neste contexto, as infecções bacterianas também geram enormes problemas de saúde ao redor do mundo devido à resistência aos antimicrobianos. Assim, o veneno da serpente *Crotalus durissus terrificus (Cdt)* possui moléculas candidatas para uso biotecnológico, entre elas a crotapotina, peptídeo não tóxico do veneno de *Cdt,* composta por três cadeias ligadas por sete pontes dissulfeto e já demonstrou capacidade antinociceptiva, anti-inflamatória e antibiótica. Desse modo o objetivo desse trabalho foi realizar ensaios biológicos em modelos de malária, leishmaniose e também modelos microbiológicos para *Staphylococcus aureus* e *Escherichia coli,* além de observar a citotoxidade da crotapotina, a fim de estudar a possível atividade biológica da crotapotina íntegra e suas subunidades. Para isso, foram realizados ensaios microbiológicos usando as bactérias propostas para a determinação da Concentração Inibitória Mínima da crotapotina e suas subunidades, ensaios *in vitro* antipromastigota contra *Leishmania amazonensis* e antimalária contra *Plasmodium falciparum,* além de ensaios de citotoxicidade da crotapotina e suas subunidades em células de hepatoma (HepG2). Nossos resultados mostraram que os peptídeos da crotapotina não são capazes de inibir o crescimento antimicrobiano em nenhum dos modelos propostos, in vitro. Por outro lado, as moléculas não interferem na viabilidade das células testadas. No entanto, os resultados demonstraram que crotapotina e suas subunidades também não possuem efeito citotóxico e lítico, além disso, não se pode descartar seus possíveis efeitos intracelulares ou mesmo seu possível comportamento de penetração celular.

Palavras-chave: *Crotalus durrissus terrificus,* toxinas, Crotapotina, peptídeos biologicamente ativos, doenças negligenciadas

ABSTRACT

The biotechnological potential of snake venoms has always been studied and recognized. These venoms have already been used from the production of the world's most selling antihypertensive drug to the production of a fibrin sealant capable of healing chronic ulcers. In addition, venoms are excellent for prospecting biological effects in various diseases models, including neglected diseases such as leishmaniasis and malaria. These have highly toxic treatments, in addition to problems of parasite resistance to existing medicines. In this context, bacterial infections also generate huge health problems around the world due to antimicrobial resistance. Thus, the snake venom *Crotalus durissus terrificus* (*Cdt*) has candidate molecules for biotechnological use, including crotapotin, a non-toxic peptide from the *Cdt* venom, composed by three peptides chains linked by seven disulfide bonds and has already demonstrated antinociceptive, anti-inflammatory and antibiotic effects. Thus, the objective of this work was to perform biological assays in models of malaria, leishmaniasis and also microbiological models for *Staphylococcus aureus* and *Escherichia coli*, in addition to observing the cytotoxicity of crotapotin, in order to study the possible biological activity of intact crotapotin and its subunits. For this, microbiological assays were performed using the proposed bacteria for the determination of the Minimum Inhibitory Concentration of crotapotin and its subunits, *in vitro* antipromastigote assays against *Leishmania amazonensis* and antimalarial against *Plasmodium falciparum*, in addition to cytotoxicity assays of crotapotin and its subunits in cells of hepatoma (HepG2). Our results showed that crotapotin's peptides are not able to inhibit antimicrobial growth in any of the proposed models, *in vitro*. On the other hand, the molecules do not interfere with the viability of the cells tested. However, the results showed that crotapotin and its subunits also do not have a cytotoxic and lytic effect; in addition, its possible intracellular effects or even its possible cellular penetration behavior cannot be ruled out.

Keywords: *Crotalus durrissus terrificus*, toxins, Crotapotin, biologically active peptides, neglected diseases

SUMÁRIO

Capítulo I

1. Introdução

1.1. Os venenos de serpentes

A fascinação do homem pelas serpentes é milenar (1,2). Na cultura suméria, há mais de 4.000 anos o Deus da fertilidade e da saúde, Ningishzida, era representado por uma serpente com cabeça de homem, sendo seu símbolo duas serpentes gêmeas entrelaçadas em um bastão, trazendo já àquela época à associação a cura e imortalidade com a figura das serpentes (3) (Figura 1). Os gregos, por sua vez, consideravam as serpentes 'a mãe de todas as coisas' (4), fazendo com que no decorrer dos séculos o homem desenvolvesse uma enorme curiosidade pela peçonha das serpentes (5).

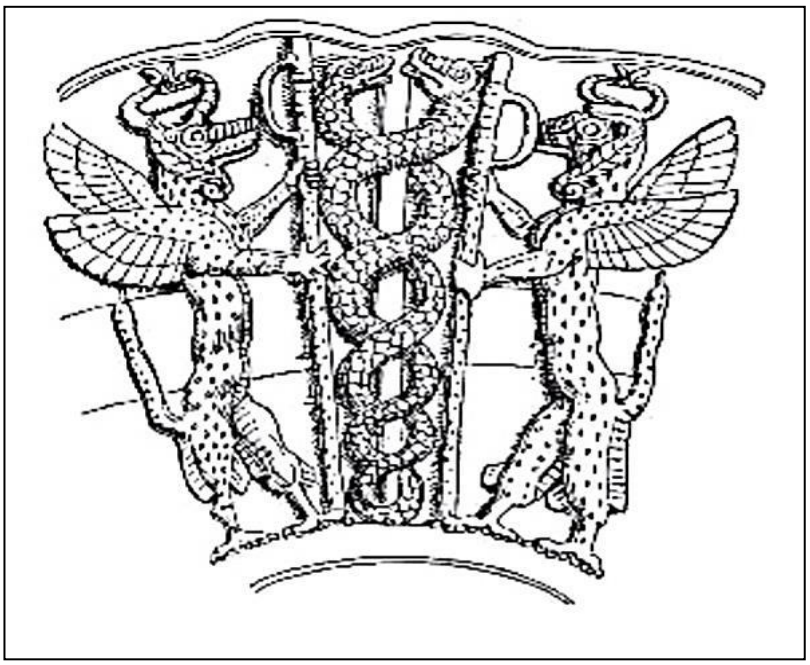

Figure 1: Representation of God Ningishzida

No último século tem sido intenso o número de trabalhos científicos desenvolvidos com peçonha de serpentes e o Brasil tem se mostrado propício para tais pesquisas (6), uma vez que apresenta riquíssima ofidiofauna, possuindo até 2018, 405 espécies de serpentes catalogadas, subdividas em 40 subespécies e 10 famílias, sendo duas famílias destas possuem serpentes peçonhentas: *Elapidae* e *Viperidae* (7).

Cerca de 90 a 95% do peso seco do veneno das serpentes é composto por proteínas, sendo a maior parte, enzimas tóxicas. Além das proteínas, peptídeos, lipídeos e carboidratos

também compõe o veneno, assim como compostos inorgânicos. As enzimas presentes no veneno são divididas em duas categorias: oxiredutases e hidrolases, sendo essa última representada pelas peptidases, lipases e glicosidases (8).

O veneno é de suma importância para a defesa do animal, visto que devido a sua alta toxicidade é utilizado como um mecanismo de caça, além de auxiliar no momento da digestão da presa (9,10). Nos acidentes ofídicos a peçonha é responsável pelos efeitos clínicos, efeitos esses que em casos graves podem levar o indivíduo a óbito, e estão diretamente atrelados à espécie da serpente (11,12).

1.1.1. Aplicações Biotécnológicas dos venenos de serpentes

Apesar dos problemas causados pelas toxinas nos casos de envenenamento é importante considerarmos que o avanço científico tem mostrado que os venenos das serpentes são uma importante ferramenta biotecnológica (13,8).

O exemplo mais comum é o Captopril®, medicamento contra hipertensão arterial mais comercializado no mundo. Cientistas observaram que, quando serpentes do gênero *Bothrops sp*. inoculavam veneno em suas presas, as vítimas desmaiavam devido a uma queda de pressão arterial. Baseado nessa observação o veneno de *Bothrops sp*. passou a ser estudado, até que nos anos 70, o medicamento Captopril® foi desenvolvido, a partir de peptídeos potenciadores de bradicina do veneno de *Bothrops jararaca*, que além de potencializar a bradicinina, molécula que mantém a pressão arterial baixa, também é capaz de inibir o conversor de Angiotensina 1 em Angiotensina 2, um potente hipertensivo, e consequentemente auxilia no controle da pressão arterial (14,15).

Além do Captopril®, ainda na classe de medicamentos para tratamento de doenças cardiovasculares, nos anos 90 foram desenvolvidos dois biofármacos derivados de veneno de serpentes: O Eptifibatide, produzido a partir do veneno da serpente *Sistrurus miliarus bardouri* e que é indicado para prevenção de infarto do miocárdio em indivíduos com angina instável, que quando administrado juntamente com ácido acetilsalicílico e heparina reduz consideravelmente a necessidade de angioplastia coronária translumial percutânea precoce (16,17,18) e o Tirofiban, derivado do veneno de *Echis carinatus*, que atua inibindo glicoproteínas IIb e IIIa, auxiliando também na prevenção de infarto do miocárdio e angina instável e diminuindo a agregação plaquetária (19,20).

Além disso, alguns venenos apresentam atividade analgésica, como por exemplo, a crotalfina (cadeia γ da crotapotina) de *Cdt* que demonstrou ser mais efetivo que a morfina para tratamento de dor, possivelmente não causando dependência ou tolerância (21,22). O mesmo ocorre para um peptídeo do veneno de *Dendroaspis polylepis*, chamado mambalgin, que tem ação inúmeras vezes maior que a morfina, e vem sendo estudo pelo Instituto de Farmacologia Celular e Molecular de Nice- França (23,24).

O selante heterólogo de fibrina é outro exemplo de aplicabilidade terapêutica dos venenos de serpentes. Produzido a partir da giroxina, uma trombina símile do veneno de *Cdt* e de fibrinogênio do sangue de grandes animais, tem se mostrado efetivo no tratamento de úlceras venosas (25,26), indo além das finalidades inicialmente descritas, como por exemplo, atuar na hemostasia em processos cirúrgicos e processos odontológicos (27).

Além dessas aplicações os venenos de serpentes têm demonstrado potencial antitumoral (28,29), tendo ação citotóxica e lítica sobre diversas linhagens de células tumorais, como por exemplo, adenoma de pulmão (29,30) e câncer de mama (31), efeito inibidor de adregação plaquetária (32) e antitrombótico (33), tratamento de doenças reumáticas e autoimunes (34,35).

Essas e diversas outras aplicações dos venenos de serpentes, mostram que tais venenos possuem um arsenal de moléculas bioativas que trazem possibilidades de usos biotecnológicos para as inúmeras doenças negligenciadas, doenças estas que possuem tratamento limitado, como por exemplo, a malária, a leishmaniose e algumas infecções bacterianas.

1.2. As doenças negligenciadas

1.2.1. Malária

A malária, também chamada de paludismo, impaludismo ou maleita é um grande problema de saúde pública por apresentar altas taxas de morbimortalidade (36,37). A doença é transmitida pela fêmea do mosquito *Anopheles* vetor do protozoário do gênero *Plasmodium* (38). Quando um indivíduo é picado, os micro-organismos presentes na saliva do mosquito penetram no sistema circulatório do hospedeiro, chegando ao fígado, onde ocorre sua reprodução (39).

São cinco as espécies de *Plasmodium* capazes de causas infecção no ser humano: *P. falciparum*, *P. vivax*, sendo esses dois os maiores causadores de infecção e morte, *P. ovale*, *P.malariae* e *P. knowlesi*, (40) sendo que a última espécie é endêmica do sudeste asiático (41).

A infecção ocorre em duas fases, uma delas ocorre no fígado, logo após a picada do mosquito infectado, onde os esporozoítos dos protozoários presentes na saliva do mosquito invadem o fígado. Todo o período de reprodução assexuada e maturação dos novos protozoários ocorre nessa fase, que pode durar de uma semana a um mês. Após a reprodução e maturação os parasitas rompem os hepatócitos, atingem a corrente sanguínea e infectam os glóbulos vermelhos. A fase em que o parasita infecta o fígado é denominado ciclo hepático do protozoário, enquanto a fase de infeção dos glóbulos vermelhos é chamada fase eritrocítica do ciclo de vida (39) (Figura 2).

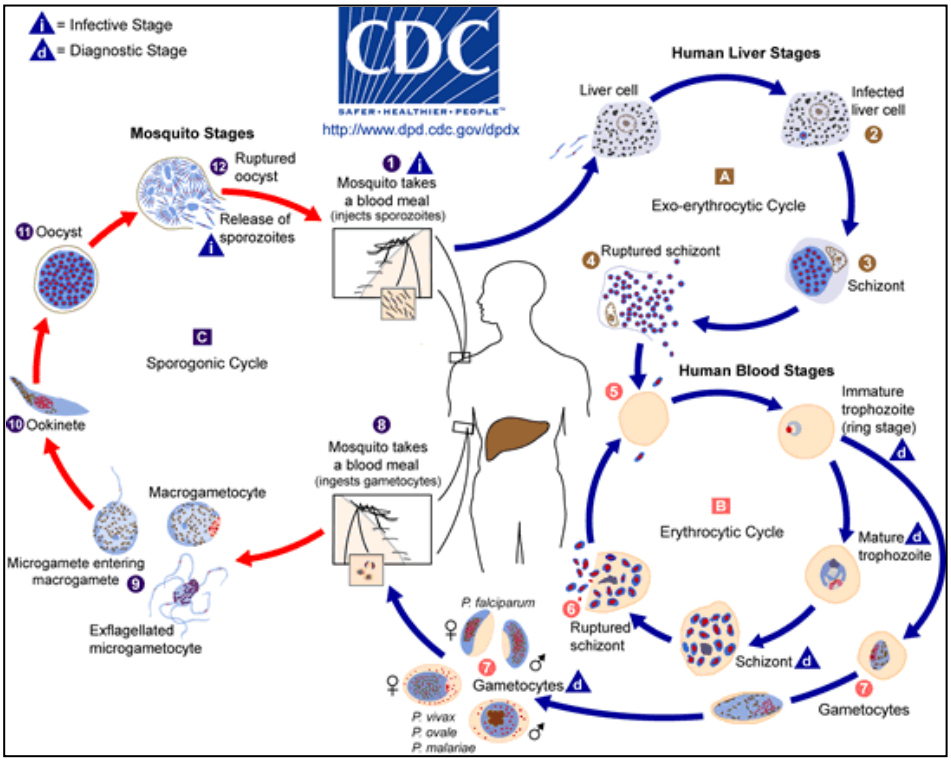

Figura 2 – Ciclo de vida do *Plasmodium*. Fonte: Centers of Disease Control and Prevention (CDC). Disponível em: https://www.cdc.gov/dpdx/malaria/index.html. Acesso em: 10 ju. 2022

Os sintomas da doença podem aparecer de alguns dias a vários após a infecção, sendo algo bastante variável. Os sintomas iniciais são bastante semelhantes aos sintomas da gripe, incluindo dores de cabeça, febre, calafrios e dores no corpo. Com a evolução da doença é muito comum a intensa sensação de frio, seguida de calafrios e essas manifestações se

mostram de forma cíclica, com intervalos de horas que estão associadas ao ciclo reprodutivo da espécie de *Plasmodium* causador da doença (42). Além disso, existe a chamada malária cerebral causada por *P. falciparum*, onde há uma série de sintomas neurológicos e pode levar a diversas complicações (43).

Atualmente o tratamento da doença é feito por meio de medicamentos via oral em casos moderados e via intravenosa ou intramuscular em casos mais graves. Existem bons medicamentos para o tratamento da doença, como por exemplo, cloroquina, primaquina e o quinino, utilizado para casos graves, porém o parasita vem desenvolvendo resistência aos fármacos anti-maláricos (44), dificultando o tratamento e aumentando a morbimortalidade.

1.2.2. Leishmaniose

Outra doença negligenciada de grande importância é a leishmaniose, doença crônica transmitida pelo mosquito do gênero *Lutzomyia* e *Phlebotomus* (45) e que possui quatro grupos clínicos: leishmaniose cutânea (46), leishmaniose cutânea difusa (47), leishmaniose mucocutânea (48) e leishmaniose visceral (49). É caracterizada por produzir um conjunto de síndromes, que são causadas por diferentes espécies de *Leishmania* e que pode atingir o homem(50) e também animais domésticos (51).

A leishmaniose cutânea caracteriza-se pela formação de úlceras na pele, o que causa certo estigma, já a leishmaniose cutânea difusa é caracterizada por lesões difusas não ulceradas por todo o corpo, sendo de difícil tratamento (52). A leishmaniose mucocutânea, por sua vez causa grandes lesões na cavidade nasal-oral e também faríngica deixando o indivíduo acometido pela doença desfigurado (48), já a forma visceral é a mais grave, caracterizada por perda de peso, febre, anemia, hepatoesplenomegalia e linfadenopatia (53). É a forma com maior importância epidemiológica devido a sua morbimortalidade, causando entre 20.000 a 40.000 mortes anualmente (54).

O Brasil possui 7 espécies de *Leishmania* e também diversas espécies do mosquito vetor da doença, sendo que os principais reservatórios para os protozoários são cães e raposas (55) e também felinos (56,57) e roedores (58). O país ainda se encontra na lista dos dez países com maior incidência da doença (59).

Os parasitas da leishmaniose possuem tropismo pelo sistema fagocitário mononuclear. No momento da picada os protozoários presentes na saliva do mosquito são fagocitados por macrófagos e então iniciam seu ciclo reprodutivo dentro dessas células. Eles se reproduzem por divisão binária e saem para o sangue ou linfa, destruindo as células e invadindo outros macrófagos (60) (Figura 3).

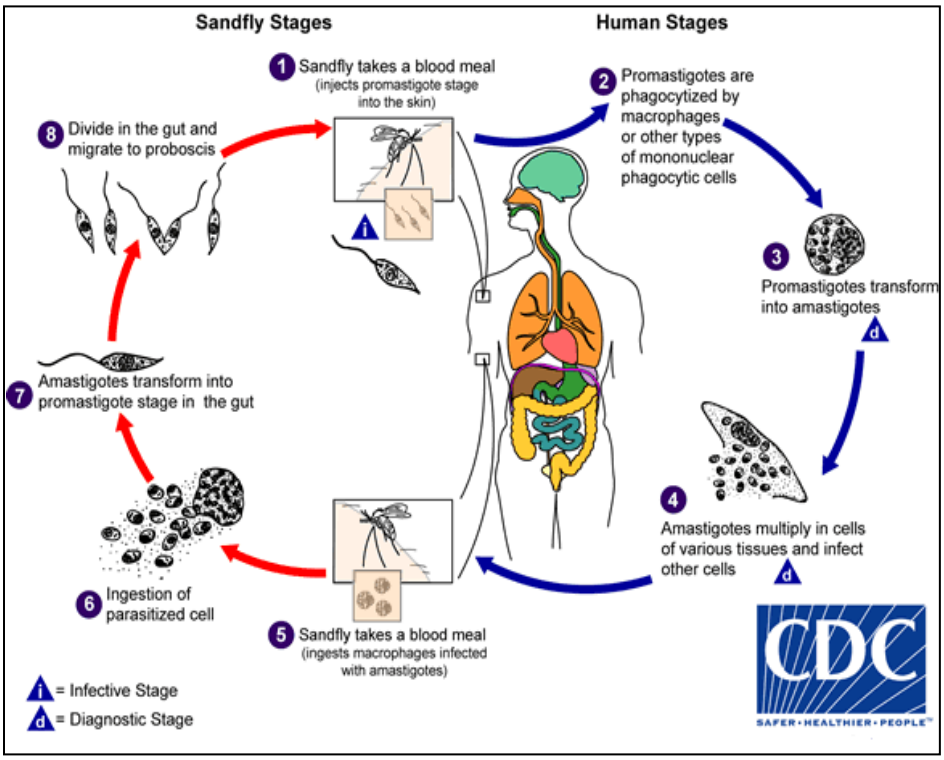

Figura 3– Ciclo de vida da *Leishmania*. Fonte: Center of Disease Control and Prevention (CDC). Disponível em: https://www.cdc.gov/dpdx/leishmaniasis/index.html. Acesso em: 10 jun. 2022.

De modo geral a elevada produção de parasitas leva a uma resposta imunológica. Quando o sistema imunológico produz uma resposta citotóxica, tem-se a forma cutânea da doença, pois há a destruição das células infectadas pelo parasita e com isso apenas uma manifestação clínica relativamente leve. Quando a resposta imunológica atua produzindo anticorpos, estes não reconhecem o parasita, que se encontra intracelular e a doença evolui para uma manifestação mais grave, a visceral, que causa principalmente hepatoesplenomegalia, e leucopenia (61).

O tratamento é feito com compostos antimônios, como a pentamidina e miltefosina e anfotericina B, porém essas drogas possuem alta toxicidade, podendo causar hepatite, pielonegrite e miocardite, além de estarem se tornando ineficazes uma vez que, assim como

na malária os parasitas vem se tornando resistentes aos medicamentos existentes, trazendo uma certa urgência para o desenvolvimento de novas drogas para o tratamento da doença (50). Estudos com toxinas animais já demonstraram eficácia sobre *Leishmania* (62).

Além das doenças negligenciadas causadas por protozoários, outro grande problema de saúde pública são as infecções bacterianas, principalmente as infecções causadas por bactérias resitentes a antimicrobianos (63).

1.3. Infecções causadas por bactérias

1.3.1. *Staphylococcus aureus*

A bactéria gram-positiva *Staphylococcus aureus*, por exemplo, é muito comum na prática clínica, uma vez que pessoas saudáveis apresenta esse tipo de bactéria na flora normal da pele, sem que esta lhe cause qualquer dano, porém em casos de imunossupressão ou ferimentos a bactéria pode causar uma série de problemas, que vão desde infecções na pele como impetigo, foliculite, terçol e furúnculo até infecções graves, como endocardite, pielonefrite, pneumonia e sepse (64,65).

Um dos maiores desafios em relação ao tratamento das infecções causadas por *Staphylococcus aureus* é o fato desta bactéria ser capaz de desenvolver mecanismos de defesa contra os antibióticos (66). A *S. aureus* resistente a meticilina (MRSA) é um exemplo clássico de bactéria resistente a antibióticos da classe das penicilinas, antibióticos comuns no tratamento de *S. aureu* (67). Estudos têm demonstrado que essa bactéria além da resistência a meticilina, muitas vezes produz biofilmes o que a torna mais virulenta (68). Além disso, MRSA é uma das principais bactérias causadoras de infecções hospitalares e sua transmissão pode ocorrer através do contato entre pessoas, principalmente através das mãos (69).

1.3.2. Escherichia coli

Além da *Staphylococcus aureus*, a bactéria gram-negativa *Escherichia coli*, também é de grande incidência. Grande parte das cepas de *E.coli* habitam o trato gastrointestinal humano e de outros animais de sangue quente, sendo inofensivas, porém alguns sorotipos dessa bactéria podem causar intoxicações alimentares graves através de transmissão oral-fecal

(70), além de infecções do trato urinário, através da migração dessas bactérias do ânus para a uretra (71).Geralmente as infecções por *E.coli* não são graves, porém em pacientes imunodeprimidos e neonatos pode haver complicações, como por exemplo, sepse (72) e meningite (73).

O tratamento das infecções causadas por *E.coli* tem sido problemático, pois assim como a *S. aureus*, essa bactéria tem se tornado resistente a um número cada vez maior de antibióticos, (74,75,76).

1.4. Moléculas de veneno de serpentes em modelos de protozoários e bactérias

No contexto de tais problemas de saúde pública, estudos já foram realizados utilizando venenos de serpentes, uma vez que estes possuem um arsenal de moléculas com potencial biotecnológico. Nas infecções bacterianas Nunes et al., 2020(77) e da Silva Caldeira et al., 2021(78), demonstraram em seus respectivos trabalhos que moléculas dos venenos de *Bothrops sp*. São capazes de inibir o crescimento de *Staphylococcus aureus* e *Escherichia coli*, além de impedir o formação de biofilme. Rheubert et al., 2020(79), por sua vez, em uma recente revisão mostrou que o veneno *Ophiophagus hannah* também possui efeito inibidor em cepas de *E.coli* e *S.aureu*, incluindo MRSA, além de demonstrar que os efeitos antibióticos dos venenos de serpente se estendem a diversas espécies de bactérias, resultados que corroboram com outros trabalhos da literatura (62).

Nas doenças parasitárias causadas por protozoários, as fosfolipases A2 de *Bohtrops marajoensis* e *Bothrops diporus* foi capaz de inibir o crescimento de trofozoítos de *Plasmodium falciparum* (80,81). Além de possuir efeito inibitório no crescimento dos parasitas causadores da Malária, o veneno de *Bothrops marajoensis* também apresenta efeito inibitório no crescimento de promastigotas de *Leishmania infantum* (80), assim como o veneno de *Bothrops jararacussu* (82) e *Naja naja oxiana* (83).

O desafio no uso de muitas das moléculas até então estudadas é fato destas serem citotóxica e lítica, desta forma, é importante estudar as moléculas não tóxicas dos venenos de serpentes, como por exemplo, a Crotapotina do veneno de *Crotalus durissus terrificus.*

1.5. Crotapotina do veneno de *Crotalus durissus terrificus*

A Crotapotina, também conhecida como Crotoxina A (84,85) - subunidade ácida e não tóxica - é um peptídeo com massa molecular 9,6 KDa, composta por 3 cadeias polipeptídicas (α, β e γ) ligadas por 7 pontes dissulfeto (86) que possuem 40, 35 e 14 resíduos de aminoácidos respectivamente (87) (Figura 4).

Seu primeiro sequenciamento foi realizado em 1985 (88), onde se observou que tal peptídeo se origina de um precursor homólogo a fosfolipase A2 (89).

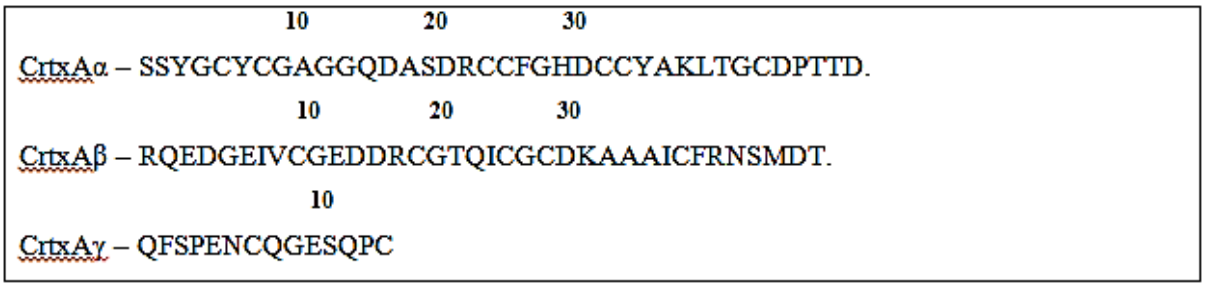

Figura 4 – Sequência das subunidades da crotapotina. **Fonte:** Arquivo pessoal

A Crotapotina foi inicialmente descrita como chaperona da fosfolipase A2, capaz de potencializar a toxicidade dessa enzima e protege-lá de realizar ligações em sítios de baixa afinidade, além de também inibir a atividade enzimática da fosfolipase A2 (86).

Apesar de ter sido descrita inicialmente como uma molécula inativa, estudos recentes demonstram que a Crotapotina possui capacidade antinociceptiva (22), anti-inflamatória (90), atuando diretamente sobre a imunidade humoral (87), além de também apresentar atividade antimicrobiana sobre bactérias gram-positivas e gram-negativas (91).

Além disso, considerando o gasto energético associado a síntese destes peptídeos (cerca de 5 ATP por ligação peptídica formada, ou seja, 2300 ATP (~1100 moléculas de glicose, considerando 100% de eficiência termodinâmica) por proteína, na média (92), muitos autores, incluindo nosso grupo, consideram que a presença de moléculas secretadas em um fluido biológico, por exemplo no veneno, consideradas ‘sem efeito biológico’ está mais associadas a um viés e/ou uma limitação técnica-criativa dos pesquisadores em descrever o modelo biológico no qual tais moléculas sejam ativas, do que propriamente tratar-se da secreção moléculas ‘inócuas’, associadas a um alto gasto energético.

Portanto, aplicar esse peptídeo e suas subunidades em modelos biológicos de doenças negligenciadas e infecções bacterianas de importância médica é crucial, uma vez que esta já tem demonstrado potencial. Além disso, explorar as diversas possibilidades das toxinas animais vem sendo uma grande forma de descoberta de moléculas candidatas para uso biotecnológico e médico e é de extrema importância ampliar os horizontes em relação ao uso de toxinas, investindo no estudo de 'moléculas negligenciadas'(93).

2. OBJETIVOS

2.1. Objetivos gerais

A presente pesquisa teve como principal objetivo avaliar a atividade da crotapotina e suas subunidadades, presentes no veneno de *Crotalus durissus terrificus*, em promastigotas de *Leishmania amazonensis*, trofozoitos de *Plasmodium falciparum*, bactérias *Staphylococcus aureus* metilicilina resistente e *Escherichia coli*, além de avaliar eveito citotóxico e lítico em células de hepatoma (HepG2).

2.2. Objetivos específicos

- Reproduzir a metodologia de de Oliveira et.al, 2017(86) para obtenção da crotapotina e suas subunidades por cromatografia líquida de fase reversa - RP-HPLC;

- Padronizar modelos experimentais para Malária, Leishmaniose e Microbiológicos para *S. aureus* e *E. coli*, frente a crotapotina;

- Estudar o efeito da administração da crotapotina e suas subunidades sobre cada modelo proposto;

3. Questão da Pesquisa

Inúmeros estudos utilizando toxinas animais já demonstraram bons resultados frente às doenças negligenciadas, porém muitas outras moléculas ainda estão a disposição na natureza para serem prospectadas.

Desta forma, estudar as moléculas não tóxicas presentes nos venenos das serpentes é de extrema importância, principalmente estudando moléculas como a crotapotina.

Finalmente, o uso de (pequenos) peptídeos como modelos de fármacos ou protótipos farmacêuticos traz a vantagem de que todo e qualquer metabólito do processamento intracelular destas moléculas não é tóxico e nem deletério ao organismo (são, ao final, aminoácidos).

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Capítulo II

The possible biotechnological role of crotapotin and its subunits: the lack of toxic effects on bacteria, protozoa, and mammal cells

Laudicéia Alves de Oliveira, Carolina Bioni Garcia Teles, Hugo Vigerelli, Ana Flávia Marques Pereira, Luciana Curtolo de Barros, Ary Fernandes Júnior, Daniel Carvalho Pimenta

Abstract

The biotechnological potential and biomedical applications of the snake venom components are widely recognized. The snake toxins have proved to be excellent for prospecting for the development of new drugs, as Captopril, Tirofiban, Eptifibatide and others and therapeutic methods, being a great source of antimicrobial peptides. Many studies have described antimicrobial effects from Crotoxin, the major toxin from *Crotalus durissus terrificus (Cdt),* a non-covalent heterodimeric neurotoxin constituted of two subunits: a catalytically active phospholipase A_2 and an acid and "non-toxic" peptide, named crotapotin. This molecule is composed of three peptide chains, connected by seven disulfide bridges. The aim of this study was evaluating the possible antimicrobial activity of crotapotin – and its subunits - in models of Methicillin resistant *Staphylococcus aureus*, *Escherichia coli* and protozoa *Leishmania amazonensis* (in vitro) and the cytotoxicity of these molecules in HepG2 cells. For this, microbiological assays were performed to determine the Minimum Inhibitory Concentration of crotapotin and its subunits, *in vitro* anti-promastigote assays against *Leishmania amazonensis* and anti-malarial against *Plasmodium falciparum*, in addition to cytotoxicity assays of crotapotin and its subunits in tumor hepatocyte cells (HepG2). Our results show that the crotapotin peptides are not able to inhibit the growth of *L.amazonensis*, *P.falciparum*, neither the bacterias *S.aureus* and *E.coli, in vitro.* On the other hand, the assayed molecules did not alter the viability of the HepG2 cells. These results demonstrate that, although crotapotin and its subunits are non-toxic, one cannot rule out their possible intracellular effects or even their possible cell-penetrating behavior.

Keywords: *Crotalus durrissus terrificus*, toxins, Crotapotin, biologically active peptides, neglected diseases

1. Introduction

Animal venom represents an evolutionary weaponry that had evolved for predatory and defensive purposes in the animal life (da Silva Caldeira et al., 2021). The last century of research has shown that animal venoms are a rich source for biotechnological and biomedical application and the snake venom is among the most complex of them all (Warrell, 2010). The venom of some species presents dozens of different toxic and non-toxic molecules (Warrell, 2010), of which 95% are proteins and peptides (Lazcano-Pérez et al., 2022). The major component and most toxic protein from the rattlesnake *Crotalus durissus terrificus* venom, Crotoxin was the first isolated toxin by Slotta and Fraenkel-Conrat (Slotta and Fraenkel-Conrat, 1938). Subsequently Fraenkel-Conrat and Singer described its composition being a heterodimeric β-neurotoxin comprise of two subunits formed by a non-covalent association of a basic phospholipase A2 (CB) and an acid non-toxic peptide (CA), known as crotapotin (Fraenkel-Conrat and Singer, 1956; Kato and Sampaio, 2021).

Crotapotin is a 9.6 kDa peptide, displaying a pI value of 3.4 and it is structurally composed by three peptides chains, connect by seven disulfide bonds, termed α, β and γ chains, presenting 40, 35 and 14 aminoacids residues, respectively (de Oliveira et al., 2017). These peptide chains originated through the cleavage of a precursor homologous to phospholipase A2 and in 1985 were sequenced for the first time. Crotapotin is always described as a chaperone from phospholipase A2, being responsible to potentiate the Phospholipase A2 (PLA2) toxicity and protect it from being inactive by binding to low-affinity sites. As an opposite effect, crotapotin inhibits the enzymatic action of PLA2 (Bon et. al, 1979).

Although it has being described as an inactive peptide, with the majority of the reported biological effects being attributed to the phospholipase A2 subunit or to the crotoxin complex, crotapotin and crotapotin peptides have been described to present biological activity, such as anti-inflammatory (Garcia et al., 2003), Hepatitis C virus infection inhibition (Shimizu et al., 2017), *in vivo* pancreatitis decrease (Leme et al., 2002), pronounced antinociceptive (Machado et al., 2014) and antimicrobial effect (De Oliveira et al., 2003). Furthermore, crotoxin has several effects that may be associated with the peptides that compose it.

Many of these effects are described based on the biological observations of the venom regarding the venomous animal life. For example, it is well known that the venom, the venom

duct, and the venom glands are sterile. This condition is associated to the presence of antimicrobial peptides (Warrell, 2010).

The antimicrobial peptides (AMPs) can be considered nature's innovative response strategy to antimicrobial infection/colonization and their widespread distribution throughout several living species are probably responsible for the successful evolution of multicellular organisms, acting as a first line of defense and protection against infections and microorganisms (Samy et al., 2007; Ratzka et al., 2012). Nowadays AMPs are being used as a novel scaffold for new drug studies as an alternative to classical medicines, due to antimicrobial resistance (Pinto et al., 2013). These peptides have been isolated from many animal venoms (Coelho et al., 2021).

Antimicrobial resistance is common phenomenon and serious public health over the world and a huge challenge for treatment of several diseases caused by multidrug-resistant bacteria, parasites, viruses and fungi causing high rates of morbidity and death (Morrison and Zembower, 2020; Zhu et al., 2022). In this context, Brazil is one of the countries bearing large numbers of neglected tropical diseases that have become multi-drug resistant, such as Leishmaniasis, due the production of pteridine reductase enzyme (PTR1) that cause loss of drug efficacy (Nikpour et al., 2021), Malaria caused by Cloroquin resistant *Plasmodium Falciparum* (Ezeani et al., 2022) and bacterial infection caused by Methicillin resistant *Staphyloccus aureus* (MRSA) and *Escherichia coli* (Gutierrez Guarnizo et al., 2021).

Leishmaniasis is a major health problem present on four continents, affecting about 12 million people worldwide. The clinical manifestation may be cutaneous, mucocutaneous or visceral; being the first, the most neglected, mutilating and stigmatizing parasitic disease that causes more than 1 million cases per year (Sezavar et al., 2021). Leishmaniasis parasites have tropism for the mononuclear phagocyte system and affect all the hemostatic systems (Borghi et al., 2017).

As well as the Leishmaniasis, the Malaria is a public health problem and the drug resistant *Plasmodium falciparum* has improved after 1970 (Badeliya et.al, 2021), and affecting about 241 million people (Huang et.al, 2019). The parasite affects the liver and the red blood cells (Tan & Blackman, 2021) causing constant cycle of fever, chills, anemia, headaches, vomiting, joint pain (Badeliya et.al, 2021) and can causes cerebral manifestation (Adams & Jensen, 2022).

Methicillin resistant *Staphylococcus Aureus* (MRSA) and *Escherichia coli* resistant became relevant widespread infections as consequence of unnecessary use of antibiotics (Zhanel et al., 2022). These resistance phenomena are responsible for the persistent infection

in humans, as well as compromise the effectiveness of therapies against other diseases such as HIV, Cancer chemotherapy and malaria (Zhu et al., 2022).

In face of all these problems, many research using snake venom are being conducted, and several molecules demonstrate effects against such diseases (Sharifi et al., 2021, Katz et al., 2020, Nunes et al., 2013, Perumal Samy et al., 2017, Diniz-Sousa et al., 2018, Zhang et al., 2013, da Silva Caldeira et al., 2021). On the other hand, many of these molecules present some degree of cytotoxic effects. In this context, Crotapotin and its subunits were investigated against *Leishmania amazonensis* to evaluate the anti-promastigote effect, MRSA and *Escherichia coli* to evaluate the antibacterial activity and cytotoxic effect in HepG2.

2. Materials and Methods

2.1. Snake venom

Cdt venom was obtained from snakes kept in the Center for the Studies of Venoms and Venomous Animals (CEVAP) of UNESP, in Botucatu (Brazil). All procedures involving snake were in accordance with the ethical standards of the institutional and national research committee. The study was approved by the responsible Ethics Committee on Animal Use of Biotechnology Institute – IBTEC (protocol no 1145/2015 – CEUA). The venom used are validated and respects the good production, handling and animal welfare practices (Santos et al., 2021).

2.2. Crotapotin, subunits isolation and identification by Mass Spectrometry

The isolation of crotapotin and subunits was performed according to de Oliveira et al., (2017). 10mg.mL−1 crude *Cdt* venom solution (0.1% trifluoroacetic acid – TFA) was centrifuged (3800 x g) and separated by RP-HPLC using a Luna C8 column (100 A, 250 × 10 mm, Phenomenex) coupled to a Shimadzu Proeminence binary HPLC system. A 20–40% linear gradiente of B (90% acetonitrile – ACN, containing 0.1% TFA) over A (0.1% TFA) was used for 40 min after initial isocratic elution for 5 min, under a constant flow of 5 mL.min−1. UV monitoring was performed at 214 nm and fractions were manually collected. The reduced and alkylated crotapotin chains were separated by a Shimpack C18 column (100 A, 10 × 4.6 mm, Shimadzu), using a 0–50% linear gradient of B, for 20 min, under constant flow of 1 mL.min−1. UV monitoring was performed at 225 nm. For MALDI-TOF mass spectrometry, analyzes were performed on an Axima Performance MS/MS instrument (Shimadzu). The samples, in solution, were mixed in a 1:1 (v:v) ratio with a supersaturated

solution of the matrix recommended for the analysis of peptides (cinnamic acid and the mixture was deposited on the sampling plate (0.4-0.8 µL) for solvent evaporation. The automatic mode of equipment control and data acquisition via equipment control software (Launchpad, Shimadzu Biotech) was used.

2.3. Antimicrobial activities assay

2.3.1 Determination of Minimum Inhibitory Concentration (MIC) and Plate Culture

MIC the resazurin microtiter assay (REMA) was performed according to Pereira et. al (2020) with modifications, aiming for MIC of crotapotin and its subunits against ATCC 33591 isolates of MRSA (methicillin-resistant *Staphylococcus aureus*) and ATCC 43895 isolates of *Escherichia coli*. Different concentrations of Crotapotin and subunits α, β and µ (from 500 to 0, 25 ug/mL) were placed in 96-well sterile microtiter plates containing Mueller-Hinton Broth (MHB, Oxoid). Inocula from overnight cultures at 37°C standardized in saline solution according to 0.5 McFarland standard (approximately 1.5×10^8 colony-forming units (CFU/mL). One hundred microliters of the inocula were added to each well, resulting in a final volume of 200 µL and approximately 10^5 CFU/mL per well. A negative control, consisting of MHB and inocula, and positive controls, consisting of BHI and oxacillin (256 ug/mL) for MRSA and BHI and ciprofloxacin (256 ug/mL) for *E.coli*, were included. Plates were incubated at 37° C for 24 h, and MIC values were defined as the lowest concentration with no visible growth after the incubation period. A solution of resazurin (0.01%) was used to indicate viable bacteria. Subcultures were performed from the microdilution assays using brain heart infusion (BHI, Oxoid) agar plates in order to obtain minimum bactericidal concentrations (MBC). Thus, the plates incubated at 37 °C for24 h, and MBC values were considered the lowest concentration with no colony growth. The assays were performed in triplicate.

2.3.2 Well diffusion test

The well diffusion test was performed in 2 plates containing Muller-Hinton Broth medium (MHB, Oxoid) inoculated with MRSA and *E.coli* at a concentration of 10^5 CFU/mL. Wells of 6 mm in diameter were made in the agar and crotapotin and the subunits, at concentrations of 1000 µg/mL and 500 µL/mL respectively, were in the wells. The plates were incubated for 24 h at 37°C and the diameter of the halo was measured.

2.3.3 Disk Diffusion Test

The microbial growth inhibition halos were measured in millimeters. Prior to the experiments, the bacterial samples were recovered in Brain Heart Infusion (BHI) broth at 36°C for 18 hours. Antimicrobial susceptibility was evaluated according to the disk diffusion method. For this, sterile filter paper discs (6 mm Ø) received 10 ug/mL of crotapotin and subunits, then the disc was applied with sterile forceps on a Petri dish containing Mueller-Hinton (MH) agar, previously inoculated with the microorganism to be tested. For the disk diffusion test, halos with a diameter ≥ 6 mm were considered to have inhibitory activity. E.test containing Oxacillin for MRSA and ciprofloxacin for *E.coli* were used as controls.

2.4 Anti Leishmania assay

2.4.1. Parasites

The promastigote forms of *Leishmania amazonensis* (strain IFLA / BR / 67 / PH8) were obtained from CLIOC / FIOCRUZ. After thawing, promastigote forms were cultured in RPMI 1640 medium supplemented with 10% Bovine Fetal Serum, 25 mM HEPES (N-2-hydroxyethylpiperazineN-2-ethanesulfonic acid), 11.1 mM D-glucose (dextrose), 23.8 mM sodium bicarbonate NaHCO3, 0.37 mM hypoxanthine, 2 mM glutamine and 40 µg / mL. This culture was maintained at 24ºC (Stauber, et al, 1958). The monitoring of the *in vitro* propagation of these promastigotes were performed by counting in an optical microscope using erythrosine B 0.04% and mirrored Neubauer chamber. Red-colored parasites were considered dead and those birefringent and mobile were considered alive. The calculation was made using the formula (Stauber et al., 1958):

number of parasites = number of parasites counted x inverse of the dilution x 10^4.

2.4.2. *In vitro* anti-promastigote activity against *Leishmania amazonensis*, (IFLA/BR/67PH8)

Promastigotes were cultured at a concentration of 1 x 10^4 parasites/mL in 96-well plates, using RPMI culture medium supplemented with 10% fetal bovine serum (FBS) and isolated crotapotin and subunits (from 200 to 6.25 µg/mL) or culture medium (negative control) for 72 hours. 3 µg/mL of pentamidine was used as a positive control. Thereafter, 20

µL of 2 mM Resazurin (Sigma®) was added. The plate was further subjected to incubation at 24°C for 5 hours and finally the fluorescence was determined at an excitation 530/25 and emission of 590/35. The percentage of dead parasites, and thus the anti-promastigote activity was calculated based on promastigotes cultured only with RPMI. The Z test was accepted under 0.5 (Rólon et.al, 2006).

2.5. Cytotoxic activity against hepatic cells (HepG2)

The HepG2 cell line (derived from a human hepatoma) and J774 were maintained in RPMI medium containing 10% fetal bovine serum and 40 mg/L of gentamicin in an incubator with 5% CO_2, 95% humidity at 37 °C. In a 96-well plate, 180 µL hepatic cells or J774 ($1x10^4$ cells) were dispensed and maintened for 18 h in humidified atmosphere with 5% CO_2 at 37°C. After, this period the cells were incubated with 20 µL of RPMI (control) or Crotapotin and subunits (500-1.56 µg/mL) under humidified atmosphere for 48 hours at the same conditions. After that, 10 µL of Rezazurin solution were added to each well. Absorbance was read in a spectrophotometer at 540 nm.

2.6. Anti malarial assays

2.6.1. *In vitro* antimalarial activity against *Plasmodium falciparum*

The parasites was synchronized with sorbitol as described by Lambros & Vanderberg (1979) to obtain the predominance of *P. falciparum* trophozoites. RPMI culture medium (180 µL/well) containing 0.05% parasitemia and 1.5% hematocrit was added to 96-well plates. Different serial concentrations of crotapotin and subunits (from 200 to 6.25 µg/mL) were also added, in triplicate. Infected erythrocytes were added as a negative control and artemisinin (Sigma®) (50-0.7813 ng/mL) as a positive control. The plate was incubated for 48 hours at 37°C. To measure the parasite's sensitivity to peptides, the fluorescence test was performed according to Smilksteins (2004). For this, the supernatant was discarded, and the red blood meal was washed with 100 µL of PBS with centrifugation at 1,500 rpm for 10 min. After washing, 100 µL of SYBR Green was added in lysis buffer (20 mM Tris, pH 7.5; 5 mM EDTA; 0.008%; w / v saponin; 0.08%, v / v Triton X-100), the solution was prepared with a volume of 0.2 µL of SYBR Green for 1 ml of lysis buffer. The mixture was homogenized and 100 µL was transferred to a 96-well plate containing 100 µL of PBS. After 30 min at room

temperature, fluorescence was measured in a fluorimeter with an excitation of 485nm and an emission of 535nm in a gain of 100.

3. Results

3.1 Crotapotin and subunits fractionation and MS

In order to obtain crotapotin, C8 RP-HPLC isolation of crude *Cdt* venom was performed according to the methods section and the profile obtained was identical to de Oliveira et al., (2017). The fraction corresponding to crotapotin acording to de Oliveira et al., 2017 (fig 1) was collected and submitted to MS in order to assess its homogeneity and molecular mass was determined corresponding to crotapotin (fig 2). The other peaks were not collected and analyzed by MS, since this molecule was previously identified by our group (de Oliveira et al., 2017). The reduction and alquilation of crotapotin were performed and the processed crotapotin was subjected to RP-HPLC to obtain the isolated chains α, β and γ (fig 3). The fractions corresponding to the chains were also submitted to MS to determine the molecular masses and were performed the molecular masses as previously described, as well as the mass of one of the isoforms present in this molecule (de Oliveira et al., 2017) (fig 4).

3.2 Minimum Inhibitory Concentration (MIC), Well diffusion test and disk diffusion test

The results shows that neither crotapotin nor α, β, and γ subunits had inhibitory values of MIC and did not interfere with the bacterial growth in values up to 500 µg/mL, on the contrary, in the presence of the peptides the relative unit of fluoresce had increased. The compound control demonstrated that the peptides were sterile (data not shown). Oxacilin was used for MRSA and Ciprofloxacin for *E.coli* as positive control, and these antibiotics were capable of inhibiting 91% and 89% of the bacterial growth for each bacteria, respectively, presenting MIC 8 µg/mL to Oxacilin e MIC 0,12 µg/mL to Ciprofloxacin (Fig 5a e 5b).

3.3. *In vitro* anti-promastigote activity against *Leishmania amazonensis,* (IFLA/BR/67PH8) and cytotoxic activity against hepatic cells (HepG2)

The crotapotin and α, β, and γ subunits were not able to present significant inhibition on the growth of *L. amazonensis* (IC<200 µg/mL) when compared to the Pentamidin 3µg/mL,

p< 0.05 (fig 6). The cytotoxic assay showed that the compounds were not toxic against HepG2 in concentration <500µg/mL, which the cells presented 100% of viability (fig 7).

3.4. *In vitro* antimalarial activity against Plasmodium falciparum W2

As well as the other assay the crotapotin and α, β, and γ subunits were not able to present significant inhibition on the growth of *Plasmodium falciparum* (IC<200 µg/mL) when compared to the Artesimin 50ng/mL, p< 0.05 (fig 8).

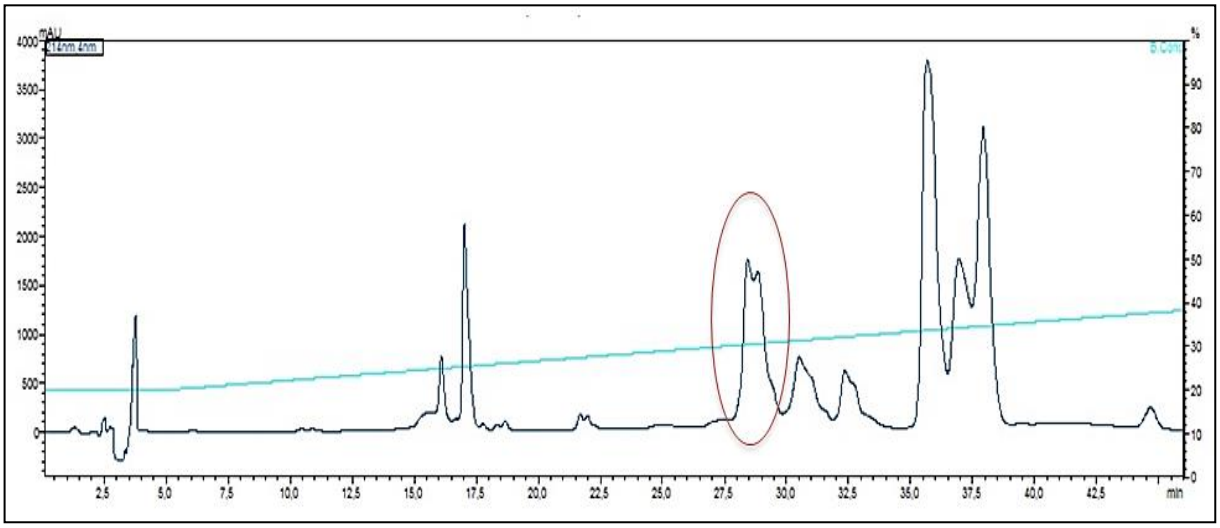

Fig. 1 RP-HPLC profile of the crude Cdt venom. The peak highlighted corresponds to the crotapotin.UV monitoring 214 nm. Inset: F3 analytical RP-HPLC demonstrating the proper molecule isolation. Chromatographic conditions are described in the Methods section.

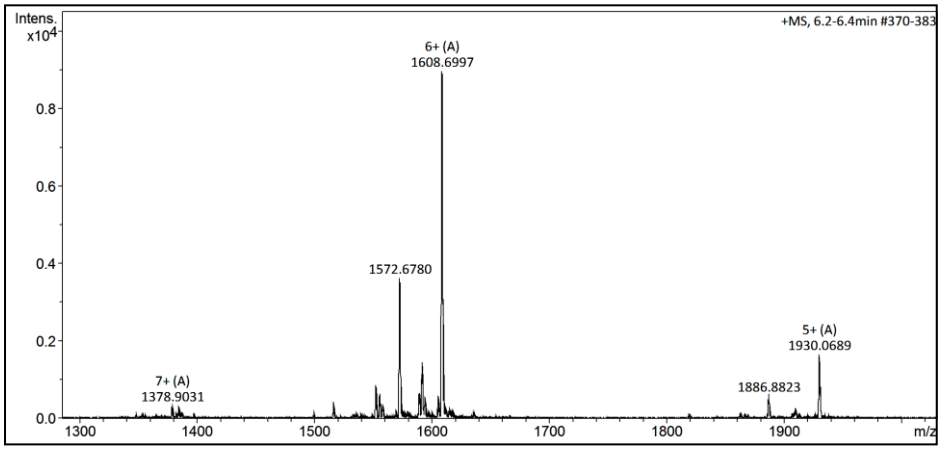

Fig 2: Crotapotin MS spectrum. The charge state of the major ions are typed over the m/z value.

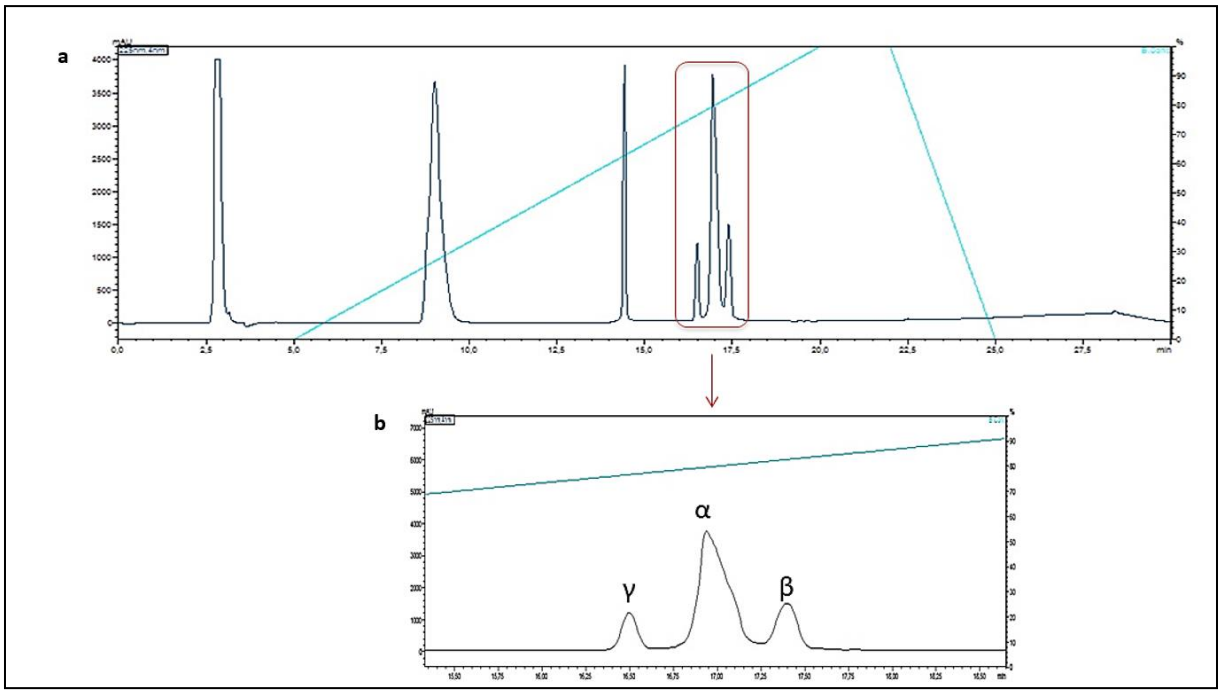

Fig 3 a) Reduced and alkylated crotapotin (F3) RP-HPLC separation chromatographic profile. **B)** Zoomed region with the identification of the individual chains. UV monitoring 225 nm. The major peaks in A correspond to the alkylation reagents

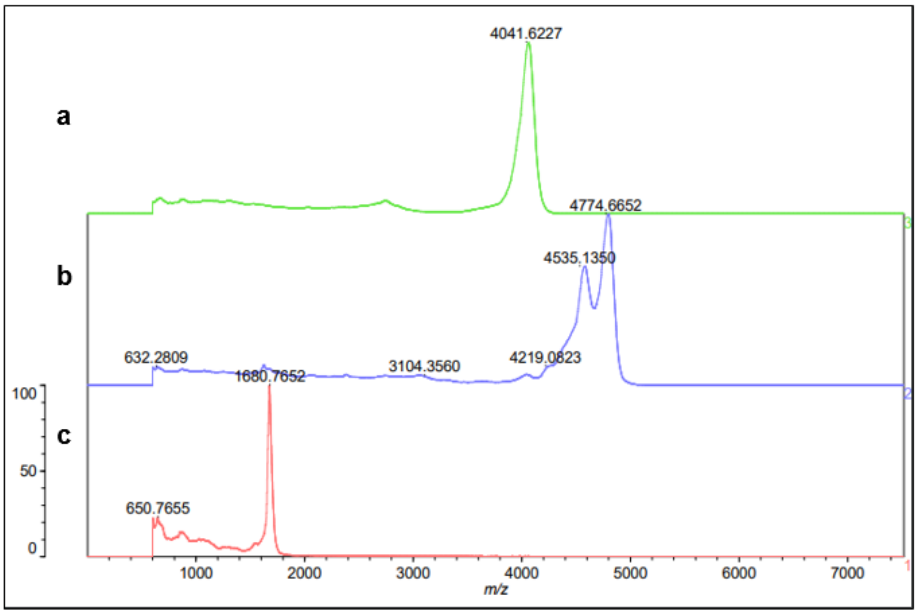

Figure 4: MALDI-TOF/MS specta of the β (a), α (b) and γ (c) chains.

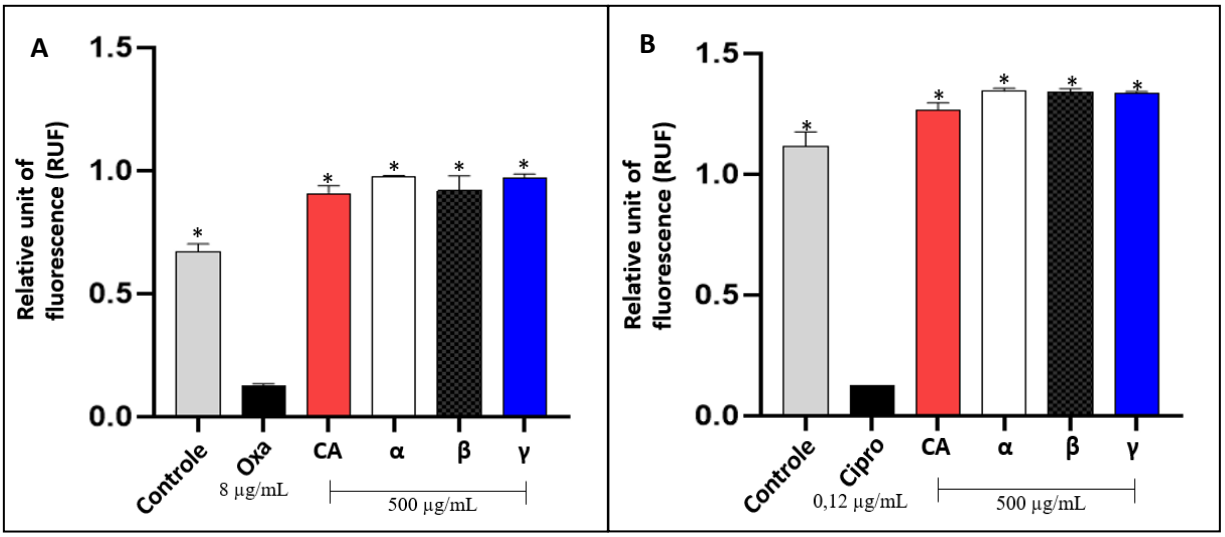

Fig 5: A) Effects of crotapotin (CA) and subunits α. β and γ on Meticilin Resistant *Staphylococcus aureus* with 500 µg/ml of the compounds. The figure represents the mean of the duplicates and the standard deviation (±). The gray bar (control) represents untreated bacterias in Mueller-Hinton Broth. The oxacilin represents 91% of bacterial death. **B)** Effects of crotapotin (CA) and subunits α, β and γ on *Escherichia coli* with 500 µg/ml of the compounds. The figure represents the mean of the duplicates and the standard deviation (±). The gray bar (control) represents untreated bacterias in Mueller-Hinton Broth. The ciprofloxacin represents 89% de bacterial death Statistical analysis was performed, ANOVA One-Way, with determination of the level of significance for $p<0.05$, through multiple comparisons (Tuckey's test). Columns marked with an asterisk (*) represent statistical differences when compared to a antibiotic.

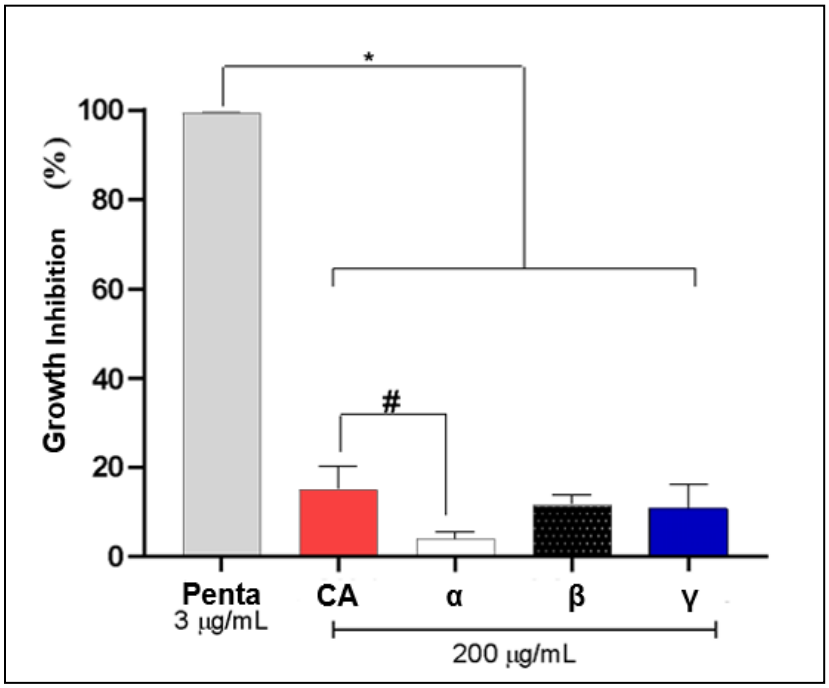

Fig 6: Inhibitory effects of Crotapotin (CA) e its subunits α, β and γ on the growth of *L. amazonensis* promastigotes. Promastigote cultures in the log phase were incubated at 24 °C for 72 h with 200 µg/mL of the compounds. The results refer to the mean and standard deviation (±) of one of the independent experiments. Statistical analysis was performed, One-Way ANOVA, with determination of the significance level for $p<0.05$, through multiple comparisons (Tuckey test). Columns marked with an asterisk (*) represent statistical differences against Pentamidine (3 µg/mL). Columns marked with asterisks (#) represent statistical diferences between them.

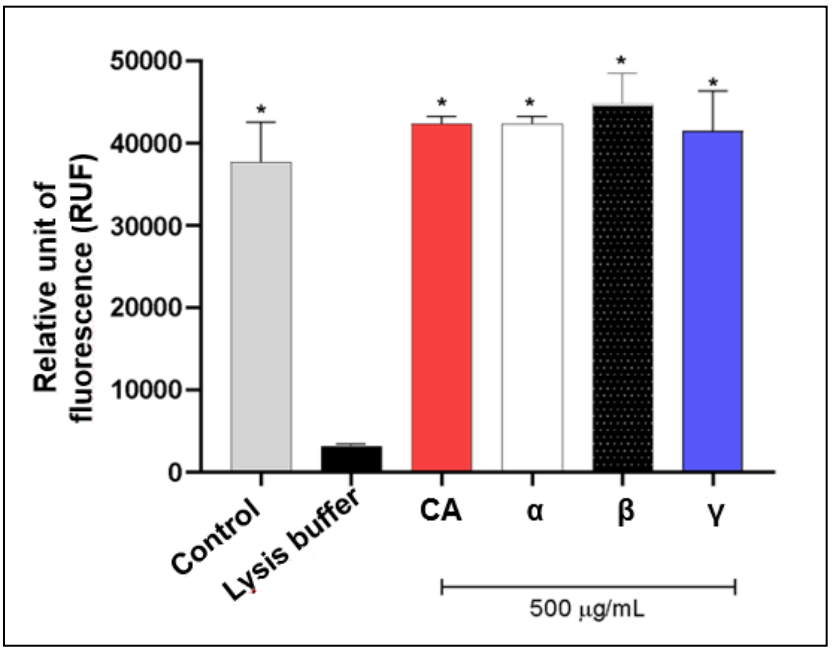

Fig 7: Effects of crotapotin (CA) and subunits α, β and γ on HepG2 cell viability after 72 hours of treatment with 500 µg/ml of the compounds. The figure represents the mean of the triplicates and the standard deviation (±) of the fluorescence of one of the two experiments performed independently. The gray bar (control) represents untreated cells maintained in RPMI medium. The lysis buffer represents 100% cell death. Statistical analysis was performed, ANOVA One-Way, with determination of the level of significance for $p<0.05$, through multiple comparisons (Tuckey's test). Columns marked with an asterisk (*) represent statistical differences when compared to a lysis buffer. There was no statistical difference between the groups and the control.

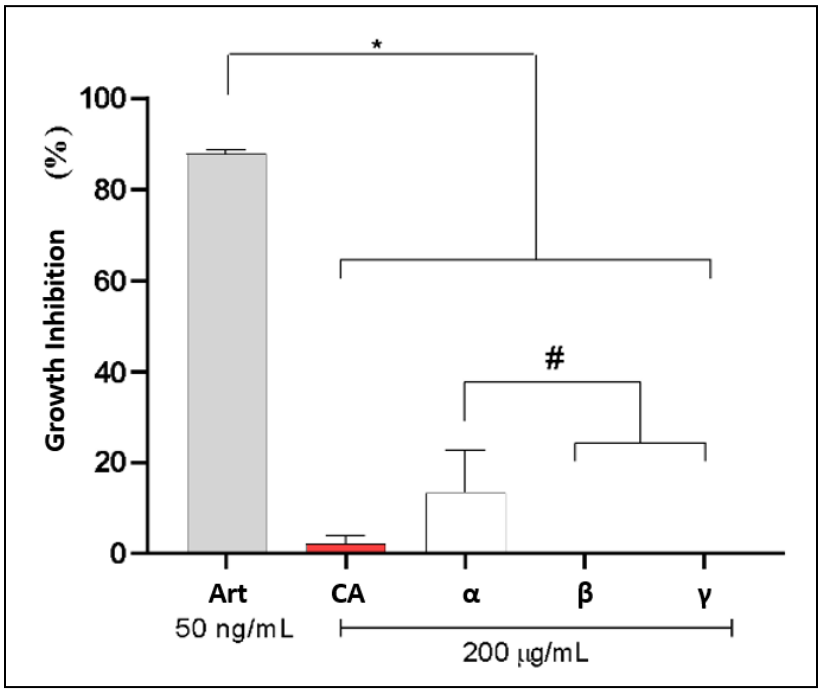

Figure 8: Inhibitory effects of Crotapotin (CA) and its subunits α, β and γ on the growth of *Plasmodium faciparum* W2. The parasites were synchronized, obtaining the predominant forms of young trophozoites and the plates with this culture were incubated at 36 ºC for 72 h with 200 µg/mL of the peptides. The results refer to the mean and standard deviation (±) of one of the independent experiments. Statistical analysis was performed, ANOVA One-Way, with determination of the level of significance for $p<0.05$, through multiple comparisons (Tuckey's test). Columns marked with an asterisk (*) represent statistical difference from Artemisinin (Art) at 50 ng/mL. Columns marked with hash marks (#) represent statistical difference between them.

4. Discussion

Antimicrobial peptides are widely expressed in snake venoms and show clinical relevance and therapeutic promise regarding efficacy and evidently do not readily induce resistance when compared to conventional antibiotics (Hancock and Sahl, 2006; Lamiyan et al., 2020). Therefore, such molecules present themselves as a possible novel and viable technology. Once Crotoxin has antimicrobial effects against bacteria, protozoa, fungus and virus (Farias et al., 2017; Canelli et al., 2020) it is important to discriminate how much of these effects are due to crotapotin and its subunits.

Our studies demonstrate that crotapotin and its subunits do not present any effect against tested Meticilin Resistant *Staphylococcus aureus* and *E.coli* bacteria or against the protozoas *Leishmania amazonenses* promastigote and *Plasmodium falciparum* trophozoites In the

protozoan model, however, the tested molecules showed a mild inhibitory effect on the growth of *L. amazonensis*, but these figures are not enough to justify the development of new drugs based on this model (Cos et al., 2006), the same goes for the results of *Plasmodium falciparum*. On the other hand, our molecules do not present toxicity on HepG2 cells, indicating that crotapotin and its subunits are not cytotoxic and lithic. It is noteworthy to mention that the lack of membrane-lytic effects do not exclude other intracellular, metabolic actions, yet unstudied and unapproached, for example, cell penetrating peptide (CPP).

Moreover, such results were obtained *in vitro*, using low peptide concentrations, and do not necessarily demonstrate the effectiveness of the use of the molecules *in vivo*, since some studies related that one of the first mechanisms to decrease the bacterial infection is the activation of the innate immune response (Krishna and Miller, 2012). Although crotapotin and it subunits were not able to lyse the bacterial membrane, this molecules have already been demonstrated to act on the immune system, particularly as an innate immune system activation agent (Castro et al., 2007; Garcia et al., 2003b; Landucci et al., 1995) and, thus, could be important to decrease the infection through other metabolic pathways. This effect on the immune system may also be important in reducing the infection caused by L. amazonensis, since one of the first immunological barriers in leishmaniasis is the activation of the complement system (Gurung and Kanneganti, 2015). However, to assess this hypothesis, amastigote *Leishmania* models need to be employed so that any crotapotin (and/or subunits) effect on the macrophage immune system could be perceived.

In a study by Shimizu et al., 2017, authors have shown that crotapotin is not able to reduce replication nor has it any virucidal effect against Hepatitis C virus (HCV); however, in presence of this peptide, authors were able to detect lower levels of intra and extracellular HCV RNA upon virus release, showing that this peptide prevents the virus from leaving the cells. Furthermore, their data show that crotapotin was able decrease lipid metabolism. Such effect on lipidic metabolism may explain *Crotalus durissus cascavella* crotapotin's action on *Xanthomonas axonopodis* (De Oliveira et al., 2003), once – for plant pathogenic bacteria - lipopolysaccharides are considered a virulence factor (Petrocelli et al., 2012). So, crotapotin-induced decrease in the lipid metabolism may correlate to the reported effect against bacteria.

Shimizu et al., 2017 also reports that both crotoxin and crotapotin always present matching biological effects, that could be either presence or absence of effect, under the same experimental conditions. This important observation suggests that at least some of the

biological effects attributed to crotoxin could be actually associated to crotapotin (or its subunits), since the intracellular environment is acid and reducing, which would lead to immediate crotoxin disassembly and crotapotin dissociation.

There is a relatively consolidated line of research that studies crotoxin antinociceptive effects (Teixeira et al., 2020). Particularly: previous reports described that such effects are specifically associated to crotapotin γ chain (Konno et al., 2008; de Freitas et al., 2021). So, besides the anti-inflammatory (Nunes et al., 2010) and immune system modulating effects attributed to crotoxin, crotapotin peptides would also play a biological role on their own (Landucci et al., 1995).

According to Fernandes et al., 2017, some crotapotin amino acid residues are exposed to the solvent, even in the heterodimeric form. Moreover, the N-terminal β chain disordered loop can partially occlude PLA2 catalytic site. This blockage and the Tyrosine31 e Tyrosine70 interaction between crotapotin and phospholipase suggests that the N-terminal sequence of phospholipase would be the major PLA_2-crotapotin interaction in crotoxin whose association (or dissociation) could be directly associated to the lithic effects of PLA2 (López-Dávila et al., 2021; P. Samy et al., 2011). On the other hand, according to the molecular modeling by (Fernandes et al., 2017), crotapotin γ chain dissociates from the PLA2 thus remaining available to interact with any molecule. Such feature may explain the biological effects attributed to this chain, in the study reported by (Teixeira et al., 2020), when studying the heterodimeric crotoxin form.

According to other researchers perspectives, crotoxin can be associated to other biological actions. (Sampaio et al., 2003) described that crotoxin is able to inhibit the macrophage function and that he lipoxygenase-derived mediators are involved in such inhibitory effect (Sampaio et al., 2006). de Araújo Pimenta et al., 2019 also related that crotoxin alters the release of mediators secreted by the macrophages. Both studies show that the macrophage inhibition mechanism is due to the release of inflammatory mediators from the macrophage, once some of these mediators are lipids, crotapotin peptides may be – at least partially - responsible for these effects. It is important to point that most of the studies where crotoxin showed some effect, the experiments were performed with high concentrations of this heterodimeric molecule, which also leads us to infer that such effects could due to the crotapotin subunits, independently or in combination.

Considering the possibility that some of the inhibitory effects observed on macrophages and attributed to crotoxin could be due to crotapotin and/or its peptides, and that several studies show the ability of crotoxin to modulate the cellular cycle (Kato and Sampaio, 2021b), we tested crotapotin peptides in HepG2 cells. Under the experimental conditions evaluated in the current study, these molecules did not interfere in the cellular viability of the studied model. Taking into account that the majority of the previously reported studied were performed under similar cellular models used higher crotoxin concentrations (de Araújo Pimenta et al., 2019), the lack of effect reported here may raise a question on whether the actual biological effects rely on the whole crotoxin or they could be separated according to the individual crotoxin molecular subunits. The main result reported here is not the lack of any cytolytic effect; rather, it is the lack of any *toxic* effect on each of the tested models (bacteria, protozoa and mammal cells).

Although complementary studies are necessary, we propose here that the non-toxic crotapotin - particularly its subunits - are, in fact, cell penetrating peptides (Sciani et al., 2017) that would passively penetrate the cells and perform their actual biological role in the intracellular medium, either by regulating metabolic processes or inducing changes in the cell cycle.

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Conclusões

A existência de venenos animais é uma demonstração de estratégia evolutiva acontecendo em um dado organismos. Toxinas são moléculas produzidas por um dado organismo e que agem sobre *outro* organismo, seja com o objtivo de predação ou para proteção do animal produtor; portanto mecanismos de regulação devem existir para evitar danos no produtor. No caso de serpentes do gênero *Bothrops* esses mecanismos já foram – pelo menos parcialmente – elucidados (Marques-Porto et al., 2008). Portanto, entender quais moléculas são responsáveis por quais mecanismos são de suma importância para, do ponto de vista de aplicações biotecnológicas, seja possível identificar bons candidados a protótipos farmacêuticos, como por exemplo, novos antimicrobianos.

Baseados nos resultados que foram obtidos no presente trabalho, podemos inferir que os afeitos antimicrobianos atribuídos a crotoxina se devem, em sua aparente maioria, a atividade fosfolipásica da crotoxina, uma vez que nem a crotapotina nem suas subunidades isoladas apresentaram efeitos mesmo em concentrações elevadas (para os parâmetros de determinação de MIC). Esta constatação não é inesperada, uma vez que fosfolipases de de venenos com atividade antimicrobiana já foram descritas por vários autores.

O resultado mais relevante até o momento, de acordo com nossa análise, foi a ausência de toxicidade da crotapotina e seus peptídeos em diferentes modelos celulares: bacteriano, protozoaário e mamífero. Como mencionado acima, os ensaios realizados foram direcionados ao monitoramento de efeitos líticos e/ou tóxicos, mas não metabólicos ou mesmo que envolvessem regulação da expressão genênica (e, portanto, alterações no perfil proteômico).

Esta ausência de toxicidade aparente abre portas para outros tipos de estudo, particularmente a avaliação da capacidade da crotapotina (e subunidades) de penetrarem as membranas celulares.

Assim, nos próximos passos deste trabalho, serão realizados ensaios que avaliarão a capacidade destes peptídeos de comportarem como Cell Penetrating Peptides. Nosso grupo já demonstrou aque algumas toxinas ‘inócuas’ possuem esse tipo de comportamento e, portanto, podem se apresentar como novas ferramentas biotecnológicas para carreadores de moléculas, em estudos de ‘drug delivery’, por exemplo. Finalmente, havendo disponibilidade e tempo, buscaremos avaliar os efeitos metabólicos da crotapotina (e subunidades) nos modelos de imunidade celular em macrófagos conduzidos pelo grupo de Sampaio e colaboradores.