UNIVERSIDADE FEDERAL DE MATO GROSSO DO SUL
FACULDADE DE CIÊNCIAS FARMACÊUTICAS, ALIMENTOS E NUTRIÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

DHÉBORA ALBUQUERQUE DIAS

Avaliação dos efeitos induzidos pelo envenenamento da peçonha de *Bothrops mattogrossensis* (jararaca-pintada) sobre as alterações do sistema purinérgico e a potencial inibitório do especiosídeo extraído da *Tabebuia aurea* (Ipê amarelo)

Campo Grande- MS
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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biotecnologia, como parte dos requisitos exigidos para a obtenção do título de Doutora em Biotecnologia.

Orientador: Prof. Dr. Edgar Julian Paredes-Gamero
Coorientador: Prof. Dr. Jeandre Augusto Otsubo Jaques

Campo Grande - MS
2024

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ATA DE DEFESA DE TESE

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

DOUTORADO

Aos três dias do mês de maio do ano de dois mil e vinte e quatro, às treze horas e trinta minutos, na UFMS, da Fundação Universidade Federal de Mato Grosso do Sul, reuniu-se a Banca Examinadora composta pelos membros: Edgar Julian Paredes Gamero (UFMS), Lucas Roberto Pessatto (UFMS), Naira Ferreira Anchieta (UCDB), Roberto Kenji Nakamura Cuman (UEM) e Sebastiao Martins de Souza Neto (UFMS), sob a presidência do primeiro, para julgar o trabalho da aluna: DHÉBORA ALBUQUERQUE DIAS, CPF 03783768101, do Programa de Pós-Graduação em Biotecnologia, Curso de Doutorado, da Fundação Universidade Federal de Mato Grosso do Sul, apresentado sob o título "Envolvimento do sistema purinérgico pelo envenenamento com a peçonha de Bothrops mattogrossensis (jararaca-pintada) e o efeito inibitório do especiosídeo extraído da Tabebuia aurea (Ipê amarelo)" e orientação de Edgar Julian Paredes Gamero. O presidente da Banca Examinadora declarou abertos os trabalhos e agradeceu a presença de todos os Membros. A seguir, concedeu a palavra à aluna que expôs sua Tese. Terminada a exposição, os senhores membros da Banca Examinadora iniciaram as arguições. Terminadas as arguições, o presidente da Banca Examinadora fez suas considerações. A seguir, a Banca Examinadora reuniu-se para avaliação, e após, emitiu parecer expresso conforme segue:

EXAMINADOR ASSINATURA

Dr. Edgar Julian Paredes Gamero (Interno)

Dr. Edson Lucas dos Santos (Externo) (Suplente)

Dra. Fabiana Fonseca Zanoelo (Interno) (Suplente)

Dr. Lucas Roberto Pessatto (Interno)

Dra. Naira Ferreira Anchieta (Externo)

Dr. Roberto Kenji Nakamura Cuman (Externo)

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RESULTADO FINAL: Aprovação

Campo Grande, 03 de maio de 2024.

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AGRADECIMENTOS

Começo por agradecer a Deus por, ao longo deste processo complicado e desgastante, me ter feito ver o caminho, nos momentos em que pensei em desistir.

Ao Professor Dr. Edgar Julian Paredes-Gamero, meu orientador por todos os anos de convivência, todos os ensinamentos, atenção e dedicação: a minha gratidão.

À minha família, principalmente meus pais Ruth Albuquerque Silva Gomes e João Batista Dias, meu irmão Iago Albuquerque Dias, pelo amor, apoio e carinho em todos os momentos. Amo vocês.

Ao meu namorado, melhor amigo e companheiro de todas as horas, Márcio Sérgio Arimura, pelo carinho, compreensão, amor e solidariedade inefável. Sendo assim, agradeço também a minha sogra Marlene Valejo Arimura. À minha cunhada Márcia Nara Arimura e os meus sobrinhos, por fazerem parte dessa história.

Aos meus amigos Bruna de Barros, Bibiana Martini, Daniel Guerra, Murilo Yonekawa, Rodrigo Mattos Silva Galeano pela amizade e companheirismo.

Aos amigos e colegas do Laboratório de Biologia Molecular e cultura celular, Kamylla Fernanda Souza de Souza e a Técnica Magali Costa pelos momentos compartilhados, pela amizade e ajuda em todos os momentos necessitados.

Aos professores Dra. Mônica Kadri, Dr. Jeandre Jaques pelos ensinamentos durante a construção do meu projeto e no decorrer do meu doutorado.

À UFMS, aos colegas, os professores e técnicos, que contribuíram de alguma forma para a realização deste trabalho.

Aos amigos e a todas as pessoas que, de uma forma ou de outra, contribuíram para a realização deste trabalho; a minha gratidão.

À CAPES, pela bolsa concedida.

À FUNDECT e CNPq pelo fomento para execução desta pesquisa.

SUMÁRIO

RESUMO

Estima-se que 5,4 milhões de pessoas em todo o mundo são picadas por serpentes. Anualmente, 100 mil pessoas morrem e 300 mil pessoas necessitam de amputações ou desenvolvem outras deficiências permanentes devido a picadas de serpentes. Os acidentes ofídicos causado por serpentes é considerado um problema grave em locais isolados no Brasil e geralmente são atribuídas as serpentes dos gêneros *Bothrops, Crotalus* e *Lachesis*. A peçonha de serpentes contém diversas toxinas proteolíticas que promovem sintomas de envenenamento locais e sistêmicos, incluindo morte celular, atividade proteolítica, neurotoxicidade, inflamação, coagulação e efeitos hemorrágicos. No local da picada, a lesão promove a lise celular e a liberação de conteúdo intracelular, com aumento dos níveis extracelulares de nucleotídeos como o ATP e seus derivados que atuam como moléculas quimiotáticas, levando à ativação do processo inflamatório e à diferenciação celular, potencializando a resposta às toxinas do veneno. A peçonha das serpentes pode liberar ectoenzimas e nucleotidases que modulam o sistema purinérgico. Além disso, a modulação de ectoenzimas do sistema purinérgico, como ectonucleosídeo trifosfato difosfohidrolase, ecto-5'-nucleotidase e adenosina desaminase, estão envolvidas no envenenamento por serpentes. A soroterapia é o tratamento de escolha para a intoxicação ofídica, mas não neutraliza as reações locais, que podem gerar sequelas graves, como perda parcial ou total da área afetada. estudo foi verificar a presença e atividade de nucleotidases no peçonha bruta de *Bothrops mattogrossensis* (BmtV) *in vitro* e caracterizar a modulação de componentes purinérgicos, diferenciação mieloide e marcadores de estresse inflamatório/oxidativo pelo BmtV *in vivo* e *in vitro*. Além disso, nosso estudo avaliou as atividades inibitórias do especiosídeo, um iridóide isolado de *Tabebuia aurea*, contra os efeitos do BmtV. A análise proteômica do conteúdo da peçonha e da atividade da nucleotidase confirma a presença de enzimas semelhantes às ectonucleotidases no BmtV. Em experimentos *in vivo*, o BmtV alterou a expressão dos componentes purinérgicos (receptor P2X7, CD39 e CD73), aumentou o número de neutrófilos no sangue periférico e elevou o estresse oxidativo/parâmetros inflamatórios, como peroxidação lipídica e atividade da mieloperoxidase. O especiosídio foi capaz de inibir a atividade da nucleotidase, restaurar o número de neutrófilos e mediar os efeitos oxidativos/inflamatórios produzidos pelo BmtV. Destacamos os efeitos produzidos pelo BmtV nos componentes do sistema purinérgico, na diferenciação mieloide e nos parâmetros de estresse inflamatório/oxidativo, enquanto o uso do especiosídeo reduziu os principais efeitos dependentes do BmtV. Esses resultados mostraram a participação do sistema purinérgico no envenenamento por BmtV e corroboraram a eficácia do especiosídio, molécula presente na casca e sementes de *Tabebuia aurea*, que é utilizada empiricamente contra inflamações causadas por envenenamento ofídico.

Palavras-chave: envenenamento ofídico; *Bothrops mattogrossensis*; nucleotidases; sistema purinérgico; especiosídio.

ABSTRACT

It is estimated that 5.4 million people worldwide are bitten by snakes. Every year, 100,000 people die and 300,000 people need amputations or develop other permanent disabilities as a result of snake bites. Snakebite accidents are considered a serious problem in isolated locations in Brazil and are generally attributed to snakes of the genera *Bothrops*, *Crotalus* and *Lachesis*. Snake venom contains various proteolytic toxins that promote local and systemic poisoning symptoms, including cell death and proteolytic activity, neurotoxicity, inflammation, coagulation and haemorrhagic effects. At the site of the bite, the injury promotes cell lysis and the release of intracellular content, with an increase in extracellular levels of nucleotides such as ATP and its derivatives that act as chemotactic molecules, leading to the activation of the inflammatory process and cell differentiation, potentiating the response to venom toxins. Snake venom can release ectoenzymes and nucleotidases that modulate the purinergic system. In addition, the modulation of ectoenzymes of the purinergic system, such as ectonucleoside triphosphate diphosphohydrolase, ecto-5'-nucleotidase and adenosine deaminase, are involved in snake envenomation. Serotherapy is the treatment of choice for snake poisoning, but it does not neutralise local reactions, which can lead to serious sequelae, such as partial or total loss of the affected area. This study aimed was of this study was to verify the presence and activity of nucleotidases in the crude venom of *Bothrops mattogrossensis* (BmtV) *in vitro* and to characterize the modulation of purinergic components, myeloid differentiation and markers of inflammatory/oxidative stress by BmtV *in vivo* and *in vitro*. In addition, our study evaluated the inhibitory activities of specioside, an iridoid isolated from *Tabebuia aurea*, against the effects of BmtV. Proteomic analysis of venom content and nucleotidase activity confirms the presence of enzymes similar to ectonucleotidase in BmtV. In *in vivo* experiments, BmtV altered the expression of purinergic components (P2X7 receptor, CD39 and CD73), increased the number of neutrophils in peripheral blood and elevated oxidative stress/inflammatory parameters such as lipid peroxidation and myeloperoxidase activity. Specioside was able to inhibit nucleotidase activity, restore neutrophil numbers and mediate the oxidative/inflammatory effects produced by BmtV. We highlight the effects produced by BmtV on the components of the purinergic system, on myeloid differentiation and on inflammatory/oxidative stress parameters, while the use of specioside reduced the main BmtV-dependent effects. These results showed the involvement of the purinergic system in BmtV poisoning and corroborated the efficacy of specioside, a molecule found in the bark and seeds of *Tabebuia aurea*, which is used empirically against inflammation caused by ophidian poisoning.

Keywords: snake envenomation; *Bothrops mattogrossensis*; nucleotidases; purinergic system; specioside.

LISTA DE ABREVIATURAS

ADO - adenosina

ADP - adenosina difosfato

AMP - adenosina monofosfato

ATP - nucleosídeo de adenosina trifosfato

BmtV - peçonha de *Bothrops mattogrossensis*

CD - cluster de diferenciação

CO_2 - dióxido de carbono

EDTA - Ácido etilenodiamino tetra-acético

FBS - Soro bovino fetal

i.p - intraperitoneal

$MgCl_2$ - cloreto de magnésio

NaCl - cloreto de sódio

NTPDase-1/CD39 - ectonucleosídeo trifosfato difosfohidrolase-1

P1/P2Y - receptores purinérgicos metabotrópicos

PBS - solução tampão fisiológica

PCA - análise de componentes principais

PE - ficoeritrina

Pi - fosfato inorgânico

RPMI - Instituto Memorial Roswell Park

MPO - mieloperoxidase

SP - especiosídeo

LISTA DE FIGURAS E TABELAS

APRESENTAÇÃO

O capítulo I é baseado em forma de artigo de revisão que contém ampla revisão de literatura atualizados, Perspectiva e conclusões e Referências.

Os resultados desta tese estão apresentados no capítulo II sob a forma de artigo.

O capítulo II foi divido nas seções: Introdução, Material e Métodos, Resultados, Discussão, Conclusão e Referências encontram-se no próprio artigo e representa a íntegra deste estudo.

CAPÍTULO I

1. OBJETIVOS – CAPÍTULO I

1.1 OBJETIVO GERAL:

Realizar uma revisão sobre o sistema purinérgico e sua possível utilização para tratamento de envenenamento ofídico.

1.2 OBJETIVOS ESPECÍFICOS

1.2.1 Apresentar uma revisão sobre os acidente ofídicos e o sistema purinégico;

1.2.2 Avaliar de que forma as toxinas, enzimas do sistema purinérgico e sua participação em casos de inoculação da peçonha;

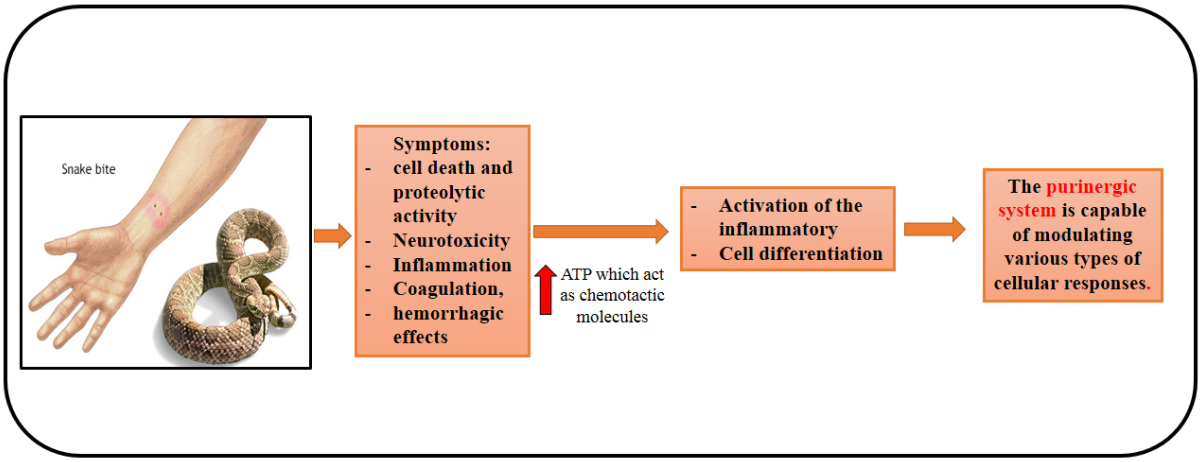
1.2.3 Analisar o envolvimento do sistema purinérgico nas respostas patogênicas à peçonha das serpentes.

CAPÍTULO I

Utilização do sistema purinérgico como estratégia terapêutica para tratamento de envenenamento ofídico

O conteúdo deste capítulo é composto pelo artigo de revisão intitulado "Use of the purinergic system as a therapeutic strategy for the treatment of snake envenomation", que será publicado no periódico Toxicon.

GRAPHICAL ABSTRACT



ABSTRACT

Snakebites are severe public health problems in tropical regions of the world. Most snake-related accidents occur because of inoculation of venom at the location of the snakebite. Snake venom contains several proteolytic toxins that promote local and systemic poisoning symptoms, including cell death and proteolytic activity, neurotoxicity, inflammation, coagulation, and hemorrhagic effects. At the bite site, injury promotes cell lysis and the release of intracellular contents, with an increase in the extracellular levels of nucleotides such as ATP. ATP and its derivatives act as chemotactic molecules, leading to the activation of the inflammatory process and cell differentiation, potentiating the response to venom toxins. Additionally, the modulation of ectoenzymes of the purinergic system, such as ectonucleoside triphosphate diphosphohydrolase, ecto-5'-nucleotidase, and adenosine deaminase, is also involved in snake poisoning. Serum therapy is the preferred treatment for snakebite poisoning, but it does not neutralize local reactions, which can generate serious sequelae such as partial or total loss of the affected area. The purinergic system may be a promising target for treating symptoms observed during snakebite envenomation as it is capable of triggering responses in most cell types. It is already known that there is a special interaction with cells of the immune system and that they can promote or inhibit inflammation. In relation to the immune system, the purinergic system acts as an inflammatory mediator, generating autocrine and paracrine purinergic signaling pathways that promote the regulation of cellular interactions, activation and leukocyte migration, mainly lymphocytes, in injured tissues. This shows that lymphocyte activity is modulated both by concentration of purines and by the expression of ectonucleotidases, which leads to responses that stimulate or inhibit inflammation.

Keywords: Purinergic Signaling, Purinergic Ectoenzymes, Venomous Snakes, Snake Toxicity, Snake Venom, P2X7 Receptor.

2. Snakebites and the purinergic system

It is estimated that 5.4 million people worldwide are bitten by snakes, approximately 100 thousand people die, and approximately 300 thousand people require amputations or develop other permanent disabilities from snakebites annually (ORGANIZATION, 2023). Thus, snakebites are a severe public health problem in tropical regions of the world and are considered neglected health problem, especially in rural and rainforest regions (FEITOSA; SAMPAIO; SACHETT; CASTRO *et al.*, 2015; GUTIÉRREZ; CALVETE; HABIB; HARRISON *et al.*, 2017; ORGANIZATION, 2023). Most of accidents in Brazil are attributed to the genus *Bothrops*, followed by *Crotalus*, *Lachesis*, and *Micrurus* (SCHNEIDER; MIN; HAMRICK; MONTEBELLO *et al.*, 2021).

Snake venoms are known to induce local and systemic toxicity, platelet disturbance, hemorrhage, and neuromuscular paralysis (SCHEZARO-RAMOS; COLLAÇO; RANDAZZO-MOURA; ROCHA *et al.*, 2017; ZHAO; ZHAO; YANG; YE, 2017). Snake venoms are composed of complex mixtures of biologically active components that vary according to the geographic location, season, and age of the snake (TASOULIS; PUKALA; ISBISTER, 2021; WILLARD; SALAZAR; OYERVIDES; WIEBE *et al.*, 2021), including mixtures of proteins with enzymatic and nonenzymatic activity, organic and inorganic molecules (WILLARD; SALAZAR; OYERVIDES; WIEBE *et al.*, 2021), and several participants in the purinergic system (KALITA; PATRA; JAHAN; MUKHERJEE, 2018; KIHELI; CHÉRIFI; AMEZIANI; SAOUD *et al.*, 2021; SAOUD; CHÉRIFI; BENHASSINE; LARABA-DJEBARI, 2017).

The release of nucleotides and nucleosides can occur via physiological mechanisms or cell membrane disruption. This release is part of a complex system, which is associated with soluble and membrane-bound enzymes, an extracellular receptor family known as the P1 and P2 or purinergic receptors which are triggered by intracellular pathways; all these components form the purinergic system (BURNSTOCK, 2015; RALEVIC; BURNSTOCK, 1998). The early concept of extracellular purinergic neurotransmission by the activation of extracellular receptors was first proposed by Geoffrey Burnstock in the 1970s (BURNSTOCK, 1972; 2015). In addition to the nervous system, the purinergic system modulates diverse physiological processes in many tissues, including pain (DI VIRGILIO; VULTAGGIO-POMA; FALZONI; GIULIANI, 2023), platelet aggregation (MACKENZIE; MAHAUT-SMITH; SAGE, 1996), endothelium-mediated vasodilation (CROSSLAND; DURGAN; LLOYD; PHILLIPS *et al.*, 2013), aging (PAREDES-GAMERO; DREYFUSS; NADER; MIYAMOTO OSHIRO *et al.*, 2007), stem cell differentiation (ADAMIAK; BUJKO; THAPA; PENSATO *et al.*, 2022; BARBOSA; LEON; NOGUEIRA-PEDRO; WASINSK *et al.*, 2011), cell proliferation

(ALVARENGA; RODRIGUES; CARICATI-NETO; SILVA-FILHO *et al.*, 2010), inflammation, and cell death (DI VIRGILIO; SARTI; COUTINHO-SILVA, 2020).

The released nucleotides, purines and pyrimidines, activate the purinergic receptor family, which is divided into three different subfamilies: adenosine (ADO) receptors, also called P1 receptors; and P2X and P2Y receptors, both of which are activated by ATP and its physiological enantiomers, such as ADP, UTP, UDP, and UDP-glucose. The P1 and P2Y receptors are G protein-coupled receptors, whereas P2X receptors are ion-gated channels (FREDHOLM; ABBRACCHIO; BURNSTOCK; DALY *et al.*, 1994; RALEVIC; BURNSTOCK, 1998). The pharmacological characteristics of sensitivity to agonists and antagonists are receptor subtype dependent and were previously reviewed (GAO; AUCHAMPACH; JACOBSON, 2022; ILLES; MÜLLER; JACOBSON; GRUTTER *et al.*, 2021; JACOBSON; DELICADO; GACHET; KENNEDY *et al.*, 2020).

Purinergic receptors activated by nucleotides are hydrolyzed by soluble, membrane-bound nucleotidases, which include the families of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), ecto-5'-nucleotidase (E-5'-NT), and alkaline phosphatases (APs) (YEGUTKIN, 2008; ZIMMERMANN; ZEBISCH; STRÄTER, 2012).

For several years, purinergic system components and their involvement in the purinergic signaling cascade activated at the bite site have been investigated. This review contributes to the understanding of snake envenomation and how the purinergic system can be used as a potential pharmacological target. Recently, the effects of *B. mattogrossensis* venom on the modulation of purinergic components, myeloid differentiation, and inflammatory/oxidative stress parameters *in vivo and in vitro* were described (DIAS; SOUZA DE SOUZA; MOSLAVES *et al.*, 2024). In this study, proteomic analysis of the venom content and nucleotidase activity confirmed the presence of ectonucleotidase-like enzymes in *B. mattogrossensis* venom. In addition, the authors also showed alterations in the protein expression of the P2X7R, CD39 and CD73 enzymes, which are related to a reduction in the neutrophil (DIAS; SOUZA DE SOUZA; MOSLAVES *et al.*, 2024).

2.1 Toxins, enzymes of the purinergic system and their participation in snakebites

The toxins present in snake venom are mainly responsible for snake envenomation. Some toxin proteins assume the fold of the phospholipase A_2 secreted by the venom (svPLA_2), which plays an important role in the pathogenesis of local tissue damage and indirectly modulates the purinergic system (CINTRA-FRANCISCHINELLI; CACCIN; CHIAVEGATO;

PIZZO *et al.*, 2010). Among them, two subgroups can be distinguished: one consists of enzymatically active svPLA$_2$ with a characteristic Asp49, a key residue for catalysis; the other subgroup includes proteins with a conserved svPLA$_2$ fold but devoid of PLA$_2$ activity because the catalytically essential Asp49 has been replaced with Lys or other amino acids (CASTRO-AMORIM; NOVO DE OLIVEIRA; DA SILVA; SOARES *et al.*, 2023). Both Asp49- and Lys49- svPLA$_2$ myotoxins induce a very rapid efflux of K^+ and ATP in murine muscle cells in culture and isolated muscles; however, quantitative and temporal evaluation of the effect of Asp49 myotoxin suggests that ATP is derived from a vesicular pool that is rapidly secreted outside of the cell, promoting nonselective large pores (CINTRA-FRANCISCHINELLI; CACCIN; CHIAVEGATO; PIZZO *et al.*, 2010). Interestingly, the use of oxidized ATP, a P2X inhibitor, reduced the cytotoxic effects of Lys-49 myotoxin, and apyrase decreased its spread signal, indicating the participation of a purinergic system that likely involves the P2X7 receptor (P2X7R) (CINTRA-FRANCISCHINELLI; CACCIN; CHIAVEGATO; PIZZO *et al.*, 2010).

Moreover, the BomoTx toxin, also known as the svPLA$_2$ protein, has been isolated from the Brazilian lancehead pit viper *B. moojeni* and is closely related to a group of Lys49 mycotoxins, which promote ATP release and indirect activation of P2X2/P2X3 purinergic receptors (ZHANG; MEDZIHRADSZKY; SÁNCHEZ; BASBAUM *et al.*, 2017). BomoTx causes nonneurogenic inflammatory pain, thermal hyperalgesia and mechanical allodynia, the last of which are completely dependent on purinergic signaling (ZHANG; MEDZIHRADSZKY; SÁNCHEZ; BASBAUM *et al.*, 2017). Furthermore, the inflammatory effect of *Bothropstoxin*-I (BthTX-I), which is isolated from *B. jararacussu* venom and is mediated by the NLRP3 inflammasome, which involves ATP and P2X7R, was also reported in macrophages (RANÉIA E SILVA; DE LIMA; LUIZ; CÂMARA *et al.*, 2021).

The activated P2X7R allows K^+ efflux, which is the key event for the recruitment and activation of NRLP3 and the subsequent activation of caspase 1 (FRANCESCHINI; CAPECE; CHIOZZI; FALZONI *et al.*, 2015). A study using intradermal treatments with the snake venom rhodocytin extracted from Malay viper (*Calloselasma rhodostoma)* reported that the induction of plasma extravasation is dependent on the lectin-type C2 receptor in mouse platelets (NAKAMURA; SASAKI; MOCHIZUKI; ISHIMARU *et al.*, 2019). *In vitro* coculture experiments revealed that rhodocytin promoted platelet activation and histamine release from mast cells via P2X7R activation (NAKAMURA; SASAKI; MOCHIZUKI; ISHIMARU *et al.*, 2019).

In addition to toxins present in the venom, nucleotides and nucleotidases are enter the extracellular space together, inducing several side and systemic effects, such as pain caused by

hyperalgesia, inhibition of platelet aggregation, potassium efflux, oxidized ATP reduced cytotoxic effects, hyperalgesia and immune response (Fig. 1).

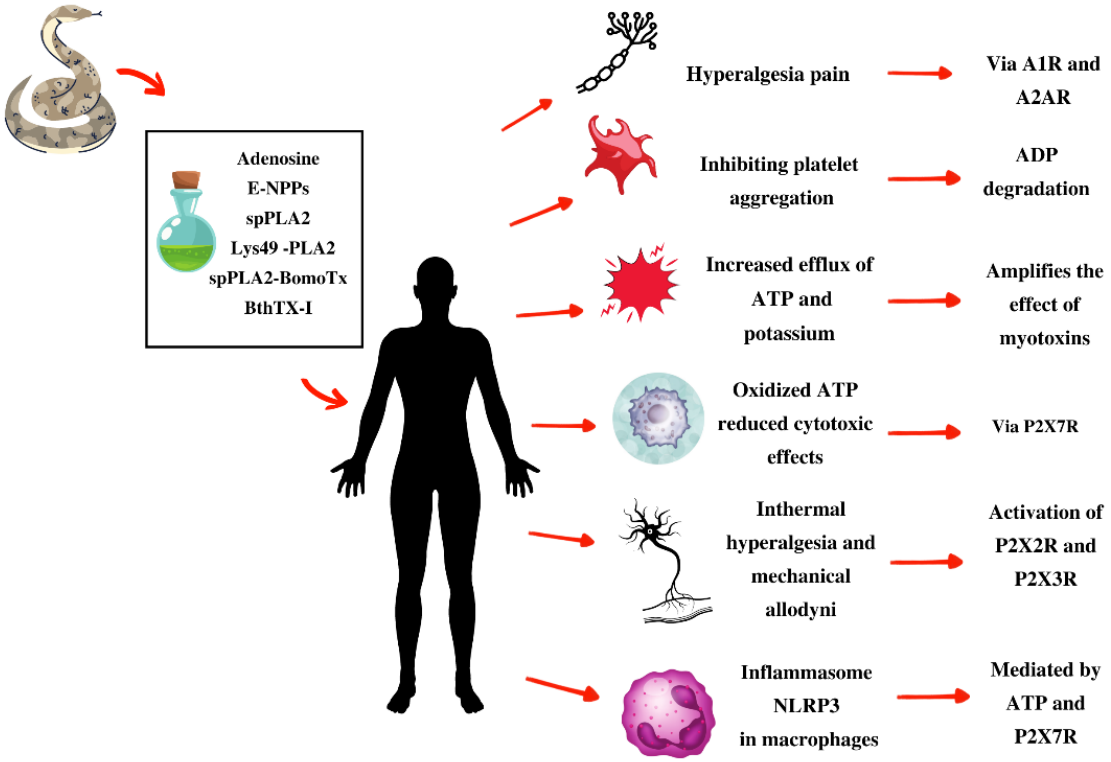


Figure 1. Prey responses after snake envenomation. The venom is composed of several molecules, such as adenosine (hyperalgesia pain), E-NPPs (Inhibiting platelet aggregation), spPLA2s (increased efflux of ATP and potassium), Lys49-PLA2 (oxidized ATP reduced cytotoxic), spPL2-BomoTx (inthernal hyperalgesia and mechanical allodyni), and BthTX-I (inflammasome NLRP3 in macrophages). Prey produces responses that involve purinergic system components, such as P2X1Rs, P2X3Rs, P2X7Rs, ATP, ADP, A1Rs and A2Rs. (The author created this figure).

Among nucleotidases, eight different genes encode members of the NTPDase family, four of which are expressed on the cell surface (NTPDase1, 2, 3 and 8), whereas the others (NTPDase4, 5, 6 and 7) are expressed as intracellular enzymes (ROBSON; SÉVIGNY; ZIMMERMANN, 2006; ZIMMERMANN; ZEBISCH; STRÄTER, 2012). Ecto-NTPDases (E-NTPDases), also known as cluster of differentiation 39 (CD39), dephosphorylate a variety of nucleoside triphosphates, such as ATP and UTP, and nucleoside diphosphates, such as ADP and UDP, to AMP in the presence of divalent cations (KUKULSKI; LEVESQUE; LAVOIE; LECKA *et al.*, 2005; ROBSON; SÉVIGNY; ZIMMERMANN, 2006).

Moreover, 5´-NT enzyme family catalyze the hydrolytic dephosphorylation of 5′-ribonucleotides and 5′-deoxyribonucleotides to their corresponding nucleosides plus phosphate; these enzymes are encoded by 7 genes and are expressed intracellularly or on the cell membrane (BIANCHI; SPYCHALA, 2003). Among these family members, extracellular 5´-NT (E-5´-NT), also known as CD73, is a glycosylated protein bound to the outer surface of the cellular membrane by a glycosylphosphatidylinositol anchor (MISUMI; OGATA; OHKUBO; HIROSE *et al.*, 1990). CD73 suppresses the activation of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome complexes to reduce the maturation of gasdermin pore-forming protein D, leading to a decrease in pyroptosis in microglia (XU; WANG; ZHONG; SHAO *et al.*, 2021). Further analyses revealed that the inhibition of the adenosine-A2B receptor decreased CD73 cell death in microglia by inhibiting gasdermin pore-forming protein D expression at the transcriptional level through the forkhead Box O1 transcription factor (XU; WANG; ZHONG; SHAO *et al.*, 2021).

Alkaline phosphatases (ALPs) are a group of phosphatidylinositol-anchored membrane proteins with wide substrate specificity and have a profound influence on organisms *in vivo*. ALP is ubiquitously expressed and catalyzes the hydrolytic removal of phosphate groups from biochemical compounds (PABIS; DUARTE; KAMERLIN, 2016; SATO; SAITOH; KIYOKAWA; IWASE *et al.*, 2021). In humans, ALP is encoded by four genes, traditionally referred to as tissue-specific genes, such as ALP, which is expressed in the intestine, placenta, placental-like germ cells, and testes, or expressed in other tissues known as tissue-nonspecific ALP (SATO; SAITOH; KIYOKAWA; IWASE *et al.*, 2021). ALP is known as a potential therapeutic target for resolving inflammation and preventing the pathology of Alzheimer's disease, as well as other inflammation-related neurodegenerative diseases, thereby decreasing the integrity and functionality of the blood–brain barrier (PIKE; KRAMER; BLAAUBOER; SEINEN *et al.*, 2015).

The nucleotide pyrophosphatase/phosphodiesterase (NPP) family includes seven isoenzymes (NPP1-7) that are widely expressed in vertebrates and dephosphorylate nucleotides such as ATP to ADP, ADP to AMP, or NAD^+ to AMP (STEFAN; JANSEN; BOLLEN, 2006). Only three members of this family (NPP1-NPP3) are capable of hydrolyzing nucleotide diphosphates, nucleotide triphosphates, nucleic acids, nucleotide sugars, choline phosphate esters and phospholipids (GODING; GROBBEN; SLEGERS, 2003). For instance, NPP1 regulation of extracellular cyclic guanosine monophosphate-adenosine (cGAMP) is a ubiquitous mechanism for attenuating signaling from the interferon gene (CAROZZA; CORDOVA; BROWN; ALSAIF *et al.*, 2022). A study revealed haploinsufficiency of

extracellular NPP1 in patients with diffuse idiopathic skeletal hyperostosis and early osteoporosis due to mutations in the ENPP1 gene (KATO; ANSH; LESTER; KINOSHITA *et al.*, 2022). The enzymes NPP2, NPP6 and NPP7 hydrolyze phospholipids and choline phosphodiesters, and NPP4 is a potent hydrolase of the substrate diadenosine triphosphate (ALBRIGHT; CHANG; ROBERT; ORNSTEIN *et al.*, 2012; GODING; GROBBEN; SLEGERS, 2003).

These findings demonstrate that the envenomation/inflammatory effects of toxin venom are direct or indirect and mediated by the activation of P2 receptors, suggesting that these toxins could be therapeutic targets for antipurinergic drugs.

2.2 Involvement of the purinergic system in snakebite pathogenic responses

As mentioned above, in injury situations such as envenomation by snake bites, the release of nucleotides and ectoenzymes of purines/pyrimidines such as E-NTPDase, E-5'-NT, and E-ADA participates in the modulation of cellular or tissue functions (CACCIN; PELLEGATTI; FERNANDEZ; VONO *et al.*, 2013; SAOUD; CHÉRIFI; BENHASSINE; LARABA-DJEBARI, 2017; TASOULIS; PUKALA; ISBISTER, 2021).

Both of these causes local damage, tissue destruction by proteolytic action, and the transformation of fibrinogen into fibrin, known as thrombin-like action (YAMASHITA; ALVES; BARBARO; SANTORO, 2014). In addition, this venom activates Factor X and prothrombin in the coagulation cascade (clotting action), causes hemorrhage, the release of hypotensive substances, and causes lesions in the basement membrane of the capillaries, which are associated with thrombocytopenia and alterations in coagulation, which are frequent in this type of accident (JORGE; RIBEIRO, 1990; RANÉIA E SILVA; DE LIMA; LUIZ; CÂMARA *et al.*, 2021; VIVAS-RUIZ; SANDOVAL; GONZALEZ-KOZLOVA; ZARRIA-ROMERO *et al.*, 2020). *Bothropic* myotoxins induce a large efflux of ATP and $K^{+,}$ propagating cell damage, pain, and other inflammatory effects (CINTRA-FRANCISCHINELLI; CACCIN; CHIAVEGATO; PIZZO *et al.*, 2010).

Snake venom metalloproteinases extracted from *B. jararaca* venom cause coagulopathy, hemorrhage, and increased plasma tissue factor levels in animals; however, metalloproteinases and serine proteinases are not directly involved in thrombocytopenia (YAMASHITA; ALVES; BARBARO; SANTORO, 2014). High levels of tissue factor in plasma, which occurs during snake envenomation, lead to coagulation syndrome and may be implicated in the appearance of hemorrhagic manifestations in severely envenomed patients (YAMASHITA; ALVES; BARBARO; SANTORO, 2014). A study showed that the venom of

B. jararaca contains an E-NPP that promotes the hydrolysis of phosphate diester bonds, acting as an inhibitor of platelet aggregation via the degradation of ADP (SANTORO; VAQUERO; PAES LEME; SERRANO, 2009). Interestingly, purified myotoxins induce rapid release of ATP, whereas crude venom of *B. asper* does so to a small extent, probably because of the high presence of nucleotidases in crude venom, increasing adenosine concentrations and activating P1 receptors (CACCIN; PELLEGATTI; FERNANDEZ; VONO *et al.*, 2013).

Lachesis venom has lower toxicity and lethal activity, but due to the large amount inoculated in accidents, its effects can be extremely severe (STEPHANO; GUIDOLIN; HIGASHI; TAMBOURGI *et al.*, 2005). Local effects are also observed and are characterized by edema, hemorrhage, ecchymosis, and necrosis, the main causes of permanent disability (PARDAL; SOUZA; MONTEIRO; FAN *et al.*, 2004). Disturbances in cardiovascular parameters in rats and in human blood cells caused by *L. acrochorda* snake venom were evaluated (ABE; ENDO; SHIBA; SATAKE, 2020). *L. acrochorda* venom induced platelet aggregation, the magnitude of which was comparable to that of the positive control ADP, and this effect was inhibited by clopidogrel, an antagonist of the P2Y12 receptor (ANGEL-CAMILO; GUERRERO-VARGAS; CARVALHO; LIMA-SILVA *et al.*, 2020).

Crotalus venom has neurotoxic, myotoxic and coagulant effects (CRUZ; VARGAS; LOPES, 2009). *C. durissus ruruima* venom contains a PLA_2 called crotapotin, which shows potent neurotoxic activity that induces macrophages to form lipid droplets and synthesize inflammatory lipid mediators (DE CARVALHO; GIANNOTTI; JUNIOR; MATSUBARA *et al.*, 2019). Crotoxin B is a catalytically active group IIA PLA_2 from the venom of *C. durissus terrificus*, a venom with immunomodulatory activity that induces the formation of lipid droplets containing 15-deoxy-delta-12, 14-prostaglandin J2, an important mediator during inflammation, regulating the transition from acute inflammation to active inflammatory resolution in macrophages (GIANNOTTI; LEIGUEZ; CARVALHO; NASCIMENTO *et al.*, 2017). In addition, characterization of small membranous vesicles present in the crude venom of *C. durissus terrificus* demonstrated the presence of E-5'-NT (SOUZA-IMBERG; CARNEIRO; GIANNOTTI; SANT'ANNA *et al.*, 2017). Another study showed that direct treatment of mouse or human blood with E-5'-NT isolated from *C. atrox* inhibits platelet aggregation through the generation of increased levels of extracellular adenosine (HART; KÖHLER; ECKLE; KLOOR *et al.*, 2008).

Cobra neurotoxin isolated from the snake *Naja Naja atra* has neurotoxic effects via short-chain peptides and has central analgesic and hyperalgesic effects via adenosine A1 and A2A receptors (ZHAO; ZHAO; YANG; YE, 2017). This study proposed a new central

analgesic mechanism by which snake neurotoxins regulate pain and locomotor behavior, with a reduction in radical oxygen species and an increase in ATP in mouse brain tissues leading to a reduction in pain inducers (ZHAO; ZHAO; YANG; YE, 2017). The increase in adenosine caused by ATP hydrolysis interrupts the transmission of pain via the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway through the activation of A1Rs by cobra neurotoxin instead of adenosine (ZHAO; ZHAO; YANG; YE, 2017).

A new apyrase, purified and characterized from Russell's viper venom (*Daboia russelii*), was called Ruviapyrase (Russell's viper apyrase). It is a high molecular weight monomeric glycoprotein with a molecular weight of 79.4 kDa. Ruviapyrase hydrolyzed ATP in greater amounts than did ADP; however, Ruviapyrase lacked E-5'-NT and phosphodiesterase activities (KALITA; PATRA; JAHAN; MUKHERJEE, 2018). The structural properties of an isolated phosphodiesterase extracted from the venom of *Vipera lebetina* with a molecular mass of approximately 120 kDa include the hydrolysis of ADP but not ATP or AMP (TRUMMAL; AASPÕLLU; TÕNISMÄGI; SAMEL *et al.*, 2014). ADP- or collagen-induced platelet aggregation was inhibited by the venom of *V. lebetina in a dose-dependent manner*. This phosphodiesterase is synthesized as a single-chain protein that is cleaved, joined by a di-sulfate bridge and forms a heterodimeric enzyme (TRUMMAL; AASPÕLLU; TÕNISMÄGI; SAMEL *et al.*, 2014).

As an alternative way to mitigate envenomation by snakebites, low-level laser therapy effectively reduced local myonecrosis caused by *B. jararacuçu* in C2C12 myoblast cells by decreasing extracellular ATP and increasing intracellular ATP (SILVA; DA SILVA; DA SILVA; VIEIRA *et al.*, 2016).

In breast and lung cancer cells, a thrombin-like enzyme called pictobin isolated from the venom of the snake *B. pictus* produced mitochondrial network fragmentation with strong NADH oxidation, mitochondrial depolarization and decreased ATP (VIVAS-RUIZ; SANDOVAL; GONZALEZ-KOZLOVA; ZARRIA-ROMERO *et al.*, 2020).

In the venom of *Cereastes cerastes*, a viper native to the deserts of northern Africa, two enzymes displaying purinergic system activity were found. The first enzyme exhibited E-5'-NT, called Cc-5'NTase (CD73-like), which displays anticoagulant activity *in vivo* via ADP hydrolysis and inhibits arachidonic acid-induced aggregation (SAOUD; CHÉRIFI; BENHASSINE; LARABA-DJEBARI, 2017). The second enzyme is a phosphodiesterase called Cc-PDE (CD39-like), which prevents platelet aggregation induced by ADP and ATP hydrolysis, reducing surface P-selectin expression and attenuating platelet function (KIHELI; CHÉRIFI; AMEZIANI; SAOUD *et al.*, 2021).

3. Perspective and conclusions

Antivenoms remain the only specific treatment that can potentially prevent the effects of snakebite envenomation. Moreover, other drugs may be used to reduce venom, such as antinecrotic and anti-inflammatory agents, to reduce the local tissue damage that can lead to severe disability and even amputation after some snake bites (ORGANIZATION, 2023). One study demonstrated the anti-inflammatory, anti-hemorrhagic and anti-myotoxic actions of *Tabebuia aurea* extract, whose main component is specioside, in mice injected with the venom of *Bothrops mattogrossensis*, formerly known as *Bothrops neuwiedi* (REIS et al.; 2014).

The components of the purinergic system present in different snake venoms participate in envenomation. Based on knowledge about purinergic signaling at the bite site, extracellular ATP may increase and potentiate the inflammatory response. Important reports have shown that in more severe cases, cells under injury conditions can release substances previously stored, such as ATP, whose increased levels activate cell death receptors. Moreover, adenosine suppresses the activity of immune cells, exerts anti-inflammatory effects and participates in the immobilization and death of prey (Figure 2). Recent studies have purified and isolated enzymes with ectonucleotidase activity from the purinergic system. The presence of activity can alter purinergic components and consequently increase inflammatory parameters. Furthermore, nucleotidase activity can vary the expression of P2 receptors.

In addition to the toxins present in venoms, nucleotides and nucleotides such as spPLA2 indirectly modulates the purinergic system, pathogenesis and cell death by increasing the efflux of K^+ and ATP in murine muscle cells. The use of ATP reduced the cytotoxic effects of myothixin Lys49-spPLA2, which may lead us to believe that the modulation occurs via P2X7R. spPLA-BomoTx promote the release of ATP and the indirect activation of P2X2/P2X3 purinergic receptors, which is mediated by the NLRP3 inflammasome, which involves ATP and P2X7R. Malayan viper rhodocytin was found to promote platelet activation and histamine release from mast cells via P2X7R activation.

The purinergic system may be a promising target for treating symptoms observed during snakebite envenomation as it is capable of triggering responses in most cell types. It is already known that there is a special interaction with cells of the immune system and that they can promote or inhibit inflammation. In relation to the immune system, the purinergic system acts as an inflammatory mediator, generating autocrine and paracrine purinergic signaling pathways that promote the regulation of cellular interactions, activation and leukocyte migration, mainly lymphocytes, in injured tissues. If shows that lymphocyte activity is modulated both by

concentration of purines and by the expression of ectonucleotidases, which leads to responses that stimulate or inhibit inflammation (Figure 2).

Recent studies have shown interesting results in inflammation induced by venom. For instance, the use of specioside, an iridoid isolated from *Tabebuia aurea*, inhibited nucleotidase activity, restored neutrophil numbers, and mitigated the oxidative/inflammatory effects produced by the venom of *B. mattogrossensis* (DIAS; SOUZA DE SOUZA; MOSLAVES *et al.*, 2024). In addition, ATP released from damaged cells may also amplify cell damage by acting on purinergic receptors in neighboring cells (CINTRA-FRANCISCHINELLI; CACCIN; CHIAVEGATO; PIZZO *et al.*, 2010).

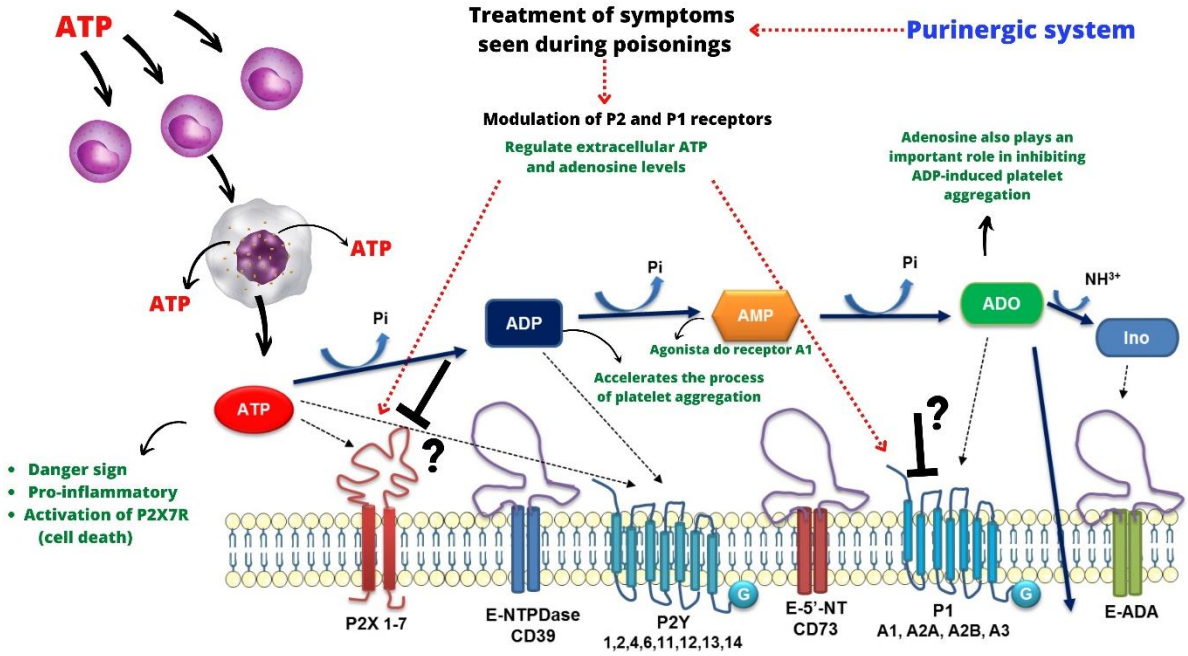


Figure 2. Therapeutic approaches based on antipurinergic system drugs may have the potential to limit the extent of muscle tissue damage. Under conditions of inflammation, there is an increase in extracellular ATP levels, which serves as a "danger" signal, triggering a series of proinflammatory responses. The increase in ATP in the initial phase of inflammation is regulated by E-NTPDase/CD39, which converts ATP into ADP to the monophosphate form, and E-5'-NT/CD73 converts AMP into adenosine. Adenosine depresses the activity of immune cells and exerts an anti-inflammatory effect. Toxins have been shown to promote ATP release and consequent activation of P2X purinergic receptors. Released ATP amplifies the effect of myotoxins, acting as a "danger signal" that spreads and causes further damage by acting on purinergic receptors. This promotes the autocrine activation of P2 receptors. E-NTPDase and CD73 ectonucleotidases promote ATP hydrolysis and the formation of adenosine, which activates P1 receptors. Adenosine can be converted to inosine by the enzyme adenosine desaminase. The responses generated can induce inflammatory responses, tissue damage, cell death, neurotoxicity and hemorrhage.

Purinergic signaling plays a fundamental role in the regulated release of nucleotides, as they are involved in inflammatory responses, tissue damage, pain and cell death. It is known that purines are present in venom and prey mediate responses on purinergic enzymes and purinergic receptors that are involved in snake poisoning. The purinergic system plays a significant role in modulating the body's responses to snake envenomation. Understanding this system better could pave the way for new therapeutic strategies aimed at mitigating the devastating effects of snakebites. Continued research is essential to develop more effective and specific treatments, benefiting the populations affected by this public health problem.

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CAPÍTULO II

5. OBJETIVOS – CAPÍTULO II

5.1 OBJETIVO GERAL:

Avaliar os efeitos induzidos pelo envenenamento da peçonha de *Bothrops mattogrossensis* (jararaca-pintada) sobre as alterações do sistema purinérgico e o potencial inibitório do especiosídeo extraído da *Tabebuia aurea* (Ipê amarelo).

5.2 OBJETIVOS ESPECÍFICOS

5.2.1 Avaliar a atividade e presença de nucleotidases na peçonha bruta da *B. mattogrossensis* (BmtV).

5.2.2 Investigar o efeito da BmtV sobre os componentes purinérgicos na expressão de P2X7R, CD39 e CD73 nas populações mielocíticas e monocíticas;

5.2.3 Avaliar os efeitos da BmtV sobre os componentes sanguíneos;

5.2.4 Avaliar o efeito do BmtV nos marcadores de estresse oxidativo/ inflamatório;

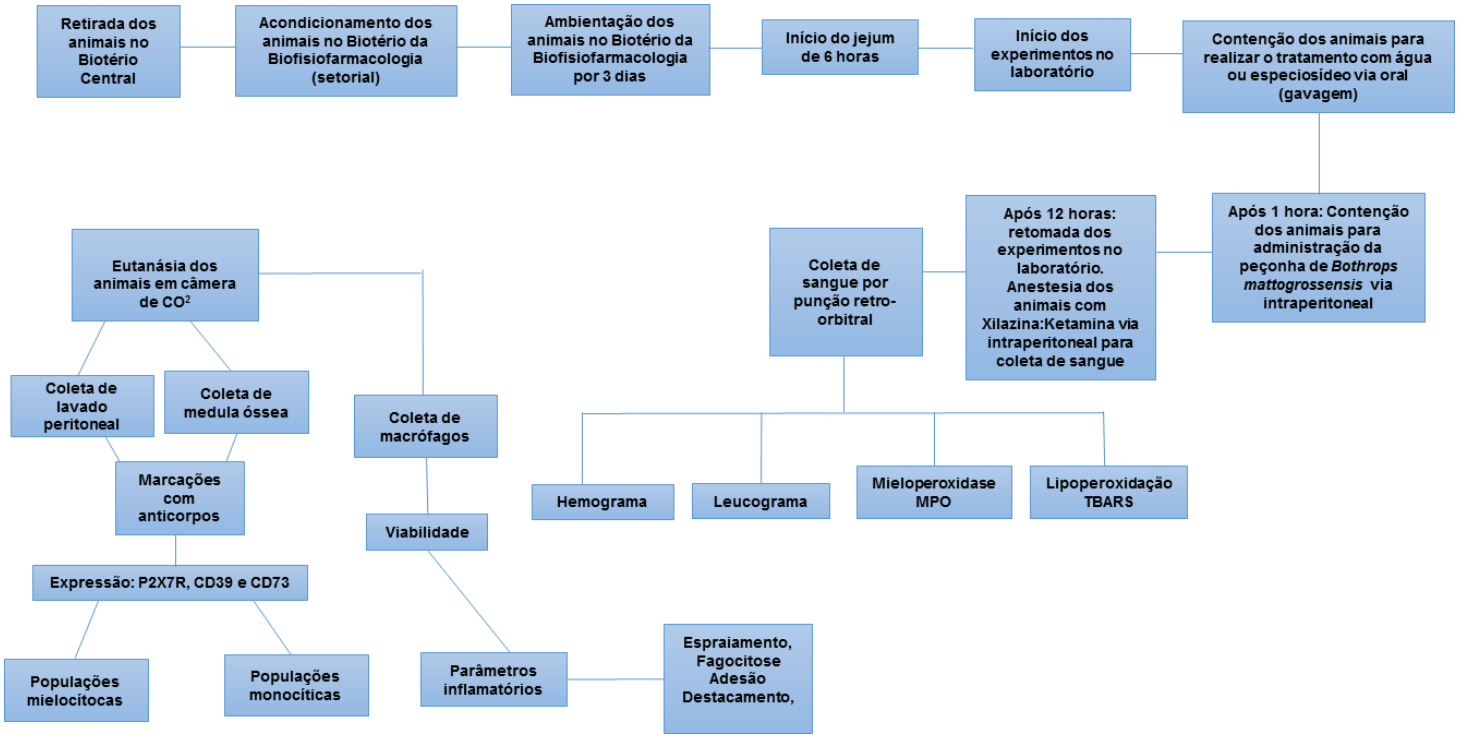
5.2.5 Avaliar a eficácia do especiosídeo como potencial redutor dos efeitos de envenenamento por BmtV.

6. DELINEAMENTO EXPERIMENTAL

Para os experimentos foram utilizados camundongos Swiss machos, com 4 a 6 semanas de idade, provenientes do Biotério Central da UFMS. Todos os procedimentos experimentais seguiram os padrões éticos de pesquisa. Os procedimentos foram previamente aprovados pelo comitê de ética da UFMS (n° 1.016/2018) e foram conduzidos de acordo com as Normas dos Institutos Nacionais da Saúde sobre o uso e cuidado de animais para fins científicos. Para os experimentos *in vivo*, os animais tratados foram divididos nos seguintes grupos:

1. Salina	Água (via oral v.o.)
	Solução salina (via intraperitoneal, i.p.)
2. BmtV	Água (v.o.)
	Peçonha 10 µg/0,5 mL (i.p.)
3. SP	Especiosídio 50 mg/Kg (v.o.)
	Solução salina (i.p.)
4. BmtV+SP	Especiosídio 50 mg/Kg (v.o.)
	Peçonha 10 µg/0,5 mL (i.p.)

A sequência cronológica dos procedimentos/tratamentos que foram realizados neste experimento em uma linha do tempo, desde a entrada dos animais até a eutanásia.



A dose da peçonha foi calculada basedada na quantidade de peçonha presentes na inoculação do gênero *Bothrops*. A dose do especiosídeo está de acordo com relatos de uso popular do extrato hidroetanólico de *Tabebuia aurea* e experimento *in vivo* previamente realizadas. O sangue foi recolhido por punção retro-orbital utilizando ácido etilenodiaminotetracético (EDTA) como anticoagulante. A separação do plasma foi efectuada por centrifugação do sangue total a 2500 g durante 10 minutos. As amostras de plasma foram congeladas e armazenadas a -80 ℃ até à análise da atividade da mieloperoxidase e dos ensaios de peroxidação lipídica. Em seguida, após eutanásia por deslocamento cervical, a medula óssea e as células peritoneais foram recolhidas em solução salina tamponada com fosfato (PBS, pH 7,2).

6.1 VARIÁVEIS EXPERIMENTAIS:

6.1.1 Atividade e presença de nucleotidases na peçonha bruta da *B. mattogrossensis* (BmtV). Os resultados são as médias ± SEM de 3 experimentos em quintuplicatas. A significância estatística foi calculada por um teste de Kruskal-Wallis, *$p < 0,05$

6.1.2 Os Efeitos da peçonha bruta de BmtV em células mieloides. Os dados são apresentados como média ± SEM de 7 experimentos independentes. A análise estatística foi realizada por ANOVA seguida do teste de Tukey.

6.1.3 Os efeitos da BmtV sobre os componentes purinérgicos na expressão P2X7R, CD39 e CD73 nas populações mielocíticas e monocíticas. Os resultados são a média ± SEM de 7 experimentos independentes. Os resultados são a média ± SEM de 7 experimentos independentes. A análise estatística foi realizada por ANOVA seguida do teste de Tukey.

6.1.4 Os efeitos da BmtV sobre os componentes sanguíneos. Os resultados são a média ± SEM de 7 experimentos independentes. Os resultados são a média ± SEM de 7 experimentos independentes. A análise estatística foi realizada por ANOVA seguida do teste de Tukey.

6.1.5 O efeito do BmtV nos marcadores de estresse oxidativo/ inflamatório; Os resultados são a média ± SEM de 7 experimentos independentes. A análise estatística foi efectuada por ANOVA seguida do teste de Tukey. Os resultados são a média ± SEM de 3-7 experimentos independentes. A análise estatística foi realizada por ANOVA seguida do teste de Tukey.

6.1.6 Os efeitos de BmtV na viabilidade celular dos macrófagos e nas características de estress. A viabilidade foi avaliada em vários momentos. O efeito do BmtV no espraiamento dos macrófagos e a atividade fagocítica foram quantificados após 12 h. Os resultados são a média ± SEM de 3-7 experimentos independentes. A análise estatística foi realizada por ANOVA seguida do teste de Tukey.

6.1.7 Efeito da peçonha BmtV no descolamento e adesão de macrófagos Os resultados foram expressos como média ± SEM de 3 experimentos em triplicatas. A análise estatística foi realizada por ANOVA seguida do teste de Tukey.

CAPÍTULO II

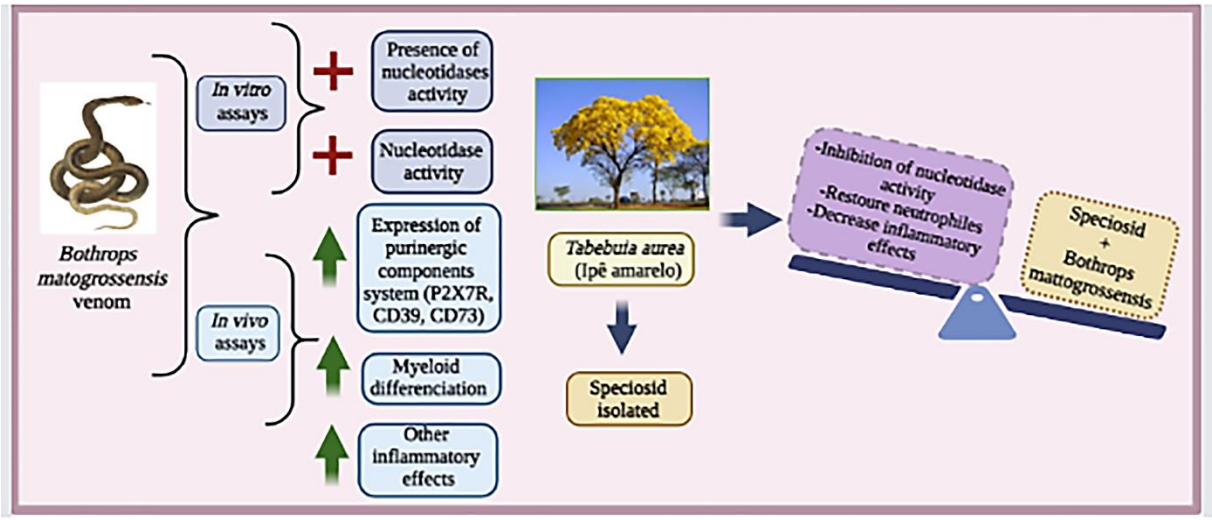
Identificação de componentes do sistema purinérgico no veneno de *Bothrops mattogrossensis* e efeito inibitório do especiosídeo extraído de *Tabebuia aurea*.

O conteúdo deste capítulo é composto pelo artigo intitulado "Identification of purinergic system components in the venom of *Bothrops mattogrossensis* and the inhibitory effect of specioside extracted from *Tabebuia aurea*", foi submetido à Purinergic Signalling em 31 de outubro de 2023 / Aceito: 16 de junho de 2024.

Referência: Dias, Dhébora Albuquerque et al. "Identification of purinergic system components in the venom of Bothrops mattogrossensis and the inhibitory effect of specioside extracted from *Tabebuia aurea*." *Purinergic signalling*, 10.1007/s11302-024-10032-z. 3 Jul. 2024, doi:10.1007/s11302-024-10032-z

Versão final publicada disponível em: https://link.springer.com/article/10.1007/s11302-024-10032-z

GRAPHICAL ABSTRACT



Identification of purinergic system components in the venom of *Bothrops mattogrossensis* and the inhibitory effect of specioside extracted from *Tabebuia aurea*

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7. Introduction

According to the World Health Organization (WHO) approximately 5.4 million people are bitten by snakes every year [1]. This results in 2.7 million cases of envenomation, 81,000 to 138,000 annual snakebite deaths worldwide, and another 400,000 patients stay permanently disabled or disfigured [1, 2]. In Brazil, 31,354 snakebites were reported in 2021; most of them were attributed to the genus *Bothrops*, followed by the genera *Crotalus, Lachesis,* and *Micrurus* [3]. Currently, snakebites are recognized as a neglected tropical disease by the WHO [1, 2].

Bothropic venom is classified as proteolytic, coagulant, and hemorrhagic [4].

In addition, components of the purinergic system, such as ectonucleotidases, purines, and pyrimidines, can be present in the venom itself or be produced by the coordinated action of several enzymes modulating the activation of P1 and P2 receptors [5]. The disruption of the cell membrane by snake venom increases the levels of extracellular nucleotides such as ATP [6]. Additionally, ATP is physiologically secreted by cells during stress, platelet aggregation, neurotransmitter release, or mechanical stimulation [7, 8].

The main components of the purinergic system are ectonucleotidases [9], extracellular nucleotides, and membrane receptors. Among extracellular receptors, P1 and P2Y receptors are G protein-coupled receptors, and adenosine (ADO), ATP, UTP, ADP, and UDP-glucose activate these receptors. P2X extracellular receptors (P2X1-7) are ligand-gated ion channels activated by ATP, ADP, and their analogs [10]. Nucleotidases hydrolyze extracellular nucleotides and can be membrane-bound enzymes such as ectonucleoside triphosphate diphosphohydrolase-1 (NTPDase-1/CD39), which converts ATP via ADP to AMP, or ecto-5′-nucleotidase (CD73), which catalyzes the conversion of AMP to adenosine [9]. The purinergic system participates in the control of many physiological functions, such as endothelial function [11], migration of inflammatory cells [12], oxidative stress aging [13], cell death [14], and myeloid differentiation [15, 16].

In Brazil, serotherapy is the primary treatment in cases of snake envenomation; nevertheless, it does not neutralize local reactions, which can generate serious sequelae such as partial or total loss of the affected area [2]. However, in cases of accidents in difficult-to-access areas, the serum is not always available, justifying the empirical use of medicinal plants. The use of plants as an antidote for snake bites is ancient and common in indigenous peaples of the Americas [17, 18].

Tabebuia aurea is a tree species belonging to the Bignoniaceae family. This species is widely distributed in tropical regions of the American continent, including Pantanal, Amazon, and Caatinga [17]. Ethnobotanical studies related that *Tabebuia aurea*, known as “paratudo”,

is maceration with alcohol, or chewing of stem bark to treat snake bites by indigenous peaples [17, 18]. In this way, a preliminary study related the potential of the hydroethanol extract of *Tabebuia aurea* in neutralizing the local effect induced by the venom as its major secondary metabolite, an iridoid named specioside (6- O-E-p-coumaroylcatalpol) [19]. The specioside is described as anti-inflammatory with a potential antivenom effect, which represents an alternative for neutralizing the local effects triggered by the venom in the absence of conventional therapy [20].

In this study, we verified the presence and activity of nucleotidases in the crude venom of *Bothrops mattogrossensis (*BmtV) *in vitro* the modulation of myeloid differentiation, components of the purinergic system, and oxidative stress/inflammatory markers were investigated *in vivo* and *in vitro*. Additionally, specioside was used as a potential molecule to reduce the envenomation effects promoted by BmtV.

8. Material and methods

8.1. Venom

Venom extracted from *Bothrops mattogrossensis* was provided by the Dom Bosco Catholic University of Campo Grande, Mato Grosso do Sul. Freeze-dried crude extracts of the venom was stored at -20 °C.

8.2. Extraction and purification of specioside

The stem bark of *Tabebuia aurea* was collected in October 2017 (Spring), in Campo Grande, Mato Grosso do Sul (20°26′37″ S, 54°38′52″ W), the plant was identified by Flavio Macedo Alves [21], and a voucher was deposited in the herbarium of the Federal University of Mato Grosso do Sul (UFMS), under number 74328. The plant material was registered at Sistema Nacional de Gestão do Patrimônio Genético under number A15EB96. Details of the hydroethanolic extraction of *Tabebuia aurea* and purification by liquid chromatography-diode array-detection-tandem mass spectrometry previously described [22].

8.3. Nucleotidase activity

A medium reaction content 160 µL of 50 mM Tris-HCl buffer (pH 8.0) and 3 mM $MgCl_2$. Twenty microliters of different concentrations (0.01, 0.05, 0.1, 1 µg/mL) were preincubated for 60 min. Additionally, enzymatic reaction in the presence of 24.5 µM specioside was also verified: 20 µL of specioside, 140 µL of Milli-Q water, 20 µL of different venom concentrations, and 20 µL of ATP, ADP, or AMP in a final volume of 200 µL. Reactions

were initiated by adding 3 mM substrates (ATP, ADP, or AMP) for 60 min. All reactions were stopped with 5% trichloroacetic acid (ATP, ADP, or AMP). The enzymatic activities were measured by the amount of inorganic phosphate (Pi) using malachite green as the colorimetric standard. The activity was expressed as nanomole of Pi released/min/mg of protein.

8.4. Identification of proteins by mass spectrometry

Venom samples pooled for proteomic analysis were subjected to reduction and alkylation, followed by trypsin digestion. Briefly, one hundred micrograms of proteins were diluted in 50 mM ammonium bicarbonate solution, incubated with urea and dithiothreitol reduction solution, followed by incubation with iodoacetamide alkylating solution for carbamidoacetylation. The linearized proteins were subjected to trypsin digestion overnight at a 1:50 ratio. The tryptic peptides were desalted using a SDB-XC (styrenedivinylbenzene polymer sorbent) stage tip. Then, the solution containing peptides was vacuum dried, and the resulting peptides were resuspended in 0.1% formic acid for later identification by LC–MS/MS. The equipment used was a Nano-Easy nanochromatograph (Thermo Fisher Scientific, USA) coupled to the orbitrap Q-exactivePlus spectrometer (Thermo Fisher Scientific, USA). The software used for protein identification was BSI Peaks (Bioinformatics Solutions Inc. USA). The database for the search was Viperidae (UniProt). The search for proteins was conducted using the SPIDER algorithm, with FDR<0.1% to identify true proteins with at least one unique peptide.

8.5. Animals

Male Swiss mice, 4 to 6 weeks old, from the Central Animal Facility of the UFMS were used. All experimental procedures followed ethical research standards. Procedures were previously approved by the UFMS ethics committee (n° 1.016/2018) and were conducted in accordance with the National Institutes of Health regulations on the use and care of animals for scientific purposes.

For *in vivo* experiments, the treated animals were divided into the following groups: Saline (orally received diluent + 0.9% sterile saline solution administered by intraperitoneal [i.p.] injection); SP diluted in water (orally received 50 mg/kg specioside + 0.9% sterile saline solution administered by i.p. injection); BmtV (orally received diluent + venom 10 µg per animal diluted in 0.9% sterile saline solution administered by i.p. injection); BmtV + SP (orally received 50 mg/kg specioside + 10 µg per animal diluted in 0.9% sterile saline solution administered by i.p. injection). Diluent (water) or specioside was offered orally 12 h before the

intraperitoneal injection of the venom. The dose of venom was calculated as the amount of venom present in snake bites in *Bothrops* genera [21, 23]. The dose of the specioside was previously reported in *in vivo* experiments [22, 23]. Then, the animals were anesthetized with a mixture of xylazine:ketamine (1:2) before blood collection. Blood was collected by retro-orbital puncture using ethylenediaminetetraacetic acid as an anticoagulant. Plasma separation was performed by centrifugation of whole blood at 2500 g for 10 min. Plasma samples were frozen and stored at -80 °C until myeloperoxidase activity and lipidperoxidation assays were analyzed. Then, after euthanasia by cervical dislocation, bone marrow and peritoneal cells were collected in phosphate-buffered saline (PBS, pH 7.2).

8.6. Flow cytometry analysis

Cells from the peritoneal cavity and bone marrow cells were collected from the femurs in PBS. Cells (10^6/sample) with anti-mouse F4/80-Alexa Fluor 647, anti-P2X7-FITC (Alomone), anti-mouse CD11b-APC/Cy7 (Becton Dickinson – BD, USA), anti-mouse Gr-1-PE/594 (BD), anti-mouse CD73-PE (BD), and anti-mouse CD39-BV42 (BD) for 20 min. Then, the samples were centrifuged and resuspended in PBS. Readings were performed on a CytoFLEX flow cytometer (Beckman Coulter, USA). The mature myeloid cell population ($CD11b^+Gr\text{-}1^+$) was classified as monocytic ($CD11b^+Gr\text{-}1^+F4/80^+$) and myelocytic ($CD11b^+Gr\text{-}1^+F4/80^+$) cells. Data were analyzed using FlowJo v10.9 software (BD).

8.7. Blood count

The blood count was evaluated by the Sysmex XP-300 automatic counter (Sysmex America, USA). The leukocyte count was determined using a smear stained with May Grünwald Giemsa and a microscope with an immersion objective (100x).

8.8. Myeloperoxidase activity

Myeloperoxidase (MPO) activity was determined by measuring the absorbance in plasma samples *in vivo* after treatments. Briefly, the reaction was started with the addition of 150 μL of reagents (0.167 mg/mL o-dianisidine hydrochloride and 0.0005% hydrogen peroxide), 50 mM monobasic phosphate, and 1% sodium azide. Then, 20 μL of plasma sample was added for 15 min, and 30 μL of 1% sodium azide was added for 10 min at room temperature. Sample readings were taken at 450 nm in an absorption plate reader (Varioskan LUX, Thermo Scientific, USA).

8.9. Lipid peroxidation assessment

Endogenous lipid peroxidation was assessed by detecting reactive thiobarbituric acid (TBARS) in plasma samples after treatments *in vivo*. Briefly, plasma was precipitated with 12% trichloroacetic acid, followed by incubation in buffer (60 mM Tris-HCl, pH 7.4, 0.1 mM diethylenetriaminepentacetic acid) and 0.73% thiobarbituric acid at 100 °C for 60 min. After cooling, the samples were centrifuged (5 min at 10,000 g), and the absorbance of the chromophore was measured at 535 nm in an absorption plate reader (Varioskan LUX, Thermo Scientific).

8.10. Assays on macrophages

Murine macrophages were obtained from peritoneal cavities of mice 96 h after administration of thioglycolate 3%. Animals were euthanized in a CO_2 chamber and the peritoneal cavity was washed with 3 mL of sterile saline solution (0.9% NaCl). For assays, 2×10^6 cells/mL were used in triplicate. Macrophages were cultivated in RPMI 1640 (Gibco Roswell Park Memorial) medium supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). For *in vitro* assays, macrophages were divided into treatment groups as follows: control (MØ = macrophage without stimulation cultivated in RPMI); SP (24.5 μM specioside); BmtV (10 μg/mL BmtV) and BmtV + SP (10 μg/mL BmtV + 24.5 μM specioside). The concentration of specioside used *in vitro* had no cytotoxicity in other cell lineages [22]. The cell viability assay was performed using 0.4% trypan blue (Sigma-Aldrich, Germany); unstained (viable) and stained (nonviable) cells were identified in an inverted microscope. The spread index was determined by counting the number of spread in cells employing phase contrast microscopy. For the phagocytosis assay, a suspension of zymosan (Sigma-Aldrich; 5 mg/mL) was incubated with mouse serum for 30 min. Afterward, the mixture was subjected to three cycles of centrifugation (700 g for 15 min) and precipitate resuspension. At the last step, the precipitate was resuspended in RPMI 1640 medium containing 5 mM glucose and 2% albumin. One hundred μL of zymosan was then incubated with peritoneal macrophages. Then, aliquots of 100 μL of samples were collected, spread on glass slides, and stained with Hema3® dye to determine phagocytosis under a light microscope.

Macrophage adhesion was quantified in RPMI supplemented medium (10% FBS) for 2 h, then the plates were washed three times with PBS and the remaining cells were fixed with paraformaldehyde 4% for 10 min. The cells present were stained in 50 μL Turk's solution (0.2% crystal violet dye in 30% acetic acid) for 30 min and washed three times with PBS. After that,

50 μL of sodium citrate (0,1 M; pH 4,2) was added and the supernatant was transferred to a reading plate. Absorbance was read at 540 nm (Varioskan LUX, Thermo Scientific). The control group was considered as 100%.

The detachment assay was performed similarly to the adhesion assay; however, macrophage suspensions were first dispensed into 96 wells and incubated for 2 h. Then, the plates were washed three times with PBS and incubated with the treatments in the diverse groups for another 2 h. The following steps were conducted in the same way as the adhesion test.

8.11. Statistical analysis

Paired Student's t-test or one-way analysis of variance (ANOVA) followed by the Tukey test were applied using GraphPad Prism 8v software (GraphPad, USA). The p-value less than 0.05 was considered statistically significant. Principal Component Analysis (PCA) was used to analyze interrelationships between variables (expression of CD39, CD73, and P2X7R of peritoneal cavity cells and bone marrow cells; plasma MPO and TBARS; viability assays, dissemination, phagocytosis, detachment and adhesion in peritoneal macrophages). The multivariate data was visualized on PCA biplots using factoextra and FactoMineR packages.

9. Results

9.1. Nucleotidase activity in crude venom extracted from *B. mattogrossensis*

The nucleotidase activity in the crude venom of *B. mattogrossensis* (BmtV) at different concentrations was tested. In contrast, a sustained increase in nucleotide hydrolysis was observed by the addition of BmtV (0.01, 0.05, 0.1, 0.5, 1.0 µg/mL) (Fig. 1A). The highest activity was observed at 1 µg/mL BmtV (ATP: 216±4, ADP: 148±10 and AMP: 250±20 nmol of Pi/min/mg of protein). The presence of 24.5 µM specioside inhibited the nucleotidase activity of 1 µg/mL BmtV (Fig. 1B). Additionally, adenosine deaminase enzyme activity was evaluated, but we did not observe ammonium release at the different concentrations used (data not shown). These results suggested the presence of nucleotidases with important activity in BmtV.

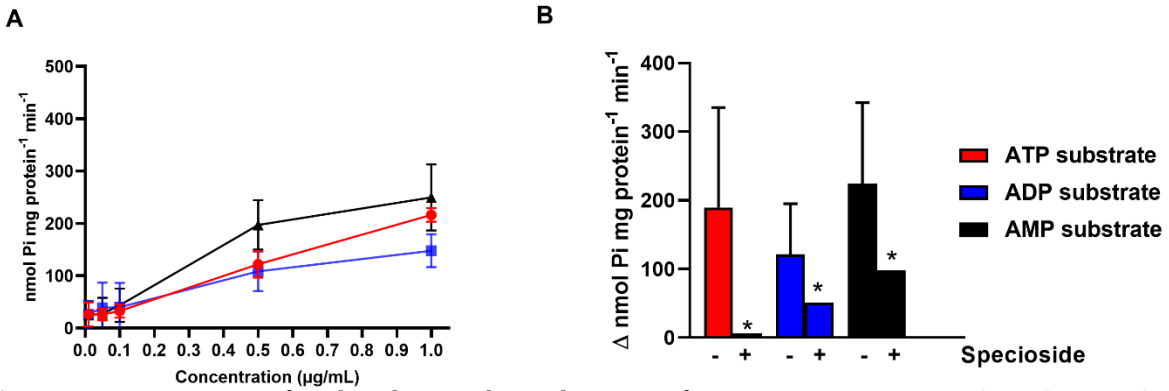

Figure 1. *In vitro* activity of nucleotidases in the crude venom of *B. mattogrossensis* using ATP, ADP, or AMP as substrates. **(A)** Results at different concentrations of BmtV (0.01, 0.05, 0.1, 0.5, 1.0 µg/mL). **(B)** Variation of enzymatic activity of 1 µg/mL BmtV in the presence of 24.5 µM specioside. The results are the mean ± SEM of 3 experiments performed in quintuplicate. Statistical significance was calculated by a Kruskal-Wallis test, *$p < 0.05$.

9.2. Analysis of proteins in the venom extracted from *B. mattogrossensis*

The venom extract, subjected to reduction and alkylation, followed by trypsinization was applied to liquid chromatography coupled to a mass spectrometer, which resulted in several protein fragments. The amino acid sequences obtained by the novo sequencing were able to partially reconstruct the 5'-nucleotidase (EC 3.1.3.5) protein (Ecto-5'-nucleotidase) after primary sequence alignments (Fig. 2). The presence of nucleotidases associated with the purinergic system in the study was validated through reconstruction guided by homologous enzymes deposited in the UniProt database, observed by coverage of 44, 36 and 27%, for *B. jararaca*, *Agkistrodon piscivorus,* and *Gloydius brevicaudus* respectively (Table 1).

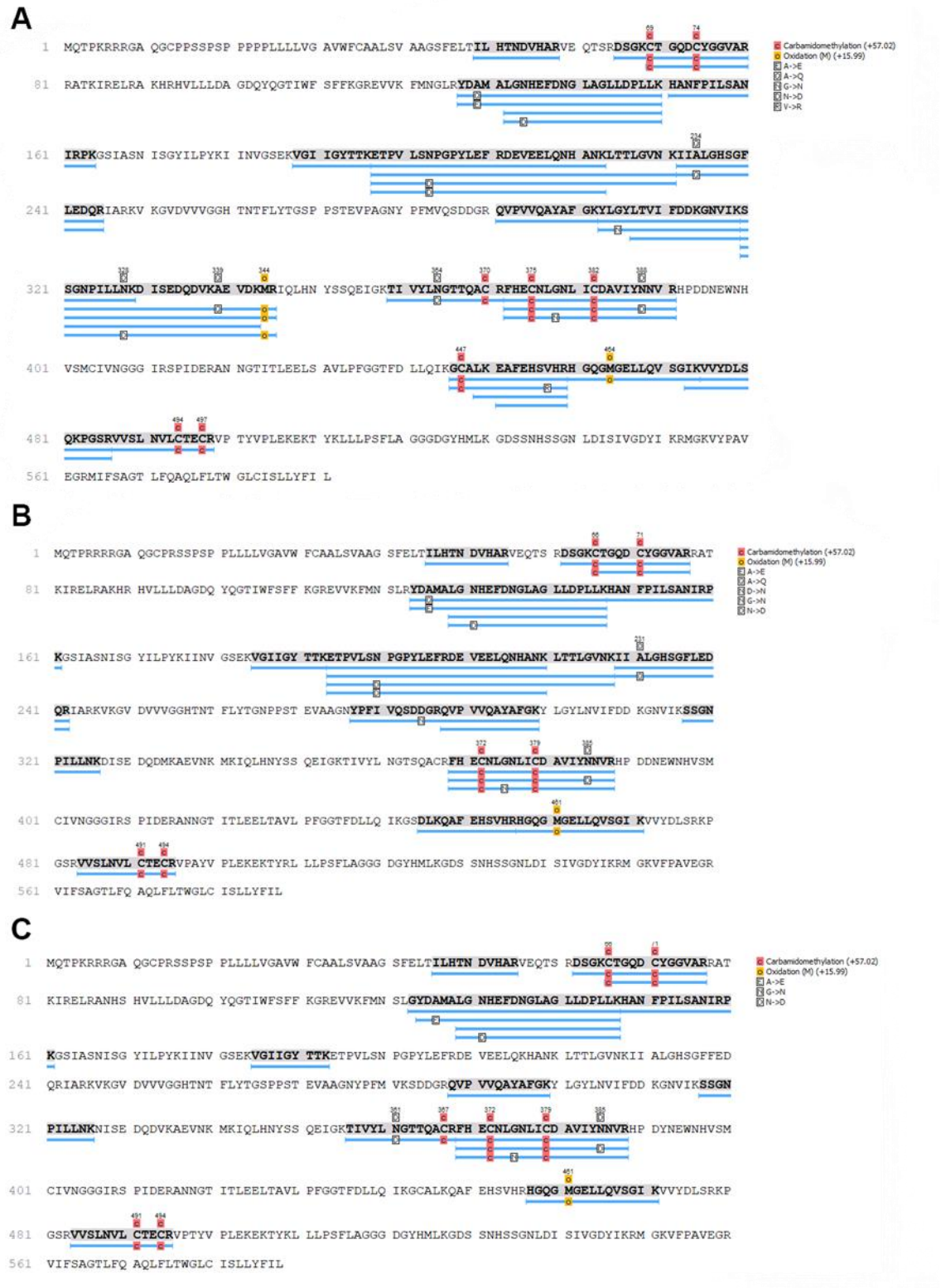

Figure 2. (A, B and C), confirmation of the fragments produced by 5'-nucleotidase in the crude venom of *B. mattogrossensis* according to the UniProt database. The fragments of this 5'-nucleotidase are represented in blue.

Table 1. Protein fragments found by mass spectrometry analysis of snake venom extracted identifying the coverage, accession number and match with species to 5'-nucleotidase.

Number of Fragments (blue lines)	Coverage (%)	Accession number	Specie	Protein families
26	44	A0A8T1N4T4	*Bothrops jararaca*	5'-nucleotidase
20	36	A0A194APL9	*Agkistrodon piscivorus*	5'-nucleotidase
15	27	B6EWW8	*Gloydius brevicaudus*	5'-nucleotidase

9.3. *B. mattogrossensis* venom promotes alterations in myeloid cells

Flow cytometry was performed to evaluate the alterations in immune cell populations induced by BmtV *in vivo*. The Gr-1$^+$Mac-1$^+$ population includes different stages of differentiated granulocytes and monocytes and plays important roles in inflammatory responses and tissue injury [15]. Myelocytic (Gr-1$^+$Mac-1$^+$F4/80$^-$) and monocytic (Gr-1$^+$Mac-1$^+$F4/80$^+$) cells extracted from the bone marrow and peritoneum were counted. The strategy for immunophenotyping of populations and the expression of proteins are shown in the supplementary material (Fig. S1).

BmtV treatment significantly decreased the percentage of bone marrow monocytic cells (Fig. 3A) and increased the percentage of peritoneal myelocytic cells (Fig. 3B). Treatment with specioside increased the percentage of myelocytic cells from both bone marrow and peritoneal (Fig. 3A) and partially reduced the increase in peritoneal myelocytic cell numbers induced by BmtV (Fig. 3B).

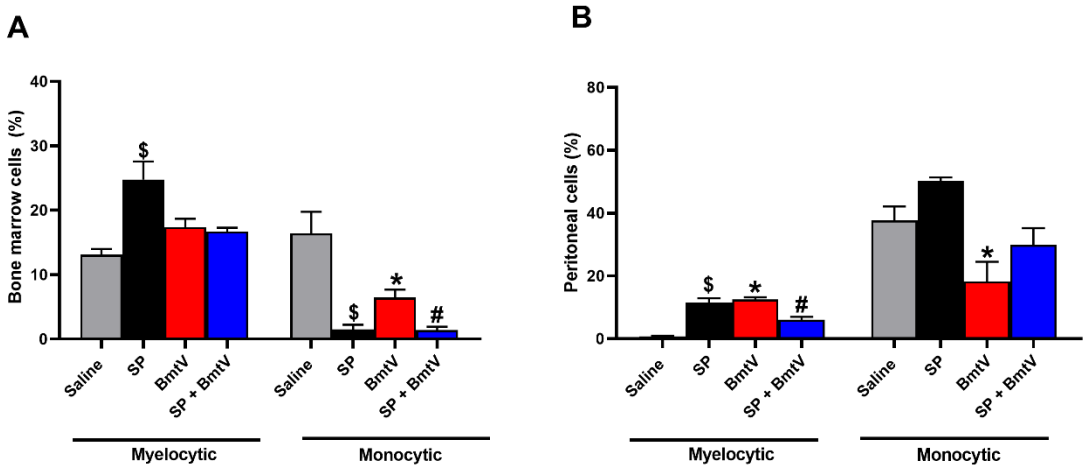

Figure 3. Effects of *B. mattogrossensis* crude venom in myeloid cells. The groups of mice were treated *in vivo* with saline (control), specioside (SP), *B. mattogrossensis* crude venom (BmtV) and SP+BmtV. Diluent or specioside were offered orally 12 h before the intraperitoneal injection of the venom. The effect of venom was evaluated after 12 h to treatment. Then, cells were collected from (A) bone marrow and (B) peritoneal and stained with Gr-1, Mac-1, and F4/80 antibodies. The percentage of myelocytic (Gr-1$^+$Mac1$^+$F4/80$^-$) and monocytic (Gr-1$^+$Mac1$^+$F4/80$^+$) cells was quantified. Data are presented as mean ± SEM of 7 independent experiments. Statistical analysis was analyzed by ANOVA followed by Tukey's test, $^{*,\#,\$}p < 0.05$. $^{\$}$Saline vs SP. *Saline vs BmtV. $^{\#}$BmtV vs SP+BmtV.

Alterations in blood components by BmtV and the effect of specioside in the presence of BmtV were also evaluated. The hemogram did not show differences among the groups (Table 2). However, differential leukocyte counts showed a significant increase in lymphocytes, eosinophils, and metamyelocytes, with a significant reduction in neutrophils by BmtV (Table 2). Treatment with specioside increased leukocytes, mainly monocytes (Table 2). Treatment with specioside, in the presence of BmtV (SP + BmtV group), reversed the decrease in neutrophil numbers, increased lymphocyte numbers (Table 2), and partially inhibited other hematological alterations. Images of cellular alterations are shown in the supplementary material (Fig. S2).

Table 2. Complete blood count in mice.

	Saline	**SP**	**BmtV**	**SP + BmtV**
Hemoglobin (g/dL)	15±4	17±4	15±2	14±2
Hematocrit (%)	45±12	51±11	50±5	46±7
Red blood cells ($x10^6$/uL)	8.5±12	7.3±11	6.5±5	13.7+1.8
Leukocytes (mm^3)	4,380±447	6,980±143$^{\$}$	5,700±895	5,530±831
Lymphocytes (mm^3)	2,332±533	2,499±80	3,883±920*	2,499±272$^{\#}$
Neutrophils (mm^3)	2,172±201	2,622±541	1,316±290*	2,879±278$^{\#}$
Monocytes (mm^3)	166±59	576±250$^{\$}$	313±95*	314±90
Eosinophils (mm^3)	44±0	0±0	124±37*	114±29
Metamyelocytes (mm^3)	0±0	0.06±0	111±0*	76±18$^{\#}$

The groups of mice were treated with saline (control), specioside (SP), *B. mattogrossessis* crude venom (BmtV) and SP+BmtV. All groups were treated for 12 h. Data are presented as mean ± SEM of 7 independent experiments. Statistical analysis was analyzed by (ANOVA) followed by Tukey's test. $^{\$,*,\#}$ P values <0.05. $^{\$}$Saline vs SP. *Saline vs BmtV. $^{\#}$BmtV vs SP+BmtV.

9.4. Evaluation of purinergic system component expression

To evaluate the effects of BmtV on the purinergic system associated with inflammation and tissue injury, the expression of P2X7R, CD39, and CD73 was quantified in myelocytic (Gr-1^+Mac-1^+F4/80^-) and monocytic (Gr-1^+Mac-1^+F4/80^+) populations (Fig. S1). P2X7R upregulation was observed after specioside treatment in monocytic peritoneal cells (Fig. 4B). Treatment with BmtV downregulated P2X7R expression in monocytic (0.2-fold) and myelocytic cells (0.56-fold) from bone marrow (Fig. 4A and B). No significant alterations of CD39 expression was observed by BmtV (Fig. 4C). The expression level of CD73 was lower in bone marrow cells than in myeloid peritoneal cells (Fig. 4E and F) Treatment with specioside

was not able to abrogate the effects elicited by BmtV, such as the expression of P2X7R (Fig. 4A). SP + BmtV group showed CD73 upregulation in bone marrow monocytic cells (Fig. 4E).

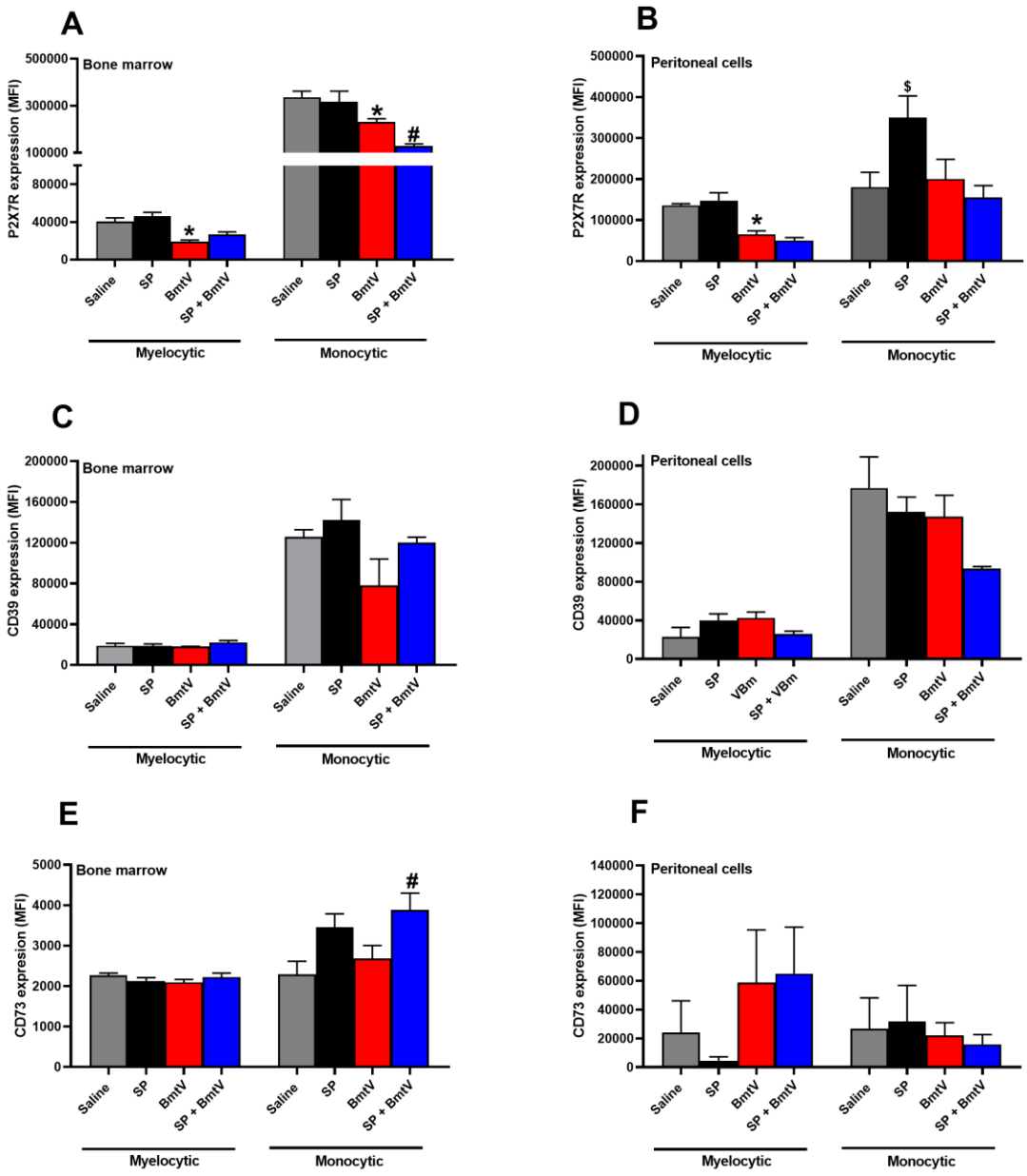

Figure 4. Expression of purinergic system components. The groups of mice were treated *in vivo* with saline (control), specioside (SP), *B. mattogrossensis* crude venom (BmtV) and SP+BmtV. Diluent or specioside were offered orally 12 h before the i.p. injection of the venom.The effect of venom was evaluated after 12 h to treatment. Then, cells were collected from (A, C, E) bone marrow and (B, D, F) peritoneal and labeled to antibodies Gr-1, Mac-1, and F4/80. The expression of (A and B) P2X7R, (C and D) CD39 and (E and F) CD73 was quantified on myelocytic ($Gr\text{-}1^{+}Mac1^{+}F4/80^{-}$) and monocytic ($Gr\text{-}1^{+}Mac1^{+}F4/80^{+}$) cells. The results are the mean ± SEM of 7 independent experiments. Statistical analysis was analyzed by ANOVA followed by Tukey's test, $^{*, \#, \$}$ $p < 0.05$. $^{\$}$Saline vs SP. *Saline vs BmtV. $^{\#}$BmtV vs SP+BmtV.

9.5 Inflammatory and oxidative stress parameters are increased by *B. mattogrossensis* venom and reduced by specioside

The levels of oxidative stress markers were quantified in plasma after treatments *in vivo* (Fig. 5). Treatment with BmtV produced an increase in TBARS (Fig. 5A) and MPO activity (Fig. 5B), which was inhibited by specioside (BmtV+SP group).

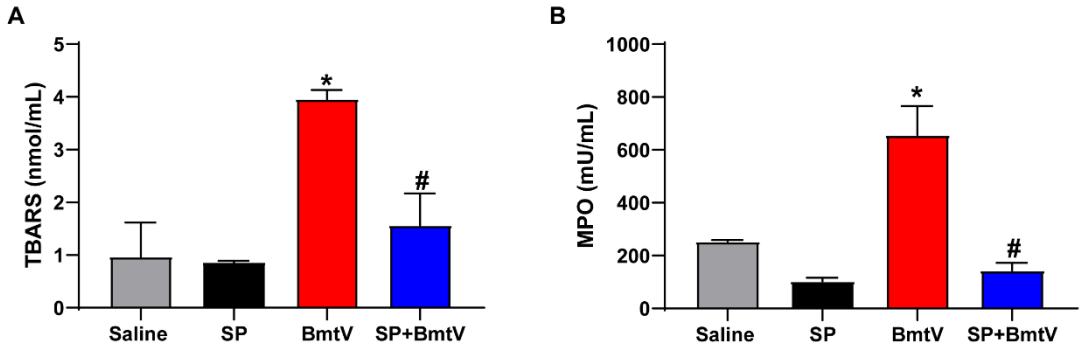

Figure 5. Evaluation of oxidative stress markers. The groups of mice were treated *in vivo* with saline (control), specioside (SP), *B. mattogrossensis* crude venom (BmtV) and SP+BmtV. Diluent or specioside were offered orally 12 h before the intraperitoneal injection of the venom. The effect of venom was evaluated after 12 h to treatment. **(A)** TBARS and **(B)** MPO were quantified in plasma. The results are the mean ± SEM of 7 independent experiments. Statistical analysis was analyzed by ANOVA followed by Tukey's test, $^{*,\#,\$}p < 0.05$. $^{\$}$Saline vs SP. *Saline vs BmtV. $^{\#}$BmtV vs SP+BmtV.

Experiments on peritoneal macrophages were done *in vitro*. Viability in macrophages was evaluated at various times (Fig. 6A). BmtV (10 μg/mL) promoted a significant decrease in cell viability in a time-dependent manner (Fig. 6A). Specioside inhibited the cytotoxicity of BmtV (BmtV+SP group). Moreover, macrophage detachment, adhesion, phagocytic activity, and spreading were also evaluated. BmtV induced an increase in the spreading (Fig. 6B), phagocytic activity (Fig. 6C), and detachment (Fig. S3A) of macrophages. The presence of specioside abrogated all effects produced by BmtV (SP+BmtV group). The adhesion of macrophages was not altered in any group (Fig. S3B).

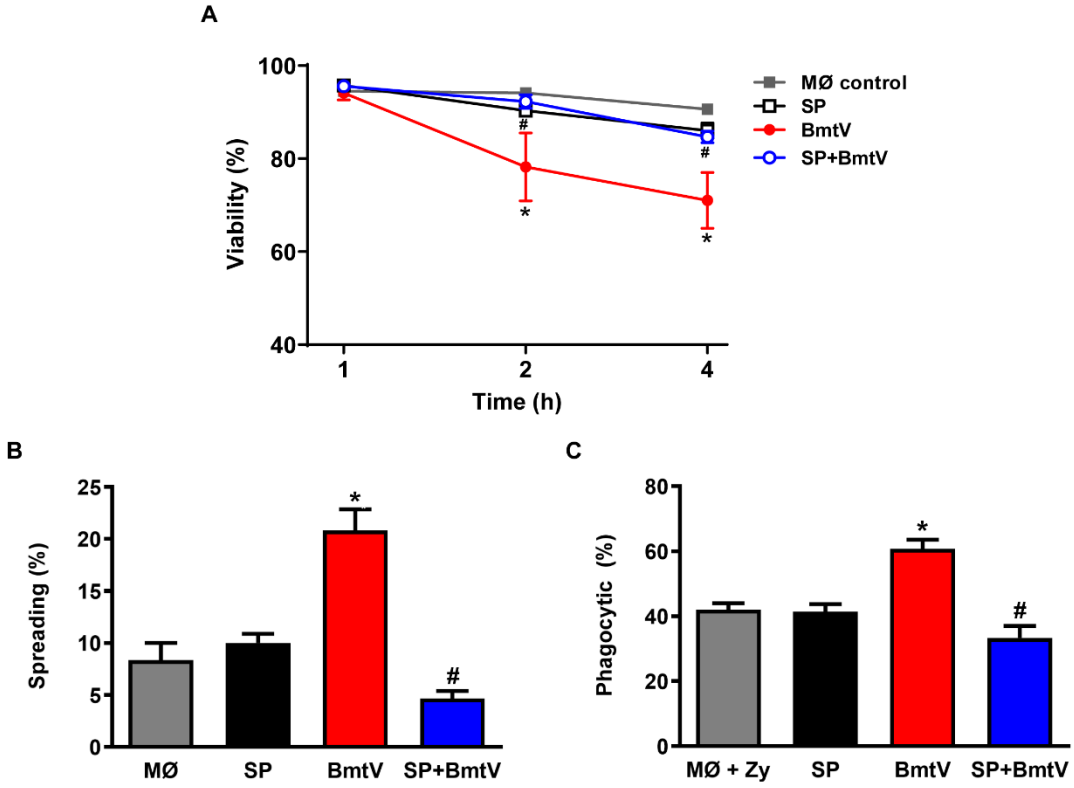

Figure 6. Effect of *B. mattogrossensis* venom in macrophages cell viability and stress characteristics. Macrophages were cultivated and treated *in vitro* with saline (control: MØ without stimulus), specioside (SP), *B. mattogrossensis* crude venom (BmtV) and SP+BmtV. **(A)** Viability was evaluated for 4 h. **(B)** Effect of BmtV on macrophage spreading and **(C)** phagocytic activity were quantified after 12 h. For quantified phagocytic activity all groups were previously stimulated with Zymosan (MØ+Zy = control) for 1 h previous to treatment. The results are the mean ± SEM of 3-7 independent experiments. Statistical analysis was analyzed by ANOVA followed by Tukey's test, $^{*,\#}$ $p < 0.05$. *Saline vs BmtV. $^{\#}$BmtV vs SP+BmtV.

10. PCA Analysis

PCA was used to conduct a multidimensional comparison among the control, SP, BmtV, and SP+BmtV groups (Fig. 7). The BmtV group had a positive correlation with the values obtained for migration, detachment, phagocytotic activity, TBARS, MPO activity, CD73 expression, and for increase in metamyelocyte numbers. On the other hand, it negatively correlated with the values obtained for cell viability on the x-axis. The SP group presented a positive correlation with increased P2X7R and CD39 expression levels and elevated numbers of leukocytes. PCA also showed that the SP+BmtV group was closer to the control group, corroborating that specioside restores the most important alterations induced by BmtV.

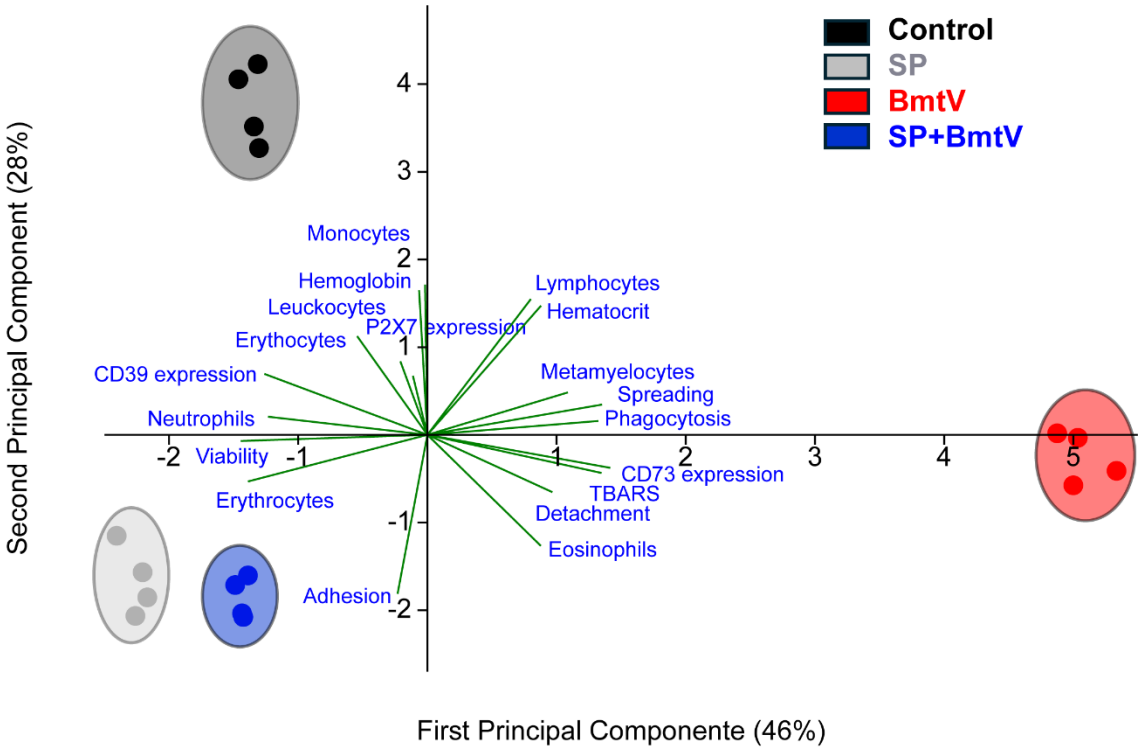

Figure 7. The biplot of principal component analysis (PCA) was performed on 19 characters. Different treatments are represented in different colors: control (black), specioside - SP (gray), *B. mattogrossensis* crude venom - BmtV (Red) and SP+BmtV (Blue). Characters are represented in arrows. The first two axes accounted for 74% of the total variation.

11. Discussion

Snakebites, currently classified as a neglected tropical disease by the WHO, have severe consequences due to the presence of many molecules in venom [1, 2]. Components of the purinergic system, such as nucleotidases, nucleotides, and P1 and P2 receptors participate in envenomation, making this system a promising target for the treatment of observed symptoms.

Nucleotidases and nucleotides are present in the venoms of distinct snake species. For example, 5´-nucleotidase activity was observed in the venoms of *Crotalus*, *Bothrops*, and *Lachesis genera* [5]. An apyrase Russell's viper venom (*Daboia russelii*), also called Ruviapyrase is a monomeric glycoprotein with a high molecular weight (79.4 kDa) that hydrolyzed ATP in greater amounts but without 5´-nucleotidase and phosphodiesterase activities [24]. In the venom of *Cereastes cerastes*, a viper native to the deserts of Northern Africa, two enzymes displayed purinergic system activity were found: the first enzyme exhibited ecto-5'-nucleotidase activity (CD73-like), which displays platelet aggregation inhibition and anticoagulant activity *in vivo* [25]; the second enzyme was a phosphodiesterase (CD39-like), which prevents ADP- and ATP-dependent platelet aggregation [26]. In this study,

we corroborated the nucleotidase activity in *B. mattogrossensis* venom and investigated the role of the purinergic system.

The purinergic system is mainly involved in innate immune responses. It is presumed that purines and ectonucleotidases participate in myeloid and macrophage differentiation by modulating P2 and P1 receptors, which are regulated by extracellular levels of ATP and adenosine, respectively [27, 28]. Cells under injury conditions can release intracellular molecules, increasing the extracellular levels of ATP that activate P2X7R [7, 8, 29].

In addition to the effects of snake venoms [25, 30] on the nervous system or the coagulation cascade, our results revealed changes in purinergic system components and an association with myeloid differentiation. Blood count showed an increase in the population of lymphocytes, eosinophils, and metamyelocytes and a decrease in the number of neutrophils by BmtV. The release of ATP by cells in the presence of BmtV could activate P2X receptors altering the percentage of the hematopoietic population from the first progenitors in the bone marrow to mature blood cells. The P2X7R is mainly associated with inflammation, immunology response, and differentiation of myeloid cells [29, 31]. Moreover, indirect ATP-induced effects, such as the generation of free radicals in macrophages [32], also promote myeloid differentiation by activation of P2X, P2Y, and P1 receptors [15, 33-35]. Studies performed on macrophages derived from bone marrow demonstrated that CD39 is constitutively expressed and is responsible for controlling their state of activation [36]. CD73 suppresses the activity of immune cells, exerting an anti-inflammatory effect [37].

In situations of homeostatic disturbance, extracellular nucleotides can trigger the production and release of reactive oxygen species and reactive nitrogen species [38, 39]. Purinergic signaling can affect the activity of antioxidant enzymes, promoting changes in the redox biology of cells [39]. This effect corroborates our results when analyzing pro-inflammatory and oxidative stress parameters, such as MPO and TBARS, which had increased activity after BmtV treatment. Increased MPO activity is associated with the accumulation of neutrophils in the tissues affected by the venom [40]. In a clinical study, an increase in TBARS levels was observed in the blood of victims bitten by *B. jararaca* and *B. jararacussu* [41].

It is known that the bark and seeds of *Tabebuia aurea* are widely used as anti-inflammatory agents [42]. In our study, specioside isolated from *Tabebuia aurea* reverted the main effects produced by BmtV, such as nucleotidase activity, alteration of myeloid cells, upregulated inflammatory and oxidative stress markers, reduction of cell viability, increase of cell spreading and phagocytosis index. However, specioside also had intrinsic activity altering blood leukocytes populations and expression of P2X7R. The inhibitory actions of specioside

are clearly shown in the PCA plot. This result agrees with those of testing the hydroethanolic extract of *Tabebuia aurea*, which shows its anti-inflammatory action [23]. Additionally, a hydroethanolic extract of *Tabebuia aurea* also reduced hemorrhage, lipid peroxidation, hyperalgesia, and neuronal injury induced by BmtV [21].

12. CONCLUSÕES

- Nossos dados confirmam a presença e atividade de nucleotidases no BmtV *in vitro*.
- Foram obsservados alterações nos componentes purinérgicos (P2X7R, CD39 e CD73) e aumento dos parâmetros inflamatórios/estresse pelo BmtV i*n vivo*.
- O uso do especiosídio reduziu os efeitos do BmtV i*n vitro* e *in vivo*, como atividade nucleotidase, variação na expressão do sistema P2, alteração no número de leucócitos e regulação positiva em parâmetros inflamatórios/oxidativos.
- Esses resultados mostraram a participação do sistema purinérgico no envenenamento por *BmtV* e corroboraram a eficácia do especiosídio, uma molécula presente na casca e sementes de *Tabebuia aurea*, que é utilizada empiricamente contra inflamações causadas por envenenamento ofídico.

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SUPPLEMENTARY MATERIAL

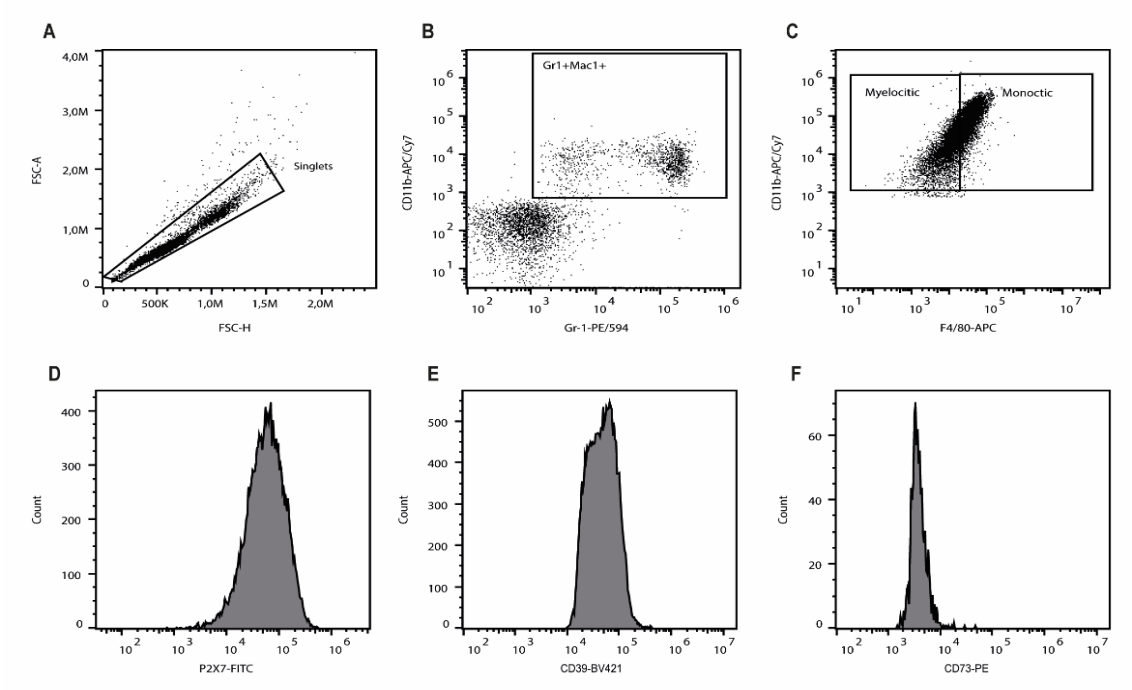

Figure S1. Strategy for evaluation of myeloid populations. **(A)** Singlets cells. **(B)** Gr-1^{+}Mac1^{+} gating was used to identified myeloid cells. **(C)** Myelocytic (Gr-1^{+}Mac1^{+}F4/80^{-}) and Monocytic (Gr-1^{+}Mac1^{+}F4/80^{+}) cells. Quantification of the expression of **(D)** P2X7R, **(E)** CD39 and **(F)** CD73 was performed on myelocytic and monocytic populations.

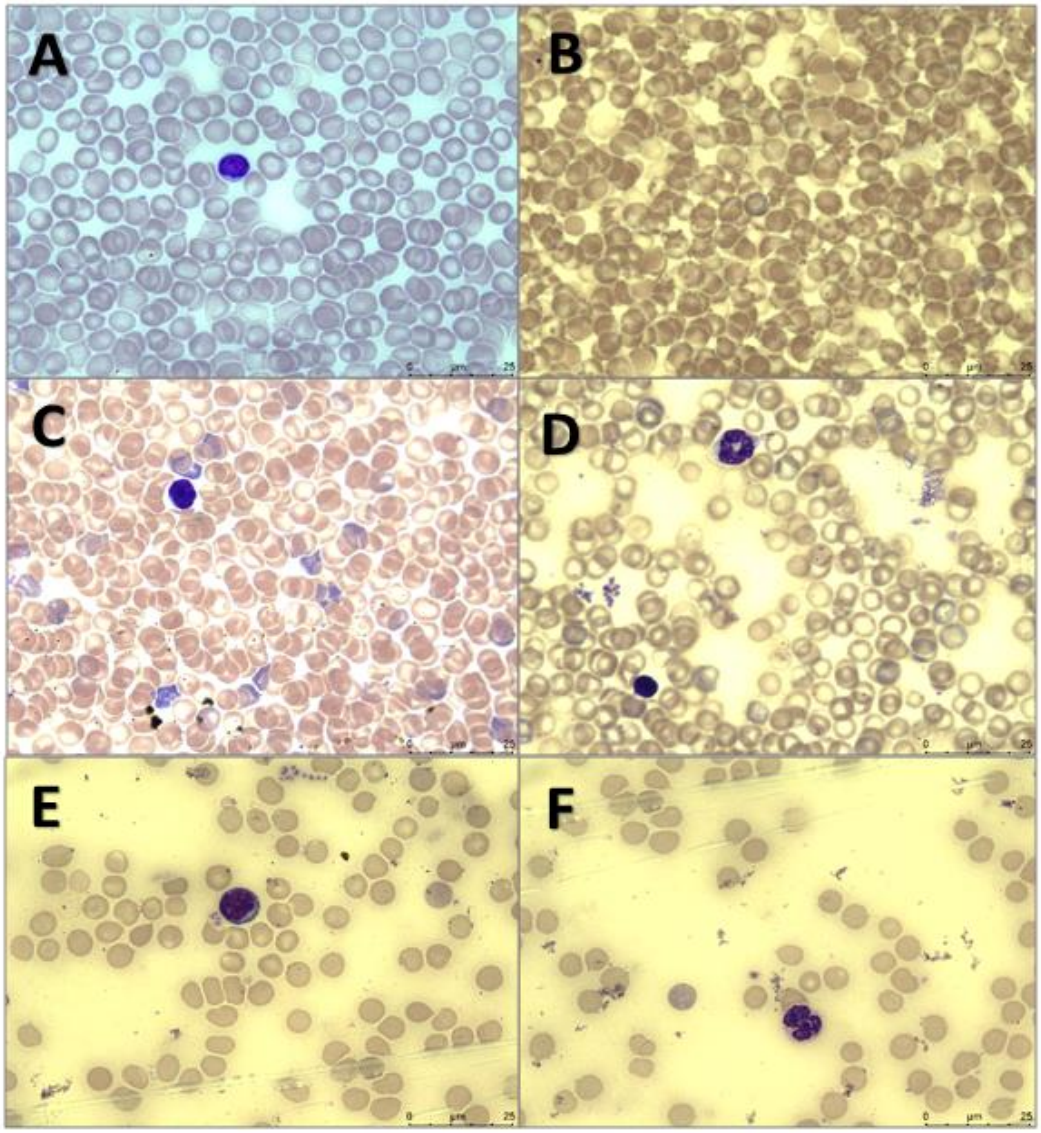

Figure S2. Blood smears of different of different groups: (A) BmtV (B), SP (C, D) and (E, F) SP+BmtV stained with May Grünwald-Giemsa. 1000x.

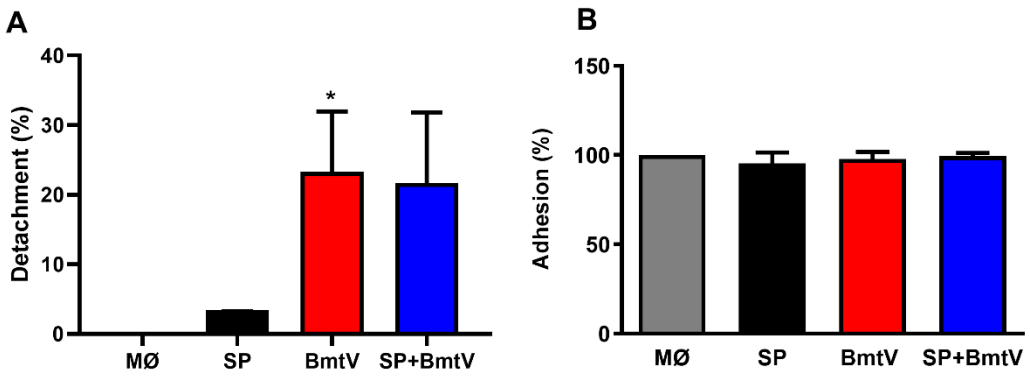

Figure S3. Effect of crude *B. mattogrossensis* venom (BmtV) on macrophage detachment and adhesion. The groups of mice were treated with saline (MØ - macrophage without stimulus), specioside (SP), *B. mattogrossensis* crude venom (BmtV) and SP+BmtV. The results were expressed as mean ± SEM of 3 experiments in triplicate. Statistical analysis was analyzed by ANOVA followed by Tukey's test, $^{*, \#, \$}$ $p < 0.05$. $^{\$}$Saline vs SP. *Saline vs BmtV. $^{\#}$BmtV vs SP+BmtV.

ANEXOS

Serviço Público Federal
Ministério da Educação
Fundação Universidade Federal de Mato Grosso do Sul

CERTIFICADO

Certificamos que a proposta intitulada "Investigação da participação do receptor purinérgico P2X7 na regulação da célula-tronco hematopoética e na linhagem mieloide", registrada com o nº 1.016/2018, sob a responsabilidade de **Edgar Julian Paredes Gamero** - que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA DA UNIVERSIDADE FEDERAL DE MATO GROSSO DO SUL/UFMS, na 2ª reunião ordinária do dia 26/03/2019.

FINALIDADE	( ) Ensino (x) Pesquisa Científica
Vigência da autorização	1º/03/2019 a 1º/03/2023
Espécie/Linhagem/Raça	*Mus musculus* / C57BL/6
Nº de animais	60 Machos + 60 Fêmeas = 120
Peso/Idade	3 - 30g / 0 – 90 dias
Sexo	Machos e Fêmeas
Origem	Biotério - UT/INBIO/UFMS

Fábio José Carvalho Faria
Coordenador da CEUA/UFMS
Campo Grande, 29 de março de 2019.

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