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ISOLAMENTO E CARACTERIZAÇÃO BIOQUÍMICA E BIOLÓGICA DE UM PEPTÍDEO ANTIMICROBIANO EXTRAÍDO DA PEÇONHA DO ESCORPIÃO *Tityus obscurus* Gervais 1843 (SCORPIONES, BUTHIDAE)

BRENNA CELINA FERREIRA DE CARVALHO

Santarém, Pará
Abril, 2017

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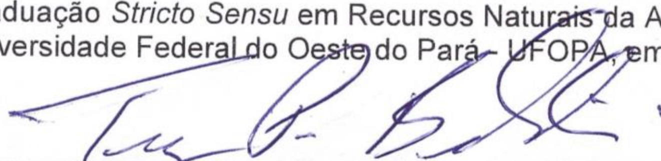

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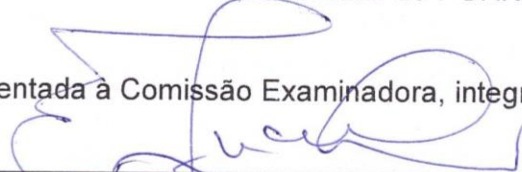

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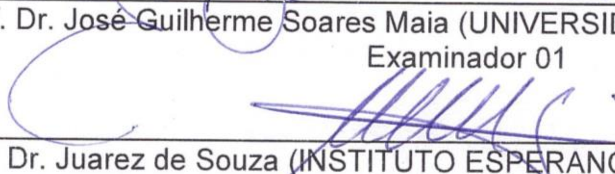

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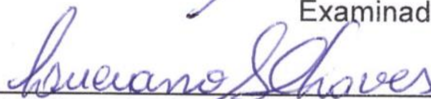

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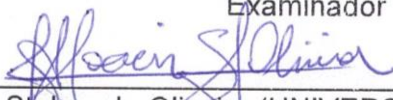

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EPÍGRAFE

“ Aprender é a única coisa de que a mente nunca se cansa, nunca tem medo e nunca se arrepende. ”

Leonardo da Vinci

CARVALHO, Brenna C. F. **Isolamento e caracterização bioquímica e biológica de um peptídeo antimicrobiano extraído da peçonha do escorpião *Tityus obscurus* Gervais 1843 (Scorpiones Buthidae)**. 2017. Nº de páginas 44. Dissertação de mestrado em Recursos Naturais da Amazônia. Área de concentração: Estudos e Manejos de Ecossistemas Amazônicos. Programa de Pós-Graduação em Recursos Naturais da Amazônia. Universidade Federal do Oeste do Pará – UFOPA, Santarém, 2017.

RESUMO

As peçonhas de escorpiões são fontes de moléculas bioativas como os Peptídeos Antimicrobianos (PAMs). Tais moléculas pertencem à imunidade inata e são ubíquas no reino animal e vegetal. Este trabalho teve o objetivo de purificar e caracterizar um peptídeo antimicrobiano extraído da peçonha do escorpião *T. obscurus* da região Oeste do Pará, Brasil. A coleta dos animais ocorreu na Floresta Nacional do Tapajós e a extração das peçonhas foi feita através da técnica de eletroestimulação usando uma fonte elétrica. O perfil eletroforético foi determinado pela eletroforese em gel de poliacrilamida 15% SDS-PAGE. O peptídeo antimicrobiano purificado (P42) teve eluição em 42 min através da Cromatografia Líquida de Alta Eficiência em fase reversa (rp-CLAE). Os ensaios antimicrobianos foram realizados com bactérias *Escherichia coli* Gram (-), *Staphylococcus aureus* Gram (+) e com fungos *Candida albicans*, *C. tropicalis* e *C. parasiplosis* através de métodos padronizados de sensibilidade por disco difusão e de determinação da Concentração Inibitória Mínima (CIM) protocolados pela Clinical and Laboratory Standards Institute (CLSI), para fungos (normas M44-A e M27-A2) e bactérias (norma M2A-9). O efeito hemolítico de P42 foi testado em eritrócitos de camundongos e para monitorar a cinética de sua citotoxicidade foram geradas imagens por microscopia confocal. A massa molecular de P42 foi obtida através da técnica de espectrometria de massas MALDI-TOF e a sua sequência de aminoácidos através da Degradação de Edman. O peptídeo (P42) foi ativo apenas contra o fungo *C. albicans* e a sua massa molecular foi de 7284,4 Da. Os valores de CIM do peptídeo contra as espécies de *Candida* foi 3,5 - 7,0 µM e para o fluconazol 6,0 - 12,0 mM. P42 não foi hemolítico em eritrócito de camundongos. A microscopia confocal detectou DNA espalhado após 3h de tratamento com o peptídeo natural. A estrutura primária desse peptídeo consistiu de 30 aminoácidos e 97% de identidade com a toxina *To4* de *T. obscurus*. Esses resultados demonstram, pela primeira vez, a existência de um PAM nativo obtido diretamente da peçonha de *T. obscurus*, expandindo as opções de possíveis novas aplicações terapêuticas.

Palavras-chave: *Candida albicans,* peptídeo antimicrobiano*, Tityus obscurus.*

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ABSTRACT

Scorpion venoms are sources of bioactive molecules such as Antimicrobial Peptides (AMPs), which are innate immunity molecules and found in the animal and plant kingdoms. In this work, it was purified and characterized a novel AMP from the venom of the Amazonian scorpion *Tityus obscurus*. Animals were collected in the Tapajós National Forest, West region of Pará state, Brazil. The venom was extracted by electrostimulation technique, using a variable power supply. Molecular mass distribution in the venom was assessed running 15% SDS-PAGE. Purification of an antimicrobial peptide (P42) migrating at 42 min was done by High-Performance Liquid Chromatography (HPLC). For biological characterization, antimicrobial activity on the Gram-negative *Escherichia coli*, Gram-positive *Staphylococcus aureus* bacteria and fungi *Candida albicans*, *C. tropicalis* and *C. parapsilosis* were performed using the standard methods of disk diffusion sensitivity and Minimum Inhibitory Concentration (MIC) of the Clinical and Laboratory Standards Institute (CLSI) for bacteria (M2A-9 standard) and fungi (M44-A and M27-A2 standards). Additionally, mouse membrane hemolytic effect of the purified P42 was determined, as well as monitoring of the kinetic cytotoxicity by confocal microscopy images. The results of mass-spectrometric analysis showed a m/z ratio of 7284.4 Da and N-terminal amino acid sequence of the first 30 residues of the peptide determined by Edman degradation, which has a 97% identity with the previously reported nucleotide sequence of To4 precursor from *T. obscurus*. P42 was only active against fungi. MIC values against *Candida* species were 3.5-7.0 µM, compared to fluconazole 6.0-12.0 mM and it did not show hemolytic effect in mouse erythrocyte. These results report, for the first time, the purification and characterization of a native AMP from the venom of the Amazonian scorpion *T. obscurus*. This kind of biological molecules constitutes a novel approach to drug development, especially against multidrug-resistant pathogens.

Key words: *Candida albicans*, antimicrobial peptide, *Tityus obscurus*.

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1. INTRODUÇÃO GERAL

Os escorpiões são animais que há muito tempo despertam muito interesse devido as suas características peculiares. Estes são animais encontrados amplamente distribuídos na Terra e que se adaptam aos mais variados ambientes. Essas vantagens de adaptação podem ser associadas a vários fatores, dentre eles à capacidade de resistir longos períodos sem alimento e com pouca umidade, à adaptação em ambientes antropizados e à autodefesa contra possíveis predadores através da inoculação de sua peçonha. As peçonhas desses animais são ricas misturas de componentes proteicos e não proteicos que estão associados aos sintomas de envenenamento. Por sua vez, o envenenamento provocado por picada de escorpião causa desde acidentes leves, moderados e até graves, podendo inclusive levar à óbito a vítima, sendo considerado um problema de saúde pública em vários países, incluindo o Brasil.

No Brasil, os escorpiões que causam acidentes graves são denominados de Escorpiões de Importância Médica e todos pertencem ao gênero *Tityus*. Na região Norte, *Tityus obscurus*, popularmente conhecido como "escorpião preto da Amazônia", é o principal responsável por vários casos de envenenamento graves. Por esse motivo, o interesse no estudo da peçonha desses artrópodes, em geral, vem crescendo gradativamente com o intuito de se aprofundar no entendimento das moléculas que compõem a peçonha. Sabe-se que as peçonhas possuem muitos componentes e entre eles destacam-se as toxinas que agem em vários tipos de canais iônicos e são os responsáveis pelos sintomas de envenenamento. Outro importante grupo de moléculas encontrado recentemente nas peçonhas escorpiônicas são os chamados Peptídeos antimicrobianos (PAMs).

Os PAMs são moléculas participantes da imunidade inata e encontradas em todos os reinos. Em escorpiões muitos desses peptídeos já foram isolados e tiveram suas atividades comprovadas contra microrganismos como fungos, bactérias, protozoários, entre outros. Portanto, os PAMs, em meio a tantos registros de resistências a antibióticos convencionais, podem ser uma alternativa de aprimoramento no estudo de novas moléculas que ajam sobre a ação de microrganismos patogênicos.

1.1 Revisão Bibliográfica

O registro fóssil mais antigo dos escorpiões os situa como os primeiros artrópodes em colonizar o ambiente terrestre há mais de 400 milhões de anos no período Siluriano (DUNLOP, 2010; LAURIE, 1898). Estes animais pertencem ao Reino Animalia, Filo Arthropoda, Subfilo Chelicerata, Classe Arachnida e Ordem Scorpiones (FET; SOLEGLAD, 2005; SOLEGLAD; FET, 2003). A sua colonização se dá em todos os continentes, exceto na Antártida, sendo este sucesso adaptativo explicado, segundo alguns autores, pelas características morfológicas, metabólicas e biológicas que apresentam estes animais, as quais lhes conferem a capacidade de ocupar uma variabilidade de micro-habitats de florestas, além de colonizarem áreas de ação antrópica ou ocupadas pelo homem (LOURENÇO, 2004; MARCUSSI et al., 2011).

Os escorpiões possuem um corpo dividido em duas grandes partes: prossoma ou cefalotórax e opistossoma ou abdômen. No cefalotórax, encontram-se os quatro pares de patas, um par de quelíceras e um par de pedipalpos. O abdômen é dividido em tronco ou mesossoma e cauda ou metassoma (Figura 1). Por sua vez, o metassoma é formado por cinco segmentos possuindo, no final, o télson. Este último é composto por uma vesícula que contém um par de glândulas responsáveis pela produção e armazenamento da peçonha e na sua extremidade superior encontra-se o aguilhão, constituindo o que se conhece como aparelho peçonhento (RUPPERT; FOX; BARNES, 1996; STAHNKE, 1970). Esse aparelho inoculador de peçonha é de grande importância para a sobrevivência dos escorpiões, pois auxilia na sua alimentação por possibilitar a captura de presas e, ao mesmo tempo, na sua autodefesa contra predadores (MARCUSSI et al., 2011).

Cerca de 2.000 espécies de escorpiões já foram descritas, as quais encontram-se agrupadas em sete famílias: Scorpionidae, Diplocentridae, Chactidae, Vaejovidae, Bothriuridae, Chaerilidae e Buthidae; esta última contém aproximadamente 500 espécies, algumas delas extremamente perigosas e consideradas de importância médica (BRAZIL; PORTO, 2010; HMED; SERRIA; MOUNIR, 2013a; MARCUSSI et al., 2011). No Brasil, as espécies responsáveis pelos casos de envenenamento graves pertencem ao gênero *Tityus,* dentro da família Buthidae. Segundo o Ministério da Saúde do Brasil, as espécies reconhecidas como de importância à saúde pública são *Tityus serrulatus*, *T. stigmurus*, *T. bahiensis, T. metuendus* e *T. obscurus (T. paraensis)* (BRASIL, 2001; PARDAL et al., 2014; RECKZIEGEL; PINTO JR, 2014).

Além destas, pelo menos outras 8 espécies do gênero *Tityus* são causadoras de acidentes em humanos, porém considerados de menor relevância (BRASIL, 2009).

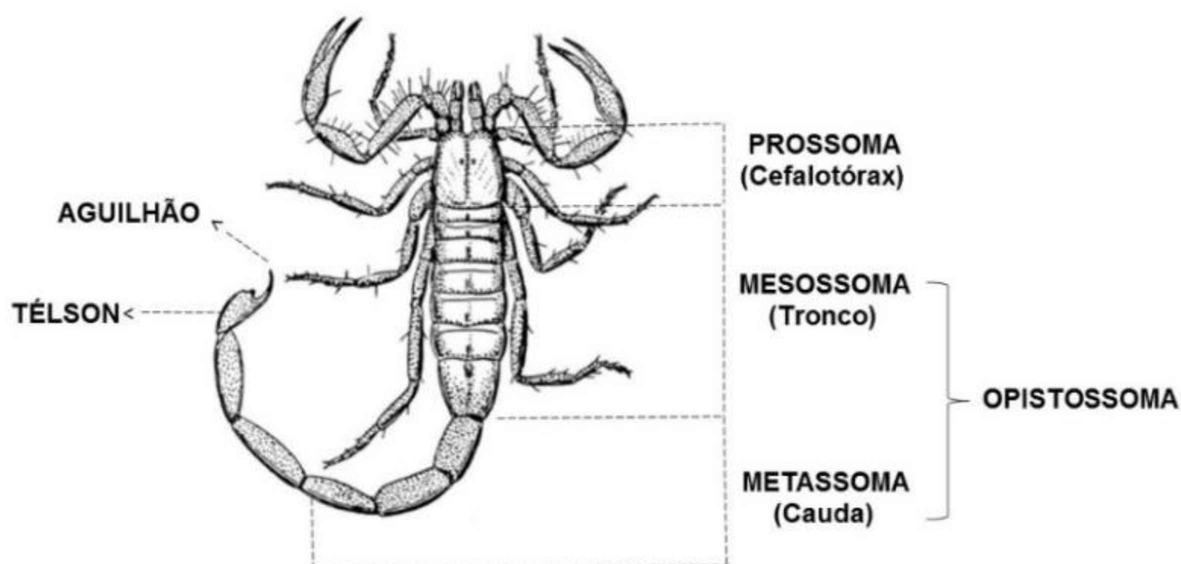

Figura 1 - Anatomia do corpo de um escorpião. Fonte: <http:// www.escorpionpedia.com/dibujos-escorpiones/>, com modificações. Acesso em 02/03/2017.

A inoculação da peçonha em humanos leva ao que se conhece como "escorpionismo", termo que designa o envenenamento causado pela picada de escorpião (CUPO et al., 1994; SANTOS et al., 2010b). Este tipo de envenenamento é considerado um problema de saúde pública e afeta países tropicais e subtropicais. Em termos gerais, a picada de escorpião provoca inicialmente uma dor intensa local, seguida de alguns sintomas moderados como sudorese, náuseas, vômitos, taquicardia, taquipneia e hipertensão leve.

Nos casos graves há uma exacerbação dos sintomas já mencionados, além de outros como sialorreia ou salivação excessiva, convulsões, insuficiência cardíaca, edema pulmonar e choque cardiogênico, podendo o óbito ser causado por complicações destes dois últimos sintomas (BAHLOUL et al., 2012). A gravidade dos envenenamentos escorpiônicos depende de alguns fatores como: a espécie e o tamanho do escorpião, a composição da peçonha inoculada, a massa corporal da vítima e a sua sensibilidade à peçonha (BAWASKAR; BAWASKAR, 2012; BRASIL, 2001; WARRELL, 2012).

Na Amazônia, principalmente na região Oeste do Pará, o escorpião *T. obscurus* (Figura 2) ou "escorpião preto da Amazônia" é reconhecido como a espécie

responsável pelos casos graves de envenenamento (PARDAL et al., 2014; PEREIRA et al., 2003). Importante destacar que apenas em 2008 a descrição desta espécie foi esclarecida como de fato sendo *Tityus obscurus* Gervais 1843, e se trata de ser o sinônimo sênior de *T. paraensis* e *T. cambridgei* (LOURENÇO; LEGUIN, 2008). De acordo com Lourenço (2011), a espécie é de grande porte, variando entre 75 e 100 mm de comprimento total e, além da sua ocorrência na Amazônia brasileira, também é encontrado no Suriname e na Guiana Francesa.

Figura 2 - Espécime macho de *Tityus obscurus* Gervais 1843. Foto: Deyanira Fuentes.

Segundo dados do Sistema de Informação de Agravos de Notificação - SINAN, em 2015 o escorpionismo ultrapassou os cinquenta mil casos em todo o Brasil (BRASIL, 2017). Dentro da região Norte, o Pará é o estado com o maior número de registros de acidentes por escorpiões, com mais de 50% dos casos reportados para a região (BRASIL, 2017; FUENTES-SILVA; SANTOS-JR; OLIVEIRA, 2014). Em um levantamento de dados para a região metropolitana de Belém, realizado entre os anos de 1998 e 2005, os acidentes escorpiônicos representaram 72,9% das notificações registradas pelo centro de Informações Toxicológicas de Belém (CIT-Belém) (MAESTRI-NETO et al., 2008). No Oeste do Pará, acidentes escorpiônicos apresentam uma alta frequência, particularmente nos municípios de Itaituba (PARDAL; CARDOSO; FAN, 1999) no município de Oriximiná, entre os moradores ribeirinhos do rio Trombetas (PARDAL et al., 2001) e nos municípios de Altamira e Brasil Novo (SPEROTTO et al., 2001). Por sua vez, no município de Santarém, no período entre 2000 e 2001 foram atendidas 72 vítimas de escorpionismo no Hospital Municipal (PEREIRA et al., 2003) e dentre estas, muitas das vítimas identificaram a espécie *T. obscurus* como o agente causador do acidente. Dessa forma, tem-se

evidenciado ser de fato esta a principal espécie envolvida nos casos de envenenamentos graves por escorpião no Oeste do Pará, o que tem despertado o interesse nas pesquisas da peçonha de *T. obscurus* (TORREZ et al., 2015).

A peçonha dos escorpiões é uma mistura de substâncias complexas compostas majoritariamente por peptídeos e proteínas, além de íons inorgânicos, aminoácidos livres e componentes orgânicos heterocíclicos como as acilpoliaminas (AL-ASMARI et al., 2016; QUINTERO-HERNÁNDEZ et al., 2013a) e dentre todos esses componentes, os peptídeos são as moléculas que agem como ferramentas na autodefesa e na captura de presas. Considerado, por alguns autores, que há cerca de 1700 espécies conhecidas de escorpiões, estima-se que exista aproximadamente 100 mil peptídeos diferentes nas peçonhas destes animais, dos quais menos de 1% tem sido isolado e identificado (TAN et al., 2006; YTHIER; STOCKMANN, 2009).

A variabilidade química na composição e concentração das peçonhas dos escorpiões depende principalmente do gênero e espécie. Entretanto, alguns estudos também têm associado as variações genéticas e ambientais como responsáveis pelas diferenças na composição da peçonha, e por consequência na sua potência (CORDEIRO et al., 2015; PUCCA et al., 2014). Os principais componentes das peçonhas de escorpiões são os peptídeos neurotóxicos, conhecidos simplesmente como neurotoxinas, os quais agem nos canais iônicos de células excitáveis (TAN et al., 2006). Eles têm sido classificados, segundo o tipo de canal, em quatro famílias: os que agem em canais para Sódio, Potássio, Cálcio e Cloreto (HMED; SERRIA; MOUNIR, 2013a). As neurotoxinas de escorpião interagem com seus alvos, os canais iônicos, modulando a função destes e, consequentemente, sendo responsáveis por dar origem aos sintomas do envenenamento (QUINTERO-HERNÁNDEZ et al., 2013a). Além das neurotoxinas, nestas peçonhas também são encontrados peptídeos antimicrobianos (PAMs), que atuam contra diferentes patógenos, entre eles bactérias, fungos, vírus, protozoários e diversos parasitas (CONDE et al., 2000; CORZO et al., 2001; FAN et al., 2011; HANCOCK; SAHL, 2006).

Os PAMs são moléculas ubíquas dos reinos vegetal e animal, constituintes evolutivamente conservados da imunidade inata dos organismos multicelulares. Desde a sua descoberta em 1981, estes peptídeos têm sido isolados a partir de uma grande variedade de tecidos e tipos celulares provenientes de artrópodes, insetos, anfíbios, plantas e mamíferos, incluindo o homem (CORDEIRO et al., 2015; STEINER et al., 1981; TORRES-LARIOS et al., 2000). Do ponto de vista biológico, eles têm

despertado o interesse por apresentar amplo espectro de atividade contra patógenos como bactérias, fungos, vírus encapsulados e protozoários (SONG; ZHENG, 2015).

O mecanismo de ação dos PAMs difere completamente dos antibióticos convencionais, visto que recentes pesquisas têm demostrado que eles rompem a estrutura da membrana, inibem a síntese de DNA/RNA, de proteínas e afeta vários processos celulares dos patógenos (AUVYNET; ROSENSTEIN, 2009; GIULIANI; PIRRI; NICOLETTO, 2007; ROSCIA et al., 2013).

Os PAMs são produzidos por uma variedade de tecidos e tipos celulares e em humanos eles são classificados em quatro famílias (Figura 3) de acordo com seu tamanho, estrutura secundária e composição de aminoácidos. De modo geral, a estrutura primária dos PAMs é constituída de 12 a 50 aminoácidos e com massa molecular abaixo de 10 kDa (HEGEDÜS; MARX, 2013; MATSUZAKI, 1999; ROSCIA et al., 2013). Muitos desses peptídeos contêm resíduos de cisteínas, os quais formam pontes dissulfeto que lhes confere estabilidade, resistência a degradações decorrentes da temperatura, das alterações de pH e de ações proteolíticas (AUVYNET; ROSENSTEIN, 2009; HEGEDÜS; MARX, 2013; ROSCIA et al., 2013).

Entretanto, independente do seu tamanho e estrutura existem duas características comuns na maioria destes peptídeos antimicrobianos. Primeira, eles são moléculas catiônicas, ou seja, contém aminoácidos com carga positiva como lisina e arginina. Segunda, aproximadamente 50% dos aminoácidos que os constituem são hidrofóbicos, conferindo características químicas importantes para o mecanismo de ação microbicida (BOMAN, 1995; GANZ et al., 1985).

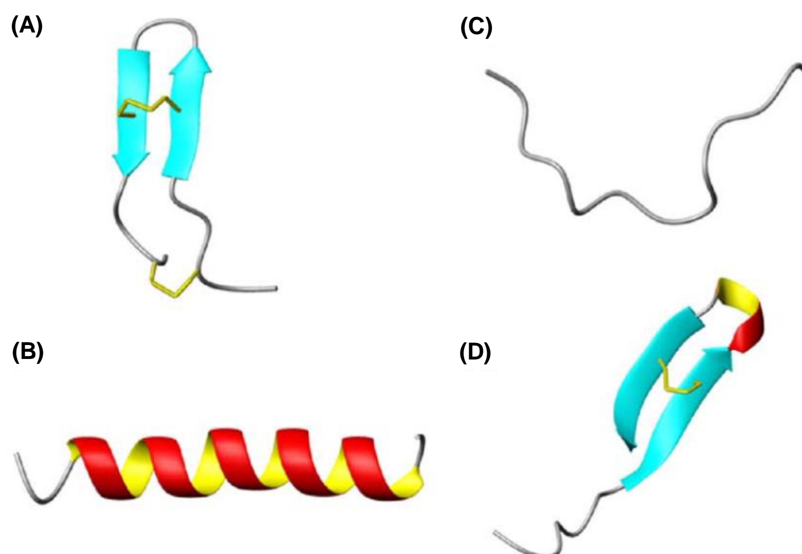

Figura 3 - Representação das estruturas das quatro famílias de Peptídeos Antimicrobianos. **(A)** Folhas-β estabilizadas por ligações dissulfeto, **(B)** estrutura em α-hélice, **(C)** estrutura estendida e **(D)** estrutura em "loop" (POWERS; HANCOCK, 2003). As estruturas **A** e **B** são as mais comumente encontradas nos PAMs (LAI; GALLO, 2009; OLIVEIRA; LACERDA, 2014; SEO et al., 2012).

Os mecanismos propostos para explicar a permeabilização da membrana por parte dos PAMs foram revistos por vários pesquisadores (LADOKHIN; WHITE, 2001; LOHNER et al., 2001; SCHREIER; MALHEIROS; DE PAULA, 2000; TEIXEIRA; FEIO; BASTOS, 2012; ZASLOFF, 2002). Tais mecanismos podem ser resumidos em três modelos, os quais são apresentados na Figura 4.

i) O modelo "barrel-stave" ou formato de barril ocorre mediante a interação eletrostática com os fosfolipídios da membrana do microrganismo. Especificamente, os peptídeos com estrutura α-hélice anfipática alinham suas regiões hidrofóbicas com a região lipídica central da membrana, enquanto que a região hidrofílica do peptídeo forma o interior do poro. Estes agregados cilíndricos, de estrutura rígida, causam a desestabilização na membrana, perda da sua seletividade e conduz, em última instância, à morte celular. Exemplo deste tipo de PAM é o alamethicin produzido pelo fungo *Trichoderma viride* (BROGDEN, 2005).

ii) No modelo do "poro toroidal", os PAMs com estrutura em α-hélice anfipática se unem à membrana formando agregados, que induzem o enovelamento da monocamada de lipídeos sobre si mesma, de forma contínua, estabilizando a formação de poros pelas interações hidrofóbicas das regiões apolares do peptídeo com as cabeças dos lipídeos da membrana e suas cadeias acílicas. Estas interações formam um poro com os grupos hidrofílicos orientados para o centro desta estrutura, capturando moléculas de água em seu interior. Este tipo de poro transmembranal é

formado por diferentes PAMs como as magaininas de anfibios e a melittina de abelhas (MATSUZAKI, 1999; RAGHURAMAN; CHATTOPADHYAY, 2007).

iii) Por outro lado, no modelo em “carpete”, as cadeias peptídicas se acumulam formando uma espécie de tapete na superfície da membrana. Primeiro, com uma orientação paralela os peptídeos são atraídos em direção às cargas eletrostáticas dos grupos fosfato em diversos sítios, cobrindo a membrana. Uma vez coberta a membrana, estes se orientam e agem como detergentes rompendo a membrana através da formação de micelas. Este tipo de mecanismo antimicrobiano é produzido pela ovispirina, peptídeo isolado de ovelha, e a cecropina 1 obtido do intestino de porco (BOMAN; AGERBERTH; BOMAN, 1993; JENSSEN; HAMILL; HANCOCK, 2006).

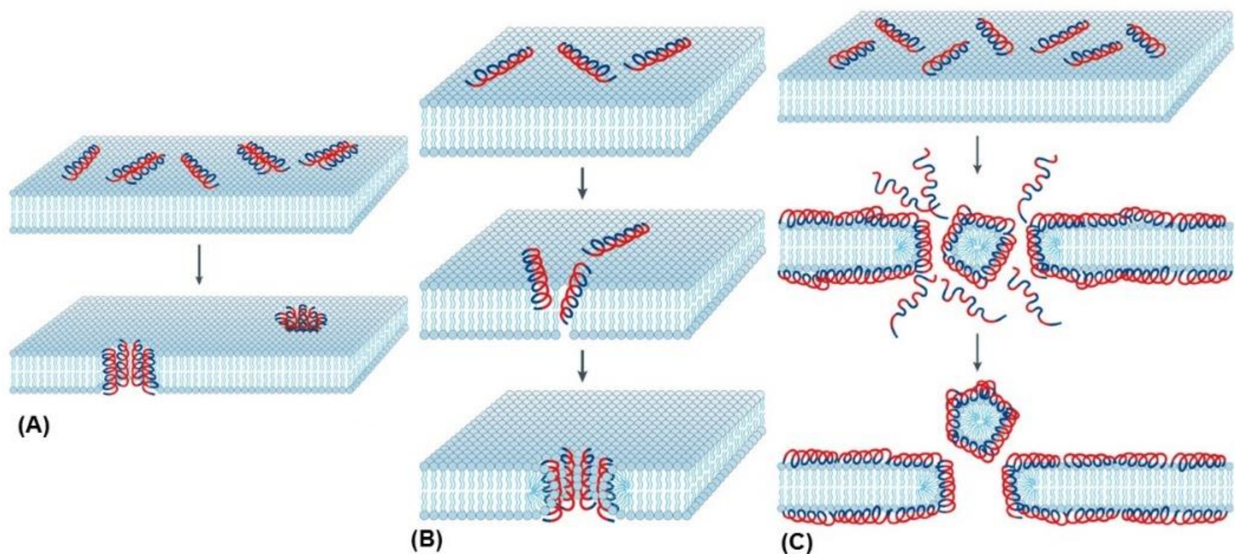

Figura 4 - Modelos dos mecanismos de ação de Peptídeos Antimicrobianos. **(A)** Modelo “Barrel-stave” ou formato de barril; **(B)** Modelo Poro Toroidal; **(C)** Modelo Carpete. Nas estruturas em hélice, o azul representa a face hidrofóbica e o vermelho a hidrofílica do peptídeo (BROGDEN, 2005).

Dentro da família Scorpionidae, o primeiro peptídeo antimicrobiano, uma defensina, foi isolado da hemolinfa da espécie *Leiurus quinquestriatus hebraeus*, (COCIANCICH et al., 1993). Posteriormente, outros PAMs de escorpião foram purificados, dentre eles a Escorpina (em inglês, *Scorpine*) com atividade antibacteriana e antiparasitária, e os Pandinin 1 e 2, extraídos da peçonha do

escorpião africano *Pandinus imperator*, ambos com atividade antibacteriana e, este último, também com ação antifúngica (Figuras 5 e 6) (CONDE et al., 2000; CORZO et al., 2001); o Hadrurin, um peptídeo com atividade antibacteriana extraído do escorpião mexicano *Handrurus aztecus* (TORRES-LARIOS et al., 2000); o IsCT extraído do escorpião de Madagascar, *Opisthacanthus madagascariensis* (DAI et al., 2002); e Imcroporin, obtido do escorpião chinês *Isometrus maculates* (ZHAO et al., 2009). A descoberta destes peptídeos nas peçonhas de escorpiões da Europa, África e América, segundo Díaz et al. (2009) confirma a sua ocorrência generalizada e a função biológica significativa como moléculas da imunidade inata.

```
Buthinin           ----------------------------------------SIVPIRCRSNRDC----------RRFCGFRGGRCTYARQCLCGY-------------
HgebetaKTx         ----------------------------KSTVGQKLKKKLNQAVDKVKEVLNKSEYMCP----VVSSFCKQHCARLGKSGQCDLLECICS--------
Vejovine           -----------------------------------------GIWSSIKNLASK----------AWNSDIGQSLRNKAAGAINKFVADKIGVTPSQAAS
Charybdotoxin      ------------------------------EFTNVSCTTSKECWSVCQRLHNT----------SRGKCMNKKCRCYS--------------------
HgeScplp1          -------------------GWMSEKKVQGILDKKLPEGIIRNAAKAIVHKMAKNQFGCFANVDVKG-DCKRHCKAEDKEGICHGTKCKCGVPISYL--
Opiscorpine1       -------------------KWFNEKSIQNKIDEKIGKNFLGGMAKAVVHKLAKNEFMCVANVDMTKSCDTHCQKASGEKGYCHGTKCKCGVPLSY---
Scorpine           -------------------GWINEEKIQKKIDERMGNTVLGGMAKAIVHKMAKNEFQCMANMDMLG-NCEKHCQTSGEKGYCHGTKCKCGTPLSY---
Heteroscorpine     -------------------GWINEEKIQKKIDEKIGNNILGGMAKAVVHKLAKGEFQCVANIDTMGNCETHCQKTSGEKGFCHGTKCKCGKPLSY---
Ctriporin          -------------------------------------FLWGLIPGAISAVTSL----------IKK-------------------------------
TsAP-2             --------------------------------------FLGMIPGLIGGLISA----------FK--------------------------------
AaeAP1             -------------------------------------FLFSLIPSVIAGLVSA----------IRN-------------------------------
AaeAP2             -------------------------------------FLFSLIPSAIAGLVSA----------IRN-------------------------------
AamAP1             -------------------------------------FLFSLIPHAIGGLISA----------FK--------------------------------
Bmkb1              -------------------------------------FLFSLIPSAISGLISA----------FK--------------------------------
AamAP2             -------------------------------------FLFSLIPSAISGLISA----------F---------------------------------
Imcroporin         --------------------------------------FFSLLPSLIGGLVSA----------IK--------------------------------
Mucroporin         --------------------------------------LFGLIPSLIGGLVSA----------FK--------------------------------
Stigmurin          --------------------------------------FFSLIPSLVGGLISA----------FK--------------------------------
HsAp1              ------------------------------SGTSEKERESGRLLGVVKRLIVC----------FRSPFP----------------------------
Heterin-1          -----------------------------------------GVWDWLKKTAKN----------VWNSDIVKQLKGKAINAAKNYVAEKIGATPS----
Pandinin1          ----------------------------------------GKVWDWIKSAAKK----------IWSSEPVSQLKGQVLNAAKNYVAEKIGATPT----
Smp43              -----------------------------------------GVWDWIKKTAGK----------IWNSEPVKALKSQALNAAKNFVAEKIGATPS----
Opistoporin1       ----------------------------------------GKVWDWIKSTAKK----------LWNSEPVKELKNTALNAAKNLVAEKIGATPS----
Parabutoporin      --------------------------------FKLGSFLKKAWKSKLAKKLR----------AKGKEMLKDYAKGLLEGGSEEVPGQ----------
Im-1               -----------------------------FSFKRLKGFAKKLWNSKLARKIR----------TKGLKYVKNFAKDMLSEGEEAPPAAEPPVEAPQ--
BmKn1              --------------------------------------FIGAVAGLLSKIF-----------------------------------------------
BmKn2              --------------------------------------FIGAIARLLSKIF-----------------------------------------------
Meucin-13          --------------------------------------IFGAIAGLLKNIF-----------------------------------------------
```

Continuação da Figura 5 - Alinhamento de sequência dos peptídeos isolados de diversos escorpiões com atividade antibacteriana. As sequencias foram obtidas da base de dados "Antimicrobial Peptide database" <http://aps.unmc.edu/AP/main.php>. Acesso em 13/03/2017 e alinhadas pela ... le/> acesso em

Figura 5 - Alinhamento de sequência dos peptídeos isolados de diversos escorpiões com atividade antibacteriana. As sequencias foram obtidas da base de dados "Antimicrobial Peptide database" <http://aps.unmc.edu/AP/main.php>. Acesso em 13/03/2017 e alinhadas pela ferramenta de bioinformática MUSCLE da base de dados European Bioinformatics Institute <http://www.ebi.ac.uk/Tools/ms/muscle/> acesso em 13/03/2017.

```
defensing      --------------------------------GFGCPLNQGACHRHCRSIRRRGG--------YCAGFFKQTCCYRN---------------------
Androctonin    --------------------------------GFGCPFNQGACHRHCRSIRRRGG--------YCAGLFKQTCTCYR---------------------
Hadrurin       -----------------------------------------GILDTIKSIASK----------VWNSKTVQDLKRKGINWVANKLGVSPQAA------
VmCT1          --------------------------------------FLGALWNVAKSVF-----------------------------------------------
VmCT2          --------------------------------------FLSTLWNAAKSIF-----------------------------------------------
Spiniferin     --------------------------------------ILGEIWKGIKDIL-----------------------------------------------
StCT1          -------------------------------------GFWGSLWEGVKSVV-----------------------------------------------
StCT2          -------------------------------------GFWGKLWEGVKSAI-----------------------------------------------
UyCT1          -------------------------------------GFWGKLWEGVKNAI-----------------------------------------------
UyCT2          --------------------------------------FWGKLWEGVKNAI-----------------------------------------------
Pantinin-3     --------------------------------------FLSTIWNGIKSLL-----------------------------------------------
Pantinin-1     -------------------------------------GILGKLWEGFKSIV-----------------------------------------------
IsCT1          --------------------------------------ILGKIWEGIKSLF-----------------------------------------------
UyCT5          --------------------------------------IWSAIWSGIKGLL-----------------------------------------------
Pantinin-2     --------------------------------------IFGAIWKGISSLL-----------------------------------------------
IsCT2          --------------------------------------IFGAIWNGIKSLF-----------------------------------------------
Hp1090         --------------------------------------IFKAIWSGIKSLF-----------------------------------------------
UyCT3          --------------------------------------ILSAIWSGIKSLF-----------------------------------------------
Smp24          --------------------------------------IWSFLIKAATKLLPS----------LFGGGKKDS--------------------------
Meucin-18      --------------------------------------FFGHLFKLATKIIPS----------LFQ--------------------------------
Pandinin2      --------------------------------------FWGALAKGALKLIPS----------LFSSFSKKD--------------------------
Heterin-2      --------------------------------------FWGALAKGALKLIPS----------LVSSFTKKD--------------------------
Bactridine1    ----------------------KDGYIIEHRGCKYSCFFGTNSWCNTECTLKKGSSGYCAWPACWCYGLPDNVKIFDSNNLKC---------------
Bactridine2    KDGYLVGNDGCKYSCFTRPGTYCANECSRVKGKDGYCYAWMACYCYSMPNWVK----------TWNRATNRCGR------------------------
BmK-AS         -------------------------DNGYLLDKYTGCKVWCVINNESCNSECKIRGGYYGYCYFWKLACFCQGARKSELWNYNTNKCNGKL-------
Cm38           -------------------------------------ARDGYIVDEKGCKFAC----------FIN--------------------------------
```

```
Androctonin     .RSVCR.... ...QIKI..C RRRGGCYYKC TNRPY..... ....
Charybdotoxin   .EFTNVSCTT SKECWSV..C QRLHNTSRGK CMNKKCRCYS ....
Ctriporin       ..FLWGL.... .......... ...IPGAISA VTSLIKK... ....
TsAP2           ..FLGM.... .......... ...IPGLIGG LISAFK.... ....
ToAP3           ..FIGM.... .......... ...IPGLIGG LISAIK.... ....
AamAP1          .FLFSL.... .......... ...IPHAIGG LISAFK.... ....
AamAP2          .FLFSL.... .......... ...IPSAISG LISAF..... ....
Con10           ..FWSF.... ...LVKA..A SKILPSLIGG GDDNKSSS.. ....
Opistoporin1    GKVWDWIKST AKKLWNSEPV KELKNTALNA AKNLVAEKIG ATPS
ToAP2           ..FFGT.... ...LFKL..G SKLIPGVMKL FSKKKER... ....
Pandinin2       ..FWGA.... ...LAKG..A LKLIPSLFSS FSKKD..... ....
Pantinin1       .GILGK.... ...LWEG... ...FKSIV.. .......... ....
NDBP5.8         .GILGK.... ...IWEG... ...VKSLI.. .......... ....
Pantinin3       ..FLST.... ...IWNG... ...IKSLL.. .......... ....
UyCT3           ..ILSA.... ...IWSG... ...IKSLF.. .......... ....
Pantinin2       ..IFGA.... ...IWKG... ...ISSLL.. .......... ....
IsCT2           ..IFGA.... ...IWNG... ...IKSLF.. .......... ....
```

Figura 6 - Alinhamento de sequências dos peptídeos isolados de diversos escorpiões com atividade antifúngica. As sequencias foram obtidas da base de dados Antimicrobial Peptide Database <http://aps.unmc.edu/AP/main.php> acesso em 20/02/2017 e alinhadas pela ferramenta de bioinformática MUSCLE da base de dados European Bioinformatics Institute <http://www.ebi.ac.uk/Tools/ms/muscle/> acesso em 13/03/2017.

Nos últimos anos várias pesquisas têm se focado fortemente no isolamento dos PAMs de diversas fontes, vislumbrando-se a possibilidade de encontrar moléculas-modelo para o desenvolvimento de novos fármacos antimicrobianos (RATES et al., 2011). Como exemplo, pode ser mencionado o peptídeo Bacteriocina, isolado a partir da bactéria *Lactobacillus plantarum* (AMORTEGUI et al., 2014); Pg-AMP1, da planta *Psidium guajava* (PELEGRINI et al., 2008); e as Phylloseptinas, presentes na pele de sapos da subfamília Phyllomedusinae (CHEN et al., 2006).

Particularmente dentro do grupo dos aracnídeos, vários peptídeos antimicrobianos têm sido isolados a partir de peçonhas e/ou hemolinfas tais como o peptídeo Juruina isolado da peçonha da caranguejeira *Avicularia juruensis* (AYROZA et al., 2012); a Gomesina e a Acanthoscurrina obtidas da caranguejeira *Acanthoscurria gomesiana* (ROSSI et al., 2012); o Rondonin de *Acanthoscurria rondoniae* (RICILUCA et al., 2012); as Lycotoxina I and II de *Lycosa carolinensis* (YAN; ADAMS, 1998) e LyeTx-I de *L. erythrognatha* (SANTOS et al., 2010a), entre outros.

Por outro lado, desde o século passado foi observado um aumento na incidência de micoses sistêmicas causadas pelo surgimento de microrganismos resistentes aos medicamentos atualmente disponíveis, afetando gravemente

indivíduos imunocomprometidos, como os acometidos pela Síndrome da Imunodeficiência Adquirida (AIDS) e o cancro, ou a aqueles submetidos a transplante de órgãos (ARMSTRONG-JAMES; MEINTJES; BROWN, 2014; ROMANI, 2004).

Dentre as infecções fúngicas sistêmicas, principalmente as oportunistas invasivas, a mais comum é a candidíase que é causada por leveduras do gênero *Candida,* sendo *C. albicans* o principal agente infeccioso na maioria dos casos. A candidíase é considerada como um problema de saúde pública que leva a altas taxas de mortalidade e morbidade em pacientes hospitalizados (CHAVES; CAVALCANTI; PORTO, 2003; MENEZES et al., 2004; NUCCI et al., 2010; PFALLER; DIEKEMA, 2007).

Em geral, em pacientes seriamente imunocomprometidos *C. albicans* se espalha pela corrente sanguínea, pelo trato gastrointestinal e no trato genital feminino (ALMIRANTE et al., 2005; COLOMBO; GUIMARÃES, 2003; GUDLAUGSSON et al., 2003; SHINOBU et al., 2007). Embora algumas espécies, como *C. glabrata* e *C. krusei*, sejam relatadas como novos microrganismos responsáveis por graves infecções fúngicas, *C. albicans* ainda é a principal responsável pela maioria e pelos mais graves casos de candidemia na América Latina (DIEKEMA et al., 2012; SOBEL, 2006). No entanto, *C. tropicalis* e *C. parapsilosis*, estão cada vez mais em foco devido que, em alguns casos, chegam a superar a patogenicidade de *C. albicans* (NUCCI et al., 2010).

Atualmente, o tratamento de candidemias é feito através de medicamentos antifúngicos sistêmicos que podem ser utilizados por via oral ou endovenosa. Dentre estes medicamentos estão a anfotericina B e os azóis, como fluconazol, itraconazol e voriconazol (PFALLER, 2012). Os azóis são compostos sintéticos que inibem o crescimento de fungos interferindo com a biossíntese de ergosterol (ergosta-5,7,22-trien-3β-ol; Figura 7), componente da membrana celular dos fungos e que cumpre as mesmas funções do colesterol na membrana das células animais (KANAFANI; PERFECT, 2008; WHITE; MARR; BOWDEN, 1998).

O antifúngico mais comumente prescrito para infecções de *C. albicans* é o fluconazol. Embora estudos relatem a capacidade deste microrganismo em desenvolver resistência de alto nível a esse medicamento (LORTHOLARY et al., 2011; OXMAN et al., 2010; WHALEY et al., 2017), o fluconazol ainda é o composto triazóico de uso endovenoso e oral mais conhecido e bem tolerado na terapêutica habitual, visto que é um medicamento seguro e tem boa atividade, especialmente contra

espécies de *Candida* em geral. Além disto, possui um baixo custo no mercado farmacêutico e apresenta poucos efeitos colaterais (COLOMBO et al., 2002; FICA, 2004; LEWIS, 2011). No entanto, em indivíduos expostos prolongadamente a este medicamento tem se registrados casos de resistência adquiridas (COLOMBO; GUIMARÃES, 2003; MAGEE; HEGINBOTHOM; MASON, 2005; TIRABOSCHI et al., 2007).

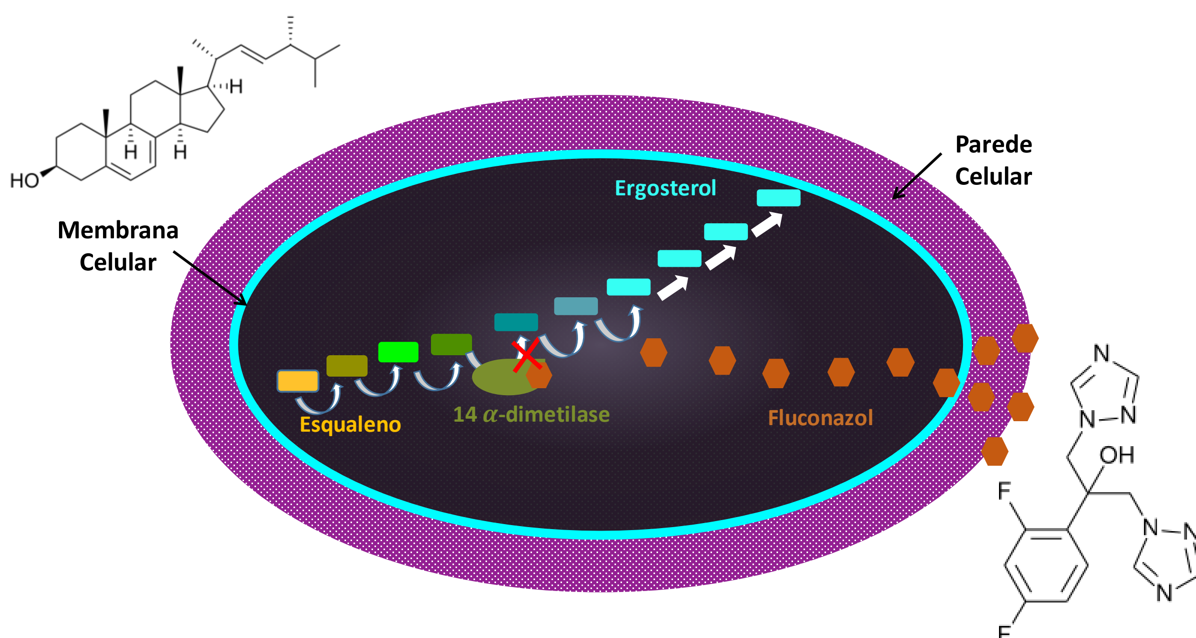

Figura 7 - Mecanismo de ação do Fluconazol na biossíntese do ergosterol. O Fluconazol age inibindo a enzima 1,4 α-dimetilase a qual catalisa a síntese do ergosterol, componente essencial da parede celular de fungos. Fonte: World-Drgus.net disponível em <http://www.world-drugs.net/generic_fluconazole.php>, com modificações. Acesso em 09/03/2017.

Outro problema de resistência aos antifúngicos está relacionado a *C. krusei*, microrganismo que apresenta uma plasticidade em desenvolver resistência a uma grande variedade de antifúngicos, principalmente ao fluconazol, além da baixa sensibilidade para anfotericina B e à 5-fluorocitosina (BARBEDO; SGARBI, 2010).

Como consequência da aparição de cepas patogênicas resistentes à ação dos medicamentos convencionais, nos últimos anos tem aumentado interesse pelos PAMs como perspectiva para o desenvolvimento de novas opções farmacoterapêuticas para o tratamento de doenças causadas por patógenos (OLIVEIRA; LACERDA, 2014; TAVARES et al., 2013). Estes peptídeos possuem um amplo espectro de atividades, apresentam baixo potencial de resistência, quando comparados com os medicamentos convencionais. Seus mecanismos de ação não se restringem à

dissociação e inibição dos componentes das membranas dos microrganismos, causando a lise celular, mas também inibem as funções do DNA e RNA, e bloqueiam a resposta celular associada ao stress, pela ação direta sobre as proteínas de "heat shock" GroEL e DnaK (BROGDEN, 2005; NGUYEN; HANEY; VOGEL, 2011). Portanto, um mecanismo de resistência contra PAMs requer mutações de um ou vários genes constitutivos, o que compromete a própria sobrevivência do microrganismo. Estudos com microrganismos isolados clinicamente têm demonstrado que a resistência a fármacos é um processo lento e que surge após longos períodos de exposição à droga, sendo improvável que uma única mutação transforme uma cepa suscetível em resistente (WHITE; MARR; BOWDEN, 1998).

Do ponto de vista da sua aplicação clínica, atualmente alguns PAMs já são reconhecidos como fármacos eficazes e, portanto, empregados rotineiramente na clínica médica. Dentre estes, pode-se mencionar a bacitracina, colistina, polimixina B, daptomicina, vancomicina e gramicidina, oriundos de diferentes organismos e utilizados no tratamento contra agentes infecciosos (ROSCIA et al., 2013). Outros PAMs mais ainda estão em fase de testes e futuramente poderão ser disponibilizados para o uso clínico (HARRISON et al., 2014; HMED; SERRIA; MOUNIR, 2013b).

Por fim, se por um lado centenas de neurotoxinas escorpiônicas já foram isoladas e caracterizadas biologicamente (KUZMENKOV; GRISHIN; VASSILEVSKI, 2015; QUINTERO-HERNÁNDEZ et al., 2013b), por outro os PAMs representam uma grande maioria de compostos desconhecidos pela ciência, pendentes de serem isolados e seus mecanismos de ação investigados. Desta forma, visto que as peçonhas são verdadeiras bibliotecas de moléculas naturais, em sua maioria ainda inexploradas, as espécies de escorpiões brasileiros, entre elas *T. obscurus* encontrada na região Amazônica, representam uma rica fonte de compostos peptídicos potencialmente úteis para o desenvolvimento de novos fármacos antimicrobianos.

1.2 Objetivos

1.2.1. Objetivo geral

Purificar e caracterizar um peptídeo antimicrobiano extraído da peçonha do escorpião *Tityus obscurus* da região Oeste do Pará.

1.2.2. Objetivos específicos

- Caracterizar por eletroforese os componentes da peçonha de *Tityus obscurus* e determinar sua concentração de proteína total.
- Fracionar a peçonha de *T. obscurus* visando obter peptídeos puros.
- Avaliar a atividade antifúngica e antibacteriana da peçonha, frações e peptídeos puros.
- Caracterizar em nível de estrutura primária, massa molecular e padrão de pontes dissulfeto o(s) peptídeo(s) antimicrobianos(s) purificados(s).
- Determinar a Mínima Concentração Inibitória (MIC) do(s) peptídeo(s) antimicrobiano(s) isolados(s).
- Determinar a ação antimicrobiana, fungicida ou fungiostática, do peptídeo de interesse.

CAPÍTULO I

FUNGICIDAL ACTIVITY AGAINST *Candida* STRAINS OF A PEPTIDE ISOLATED FROM THE AMAZONIAN SCORPION *Tityus obscurus*

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Fungicidal activity against *Candida* strains of a peptide isolated from the Amazonian scorpion *Tityus obscurus*

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Abstract
Scorpion venoms are sources of bioactive molecules such as Antimicrobial Peptides (AMPs), which are innate immunity molecules and found in the animal and plant kingdoms. In this work, it was purified and characterized a novel AMP from the venom of the Amazonian scorpion *Tityus obscurus*. Animals were collected in the Tapajós National Forest, Pará state, Brazil. The venom was extracted by electrostimulation technique, using a variable power supply. Molecular mass distribution in the venom was assessed running 15% SDS-PAGE. Purification of an antimicrobial peptide (P42) migrating at 42 min was done by High-Performance Liquid Chromatography (HPLC). For biological characterization, antimicrobial activity on the Gram-negative *Escherichia coli*, Gram-positive *Staphylococcus aureus* bacteria and fungi *Candida albicans*, *C. tropicalis* and *C. parapsilosis* were performed using the standard methods of disk diffusion sensitivity and Minimum Inhibitory Concentration (MIC) of the Clinical and Laboratory Standards Institute (CLSI) for bacteria (M2A-9 standard) and fungi (M44-A and M27-A3 standards). Additionally, mouse membrane hemolytic effect of the purified P42 was determined, as well as monitoring of the kinetic cytotoxicity by confocal microscopy images. The results of mass-spectrometric analysis showed a m/z ratio of 7284.4 Da and N-terminal amino acid sequence of the first 30 residues of the peptide determined by Edman degradation, which has a 97% identity with the previously reported nucleotide sequence of To4 precursor from *T. obscurus*. P42 was only active against fungi. MIC values against *Candida* species were 3.5-7.0 µM, compared to fluconazole 6.0-12.0 mM and it did not show hemolytic effect in mouse erythrocyte. These results report, for the first time, the purification and characterization of a native AMP from the venom of the

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48 Amazonian scorpion *T. obscurus*. This kind of biological molecules constitutes a novel approach to
49 drug development, especially against multidrug-resistant pathogens.
50
51 **Introduction**
52 Scorpion venoms are source of three major components, the first of them are constituted by large
53 proteins as enzymes, which include proteases, PLA2, hyaluronidases, phosphatases and
54 acetylcholinesterases (Nabi et al., 2015). Following appear the neurotoxins, that are comprised by
55 peptides between 4 - 8 kDa and that are classified, according to their target ion channels, in peptides
56 modulating/blocking sodium, potassium, chloride, or calcium-gated channels (Kuzmenkov et al., 2015;
57 Quintero-Hernández et al., 2013; Sunagar et al., 2013). These peptides represent the bulk of the venom
58 and they have been for a long time ago foremost spotlight of toxinology studies (Possani et al., 2000).
59 Later is found the small molecules represented by free amino acids, neurotransmitters, nucleotides,
60 lipids and several ions (Al-Asmari et al., 2016; Díaz-García et al., 2015; Possani et al., 2000).
61 Among scorpion peptides, several antimicrobial peptides (AMPs) have been identified over the past
62 two decades in both hemolymph and venom fluids. The first of them was a hemolymph defensin of the
63 scorpion *Leiurus quinquestriatus* (Cociancich et al., 1993) and since their discovery tens of AMPs in
64 venoms from Eurasian, African and the American scorpions have been purified. To date, the number
65 of AMPs isolated from six kingdoms overcome the 2800 (Wang et al., 2016), being only 67 scorpion
66 peptides "http://aps.unmc.edu/AP/main.php". Particularly, within South American species belonging
67 to the *Tityus* genus were isolated from venoms four AMPs; from *Tityus serrulatus* venom, TSp1 and
68 TSp2 exhibiting antibacterial and antifungal activity (Guo et al., 2013) and from Venezuelan scorpion
69 *Tityus discrepans,* Bactridine 1 and 2 with only antibacterial activity (Diaz et al., 2009). More recently
70 five synthetic putative AMPs obtained from *T. obscurus* venom gland transcripts were demonstrated
71 to possess activity against *Candida* spp. and *Cryptococcus neoformans* strains (Guilhelmelli et al.,
72 2016). Antimicrobial peptides are ubiquitous molecules that belong to the innate immune system. They
73 are germline-encoded recognition components that belong to the innate immunity system and
74 constitute the first line of defense against microbes (Hancock and Sahl, 2006; Ortiz et al., 2015). In
75 scorpions, AMPs are positively charged amphipathic peptides that can be conveniently divided in
76 cysteine-containing antimicrobial peptides with disulfide bridges and antimicrobial peptides without
77 cysteine residues (Harrison et al., 2014; Zeng et al., 2005). The probable mechanisms of these peptides,
78 which cause membrane disruption and consequently the antimicrobial activity, were proposed like a
79 hybrid between the classical carpet model and toroidal model (Bobone et al., 2012; Marks et al., 2011).
80 On the other side, over the last decades have been observed a significant increase in the emergence of
81 several opportunistic infections associated with AIDS and others immunosuppressive conditions,
82 which has caused rising in patient morbidity and mortality. The resistance to antimicrobial-drugs has
83 important consequences for morbidity and mortality, fact that has attracting the attention of both
84 medical and research communities. Overall, the yeast *Candida* spp. are responsible for fungemia
85 worldwide, some of them exhibiting resistance to fluconazole and variable susceptibility to other azoles
86 (Lockhart et al., 2017; Perea et al., 2001; Strollo et al., 2017). *Candida albicans* is the major
87 opportunistic yeast associate to fungal infections in HIV-infected patients, in urinary infections and
88 peritonitis, by development molecular strategies to antifungal-drug resistance (Perea et al., 2001).
89 Other identified multidrug-resistant yeasts that causes invasive infections were *C. auris*, *C. glabrata*
90 and *C. krusei* (Lortholary et al., 2011; Pappas et al., 2010). Therefore, currently the interest by AMPs
91 has growing as a perspective for development more efficacious agents to combat bacterial and fungal
92 pathogens, mainly due to their broad spectrums of activity linked to the diverse mechanisms of action
93 and the uncommon acquisition of resistance to them (Andersson et al., 2016; Perron et al., 2015;
94 Samuelsen et al., 2005). In this concern, scorpion venoms are considered a rich source of new
95 molecules to be employed as template for the development of new drugs (Rates et al., 2011).

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96 The aim of this study was purifying a fungicidal peptide from *T. obscurus* venom and determine the
97 antimicrobial activity against *Candida* fungi and both gram-positive and gram-negative bacteria. In
98 addition, the anti-candida activity, killing kinetics and cytotoxicity was investigated by fluorescence
99 employing the pure peptide labeled by Rhodamin-123. To the best of our knowledge this is the first
100 report characterizing a native AMP obtained directly from the venom of *T. obscurus*.
101
102 **Materials and methods**
103 **Chemicals**
104 Except otherwise stated all chemicals were purchased from Sigma-Aldrich (Brazil).
105
106 **Animals and Venom collection**
107 Animals were collected at the Federal Reserve Floresta Nacional do Tapajós, under license 14.018-9
108 for capture and transport from the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais
109 Renováveis and the Instituto Chico Mendes de Conservação da Biodiversidade IBAMA/ICMBio. The
110 species were maintained in individual plastic cage and they were milked each month by electrical
111 stimulation. Venom was collected and stored at -20°C until used.
112
113 **SDS-PAGE**
114 SDS–PAGE was performed according to the method of (Laemmli, 1970) under reducing conditions
115 and a separating gel contained 15% acrylamide. Following electrophoresis, scorpion proteins were
116 stained with Coomassie R-250 Brilliant Blue and their relative molecular weight were determinate by
117 comparing with the protein migration of a low molecular-weight standards (Bio-Rad, Hercules, USA).
118
119 **Peptide Purification**
120 Crude venom was solubilized in ultrapure Milli-Q water, centrifuged at 15142 x g, 8 ºC during 5 min.
121 The supernatant was separated and its protein concentration was determined using both the Micro BCA
122 protein Assay Kit from the Thermo Scientific Pierce (Rockford, USA) and the UV spectrophotometric
123 method by reading at 280 nm. Aliquots containing approximately 200 micrograms of proteins were
124 loaded onto a Thermo Scientific C-18 reverse phase column previously equilibrated with 0.1% aqueous
125 trifluoroacetic acid (TFA). Peptides were eluted using a linear gradient from 0% to 60% of acetonitrile
126 containing 0.12% TFA, at a flow rate of 1 ml/min. Peptide with antimicrobial activity was further
127 purified using the same gradient conditions.
128
129 **Hemolysis Assay**
130 Mouse fresh collected blood was rinsed three times with isotonic saline solution (0.85% NaCl) and
131 centrifuged at 806 x g for 5 min each time. Purified peptide was measured by incubating in two
132 concentrations with a 0,5% (v/v) mouse erythrocytes suspension. Solution at 1% (v/v) Triton-X 100
133 was used as the positive control and it was considering 100% hemolysis. Suspension of erythrocytes
134 in saline solution was used as negative control. The mixture was incubated in microtubes for 60 min at
135 37°C under shaking. After centrifugation, the supernatant was separated and the absorbance measured
136 at 540 nm.
137
138 **Antimicrobial assays**
139 *Escherichia coli (CCCD-E005), Staphylococcus aureus (CCCD-S007), Candida parapsilosis* (CCCD-
140 CC004), *Candida tropicalis* (CCCD-CC002) and *Candida albican*s (ATCC10231) were commercially
141 acquired. Antimicrobial activity of the venom and peaks were evaluated by the agar disk diffusion
142 assay from the Clinical and Laboratory Standards Institute (CLSI) (formerly the National Committee
143 for Clinical Laboratory Standards - NCCLS) according to the M44-A and M2A-9 reference methods

144 for fungi and bacteria, respectively (NCCLS, 2004, 2011).
145 Microorganisms were activated by inoculating a loop of the strain in the nutrient broth and incubated
146 on rotary shaker overnight. Then, 0.1 mL of inoculum (10^7 - 10^8 mL as per McFarland standard) was
147 added to the Mueller Hinton agar media. Subsequently, 100 µg of fractions and 40 µg purified peptide
148 was applied on the disc (d: 0.55 cm). After 24 hours of incubation at 37 ± 0.1°C, microbial growth was
149 determined by measuring the diameter of the inhibition zone. For antifungal activity investigation,
150 yeasts (0.5-2.5 × 10^6 /mL) were cultivated on Sabouraud 2%-dextrose agar. Peptide solution was
151 applied as mentioned above. After cultivation for 24 hours at 35 ± 0,1°C, the growth was determined
152 by measuring the diameter of the inhibition zone. Penicillin G and fluconazole were used as positive
153 controls. Saline solutions soaked disks were used as negative control.
154
155 **MIC assay**
156 Minimal Inhibitory Concentration (MIC), defined as the lowest concentration of a drug that completely
157 inhibits the growth, was determined for *Candida* strains according to the document M27-A3 (NCCLS,
158 2002). Briefly, sterile multiwell plates (96 wells) containing 0,05 mL of inoculum containing between
159 1 x 10^3 and 5 x 10^3 CFU/mL were mixtured with 0,05 mL of the 2x peptide concentrations or
160 Fluconazole into the wells of Rows 1 to 10 with a multichannel pipette. Row 1 contained the highest
161 drug concentration (either 14, 3 µM peptide or 7,5 mM fluconazole) and row 10 contained the lowest
162 drug concentration (either 0,056 µM peptide or 0,014 mM fluconazole). Row 11 with the growth
163 control wells contained 50 µL of sterile drug-free medium and 50 µL of the corresponding inoculum
164 suspension. Row 12 of the microdilution plate with drug-free medium only was used to perform the
165 sterility control. The microdilution plates were incubated at 35 °C during 48h and read the OD at 530
166 nm.
167
168 **Rhodamine Labeling peptide**
169 Peptide was labeled as described in Bark and Hahn (2000), with the following modifications. Peptide
170 (0.53 mmol) was dissolved in a clean Eppendorf at 2.1 mM concentration of 100 mM sodium
171 carbonate/bicarbonate buffer at pH 9.5 (250 µL volume). A 10-fold excess of Rhodamine-123 dye (5.3
172 mmol) was dissolved in 0.05 M sodium borate. The solution of activated dye was added in two aliquots
173 of 125 mL each, over 2 min. After the addition was completed, the reaction was left in the dark at room
174 temperature, with either gentle mixing or inversion by hand every 15 min. After 4 h, the peptide was
175 purified by same HPLC purification described for the native peptide.
176
177 ***In vitro* confocal laser-scanning microscopy**
178 Fungi were seeded on glass microscope slides and subsequently incubated at different times, in
179 triplicate for each time (0, 15, 30, 60, 90 min, 3, 6, 12 and 24 h), with 7,5 µM of purified peptide,
180 fluconazole and Fluorescent Rhodamine123 peptide. Then they were fixed with 4% paraformaldehyde
181 and washed with PBS. Subsequently, fungi were stained with 2µg/mL Hoechst 33342. Inoculum
182 suspension was considered as negative control of antifungal activity. Images were acquired using a
183 Zeiss LSM 780 – NLO microscope and LSM 5 Image examiner software.
184
185 **Amino acid sequencing and Mass-spectrometry analysis**
186 N-terminal sequencing of native antifungal peptide was performed by automatic Edman degradation
187 in a Shimadzu PPSQ-33A Protein Sequencer (Shimadzu, Tokyo, Japan). Database searching and
188 protein identification were performed using the Basic Local Alignment Search Tool - Blast (Altschul
189 et al., 1990) at the National Center for Biotechnology Information (NCBI) site
190 “https://www.ncbi.nlm.nih.gov”. Mass spectrometric measurements were performed using a matrix

191 assisted laser desorption ionization–time of flight MALDI–TOF Omniflex mass spectrometer from
192 Bruker Daltonics (Bremen, Germany) equipped with a pulsed nitrogen laser (= 337 nm, 10 ns pulse
193 width). Spectra were acquired in positive-reflection mode with a 19 kV accelerating voltage. The
194 proteins were dissolved in a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic
195 acid) in 30% acetonitrile, 0.1% trifluoroacetic acid. Carbonic anhydrase (28 kDa) and bovine serum
196 albumin (66 kDa) were used for internal calibration.
197
198 **Sequencing alignment**
199 The primary sequences of the members of *Tityus* toxins were retrieved from the Universal Protein
200 Resource Knowledgebase "https://www.uniprot.org" (The Uniprot Consortium, 2004) and NCBI
201 "https://www.ncbi.nih.nlm.gov". The multiple sequence alignment and percentage of identity of
202 purified peptide were compared using the program Muscle (Edgar, 2004) at the Expasy Bioinformatics
203 Resource Portal "http://www.expasy.org". Alignment images were built using Chimera
204 "https://www.cgl.ucsf.edu" (Pettersen et al., 2004).
205
206 **Results**
207 **Peptide purification and characterization**
208 The average protein concentration of *T. obscurus* venom determined by Micro BCA protein Assay Kit
209 assay was 113 µg/mL. The venom electrophoretic profile showed in Figure 1 is closely related with
210 other scorpion venoms of the *Tityus* species. Venom loaded and ran on 15% glycine SDS-PAGE
211 allowed to separate both high- and low- molecular-weight proteins on the same gel. Multiple proteins
212 between 31 to 100 kDa were detected with a major band at 31 kDa. The peptides migrated around 14
213 kDa as a broad diffuse band.
214
215 **Purification and peptide mass determination**
216 Soluble venom of *T. obscurus* was separated in at least 50 different components by HPLC (Figure 2A).
217 The peak eluted at 42.2 min showed antifungal activity against *Candida albicans* and was further
218 purified until homogeneity as shown in the Figure 2B, being the pure peptide designed as P42. This
219 graphic shows the elution of a major component which was the peptide under study. Additional minor
220 peaks in the chromatogram profile were discarded. The antifungal peak was analyzed by mass
221 spectrometry and it showed two signal with molecular mass of 4169 Da and 7284.4 Da (Figure 3).
222
223 **Antimicrobial Activity**
224 Antimicrobial activity of the venom and HPLC peaks on gram-negative *Escherichia coli* and gram-
225 positive *Staphylococcus aureus* bacteria as well as fungal *Candida* spp. was assessed by using agar
226 disk diffusion method. Zones of inhibition were observed after 24 hours of treatment with peptide and
227 controls. Not antibacterial or antifungal effect of the venom was detected using directly 10 µL of fresh
228 crude venom of soluble venom. However, the peak from HPLC eluted at 42.2 minutes showed
229 antifungal activity when 40 µg of peptide was assayed.
230
231 **MIC**
232 **Fungicidal activity**
233 Fungicidal activity of the P42 was assayed against three fungi, *C. albicans*, *C. tropicalis and C.*
234 *parapsilosis* and expressed as MIC. The peptide displayed identical MICs against all *Candida* spp.
235 tested (3.5 – 7.0 µM). In contrast, the MIC of fluconazole, employed as positive control, against both
236 *C. tropicalis* and *C. albicans* was 6.0 – 12.0 mM, while *C. parapsilosis* was more resistant for this
237 drug, showing higher values, 191 – 96 µM (Figures 4A,B). To investigate fungal activity against *C.*

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238 *albicans*, cells were incubated at different times with free-antifungal medium, fluconazole, natural
239 peptide and fluorescent Rhodamine-peptide. Confocal images showed the spread fluorescent-DNA
240 after 3 h of treatment with natural peptide. After 24 h of treatment with Rhodamine-peptide cells did
241 not show rhodamine fluorescence (Figure 5).
242
243 **Hemolytic activity**
244 Mouse membrane hemolytic effect of venom and purified peptide were studied. The percentage of
245 hemolysis was determined by subtracting the negative control to the ratio between hemoglobin free in
246 solution after incubation with Triton X-100 solutions and total hemoglobin released after incubation
247 of the erythrocytes with venom or peptide. Neither venom nor peptide caused mouse erythrocyte lysis
248 after 60 min incubation in the tested concentrations (Figure 4C). Protein venom concentration was
249 assayed up to 0,5 mg/mL, whereas purified peptide was analyzed at both 137 μM (1 mg/mL) and 34
250 μM (0,25 mg/mL). The percent hemolysis calculated for the negative control, when erythrocytes were
251 incubated with saline isotonic solution, was 6%.
252
253 **Structural analysis**
254 The P42 was used for primary structure determination by automatic Edman degradation and we
255 obtained by direct sequencing the first 30 amino acids at the N-terminal end of the peptide. Blastx
256 analysis of sequence showed 97% identity with previously precursor sequence To4 from the mRNA
257 gland of *T. obscurus* (Guerrero-Vargas et al., 2012). Amino acid differences in the purified peptide
258 was localized at position S28T as can be observed in the Figure 6.
259
260 **Discussion**
261 Scorpions are ancient animals that have subsisted to the present. They have adapted in almost all
262 environments, developed successful biological and chemical strategies for survive and colonize.
263 Natural toxins contributed on this survival strategies because these molecules constitute great libraries
264 of molecules, most yet unknown, present in scorpion venom glands (Al-Asmari et al., 2016; Ortiz et
265 al., 2015). The research about scorpion toxins have been important to discovery of the mechanisms of
266 several ion channels. They have been largely employed as probes for identification of distinct types of
267 ion channels, important tools for understanding their physiology (Catterall, 2012; Possani et al., 1999),
268 as well as the identification of novel molecules with pharmacological use and they also might offer a
269 promising scaffold for new drugs development. Among them, the AMPs from scorpion venoms
270 represent an effective strategy against invading pathogens, protecting the venom gland from infection
271 and facilitating the action of other neurotoxins (Harrison et al., 2014).
272 AMPs are molecules belonging to innate immune system that are presents in the animal and plant
273 kingdoms. In the last decade, many antimicrobial peptides have been isolated from plants, vertebrates
274 and invertebrates (Bulet et al., 1999; Vizioli and Salzet, 2002; Zasloff, 2002), but the need to discover
275 new antimicrobial substances is still urgent, due to the progressive development of resistance by
276 pathogenic microorganism against conventional antibiotics. In this concern, herein, we isolated and
277 characterized a native AMP from the venom of *T. obscurus*, a big black scorpion of medical importance
278 in the Brazilian Amazon region. According to the liquid chromatographic venom profile, it is composed
279 by at least 50 different protein compounds and the rp-HPLC profile of *T. obscurus* obtained in this
280 work is similar to others previously reported (Batista et al., 2000, 2002a, 2004). A peptide eluting with
281 the retention time at 42.4 min was purified, being named as P42. The sequence analysis of its first 30
282 N-terminal amino acids (KDGYLMEYGG CKMSCLMKKG TFCAEECTRM) showed 97% identity
283 of amino acid sequence with the previously reported nucleotide sequence of *To4* precursor (accession
284 code P60215.2) also from *T. obscurus* (Guerrero-Vargas et al., 2012). However, we compare the

285 molecular mass of P42, 7284.4 Da, and it was different from that obtained in previous works, where
286 masses of 7254.6 and 7259.0 Da were obtained (Batista et al., 2002b; Guerrero-Vargas et al., 2012).
287 This difference could be due to P42 be an isotoxin, as is common in other species of invertebrates
288 (Oliveira et al., 2012). However, complete sequence determination is necessary.
289 The P42 is a scorpion AMP from *T. obscurus* that did not exhibit antibacterial activity against gram-
290 negative *E. coli* or gram-positive *S. aureus*, but it was active against the three Candida species tested.
291 Interestingly, P42 is a non-hemolytic peptide and, when tested against *Candida* spp., the ratio of
292 antimicrobial activity to hemolytic activity is defined as the therapeutic index, and a high therapeutic
293 index is necessary for avoiding hemolysis of host cells (Malmsten et al., 2011). To solve the issue of
294 hemolysis, it is important to use non-hemolytic AMPs as seed compounds.
295 When compared the cytotoxicity between P42 and Fluconazole, against *Candida* spp., it was a
296 thousand-fold greater (Figure 5). It is worthy of mention that fluconazole is the most widely used azole
297 in systemic mycoses. However, in recent years there have been increasing reports of fluconazole-
298 resistant *Candida* species, many of which are cross-resistant to other antimycotics (Colombo et al.,
299 2002; Diekema et al., 2012; Oxman et al., 2010; Perea et al., 2001), causing problems in the clinical
300 management of infections, especially in immunocompromised individuals.
301 Otherwise, live-cell images of peptide-induced killing of *C. albicans* show changes in cell structure
302 (Figure 5) along with fluorescent labeled-DNA spread, in contrast with the controls, which may be the
303 result of disruption of cell membrane throughout one of the probable mechanism suggested to
304 antifungal activities (Brogden, 2005).
305 Recently, others AMPs isolated from the *Tityus* species has also displayed antimicrobial activity. And
306 more recent, Melo et al. (2015) reported the peptide Stigumurim, that was synthesized from a cDNA
307 library of the venom gland of *T. stimurus*. It was active against *C. albicans*, and *C. glabrata* with the
308 MIC between 34.8 μM and 69.4 μM. Díaz et al. (2009) isolated six AMPS from *Tityus discrepans*
309 venom and named bactridines (bactridines 1- 6). These AMPs were active against a wide range of
310 Gram positive and Gram negative bacteria at concentrations from 20 to 80 μM depending on the
311 bacteria and peptide tested.
312
313 **Conclusion**
314 A novel antimicrobial peptide was identified in the venom of the black Amazonian scorpion, *Tityus*
315 *obscurus*, and designed P42. Its first 30 amino acids sequenced were identical to the sequence of To4,
316 also from this scorpion, but the molecular mass of this peptide differs and, therefore, we assume that
317 the P42 could be To4 or an isotoxin. This native peptide exhibits antifungal activity against *C. albicans,*
318 *C. tropicalis* and *C. parapsilosis* being one thousand-fold active than fluconazole, and it was ineffective
319 against *E. coli* and *S. aureus*, Gram-negative and Gram-positive bacteria, respectively. The P42 has no
320 hemolytic effect on mouse membrane erythrocytes. Confocal images were not clear to determine the
321 activity on membrane; however, these studies are in progress. Although preliminary, the data presented
322 demonstrate the potential of *T. obscurus* antimicrobial peptides as template for the rational design of
323 pharmaceutical drug from natural molecules.
324
325 **Conflict of Interest**
326 The authors declare that the research was conducted in the absence of any commercial or financial
327 relationships that could be construed as a potential conflict of interest.
328
329 **Author Contributions**
330 DFS and JSO Conceived and designed the experiments, BCFC Performed the most of experiments.
331 HC, collected and identified scorpion species, KCFB and ECA contributed to sequence of peptide.
332 DFS and JSO wrote the paper.

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339 and the Core facility for Scientific Research of the University of São Paulo (CEFAP-USP) for the mass
340 spectrometry and confocal analysis.
341
342 **Legends**
343 **Figure 1. SDS-PAGE of the *T. obscurus* venom.** Electrophoretic profile of 113 µg of protein of the
344 soluble venom under reduced conditions on 15% glycine gel. It can be observed multiple proteins
345 between 31 to 100 kDa, and a major band at 31 kDa. Peptides compose the most abundant molecules
346 in the venom and migrated around 14 kDa as a broad diffuse band.
347
348 **Figure 2. HPLC of the *T. obscurus* venom and isolation of the antifungal peptide P42 (To4). (A)**
349 Fractionation of 230 µg soluble venom by using a Thermo Scientific C-18 reverse phase column,
350 previously equilibrated with 0.1% aqueous trifluoroacetic acid (TFA), followed by a linear gradient
351 from 0% to 60% of acetonitrile containing 0.12% TFA, flow rate of 1 ml/min and UV monitoring at
352 216 nm. **(B)** The antimicrobial peptide (asterisks) was isolated by an additional re-chromatographic
353 separation step using the same system and conditions, where small contaminants were eliminated.
354
355 **Figure 3. Mass spectrum of the native AMP isolated from *T. obscurus* venom.** The molecular ion
356 mass of 7284.4 ($M + H^+$), determined by MALDI-MS in positive reflectron mode, corresponds to the
357 pure AMP peptide eluted at 42.2 min; the mass 4169.1 ($M + H^+$) possibly corresponds to a fragment
358 of this peptide.
359
360 **Figure 4. Minimal Inhibitory Concentration** (MIC) determination of the AMP from *T. obscurus*
361 venom. **(A)** Inhibition growth curves of *C. albicans*, *C. tropicalis* and *C. parapsilosis* in presence of
362 the Fluconazole and pure *T. obscurus* AMP. The peptide displayed identical MICs against all *Candida*
363 spp. (3.5 and 7.0 µM) being a thousand-fold active when compared to Fluconazole (between 12 and 6
364 mM); *C. parapsilosis* was more resistant for this drug, showing a higher MIC value (191 – 96 µM).
365 **(B)** Plates of *Candida* spp. cultures after 48h incubation showing the MIC of the pure *T. obscurus*
366 peptide and their sterility controls. **C)** P42 had no hemolytic effect on mouse erythrocytes after 60 min
367 incubation; saline solution (0.85% NaCl) employed as negative control produced only 6% percent of
368 hemolysis and 100% of this effect was obtained by 0.5% Triton X-100.
369
370 **Figure 5. Confocal microscopy of P42 antifungal activity.** Confocal laser-scanning microscopy
371 images of *Candida albicans* without treatment (a), treated with natural peptide (b), and cell stained
372 incubated with natural peptide and staining with blue fluorescent DNA-dye Hoechst 33342 (c).
373
374 **Figure 6. N-terminal amino acid alignment of P42.** The amino acid sequence of the P42 was
375 compared pairwise with To4 toxin from *T. obscurus* (Accession code: P60215.2). Sequence logo on
376 the top shows the consensus sequence alignment including the modified amino acid at the 28th position.
377 In last line, asterisks indicate amino acids in equivalent positions. Amino acid comparison of P42 to
378 To4 from *Tityus obscurus* shares 97% sequence identity. Cysteine residues in P42 are shown in black
379 boxes.

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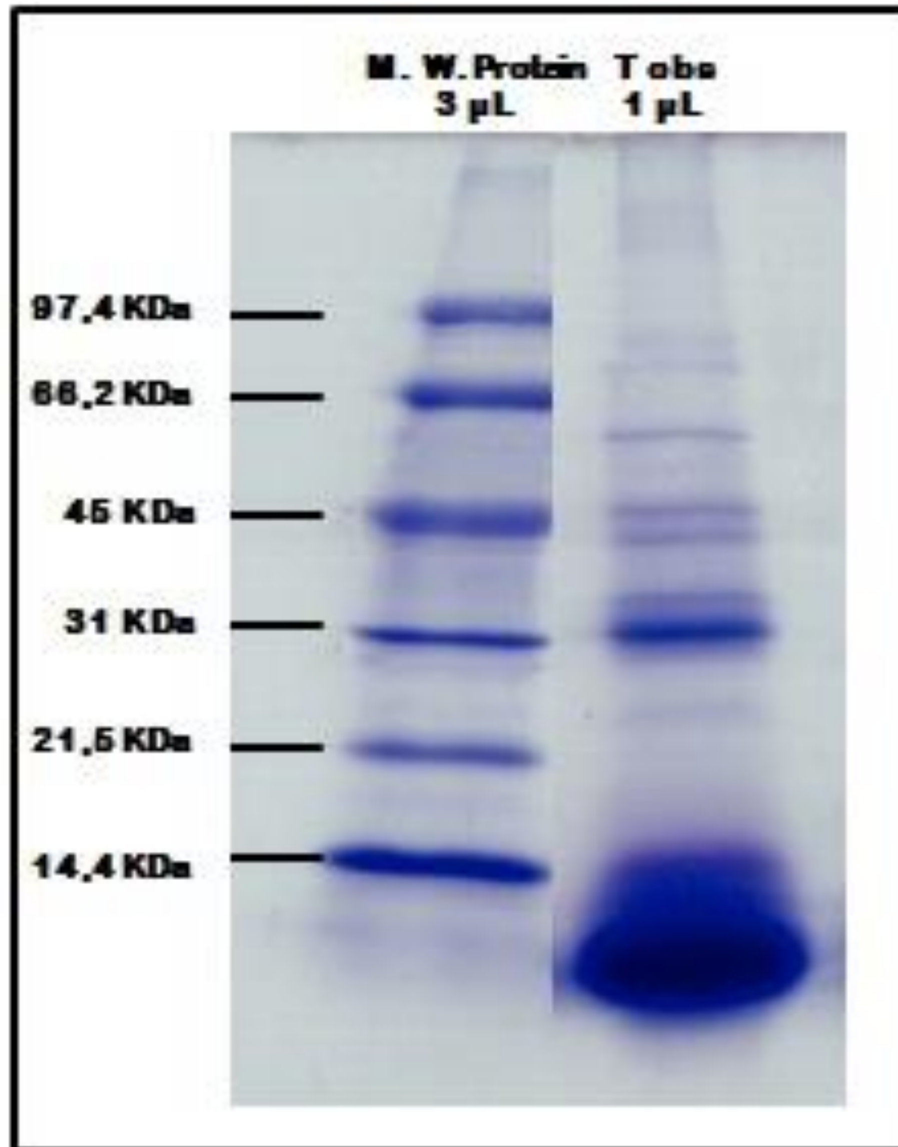

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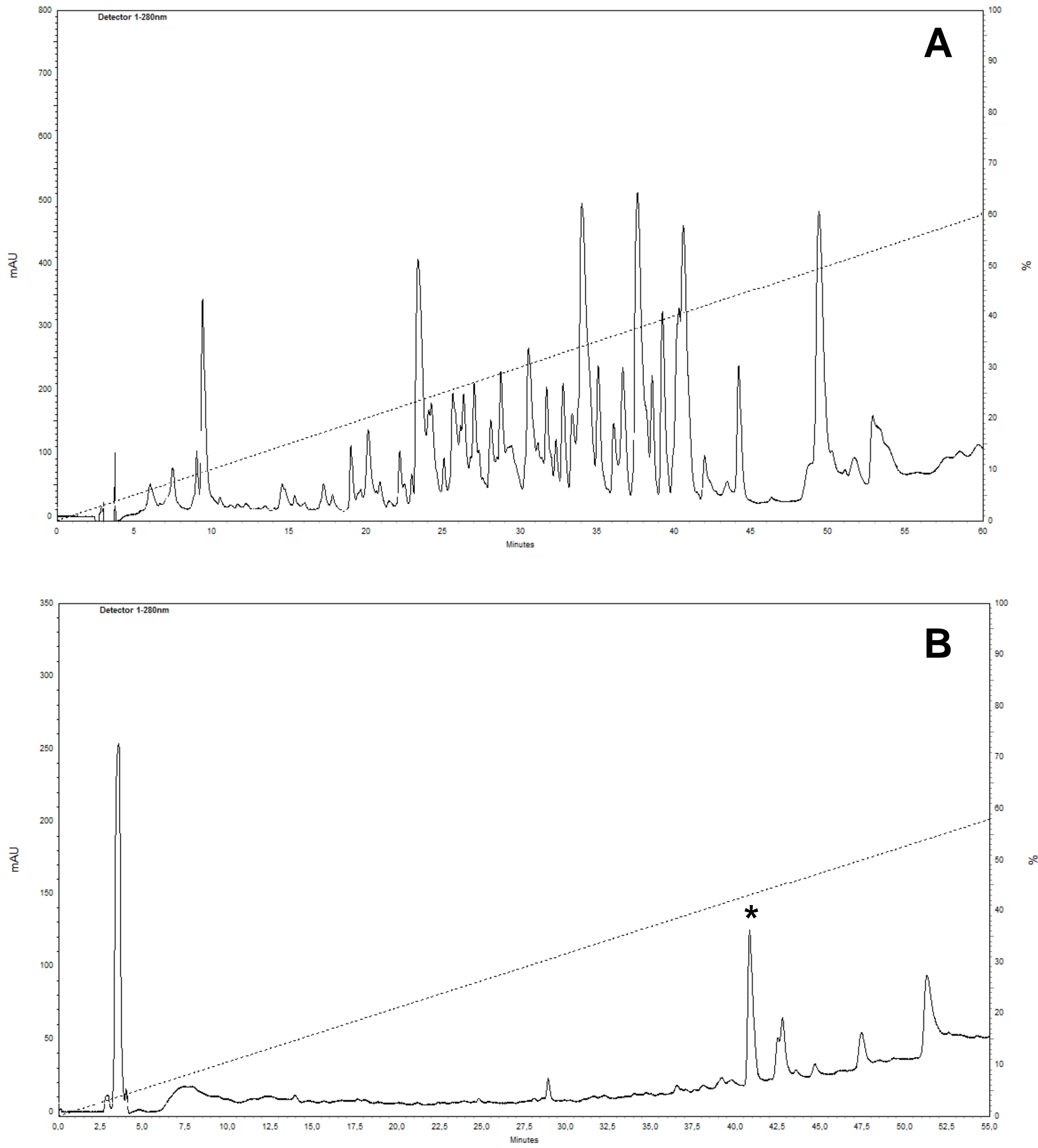

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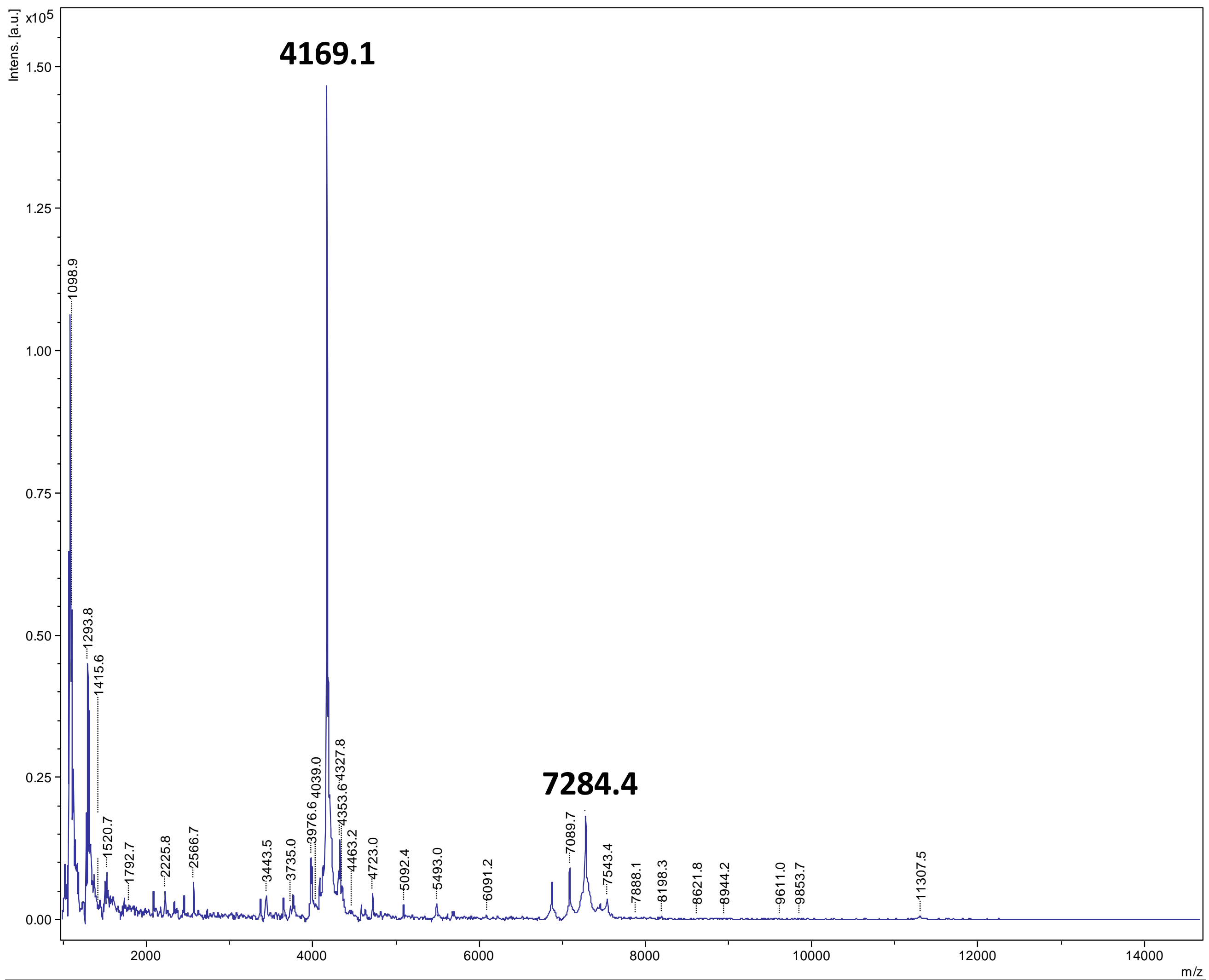

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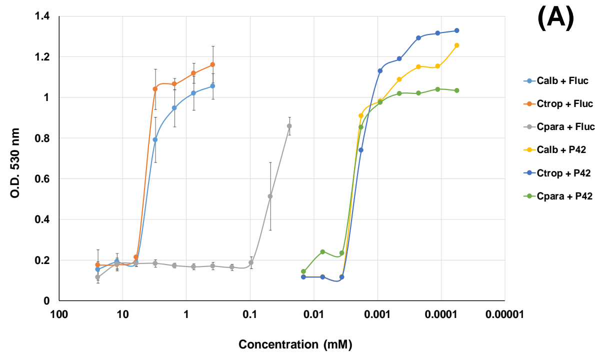

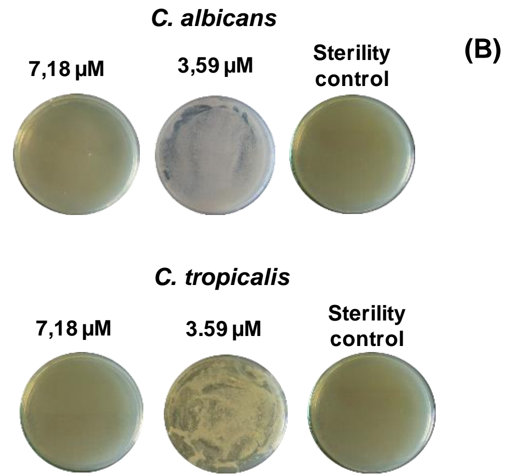

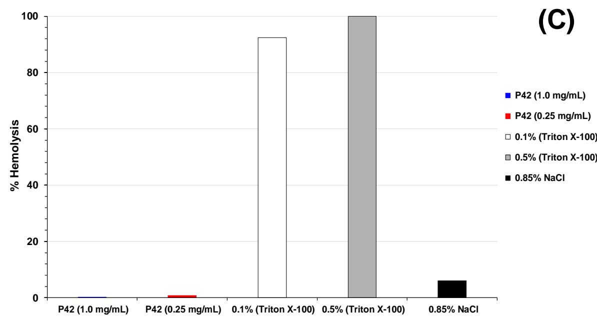

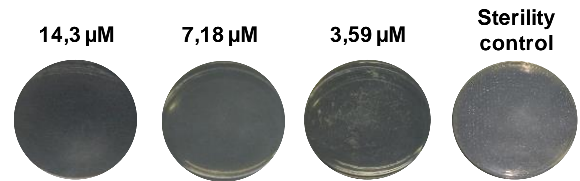

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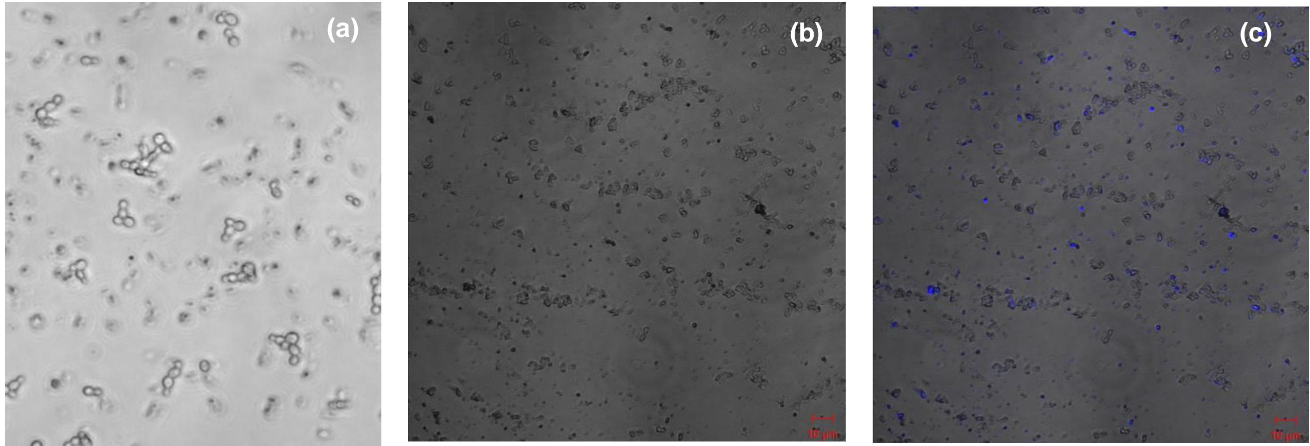

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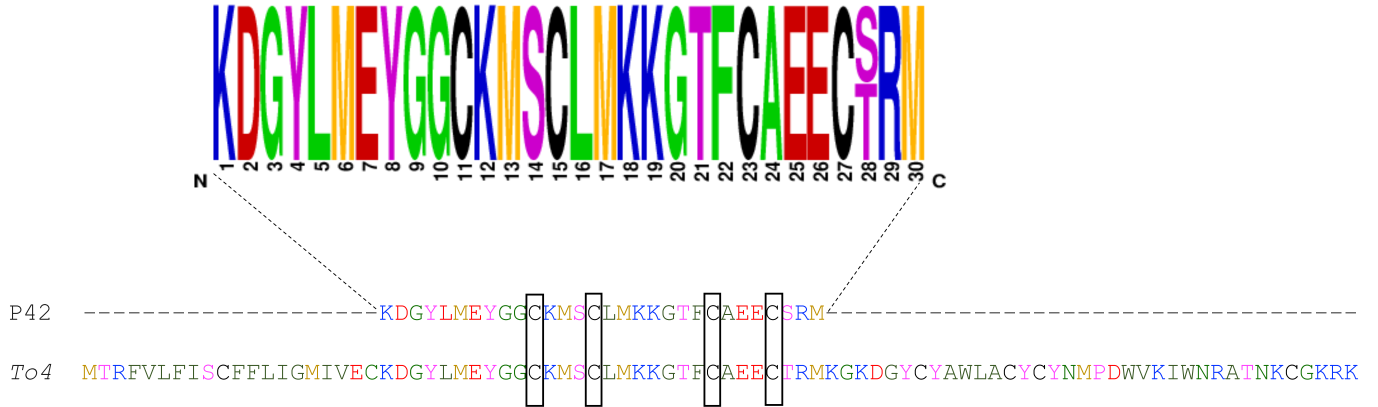

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3. CONCLUSÃO

A peçonha do escorpião *T. obscurus* é um líquido opalescente e altamente viscoso, cuja concentração média de proteína, calculada pelo método colorimétrico de BCA foi de 113 mg/mL. As proteínas desta peçonha apresentam massas moleculares distribuídas entre 31 a 100 kDa, enquanto que os peptídeos, com massas moleculares abaixo de 14 kDa, representam a maior parte dos constituintes. Da peçonha deste escorpião foi purificado um peptídeo com tempo de eluição de 42,2 min, através do fracionamento por rp-CLAE, o qual foi designado de P42. O peptídeo não apesentou atividade contra as bactérias Gram-negativa *Escherichia coli* e Gram-positiva *Staphylococcus aureus*. No entanto, houve atividade contra os fungos *Candida albicans*, *C. tropicalis* e *C. parapsilosis.* O peptídeo P42 tem uma massa molecular de 7284,4 Da e sua sequência foi determinada para os seus 30 primeiros aminoácidos. A análise da sua sequência mostra 97% de identidade com o precursor *To4*, peptídeo traduzido a partir do RNAm da glândula de peçonha de *T. obscurus*.

Os testes de Concentração Inibitória Mínima (CIM) do peptídeo puro apresentaram os valores de 3,5 - 7,0 µM para todas as espécies de *Candida* testadas. Em contraste, o medicamento fluconazol apresentou números de CIM maiores, entre 6,0 - 12,0 mM para *C. albicans* e *C. tropicalis* e teve *C. parapsilosis* como a cepa mais sensível com 96 - 191 µM. Estes dados mostram que o P42 apresenta valores de CIM 1000 vezes menores do que os valores de fluconazol. Por outro lado, tanto a peçonha quanto o peptídeo purificado não apresentaram atividade hemolítica em eritrócitos da membrana de camundongos. Na interação do peptídeo com a membrana celular do fungo, através de imagens de microscopia confocal, foi observado DNA fluorescente espalhado após 3h de tratamento com peptídeo natural. Após 24h de tratamento do peptídeo com Rodamina, não foi observada a fluorescência da mesma. Entretanto, estes resultados foram preliminares e necessitam de complementação.

Neste trabalho foi apresentado uma nova molécula com atividade antifúngica extraída da peçonha do escorpião preto *T. obscurus* comumente encontrado na região Oeste do Pará. Os resultados alcançados são de fundamental importância para o estudo de novas moléculas extraídas de animais da região amazônica. Além disto, se configura como uma pesquisa inédita, pois até o momento nenhum trabalho com intuito de se isolar moléculas com atividade antimicrobiana havia sido realizado a partir da peçonha dessa espécie. Dos peptídeos nativos, somente aqueles com ação em em canais iônicos é que tem sido isolados e caracterizados. O estudo de um novo peptídeo antifúngico expõe uma nova possibilidade de investigação para a produção de possíveis novos medicamentos, tendo em vista a resistência das drogas convencionais, e como forma de tentar ampliar as opções terapêuticas contra doenças causadas por fungos patogênicos.

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5. ANEXOS

ANEXO A – Instruções para os autores para submissão de artigos no periódico Frontiers in Microbiology.

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	Abstract (max. length)	Running title (5 words)	Figures and/or tables (combined)	Manuscript max. length	Peer review	Author fees	Submitted to PubMed Central or other indexing databases
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Case Report	350 words	✓	4	3'000 words	✓	✓	✓
Clinical Trial	350 words	✓	15	12'000 words	✓	✓	✓
Code	250 words	✓	3	3'000 words	✓	✓	✓
Community Case Study	350 words	✓	5	5'000 words	✓	✓	✓
Conceptual Analysis	350 words	✓	10	8'000 words	✓	✓	✓
CPC	250 words	✓	6	2'500 words	✓	✓	✓
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Data Report	✗	✓	2	3'000 words	✓	✓	✓
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- Studies involving animal research
- Clinical Trial Registration
- Materials and Data Policies
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				words*			
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Evaluation	350 words	✔	5	6'000 words	✔	✔	✔
Field Grand Challenge	✘	✔	1	2'000 words	✔	✘	✔
Focused Review [(1)]	350 words	✔	5	5'000 words	✔	✘	✔
Frontiers Commentary [(1)]	✘	✘	1	1'000 words	✔	✘	✔
General Commentary	✘	✘	1	1'000 words	✔	✘	✔
Hypothesis and Theory	350 words	✔	15	12'000 words	✔	✔	✔
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Acknowledgments

This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

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Data:
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Any data	FigShare Dryad Digital Repository	None

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The International Code of Zoological Nomenclature, in a recent 2012 amendment to the 1999 Zoological Code (http://iczn.org/content/electronic-publication-made-available-amendment-code), allows all electronic-only papers, such as those published by the Frontiers journals, to have valid new taxon names and nomenclatural acts. However, these new names or nomenclatural acts must be registered in ZOOBANK (http://zoobank.org/) and have associated Life Science Identifiers (LSIDs). Registration must be done by the authors before publication. Should your manuscript include any zoological new taxon names and/or nomenclatural acts, please ensure that they are registered prior to final publication.

Inclusion of Proteomics Data

Authors should provide relevant information relating to how peptide/protein matches were undertaken, including methods used to process and analyze data, false discovery rates (FDR) for large-scale studies, and threshold or cut-off rates for peptide and protein matches. Further information should include software used, mass spectrometer type, sequence database and version, number of sequences in database, processing methods, mass tolerances used for matching, variable/fixed modifications, allowable missed cleavages, etc.

Authors should provide as supplementary material information used to identify proteins and/or peptides. This should include information such as accession numbers, observed mass (m/z), charge, delta mass, matched mass, peptide/protein scores, peptide modification, miscleavages, peptide sequence, match rank, matched species (for cross-species matching), number of peptide matches, etc. Ambiguous protein/peptide matches should be indicated.

For quantitative proteomics analyses, authors should provide information to justify the statistical significance, including biological replicates, statistical methods, estimates of uncertainty, and the methods used for calculating error.

For peptide matches with biologically relevant post-translational modifications (PTMs) and for any protein match that has occurred using a single mass spectrum, authors should include this information as raw data or annotated spectra, or submit data to an online repository (recommended option; see table below).

Raw or matched data and 2-DE images should be submitted to public proteomics repositories such as those participating in ProteomeXchange. Submission codes and/or links to data should be provided within the manuscript.

4. Figure and Table Guidelines

General Style Guidelines for Figures

The maximum number of figures and tables for all article types are shown in the Summary Table. Frontiers requires figures to be submitted individually, in the same order as they are referred to in the manuscript, the figures will then be automatically embedded at the end of the submitted manuscript. Kindly ensure that each table and figure is mentioned in the text and in numerical order.

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Tables should be inserted at the end of the manuscript. If you use a word processor, build your table in word. If you use a LaTeX processor, build your table in LaTeX. An empty line should be left before and after the table.

Please note that large tables covering several pages cannot be included in the final PDF for formatting reasons. These tables will be published as supplementary material on the online article abstract page at the time of acceptance. The author will notified during the typesetting of the final article if this is the case. A link in the final PDF will direct to the online material.

Figure and Table Legends

Figure and table legends are required to have the same font as the main text (12 point normal Times New Roman, single spaced). Legends should be preceded by the appropriate label, for example "Figure 1" or "Table 4". Figure legends should be placed at the end of the manuscript (for supplementary images you must include the caption with the figure, uploaded as a separate file). Table legends must be placed immediately before the table. Please use only a single paragraph for the legend. Figure panels are referred to by bold capital letters in brackets: (A), (B), (C), (D), etc.

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Figure images should be prepared with the PDF layout in mind, individual figures should not be longer than one page and with a width that corresponds to 1 column or 2 columns.

- **All articles are prepared using the 2 column layout:** 2 column articles can contain images 85 mm or 180 mm wide.

Format

The following formats are accepted:
TIFF (.tif) TIFF files should be saved using LZW compression or any other non-lossy compression method.
JPEG (.jpg)
EPS (.eps) EPS files can be uploaded upon acceptance

Color Image Mode

Images must be submitted in the color mode RGB.

Resolution Requirements

All images must be uploaded separately in the submission procedure and have a resolution of **300 dpi at final size**. Check the resolution of your figure by enlarging it to 150%. If the resolution is too low, the image will appear blurry, jagged or have a stair-stepped effect.

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Chemical Structures

Chemical structures should be prepared using ChemDraw or a similar program according to the guidelines given below:

Drawing settings: chain angle, 120° bond spacing, 18% of width; fixed length, 14.4 pt; bold width, 2.0 pt; line width, 0.6 pt; margin width 1.6 pt; hash spacing 2.5 pt. Scale 100%Atom Label settings: font, Arial; size, 8 pt.

Assign all chemical compounds a bold, Arabic numeral in the order in which the compounds are presented in the manuscript text. Figures containing chemical structures should be submitted in a size appropriate for incorporation into the manuscript.

Legibility

Figures must be legible. Check the following:

- The smallest visible text is no less than 8 points in height, when viewed at actual size.
- Solid lines are not broken up.
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- Text is legible and of high quality.
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